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Image analyser-assisted morphometry of the locus coeruleus in Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis

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Summary

Several observations suggest that neuronal shrinkage rather than cell death is the major phenomenon in neurodegenerative diseases. In order to make this distinction, smaller cells should also be included in cell counts. Also, morphometric determination of total cell numbers of brain structures is required. Morphometry was performed on the locus coeruleus using a newly developed method to delineate this nucleus from five patients who had died with Alzheimer's disease, five with Parkinson's disease, five with amyotrophic lateral sclerosis and from five control subjects who had died from causes that would not have affected the locus coeruleus. The length and volume of the locus coeruleus and its total number of large pigmented neurons, small unpigmented neurons and glial cells were determined. Since reliable delineation of the boundaries of the locus coeruleus is a requirement for the determination of total cell numbers, an image analyser-assisted procedure was developed. In Alzheimer's disease we found an 82% decrease in the number of large pigmented neurons and a 39% decrease of small unpigmented neurons. In Parkinson's disease, we found a 39% decrease of large pigmented neurons but also a 44% (though not significant) increase of small unpigmented neurons, which is indicative of a shift from large pigmented neurons to small unpigmented neurons in Parkinson's disease. The large pigmented/small unpigmented neuron number ratio was greatly and significantly reduced in Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis. These findings support the hypothesis that the decrease of large pigmented neurons of the locus coeruleus in some neurodegenerative diseases is not entirely due to cell death, but rather to cell shrinkage and a loss of phenotype. This hypothesis may have consequences for the development of therapeutic strategies since atrophied cells can be activated. On the other hand our data confirm that, at least in Alzheimer's disease, large pigmented neurons do also undergo cell death.

Keywords: image analysis; locus coeruleus; Alzheimer's disease; Parkinson's disease; amyotrophic lateral sclerosis

Introduction

The locus coeruleus is the major source of noradrenaline in the brain. It sends efferent projections to almost every brain region, and may thus influence such different functions as, for example, the level of vigilance (Aston-Jones *et al.*, 1991), selective attention (Mason, 1981), cerebral blood flow (Katayama *et al.*, 1981) and capillary wall permeability (Harik and McGunigal, 1984).

The locus coeruleus is affected in ageing, Alzheimer's disease and Parkinson's disease (Vijayashankar and Brody, 1979; Tomlinson *et al.*, 1981; Mann and Yates, 1983) and even more in patients with a combination of both Alzheimer's disease and a depressive disorder (Zubenko and Moossy, 1988). Neurofibrillary tangles are generally seen in the locus coeruleus in Alzheimer's disease and Lewy bodies in Parkinson's disease. Both changes, however, also appear in increasing numbers in old age (Tomonaga, 1983). No marked cytoskeletal changes of the amyotrophic lateral sclerosis type were seen in motor neuron disease (Mann *et al.*, 1983), compared with an age-matched control group. The occurrence of these neuropathological changes was found to be accompanied by the disappearance of large pigmented neurons of the locus coeruleus. Loss of up to 40% of large pigmented neurons in the locus coeruleus has been reported during ageing (Vijayashankar and Brody, 1979; Chan-Palay and Asan, 1989*a*), up to 87% in Alzheimer's disease (Bondareff *et al.*, 1982; Marcyniuk *et al.*, 1986*a*; Chan-Palay

and Asan, 1989*b*) and up to 94% in Parkinson's disease (Mann, 1983; Chui *et al.*, 1986; Zweig *et al.*, 1993), whereas no cell loss was found in the locus coeruleus in amyotrophic lateral sclerosis (Mann *et al.*, 1983).

These data did not, however, reveal whether the disappearance of large pigmented neurons of the locus coeruleus in Alzheimer's disease or Parkinson's disease was due to cell death or rather to neuronal shrinkage and depigmentation, causing a shift from large pigmented neurons to small unpigmented neurons. Such a shift has been described earlier in the nucleus basalis of Meynert in Alzheimer's disease where the large cholinergic neurons seem to change into smaller neurons (Rinne *et al.*, 1987). Furthermore, in a recent study (Regeur *et al.*, 1993) the assumed cortical cell loss in Alzheimer's disease could not be confirmed when the volume of the cortex was taken into account. In order to investigate the possibility that not only cell death, but also change of phenotype (i.e. neuronal shrinkage and depigmentation), takes place in the locus coeruleus, we determined the total number of large pigmented neurons, small unpigmented neurons, glial cells and the large pigmented/small unpigmented neuron number ratio in this nucleus in Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis and age-matched controls. The small unpigmented neurons and glial cells were counted within an area covering the locus coeruleus. This area was defined by the distance between the large pigmented neurons in the locus coeruleus. In this way all cell types were counted within the same area.

Since pigmented neurons, especially in the rostral part of the locus coeruleus, are scattered in such a way that the boundaries cannot be determined in a reproducible way, a new standardized image analyser-assisted method was developed to set these boundaries. This method is based upon the estimation of the mean distance between all large pigmented neuron profiles in the centre of the locus coeruleus, multiplied by a constant as a specific descriptor of the entire nucleus. Apart from the advantage of a tremendous reduction in the observer's bias during outlining of the locus coeruleus, this strategy results in outlines that fairly accurately fit the anatomical borders of the locus coeruleus.

The delineation of the locus coeruleus borders by the image analyser, together with the availability of a scanning stage on the microscope, allowed the implementation of a systematic random sampling strategy. In this way an accurate and easy-to-handle measurement of small structures, such as cell nucleus profiles (micrometre range), could be performed at high magnification in an area as large as the locus coeruleus (millimetre range).

Material and methods

Subjects

In order to obtain homogeneous age groups, we selected patients who had died suffering from a neurodegenerative disease with onset before the age of 65 years. The clinical diagnosis Alzheimer's disease ($n = 5$), Parkinson's disease ($n = 5$) and amyotrophic lateral sclerosis ($n = 5$) had been established by two neurologists. Five subjects with a pathology which was not expected to have affected the locus coeruleus served as controls, whilst the amyotrophic lateral sclerosis group was selected as a disease control group, since this condition does not show cell loss (Mann *et al.*, 1983). Two neuropathologists confirmed the diagnosis by autopsy. Patients were matched for age and sex. Some of the material was obtained from a brain collection at the Utrecht University Clinic, some from the Netherlands Amyotrophic Lateral Sclerosis Bank and some from the Netherlands Brain Bank, Amsterdam (coordinator Dr R. Ravid). The brains were fixed in 4% formaldehyde at room temperature for at least 1 month. The medulla oblongata, which contains the locus coeruleus, was divided mid-sagitally into a left and a right part, dehydrated in graded ethanol and embedded in paraffin via toluene. One half was used for neuropathology, and the other half for morphometry. No distinction was made between left and right, since the bilaterally symmetrical distribution of cells in the locus coeruleus has been well established (German *et al.*, 1988; Baker *et al.*, 1989). The clinical characteristics of all subjects are shown in Table 1.

Staining procedure

For the neuropathology of the locus coeruleus, 6 μm paraffin sections of the rostral, middle and caudal parts were stained with Klüver–Barrera, Nissl, Bodian and 0.1% haematoxylin with 0.2% eosin for 10 min and 25 s, respectively.

For morphometry, serial 6 μm sections were cut transversely on a Leitz microtome. Starting with a randomly chosen section (Uylings *et al.*, 1986), every 100th section was mounted on uncoated object slides, deparaffinized in xylene, hydrated via a graded series of alcohols, and stained with haematoxylin–eosin. Between the two sections that contained the highest number and density of large pigmented neuron profiles, every 50th section was stained.

Morphometry

The analyses were performed on an image analysis system (Kontron-IBAS 2000). The image analyser was connected to a Bosch TYK9B TV camera equipped with a chalnycon tube mounted on a Zeiss microscope with planapo objectives and a scanning stage under control of both a joystick and the image analyser. The critical minimum of the number of sections that had to be measured was determined. A mean large pigmented neuron profile distance estimate was determined for the locus coeruleus in each patient separately in five sections 300 μm apart through the area of the locus coeruleus with the highest number and density of large pigmented neuron profiles. From the stored 512×512 grey value image a 256×256 pixel area covering the locus coeruleus was selected (Fig. 1A). The analysis was performed in the corresponding area in the zoomed binary profile image

Table 1 *Clinical characteristics*

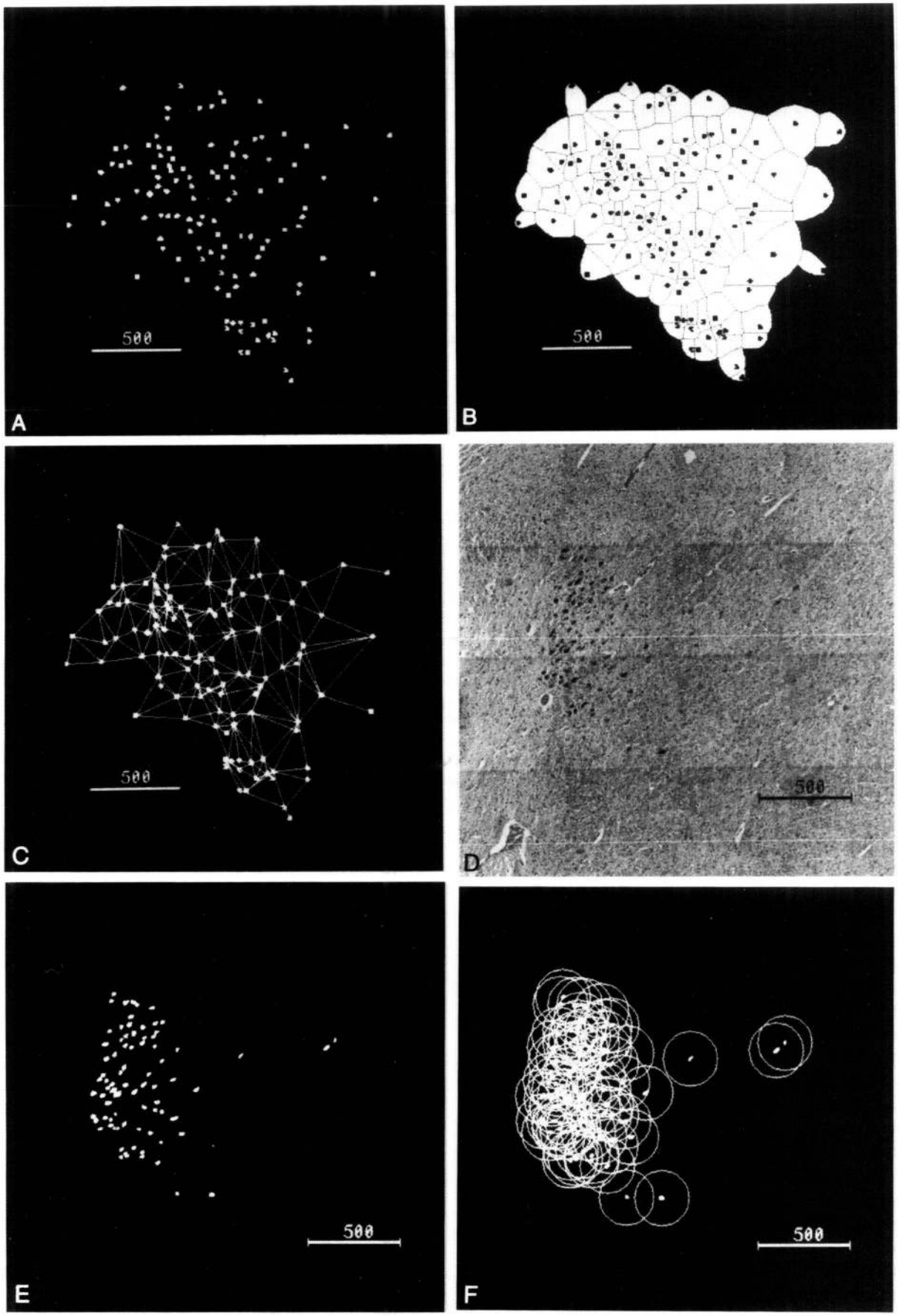
Patient*	Diagnosis	Sex	Age (years)	Age at onset (years)	Brain weight (g)	Fixation time (months)
1	Control	M	60.3		1425	1
2	Control	F	70.9		1300	1
3	Control	F	64.5		1060	6
4	Control	F	59.8		1040	4
5	Control	M	42.2		1340	3
Mean ± SEM			59.5 ± 4.8		1233 ± 77.5	3 ± 1.0
6	AD	M	66.3	59	1280	120
7	AD	F	74.5	< 65	975	240
8	AD	F	60.5	59	1215	324
9	AD	F	56.8	51	1180	11
10	AD	M	45.9	34	1130	1
Mean ± SEM			60.8 ± 4.8	50.8 ± 5.1	1156 ± 51.4	139.2 ± 63.3
11	PD	M	69.6	63	1350	84
12	PD	F	69.3	66	1325	240
13	PD	F	61.8	< 41	1270	132
14	PD	F	58.5	56	1060	348
15	PD	M	49.1	43	1390	240
Mean ± SEM			61.7 ± 3.8	53.8 ± 5.1	1279 ± 58.1	208.8 ± 46.3
16	ALS	M	70.8	66	1530	5
17	ALS	F	66.9	64	1360	5
18	ALS	F	63.3	60	1290	9
19	ALS	F	59.5	58	1430	1
20	ALS	F	45.5	42	1310	1
Mean ± SEM			61.2 ± 4.4	58 ± 4.3	1384 ± 43.8	4.2 ± 1.5

AD = Alzheimer's disease; PD = Parkinson's disease; ALS = amyotrophic lateral sclerosis; M = male; F = female.
*1, lymphadenopathy, respiratory insufficiency; 2, mamma carcinoma, small cerebral infarction, respiratory insufficiency; 3, contusio cerebri, cerebral infarctions, hypokalaemia; 4, cerebral infarctions, diabetes type II, liver cirrhosis; 5, AIDS, mild virus encephalitis (no dementia); 6, pneumonia; 7, focal cerebral gliosis, pneumonia; 8, rigidity, but no lewy bodies normal substantia nigra; 9, pneumonia; 10, depression, brother died of Alzheimer's disease at age 42; 11, frontal lobectomy, psychosis; 12, sepsis, no dementia; 13, frontal lobe meningioma, debilitas mentis from age 23; 14, psychosis, pneumonia; 15, frontal atrophy (no dementia), sepsis; 16, respiratory insufficiency; 17, some neurofibrillary tangles in substantia nigra and locus coeruleus (no dementia), pneumonia; 18, pneumonia; 19, gliosis in brainstem, respiratory insufficiency; 20, respiratory insufficiency.

and consisted of two steps: in the first step the neighbours of each large pigmented neuron profile were determined by a tessellation of the binary profiles (Fig. 1B). The mean profile distance was determined from this image by the automatic drawing of lines between each profile and its neighbours (Fig. 1C) and calculating the mean length of these lines.

The fields to be measured in the locus coeruleus area were determined as follows. For each section to be analysed the area covered by the locus coeruleus was determined by loading the 16 areas obtained with a ×125 magnification (Fig. 1D), then marking the large pigmented neurons (Fig. 1E) and then putting a filled circle with a radius of mean profile distance times a factor of 2.25 around the 'centre of gravity' of each large pigmented neuron profile in the stored binary image (Fig. 1F). The locus coeruleus boundaries were defined as the outer borders of the clusters of circles containing more than two large pigmented neurons (Fig. 1G). These boundaries were superimposed on the reconstructed image and the locus coeruleus area was measured (Fig. 1H). The factor of 2.25 was necessary to obtain clusters of circles instead of several individual circles not touching each other. We determined the percentage of large pigmented neurons within the locus coeruleus boundaries (i.e. within clusters containing more than two large pigmented neurons) when using a series of different multiplication factors. With a factor of 2.25, more than 90% of all large pigmented neurons in the brainstem section fall within the locus coeruleus boundaries as defined above. This was the case in both the control group and all disease groups. The mean profile distance of a patient times the factor 2.25 was called the 'patient specific locus coeruleus cluster factor'.

In order to take samples for counting cells at high magnification, a grid with corresponding areas at ×500 magnification was superimposed on the locus coeruleus area. From the parts of this grid covering the locus coeruleus area an area-weighted non-selective sample, the size of which was determined by the investigator, was taken (Fig. 1I). In the present study we counted large pigmented and small unpigmented neurons in 100% and glial and remaining cells in 10% of the measuring fields. The position of each of the fields belonging to this sample was visualized and expressed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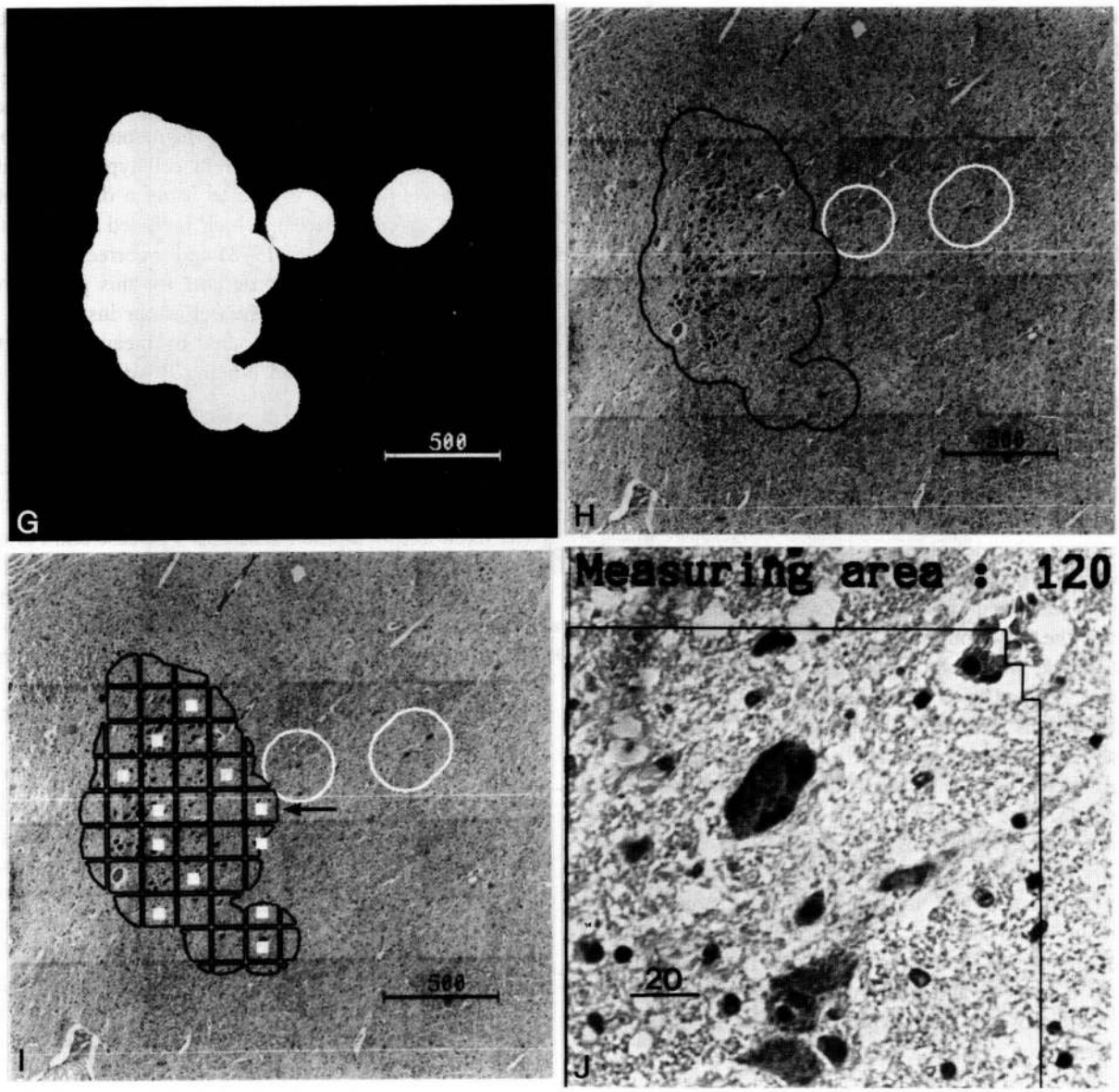

Fig. 1 A–J The image analyser-assisted procedure to determine the mean profile distance (**A–C**) the area of the locus coeruleus and the number of the cell profiles in each section (**D–J**). (**A**) Marking of the profiles of the large pigmented neurons in the five sections with the highest large pigmented neuron number and density after loading ×125 magnified areas covering the locus coeruleus. Bar = 500 μm. (**B**) Determining of the neighbours of each large pigmented neuron profile by a tessellation of the binary cell profiles. Bar = 500 μm. (**C**) Automated drawing of lines between each cell profile and its neighbours and calculating the mean length of these lines. Bar = 500 μm. (**D**) Loading the 16 areas (×125 magnification) covering the section in which cell counts are to be performed. Bar = 500 μm. (**E**) Marking the profiles of the large pigmented neurons in this section. Bar = 500 μm. (**F**) Putting a circle around each large pigmented neuron profile with the diameter of the patient-specific cluster factor (i.e. the mean profile distance×2.25). Bar = 500 μm. (**G**) Defining the locus coeruleus boundaries in the binary image by selecting the clusters of circles containing more than two neurons. Bar = 500 μm. (**H**) Superimposing the locus coeruleus boundaries on the reconstructed area covering the locus coeruleus. Bar = 500 μm. (**I**) Superimposing a grid, corresponding to areas to be examined at ×500 magnification, on the locus coeruleus area and determining an area-weighted systematic sample [the arrow indicates the field shown in (**J**)]. Bar = 500 μm. (**J**) Superimposing the actual field to be measured, enclosed by the locus coeruleus boundaries (the right line and the upright corner) and the exclusion lines according to Gundersen (the right line and the upper line), on the 'on-line' image and manual outlining of cell nucleus profiles. Bar = 20 μm.

in *x*, *y* coordinates of the scanning stage in the original section. After the (re)positioning of this section on the stored images and after putting a ×40 objective (×500 magnification) on the microscope, the scanning stage moved automatically to the positions included in the sample. At each position the outline of the actual area to be measured, together with the exclusion lines according to Gundersen *et al.* (1988) were displayed, superimposed on the 'on-line'

image (Fig. 1J). At each location, nuclear profiles of different cell types could be outlined manually in this on-line image.

The total number of large pigmented neurons, small unpigmented neurons, glial cells (i.e. both astrocytes and oligodendrocytes) and 'remaining' cells (those not falling into these catagories, *see* definition below) and the large pigmented neuron:small unpigmented neuron number ratio was determined for each subject. The large pigmented neurons were defined as those cell profiles containing dark pigment, i.e. neuromelanine. This group mainly consisted of profiles containing a nucleus with a mean diameter of ~11 μm, but, since only a part of the nucleus was present in the section, profiles with dark pigment and smaller nuclear profiles were also included. All large neurons appeared to have at least some pigment, except for the motor neurons of the tractus mesencephalicus of the trigeminal nerve and the neurons of the surrounding brainstem nuclei, containing the much lighter staining lipofuscin. These two cell types were excluded from the countings. Our definition of large pigmented neurons is in agreement with that in other studies (Iverson *et al.*, 1983; Bondareff and Mountjoy, 1986; Zweig *et al.*, 1988) and resembles the description of the medium-sized pigmented neurons in the studies by Vijayashankar and Brody (1979) and Baker *et al.* (1989). Small unpigmented neuron profiles were defined as profiles without the dark neuromelanine pigment. The nucleus contained a distinct nucleolus or no chromatin at all, since only a part of the nucleus was present in a section. The morphology of the small unpigmented neurons was described by Baker *et al.* (1989). In our study no subdivision has been made in the small neuron group between 'pigmented' and 'unpigmented' small neurons, since pigment in small neurons is generally lipofuscin rather than neuromelanine (Baker *et al.*, 1989). If neuron profiles contained a nucleus and neuromelanine, detected with the aid of the microscrew, they were counted as large neurons. All small neuron profiles contained some cytoplasm. Glial cell profiles were defined as profiles containing a nucleus with scattered chromatin (astrocytes) or a nucleus filled with dark chromatin (oligodendrocytes), both without visible cytoplasm. Since not all small unpigmented neurons could be distinguished with certainty from glial cells, we created a separate group of 'remaining' cells. The 'remaining' cell profiles were defined as astrocyte-like profiles with a nucleus containing one distinct patch of chromatin, which may or may not have been a nucleolus, or as oligodendrocyte-like profiles with surrounding material which may have been a nucleus or cytoplasm (Fig. 2). The use of the microscrew of the microscope facilitated the distinction between the different cell groups.

The total volume of the locus coeruleus was determined by integrating the locus coeruleus areas in all sections (Van Eden *et al.*, 1984). The locus coeruleus was subdivided into a rostral, a middle and a caudal part, according to Chan-Palay (1989*a*). The rostral part extends from the point where a cluster covers more than two large pigmented neuron profiles to the decussation of the trochlear nerve. From here the middle part extends caudally to the point where the inferior part of the mesencephalic trigeminal tract is no longer enveloped by the main body of the locus coeruleus. The caudal part extends from here to the point where the cluster covers less than three large pigmented neuron profiles. The total number of the different cell types per unit locus coeruleus volume was estimated using a discrete unfolding procedure (Weibel, 1979), which included the modification proposed by Cruz-Orive (1978) and a correction for section thickness. The computer programs for this procedure were developed by Dr R. W. H. Verwer at our institute.

Subject groups were compared by means of two tailed t test, the level of significance being $P < 0.05$.

Results

Neuropathology

The fixation time in the Alzheimer's disease and Parkinson's disease groups was longer ($P < 0.01$) than in the control and amyotrophic lateral sclerosis groups (Table 1). This difference did not influence the outcome of the morphometry by extra shrinkage in the groups with longer fixation times, because the locus coeruleus volume of the Alzheimer's disease brain that was fixed for only 1 month was within the range of the other locus coeruleus volumes in the Alzheimer's disease group. The observation that the mean diameter of the cell nucleus of the different cell types did not differ significantly between disease groups with short and disease groups with long fixation times ($P > 0.1$) points in the same direction.

Extraneuronal pigment was present in subjects of each group, but most extensively in Alzheimer's disease and Parkinson's disease (Table 2). Lewy bodies were seen in the locus coeruleus of all Parkinson's disease and of one Alzheimer's disease patient (Patient 9). The latter case did not reveal parkinsonian symptoms during life or signs of degeneration of the substantia nigra or Lewy bodies in the cortex. Neurofibrillary tangles were sporadically present in the locus coeruleus of subjects of each group, whereas in Alzheimer's disease their presence was more abundant. Neuritic plaques were not found.

Morphometry

The mean length of the rostro-caudal axis of the locus coeruleus, which was 12.7 ± 1.1 mm in the control group, was 34% smaller ($P < 0.05$) in the Alzheimer's disease group. The loss in length was more severe in the rostral part than in the middle and caudal parts (83%, 23% and 15%, respectively). No significant difference in length was observed between controls, Parkinson's disease and amyotrophic lateral sclerosis (Table 3). The mean volume of the locus coeruleus, which was 10.5 ± 0.9 mm^3 in the control group, was 22% smaller in the Alzheimer's disease group ($P < 0.05$) and 22% larger in the amyotrophic lateral sclerosis group ($P < 0.05$). The volume of the Parkinson's disease group

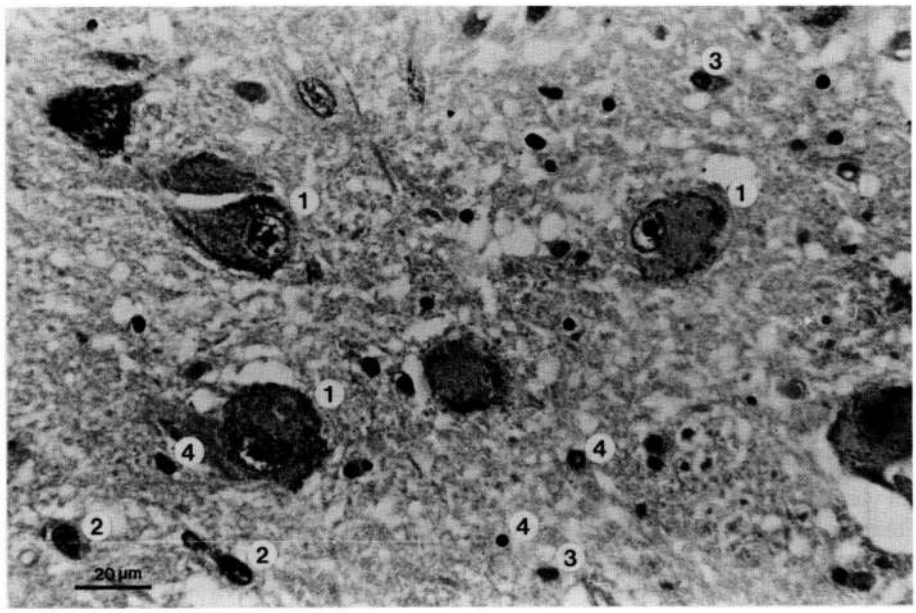

Fig. 2 The different cell types in the locus coeruleus of a control subject. Haematoxylin–eosin; 1 = large pigmented neuron; 2 = small unpigmented neuron; 3 = 'remaining' cell; 4 = glia cell.

Table 2 *Neuropathological characteristics*

	Controls	AD	PD	ALS
Extraneuronal pigment	+	++	++	+
Lewy bodies	–	–	++	–
Neurofibrillary tangles	+	++	+	+
Neurite plaques	–	–	–	–

– = absent; + = present; ++ = abundant; AD = Alzheimer's disease; PD = Parkinson's disease; ALS = amyotrophic lateral sclerosis.

was not significantly different from that of the controls ($P > 0.1$, *see* Fig. 3). The locus coeruleus volume was smaller in Alzheimer's disease ($P < 0.001$) and in Parkinson's disease ($P < 0.01$) when compared with the amyotrophic lateral sclerosis group (Fig. 3). The larger volume in amyotrophic lateral sclerosis may, at least partly, be explained by allometric relationships between the size of the brain and brain structures since brain weight, though not significant ($P > 0.1$, *see* Table 1), was 11% greater.

The intra-observer reliability was determined by measuring the same section five times. The intra-observer coefficient of variation, i.e. standard deviation/mean value, in the measurement for large pigmented neurons was 7.7%, for small unpigmented neurons 4.4%, for glial cells 6.3%, for 'remaining' cells 7.5% and for the locus coeruleus area 1.3%. In the present study the inter-observer reliability was not determined since there was only one observer (W.H.).

The total number for all cell types for the entire locus coeruleus, when counting one out of every 200 sections, did not change when one out of every 100 were counted. The large pigmented neurons and small unpigmented neurons were counted in all fields selected for measurement per section. No significant difference in the total number of glial cells was found when counting 100% or only 10% of the measured fields.

The mean number of large pigmented neurons in the control group was 16 474±2243. In Alzheimer's disease we found an 82% decrease ($P < 0.001$) and in Parkinson's disease a 39% decrease in the number of large pigmented neurons, whereas no significant difference was found in the amyotrophic lateral sclerosis group compared with the control group (Fig. 4). The large pigmented neuron number was decreased in Alzheimer's disease ($P < 0.001$) and in Parkinson's disease ($P < 0.05$) compared with the amyotrophic lateral sclerosis group (Fig. 4). The mean number of small unpigmented neurons, which was 13 756±1705 in the control group in the locus coeruleus, was 82% larger in the amyotrophic lateral sclerosis group ($P < 0.001$). A non-significant 44% increase was found in Parkinson's disease. In Alzheimer's disease a 33% decrease was seen (Fig. 5). The small unpigmented neuron number was decreased in Alzheimer's disease ($P < 0.001$), but was not significantly different in Parkinson's disease ($P > 0.05$) compared with the amyotrophic lateral sclerosis group (Fig. 5). The large pigmented/small unpigmented neuron number ratio was strongly reduced in Alzheimer's disease ($P < 0.01$), in Parkinson's disease ($P < 0.01$) and in amyotrophic lateral sclerosis ($P < 0.01$) compared with the control group (Fig. 6). The reduction in amyotrophic lateral sclerosis was mainly caused by the higher small unpigmented neuron number. The larger locus coeruleus volume and the higher number of small unpigmented neurons in amyotrophic lateral sclerosis, compared with the control group, could not be explained by the disease process.

Table 3 *Morphometrical characteristics*

Patient	Diagnosis	Volume (mm^3)	Length (mm)	LP neuron	No. of SU neurons	No. of Glia cells	Glia cell density (per mm^3)	No. of rest cells	Density of rest cells
1	Control	9.09	10.8	10 580	12 318	716 196	78 815	23 930	2633
2	Control	10.41	13.2	19 228	11 771	878 815	84 388	33 391	3264
3	Control	10.83	10.8	13 942	9303	663 757	61 306	42 105	3889
4	Control	8.42	12.0	15 083	18 488	594 869	70 658	41 276	4903
5	Control	13.60	16.8	23 541	16 900	1235 967	90 867	46 445	3415
Mean ± SEM		10.47 ± 0.90	12.7 ± 1.1	16 475 ± 2243	13 756 ± 1704	817 921 ± 114 529	77 207 ± 5179	37 549 ± 3949	3621 ± 378
6	AD	9.34	8.4	2317	15 895	520 782	55 782	78 838	8445
7	AD	6.89	6.0	1965	8086	538 084	78 108	18 545	2692
8	AD	8.64	9.6	3642	6337	450 717	52 191	19 615	2271
9	AD	7.58	7.2	3250	5741	582 121	76 838	4395	580
10	AD	8.05	10.8	3671	9908	1069 414	132 830	13 878	1724
Mean ± SEM		8.10 ± 0.42	8.4 ± 0.8	2969 ± 351	9193 ± 1827	632 223 ± 111 326	79 150 ± 14 424	27 054 ± 13 222	3142 ± 1372
11	PD	10.09	14.4	12 554	17 784	671 978	66 618	1437	142
12	PD	7.47	10.8	8282	21 672	554 258	74 248	15 633	2094
13	PD	11.29	12.0	15 645	26 621	538 954	47 729	33 807	2993
14	PD	8.03	10.8	8019	19 403	406 731	50 677	27 968	3485
15	PD	6.27	6.0	5699	13 575	436 755	69 647	22 461	3582
Mean ± SEM		8.63 ± 0.91	10.8 ± 1.4	10 040 ± 1785	19 811 ± 2156	521 735 ± 47 103	61 784 ± 5298	20 261 ± 5583	2459 ± 636
16	ALS	12.00	12.0	13 973	23 078	808 032	67 364	33 621	2803
17	ALS	12.63	13.2	18 981	29 357	929 757	73 621	42 779	3387
18	ALS	12.71	9.6	14 529	26 408	800 792	63 015	43 682	3437
19	ALS	12.64	12.0	12 631	23 389	774 744	61 293	36 253	2868
20	ALS	13.66	12.0	15 547	22 862	841 027	61 587	37 785	2767
Mean ± SEM		12.73 ± 0.27	11.8 ± 0.6	15 132 ± 1071	25 019 ± 1261	830 870 ± 26 887	65 376 ± 2330	38 824 ± 1924	3053 ± 148

LP = large pigmented; SU = small unpigmented; AD = Alzheimer's disease; PD = Parkinson's disease; ALS = amyotrophic lateral sclerosis.

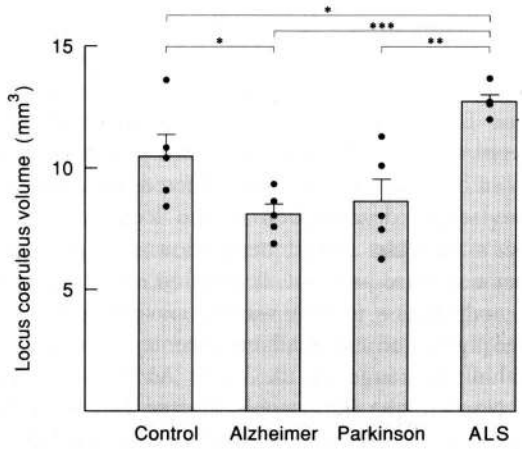

Fig. 3 Distribution of the volume of the locus coeruleus plotted separately for the control subjects, Alzheimer's disease patients, Parkinson's disease patients and patients with amyotrophic lateral sclerosis (ALS).

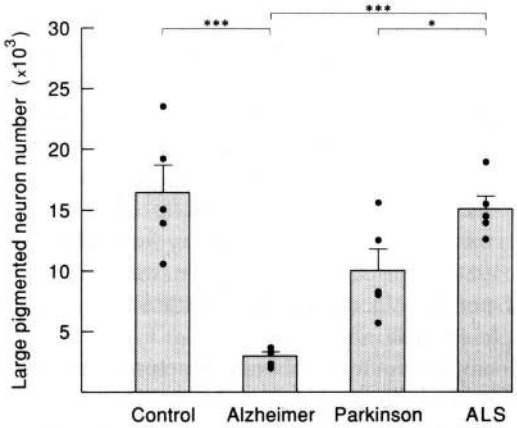

Fig. 4 Distribution of the number of large pigmented neurons in the locus coeruleus plotted separately for the control subjects, Alzheimer's disease patients, Parkinson's disease patients and patients with amyotrophic lateral sclerosis (ALS).

In the control group the mean glial cell number was 817 921±114 529. The mean 'remaining' cell number was 37 549±3949, which is 4.6% of the glial cell number. Glial and 'remaining' cell numbers in Alzheimer's disease and amyotrophic lateral sclerosis were not significantly different from those in the control group (Table 3). In Parkinson's disease a significant decrease in the number of glial cells ($P < 0.05$) and 'remaining' cells was found. As no such difference was found in the glial and 'remaining' cell density (Table 3), we assume that this decrease was due to the smaller volume in the Parkinson's disease group.

Discussion

The newly developed image analysis method to delineate the locus coeruleus yielded reproducible results and allowed the estimation of total locus coeruleus cell numbers. The major finding as reported in the literature, i.e. the decrease in the

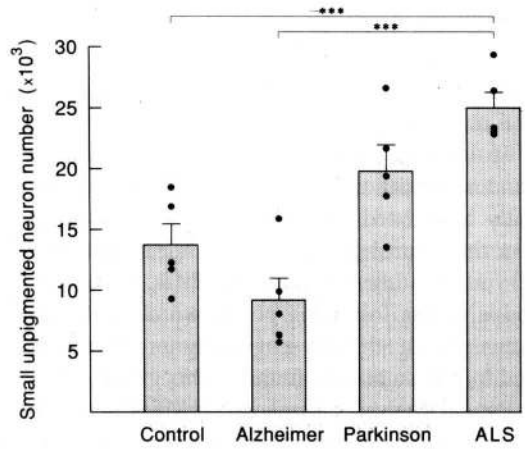

Fig. 5 Distribution of the number of small unpigmented neurons in the locus coeruleus plotted separately for the control subjects, Alzheimer patients, Parkinson patients and patients with amyotrophic lateral sclerosis (ALS).

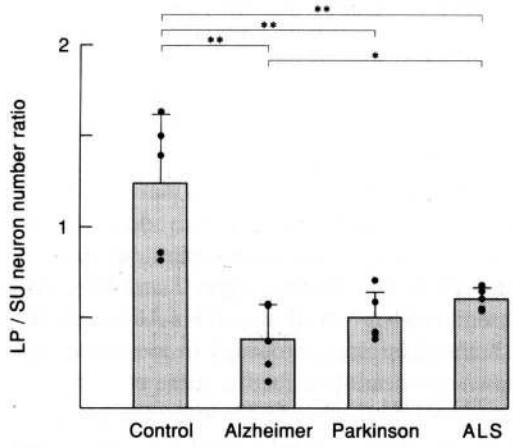

Fig. 6 Distribution of the large pigmented/small unpigmented neuron number ratio in the locus coeruleus plotted separately for the control subjects, Alzheimer's disease patients, Parkinson's disease patients and patients with amyotrophic lateral sclerosis (ALS).

number of large pigmented neurons in the locus coeruleus in Alzheimer's disease and Parkinson's disease, was confirmed by this procedure. Various papers indicate that in neurodegenerative diseases, cell death may not be the major phenomenon. There appears to be no neuronal loss in the cerebral cortex, e.g. in Alzheimer's disease (Regeur *et al.*, 1993). The possibility that the disappearance of large pigmented neurons in some neurodegenerative diseases does not result entirely from cell death, but can, at least partly, also be explained by shrinkage and depigmentation, does not hold for Alzheimer's disease since numbers of both large pigmented and small unpigmented neurons decrease. However, this possibility was supported for Parkinson's disease. The 40% decrease in the number of large pigmented neurons in Parkinson's disease was accompanied by a similar, though not significant, increase of small unpigmented

neurons. The significant decrease in large pigmented/small unpigmented neuron number ratio reinforced the possibility of a shift from large pigmented neurons to small unpigmented neurons.

Some of the subjects in the present study had co-existing clinical and neuropathological diagnoses (Table 1), which are not usually associated with the locus coeruleus. Subjects chosen for the control group were not ideal. However, ideal controls, younger than 65 years are difficult to obtain, but on the basis of the following points we are convinced that the conclusions of the present study are not essentially influenced by the pathology found in this group. One control subject (Patient 5) had a viral encephalitis and AIDS and hence the higher glial cell number found may be due to the inflammatory process. Also the slightly higher large pigmented neuron number found in this patient may be explained by the young age at death (42 years), since younger subjects are known to have higher numbers of large pigmented neurons (Vijayashankar, 1979). The small neuron number was within the range of the other control subjects. Eliminating this subject from the statistical calculations did not alter our conclusions. Three of our controls had cerebral infarctions without dementia (Patient 2 in the right frontal lobe, Patient 3 in all cortical lobes on both sides, and Patient 4 in the right occipital lobe, in the left putamen and in the left cerebellum). Mann *et al.* (1982) showed that multiple cerebral infarctions, even when giving rise to dementia, do not influence cell counts in the locus coeruleus. One control subject (Patient 4) had diabetes type II and liver cirrhosis, due to chronic non-A non-B hepatitis. Although this may influence brain physiology, a change in locus coeruleus cell number has never been described in these conditions and is, from a pathophysiological point of view, very unlikely. Also, the cell numbers of this control fell fully within the range of the others. Finally, if the pathology in the control group had had any influence on the locus coeruleus, it would have been manifest as a decrease in large pigmented neuron number. The difference between 'ideal' controls on the one hand and Alzheimer's disease and Parkinson's disease subjects on the other hand would thus have been even more pronounced. This suggests that our figures for the decrease in large pigmented neuron numbers in Alzheimer's disease and Parkinson's disease of 82 and 39%, respectively, compared with the control group, are not over-estimated. Further support for co-existing diagnoses having no influence on locus coeruleus cell counts came from the fact that the large pigmented neuron counts in the control group of our study is in agreement with the findings of almost all other studies. Finally, we added to the control group a 'disease control group', which consisted of subjects with amyotrophic lateral sclerosis, who appeared to have similar large pigmented neuron numbers to the controls. Mann *et al.* (1983) also found no change in large pigmented neuron number in the locus coeruleus in this condition. Using either the control group or the disease control group as controls, the main conclusions from the present study, concerning the large pigmented neurons, were similar. One Alzheimer's disease patient (Patient 8) showed rigidity of the limbs, without further clinical or neuropathological signs of Parkinson's disease or Diffuse Lewy Body disease as described by Hughes *et al.* (1992). One Alzheimer's disease patient (Patient 10), with an onset age of 34 years, suffered from depression. The more severe degeneration in the locus coeruleus in Alzheimer's disease with depression compared with Alzheimer's disease without depression, as described by Zubenko and Moossy (1988), was not obvious for this case, since large pigmented and small unpigmented neuron numbers were within the range of the other Alzheimer's disease patients. None of the Parkinson's disease patients suffered from depression or dementia. It has been assumed that cell loss in the locus coeruleus is due to a secondary retrograde degeneration caused by a pathological event in the cortex, i.e. in the terminal fields (Marcyniuk *et al.*, 1986*a*). The large pigmented neuron number in the Parkinson's disease cases with frontal lobe pathology (Patients 11, 13 and 15) was, however, within the range of the other Parkinson's disease cases. This observation suggests a primary degenerative process in the locus coeruleus itself, rather than a secondary reaction to frontal lobe involvement. The possibility that primary damage in the cortex may lead to secondary retrograde damage in the locus coeruleus was also refuted by the finding that in leucotomized subjects no significant changes in the locus coeruleus were found (Lohr and Jeste, 1988). This also may explain why Mann *et al.* (1982) did not find locus coeruleus pathology in subjects with multiple cortical infarctions.

Estimation of the volume of a brain structure is an essential step in determining its total cell number. Thus, in order to determine total cell numbers of the locus coeruleus, its boundaries had to be delineated. Cell density or 'nearest neighbour distances', without reference to the volume of a structure (e.g. Förstl *et al.*, 1992), may be misleading measurements for total cell number, since some cell populations tend to remain at a rather constant distance from each other, even when extreme cell loss is present (Swaab and Uylings, 1987). In order to test the hypothesis that the decrease in large pigmented neuron number in some neurodegenerative diseases is, at least partly, due to a shift from large pigmented to small unpigmented neurons, both cell types had to be counted in the same volume.

The counting of cell types in the locus coeruleus, such as small unpigmented neurons and glial cells that are also present in surrounding structures, has proved unreliable when carried out within arbitrary boundaries (Baker *et al.*, 1989). Moreover, delineation of the locus coeruleus boundaries by hand did not provide reproducible values. This is mainly due to the fact that, especially rostrally, it is difficult to define the nucleus because there are only a few cells which are scattered over a large surface (Vijayashankar and Brody, 1979). The lateral, ventral and dorsal borders also provide problems in the other parts of the locus coeruleus. The newly developed IBAS-assisted method, using the mean profile

distance of the patient as an objective measure, has made it possible to determine the boundaries of the nucleus in an automated and very reproducible way. Although these boundaries coincide with the borders seen under the microscope, the question arises, as it does with every border in the brain, as to what degree they remain artificial.

Cell types such as glial cells, which are extremely abundant, can only be measured if samples are taken. However, when samples are taken by hand, the observer may become biased, e.g. by an unconscious preference for a field that contains a large pigmented neuron profile. In that case no representative estimate would be obtained, since glial cells are often preferentially located around neurons. Another disadvantage of sampling by hand is that the edge of the locus coeruleus tends to be avoided in order to make sure measurements are only carried out inside the nucleus. This can certainly influence the outcome of the measurements, since the cells in different parts of the locus coeruleus are not evenly distributed and are not equally affected in Alzheimer's disease (Marcyniuk *et al.*, 1986*b*). The IBAS-steered scanning stage allows weighted random samples to be taken from an area in such a way that the sample size can be adjusted to the frequency in which a structure is present. Apart from the fact that an IBAS and a scanning stage are required, another disadvantage of the procedure is that it is quite laborious, since the areas of all measured fields and cell profiles are stored individually. However, it does allow the detection of selective cell loss in particular parts of the section. In the present study we did not use the option of a three-dimensional reconstruction of the locus coeruleus. Had spheres instead of circles been put around the identified large pigmented neuron profiles, the chance of a cluster of two profiles touching a third would have increased, resulting in a larger locus coeruleus volume. The distance to the next section, however, is much larger than the radius of the spheres. Thus the use of spheres instead of circles would, in our material, not have resulted in a marked enlargement of the locus coeruleus volume.

The differences in length of the locus coeruleus in our study are in agreement with other studies for the control and Alzheimer's disease group (Vijayashankar and Brody, 1979; Marcyniuk *et al.*, 1986*a*) and for the control and Parkinson's disease group (Chan-Palay and Asan, 1989*a*, *b*). The smaller volume of the locus coeruleus in the Alzheimer's disease group, compared with the control group, is partly due to the shorter length (Table 3) and partly to the smaller area per section. The finding that a brain nucleus shrinks when neurons degenerate is consistent with similar observations, e.g. in the suprachiasmatic nucleus in Alzheimer's disease (Swaab *et al.*, 1985; Swaab and Uylings, 1987).

The total number of large pigmented neurons in the locus coeruleus as determined in our study was quite similar to that reported in other studies (Vijayashankar and Brody, 1979; Bondareff *et al.*, 1982; German *et al.*, 1988, 1992). Only in Chan-Palay's study (1989*a*) and in the one subject described in Baker's study (1989) were cell counts in the different groups clearly higher than in the other studies, including ours. This difference is unlikely to be due to the finding that some of the tyrosine hydroxylase (TH)-positive large neurons do not contain neuromelanine, since this subpopulation only accounts for ~5% of the cells (Chan-Palay and Asan, 1989*a*). Further, a discrepancy between neuromelanine as a marker on the one hand and TH or dopamine-β-hydroxylase (DBH) immunoreactivity on the other, would be in disagreement with studies of Iverson (1983) and of Baker *et al.* (1989), who found equal or higher pigmented cell counts using conventionally stained sections compared with cell counts using sections stained immunocytochemically. However, in both studies (Baker *et al.*, 1989; Chan-Palay and Asan, 1989*a*) thick sections were used (50 μm and 70 μm, respectively) and no correction factor was used for truncation of individual cells, split cells or cell size, asssuming that only whole cells were counted. This may well account for the higher numbers they reported. The large pigmented neuron count in the Alzheimer's disease group in the present study was low but fell entirely within the range of the Alzheimer's disease type I group (i.e. young age at onset) as proposed by Bondareff *et al.* (1982).

The distinction between large pigmented and small unpigmented neurons was irrespective of size. A section always contains a mixture of entire cells and cut segments of cells. The main advantage of the discrete unfolding procedure we used is that segments of cells are also taken into account in order to estimate the cell number. The distinction between a large segment of a small neuron and a small segment of a large neuron was thus based on the presence or absence of neuromelanin pigment and not on profile size. The distinction between small unpigmented neurons and glial cells may be made on the basis of glial fibrillary acidic protein (GFAP) immunocytochemistry if astrocytes are GFAP-positive. However, a proportion of the astrocytes is not GFAP-positive (e.g. Velasco *et al.*, 1982) so that we opted for a conventional stain. Also, since it is not always possible to make a distinction between small unpigmented neurons and glial cells, we introduced a 'remaining group'. In the present study the intra-observer coefficient of variation in the measurement for the different cell types is excellent. Counting in the locus coeruleus area and estimates of its volume showed an especially high intra-observer reliability, mainly due to the automated way of delineating the brain nucleus.

Our definition of small unpigmented neurons is in agreement with the description of Vijayashankar and Brody (1979) and Baker *et al.* (1989), but they have never been counted in the locus coeruleus, because they are indistinguishable from the cells surrounding the locus coeruleus (Baker *et al.* 1989). With the automated outlining and sampling procedure, as described above, it became possible to count this cell type within the locus coeruleus boundaries as well. A decrease was seen in Alzheimer's disease, which was partly accounted for by the decreased locus coeruleus volume (Fig. 3). However, despite the volume

of the locus coeruleus also being smaller in Parkinson's disease, an increase in small unpigmented neuron number was found, suggesting a shift from large pigmented neurons to small unpigmented neurons. Furthermore, the significant decrease of the large pigmented/small unpigmented neuron number ratio in the disease groups shows that a decrease in large pigmented neuron number is accompanied by a less severe decrease in small unpigmented neuron number as in Alzheimer's disease or even an increase as in Parkinson's disease.

This possibility of a shift from large pigmented neurons to small unpigmented neurons due to shrinkage and depigmentation may have consequences for therapeutic strategies (Swaab, 1991), since atrophied cells can (theoretically) be activated. In order to measure this shift, not only large pigmented but also the small unpigmented neuron numbers had to be determined. This is only possible if different cell types can be measured in the same area and within reliable locus coeruleus boundaries, i.e. with an automated procedure as described in the present paper.

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References

Aston-Jones G, Chiang C, Alexinsky T. Discharge of noradrenergic locus coeruleus neurons in behaving rats and monkeys suggests a role in vigilance. [Review]. Prog Brain Res 1991; 88: 501–20.

Baker KG, Törk I, Hornung JP, Halasz P. The human locus coeruleus complex: an immunohistochemical and three dimensional reconstruction study [published erratum appears in Exp Brain Res 1990; 79: 664]. Exp Brain Res 1989; 77: 257–70.

Bondareff W, Mountjoy CQ. Number of neurons in nucleus locus ceruleus in demented and non-demented patients: rapid estimation and correlated parameters. Neurobiol Aging 1986; 7: 297–300.

Bondareff W, Mountjoy CQ, Roth M. Loss of neurons of origin of the adrenergic projection to cerebral cortex (nucleus locus coeruleus) in senile dementia. Neurology 1982; 32: 164–8.

Chan-Palay V, Asan E. Quantitation of catecholamine neurons in the locus coeruleus in human brains of normal young and older adults and in depression. J Comp Neurol 1989a; 287: 357–72.

Chan-Palay V, Asan E. Alterations in catecholamine neurons of the locus coeruleus in senile dementia of the Alzheimer type and in Parkinson's disease with and without dementia and depression. J Comp Neurol 1989b; 287: 373–92.

Chui HC, Mortimer JA, Slager U, Zarow C, Bondareff W, Webster DD. Pathologic correlates of dementia in Parkinson's disease. Arch Neurol 1986; 43: 991–5.

Cruz-Orive LM. Particle shape–size distribution: the general spheroid problem. II. Stochastic model and practical guide. J Microsc 1978; 112: 153–67.

Förstl H, Burns A, Luthert P, Cairns N, Lantos P, Levy R. Clinical and neuropathological correlates of depression in Alzheimer's disease. Psychol Med 1992; 22: 877–84.

German DC, Walker BS, Manaye K, Smith WK, Woodward DJ, North AJ. The human locus coeruleus: computer reconstruction of cellular distribution. J Neurosci 1988; 8: 1776–88.

German DC, Manaye KF, White CL 3d, Woodward DJ, McIntire DD, Smith WK, et al. Disease-specific patterns of locus coeruleus cell loss. Ann Neurol 1992; 32: 667–76.

Gundersen HJG, Bendtsen TF, Korbo L, Marcussen N, Möller A, Nielsen K, et al. Some new, simple and efficient stereological methods and their use in pathological research and diagnosis. [Review]. APMIS 1988; 96: 379–94.

Harik SI, McGunigal T Jr. The protective influence of the locus ceruleus on the blood-brain barrier. Ann Neurol 1984; 15: 568–74.

Hughes AJ, Daniel SE, Kilford L, Lees AJ. Accuracy of clinical diagnosis of idiopathic Parkinson's disease: a clinico-pathological study of 100 cases [see comments]. J Neurol Neurosurg Psychiatry 1992; 55: 181–4. Comment in: J Neurol Neurosurg Psychiatry 1993; 56: 938–9.

Iversen LL, Rossor MN, Reynolds GP, Hills R, Roth M, Mountjoy CQ, et al. Loss of pigmented dopamine-beta-hydroxylase positive cells from locus coeruleus in senile dementia of Alzheimer's type. Neurosci Lett 1983; 39: 95–100.

Katayama Y, Ueno Y, Tsukiyama T, Tsubokawa T. Long lasting suppression of firing of cortical neurons and decrease in cortical blood flow following train pulse stimulation of the locus coeruleus in the cat. Brain Res 1981; 216: 173–9.

Lohr JB, Jeste DV. Locus coeruleus morphometry in aging and schizophrenia. Acta Psychiat Scand 1988; 77: 689–97.

Mann DMA. The locus coeruleus and its possible role in ageing and degenerative disease of the human central nervous system. [Review]. Mech Ageing Dev 1983; 23: 73–94.

Mann DMA, Yates PO. Pathological basis for neurotransmitter changes in Parkinson's disease. Neuropathol Appl Neurobiol 1983; 9: 3–19.

Mann DMA, Yates PO, Hawks J. The noradrenergic system in Alzheimer and multi-infarct dementias. J Neurol Neurosurg Psychiat 1982; 45(2): 113–19.

Mann DMA, Yates PO, Hawkes J. The pathology of the human locus ceruleus. Clin Neuropathol 1983; 2: 1–7.

Marcyniuk B, Mann DMA, Yates PO. Loss of nerve cells from locus coeruleus in Alzheimer's disease is topographically arranged. Neurosci Lett 1986a; 64: 247–52.

Marcyniuk B, Mann DMA, Yates PO. The topography of cell loss from locus coeruleus in Alzheimer's disease. J Neurol Sci 1986b; 76: 335–45.

Mason ST. Noradrenaline in the brain: progress in theories of behavioural function. [Rreview]. Prog Neurobiol 1981; 16: 263–303.

Regeur L, Badsberg-Jensen G, Pakkenberg H, Evans SM, Pakkenberg B. No global neocortical nerve cell loss in brains from patients with senile dementia of Alzheimer's type. Neurobiol Aging. In press.

Rinne JO, Paljärvi L, Rinne UK. Neuronal size and density in the nucleus basalis of Meynert in Alzheimer's disease. J Neurol Sci 1987; 79: 67–76.

Swaab DF. Brain aging and Alzheimer's disease, 'wear and tear' versus 'use it or lose it' [see comments]. [Review]. Neurobiol Aging 1991; 12: 317–24. Comment in: Neurobiol Aging 1991; 12: 325–40, 349–52.

Swaab DF, Uylings HBM. Comments on review by Coleman and Flood, 'Neuron numbers and dendritic extent in normal aging and Alzheimer's disease.' Density measures: parameters to avoid. Neurobiol Aging 1987; 8: 574–6.

Swaab DF, Fliers E, Partiman TS. The suprachiasmatic nucleus of the human brain in relation to sex, age and senile dementia. Brain Res 1985; 342: 37–44.

Tomlinson BE, Irving D, Blessed C. Cell loss in the locus coeruleus in senile dementia of Alzheimer type. J Neurol Sci 1981; 49: 419–28.

Tomonaga M. Neuropathology of the locus ceruleus: a semi-quantitative study. J Neurol 1983; 230: 231–40.

Uylings HBM, van Eden GG, Hofman MA. Morphometry of size/volume variables and comparison of their bivariate relations in the nervous system under different conditions. J Neurosci Meth 1986; 18: 19–37.

Van Eden CG, Uylings HBM, Van Pelt J. Sex-difference and left-right asymmetries in the prefrontal cortex during postnatal development in the rat. Brain Res 1984; 314: 146–53.

Velasco ME, Roessmann U, Gambetti P. The presence of glial fibrillary acidic protein in the human pituitary gland. Journal of Neuropathol Exp Neurol 1982; 41: 150–63.

Vijayashankar N, Brody H. A quantitative study of the pigmented neurons in the nuclei locus coeruleus and subcoeruleus in man as related to aging. J Neuropathol Exp Neurol 1979; 38: 490–7.

Weibel ER. Stereological methods. Vol. 1: practical methods for biological morphometry. London: Academic Press 1979: 186–96.

Zubenko GS, Moossy J. Major depression in primary dementia: clinical and neuropathologic correlates. Arch Neurol 1988; 45: 1182–6.

Zweig RM, Ross CA, Hedreen JC, Steele C, Cardillo JE, Whitehouse PJ, et al. The neuropathology of aminergic nuclei in Alzheimer's disease. Ann Neurol 1988; 24: 33–42.

Zweig RM, Cardillo JE, Cohen M, Giere S, Hedreen JC. The locus ceruleus and dementia in Parkinson's disease. Neurology 1993; 43: 986–91.

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