The substantia nigra of the human brain
I. Nigrosomes and the nigral matrix, a compartmental organization based on calbindin D$_{28K}$ immunohistochemistry

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Summary
Parkinson’s disease is characterized by massive degeneration of dopamine-containing neurons in the midbrain. However, the vulnerability of these neurons is heterogeneous both across different midbrain dopamine-containing cell groups and within the substantia nigra, the brain structure most affected in this disease. To determine the exact pattern of cell loss and to map the cellular distribution of candidate pathogenic molecules, it is necessary to have landmarks independent of the degenerative process by which to subdivide the substantia nigra. We have developed a protocol for this purpose based on immunostaining for calbindin D$_{28K}$, a protein present in striatonigral afferent fibres. We used it to examine post-mortem brain samples from seven subjects who had had no history of neurological or psychiatric disease. We found intense immunostaining for calbindin D$_{28K}$ associated with the neuropil of the ventral midbrain. Within the calbindin-positive region, there were conspicuous calbindin-poor zones. Analysed in serial sections, many of the calbindin-poor zones seen in individual sections were continuous with one another, forming elements of larger, branched three-dimensional structures. Sixty per cent of all dopamine-containing neurons in the substantia nigra pars compacta were located within the calbindin-rich zone, which we named the nigral matrix, and 40% were packed together within the calbindin-poor zones, which we named nigrosomes. We identified five different nigrosomes. This organization was consistent from one control brain to another. We propose that subdivision of the human substantia nigra based on patterns of calbindin immunostaining provides a key tool for analysing the organization of the substantia nigra and offers a new approach to analysing molecular expression patterns in the substantia nigra and the specific patterns of nigral cell degeneration in Parkinson’s disease.

Keywords: Parkinson’s disease; substantia nigra; dopamine; calbindin; basal ganglia

Abbreviation: TH = tyrosine hydroxylase

Introduction
Dopamine-containing neurons of the substantia nigra are severely affected by the degenerative process that occurs in Parkinson’s disease. Evidence suggests that the loss of these neurons is heterogeneous, so that the ventrolateral part of the substantia nigra is almost completely destroyed, whereas its dorsal part is only partly damaged (Hassler, 1938). Such lesion selectivity may be critical for understanding the mechanisms of neuronal death in this disease, but all previous anatomical subdivisions of the nigral complex in the human brain have been based on the use of the dopamine-containing cells themselves as landmarks, and there has been no consensus in the literature on the major nigral cell groups because of difficulties in delineating subgroups of these neurons. Moreover, any such subdivisions based on the disposition of dopamine-containing neurons are of questionable utility in analysing brains from patients with Parkinson’s disease, in which the loss of dopamine-containing neurons progressively effaces these anatomical reference marks.

In this study, we developed a method based on immunohistochemistry for calbindin D$_{28K}$, a protein that is present in striatogniral afferent fibres and that stains the neuropil of the substantia nigra, thus providing landmarks independent of dopamine-containing neurons by which to subdivide this region in a consistent and reproducible way. With this method, we were able to determine the numbers of dopamine-containing neurons in reliably identifiable subgroups of the substantia nigra of the human brain.
Material and methods

Brain samples

Specimens were collected post-mortem from the brains of seven subjects without history of neurological or psychiatric disease [mean age ± standard error of the mean (SEM) = 84 ± 3 years]. Pathological examination of one hemisphere of each brain ruled out generalized degenerative or vascular disease. Focal abnormalities would not have been detected at this stage.

In order to ensure that the entire rostrocaudal extent of the nigral complex could be sampled, brainstems were removed from the cerebrum at the level of the mamillary bodies, and the cerebellum was detached. The brainstems were transected in the sagittal plane to provide one-half of the substantia nigra and the ventral tegmental area for analysis, and they were then blocked transversely at the level of the upper pons. After dissection, the midbrain blocks were fixed for 72 h in 4% paraformaldehyde containing 15% saturated picric acid solution, and they were then washed in 0.1 M dibasic potassium phosphate buffer (pH = 7.4), cryoprotected at 4°C in phosphate buffer containing, consecutively, 0, 5, 10, 15 and 20% sucrose (24 h each), frozen in powdered dry ice, and stored at –80°C until further processing. Serial

40-µm-thick sections were cut from the tissue blocks on a sliding microtome. The sections were immersed free-floating in 0.25 M Tris buffer containing 0.1% sodium azide and were then stored at 4°C. One midbrain was cut parasagittally and another horizontally, in a plane perpendicular to the coronal axis of the brainstem. The five other midbrains were cut transversely (Fig. 1).

Calbindin D_{28K} and tyrosine hydroxylase immunohistochemistry

Five midbrains (three transversely cut, one sagittally cut and one horizontally cut) were studied extensively. Every third section was stained for calbindin D_{28K} immunoreactivity, and every ninth section was stained for tyrosine hydroxylase (TH) immunoreactivity. Each TH-stained section was immediately adjacent to a calbindin-stained section. For the two other midbrains, fewer sections were prepared for analysis; every ninth section was immunostained for calbindin D_{28K} and every 36th for TH.

Immunohistochemistry was performed according to the double bridge PAP (peroxidase–antiperoxidase) method described previously (Graybiel et al., 1987). Briefly, primary incubations were preceded by successive 8-min exposure to 20% methanol with 3% hydrogen peroxide, 5-min exposure to 0.2% Triton X-100, and 30 min exposure to a 1:30 dilution of normal goat serum in 0.25 M Tris–HCl, pH 7.4, containing 0.9% NaCl (Tris-buffered saline; TBS). Primary incubations were carried out at +4°C for
Mapping and quantitative analysis of dopamine-containing neurons

Dopamine-containing neurons were identified by their TH content. Many of them contained a visible nucleus, but a few large neuronal profiles (>10 µm in diameter) in which the dense immunostaining prevented the nucleus from being visible were also counted. The locations of the neurons were plotted with the aid of an image analysis system (HistoRag, Biocom, Les Ulis, France) that allowed us to generate and to print precise maps of the distribution of all TH-positive neurons. Quantitative analysis of dopamine-containing neurons was performed on the five transversely cut brains. First, the midbrain was divided into the six dopaminergic regions previously identified by Hirsch et al. (1988) and shown in Fig. 2. Next, the substantia nigra was subdivided by using patterns of calbindin staining as a template. Sections adjacent to the TH-immunostained sections, stained for calbindin immunoreactivity, were used to delineate the calbindin-poor and calbindin-rich zones observed (see Results), and the outlines of these zones were projected onto the maps of TH-positive neurons. Adjacent sections were aligned by

2 days with mouse anti-calbindin D_{28k} antiserum (1 : 500; Sigma, St Louis, Mo., USA) or rabbit anti-TH (1 : 500; Eugene Tech, Allendale, NJ, USA). Successive secondary (goat anti-mouse, 1 : 800 for calbindin, and goat anti-rabbit, 1 : 400 for TH) and tertiary (mouse PAP, 1 : 200 for calbindin, and rabbit PAP, 1 : 100 for TH) incubations followed (30 min, each at room temperature). All incubation solutions contained 1% normal goat serum and 1% normal human serum in TBS. Primary incubation solutions also contained 0.01% thimerosal. Steps were separated by buffer washes. Sections were developed in 0.05% diaminobenzidine.
Fig. 5 Reproducibility of calbindin immunostaining at the same rostral (R), intermediate (I) and caudal (C) levels in two control midbrains (A and B). Symbols indicate different calbindin-poor zones (★, nigrosome 1; ●, nigrosome 2; ▲, nigrosome 3; ○, nigrosome 4. RN = red nucleus; CP = cerebral peduncle; III = exiting third cranial nerve fibres. Scale bar = 3 mm.

Results
Calbindin staining patterns in the ventral midbrain

Calbindin staining was intense across all of the ventral midbrain and appeared to be associated with the neuropil rather than with the cell bodies of the substantia nigra. Calbindin-positive neuropil was present throughout the substantia nigra pars reticulata and most of the substantia nigra pars compacta, the main region containing TH-positive neurons. Some TH-positive neurons appeared above the

\[ N_r = N_s \times (S_t/S_s) \]

where \( N_r \) = total number of neurons, \( N_s \) = number of neurons counted, \( S_t \) = total number of sections through the region and \( S_s \) = number of sections in which neurons were counted (Konigsmark, 1970). To evaluate rostrocaudal variations, midbrains were analysed in three domains: anterior to the level of exiting third cranial nerve fibres; at this level; and posterior to it (Fig. 1).
Fig. 6A

...calbindin-rich region, and for ease of description we termed this zone the 'substantia nigra pars dorsalis' (Fig. 3).

Within this large calbindin-positive zone, there were smaller but conspicuous calbindin-poor zones. These had variable shapes, some being long and thin, others being rounded or branched. Analysed in serial sections, many of...
Fig. 6B
Fig. 6 (A–C) Charts of dopamine-containing neurons (right) and photographs of calbindin D28K immunostaining in adjacent transverse sections (left). Values indicate distance (in mm) to the level of rostral exiting third cranial nerve fibres; positive and negative distances denote rostral and caudal distances, respectively, to this level. Symbols indicate the five different invaginated pockets of low calbindin staining (nigrosomes) identified within the calbindin-positive neuropil (★, nigrosome 1; ●, nigrosome 2; ▲, nigrosome 3; ○, nigrosome 4; ■, nigrosome 5). RN = red nucleus; CP = cerebral peduncle; DBC = decussation of the brachium conjunctivum; III = exiting fibres of third cranial nerve. Scale bar = 3 mm.
the calbindin-poor zones seen in individual sections were continuous with one another, forming elements of branched, three-dimensional subsystems within the region as a whole.

Zones of low calbindin immunoreactivity were evident in both sagittal and horizontal sections (Fig. 4). We chose for the quantitative analysis a plane that intersected perpendicularly most of these zones and that could also be defined by accessible anatomical landmarks: a transverse plane passing (i) between the superior and the inferior colliculus and (ii) through the border between the pons and the midbrain (Fig. 1). Figure 5 illustrates the patterns of calbindin immunoreactivity in sections cut in this plane for two brains at three rostrocaudal levels.

**Three-dimensional organization of nigral compartments delineated by calbindin immunostaining**

To determine the three-dimensional organization of the calbindin-poor zones distributed within the calbindin-positive neuropil of the nigral complex, we analysed the staining patterns in near-serial sections (Figs 6 and 7). We were able to identify five different calbindin-poor zones, which we named ‘nigrosomes’ and numbered from 1 to 5. All five nigrosomes appeared in individual sections as invaginated pockets of low calbindin staining embedded in a calbindin-rich surround (Figs 6 and 7). Nigrosome 1 (★) was the largest of the five. Its shape was that of a lens with its concavity dorsal and its main axis parallel to the rostrocaudal axis of the substantia nigra. It was present from the caudal part of the calbindin-positive region to the level of the rostral exit of third cranial nerve fibres. Nigrosome 1 was situated in the ventral third and the lateral two-thirds of the calbindin-rich neuropil, at all but the caudal level, where it had a more dorsal and lateral position. Nigrosome 2 (●) looked like a cylinder with its main axis parallel to that of nigrosome 1. It was centred in the medial third of the calbindin-rich zone of neuropil, and its rostral border was slightly more caudal than that of nigrosome 1. Nigrosome 3 (▲) corresponded to a depression in the lateral and caudal part of the calbindin-positive neuropil. Its rostrocaudal extent was...
Human substantia nigra

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Fig. 8 Summary of midbrain subdivisions illustrated at three representative transverse levels. CGS = central grey substance; M = medial group; Mv = medioventral group; A8 = dopaminergic group A8; SNpd = substantia nigra pars dorsalis; SNpl = substantia nigra pars lateralis; N = nigrosome. RN = red nucleus; DBC = decussation of the brachium conjunctivum; CP = cerebral peduncle; III = exiting third cranial nerve fibres.

almost the same as that of nigrosome 2, despite some variations from one brain to another that we attribute to differences in plane of sectioning. Nigrosome 4 (●) was well developed in the dorsal and mid-lateral part of the calbindin-rich neuropil. Its shape was that of a dorsoventrally flattened cylinder with a main axis parallel to that of nigrosome 1. Nigrosome 4 was located in the dorsal third of the calbindin-rich neuropil, and was visible from the level of the middle extent of the exit zone of third cranial nerve fibres to levels rostral to the exiting of these fibres. Nigrosome 5 (●) corresponded to a group of calbindin-poor pockets located in the rostral part of the midbrain (anterior to the exiting third cranial nerve fibres) and situated ventral and medial to nigrosome 4. Schematic diagrams of these five nigral subdivisions (nigrosomes 1–5) are shown for three rostrocaudal levels in Fig. 8.

Reproducibility of the calbindin immunostaining patterns
Analysis of sets of serial sections showed that the patterns of calbindin immunostaining were reproducible from one subject to another. Even though variations in the plane of sectioning, and probable inter-individual differences, sometimes prevented strictly identical patterns in equivalent sections from different subjects being obtained, the general three-dimensional architecture of the nigrosomes was reproduced in remarkable detail from brain to brain.

Quantitative analysis of dopamine-containing neurons in relation to calbindin-defined compartments
Nearly 200 000 dopamine-containing neurons per half-midbrain were counted for five brains (exact mean ± SEM = 199 251 ± 18 446). Two-thirds of these neurons were located in the substantia nigra, defined as being ventral to the medial lemniscus and lateral to the medial edge of the red nucleus, or, in the caudal midbrain, the decussation of the brachium conjunctivum (Fig. 2). Eighty per cent of these nigral dopamine-containing neurons were within the calbindin-rich region of the ventral midbrain that we defined as the substantia nigra pars compacta, and 20% were dorsal to this zone, in the region that we termed the substantia nigra pars dorsalis (Figs 8 and 9).

Among dopamine-containing neurons located in the substantia nigra pars compacta, 60% were sparsely distributed within the large region of intense calbindin staining, which we named the nigral 'matrix'. The other 40% of the dopamine-containing neurons were included within the different nigrosomes, with a majority (60%) in nigrosome 1. The intranigrosomal neurons tended to be densely packed together (Figs 6 and 7). Table 1 shows the mean numbers of dopamine-containing neurons estimated for the different subdivisions of the substantia nigra. Table 2 and Fig. 10 indicate how the distribution of dopamine-containing neurons varied along the rostrocaudal axis of the substantia nigra.
Fig. 9 Distribution of TH-positive neurons in the substantia nigra, including the calbindin-rich region (substantia nigra pars compacta) and the zone above it (substantia nigra pars dorsalis and pars lateralis) containing TH-positive neurons (●, compared with the distribution of TH-positive neurons included within the calbindin-rich zone neuropil (▼). Symbols show the mean numbers of neurons and bars indicate the SEM calculated from the three midbrains studied in near-serial sections. Values on the abscissa indicate distance (in mm) to the most rostral level of the exiting third cranial nerve fibres. Values on the ordinate indicate the number of TH-positive neurons. The areas under the curves correspond to the total number of neurons in the regions indicated; the area of the indicator box corresponds to 5500 TH-positive neurons.

Discussion
Understanding the anatomy of the substantia nigra is crucial to analysing its pathology. In this study, we developed a compartmental analysis of the human substantia nigra based on calbindin D$_{28K}$ immunohistochemistry. The compartmental organization that we describe provides, for the first time, a reliable and easily reproducible tool with which to divide the human substantia nigra into major subdivisions. In the second paper of this series, we demonstrate that this classificatory scheme, independent of the distribution of dopamine-containing neurons, makes it possible to study with great accuracy the pathology of the substantia nigra in Parkinson’s disease.

The substantia nigra complex defined by calbindin immunohistochemistry
The A8, A9 and A10 dopaminergic cell groups of the human midbrain are directly continuous with one another. Thus, other than on a rough topographic basis, the outlines of the substantia nigra pars compacta are difficult to assess, as reflected by the lack of a consensual definition of the nigral complex despite many attempts to develop one. For some authors, the medial lemniscus constitutes the dorsal border of the substantia nigra (Hirsch et al., 1988; Fearnley and Lees, 1991). Others have suggested defining the substantia nigra by its dense substance P immunoreactivity (Gibb, 1992; McRitchie et al., 1995). The ventral midbrain region containing calbindin D$_{28K}$ immunoreactivity probably provides an equivalent definition, given the regional similarity in distribution of immunostaining for substance P and calbindin (Gibb, 1992). In the present study, we combined these criteria. We defined the substantia nigra as the midbrain region ventral to the medial lemniscus and the substantia nigra pars compacta as the region containing calbindinpositive neuropil. As no data are available on the projection sites of dopamine-containing neurons located dorsal to the calbindin-immunoreactive region but ventral to the medial lemniscus, it is difficult to classify these neurons as lying either in the substantia nigra pars compacta or in the dopaminergic group A8. Thus, we considered these neurons as a separate group, and termed the region the ‘substantia nigra pars dorsalis’.

Table 3 summarizes the mean numbers of dopamine-containing neurons estimated to occur outside the substantia nigra, in regions of the midbrain containing TH-positive neurons, together with their rostrocaudal variations (Fig. 11).

The estimated numbers of neurons in each midbrain group identified were similar (not significantly different) for the three midbrains studied by near-serial section analysis and the two midbrains studied on the basis of less closely spaced sections.

Neurochemical compartments in the human substantia nigra pars compacta
The main finding of this study is the compartmentation of calbindin-positive neuropil in the substantia nigra pars compacta. We found that six subgroups of dopamine-containing neurons could be delineated in the substantia nigra pars compacta according to their locations, within either the calbindin-rich zone, named the ‘matrix’, or different calbindin-poor pockets, named ‘nigrosomes’, that are embedded in the matrix. The organization of the calbindin-poor zones into five main nigrosomal pockets was reliably and constantly found in the seven brains analysed in this study. In each brain, the nigrosomes appeared to be largely
Table 1 Quantitative analysis of dopamine-containing neurons in the substantia nigra

<table>
<thead>
<tr>
<th>Substantia nigra</th>
<th>SNpl</th>
<th>SNpd</th>
<th>Matrix</th>
<th>Nigrosomes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2129 ± 239</td>
<td>30 164 ± 6744</td>
<td>58 982 ± 7894</td>
<td>43 725 ± 4046</td>
</tr>
<tr>
<td></td>
<td>Total SNpc = 102 707 ± 10 622</td>
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Nigrosomes

<table>
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<th>N3</th>
<th>N4</th>
<th>N5</th>
</tr>
</thead>
<tbody>
<tr>
<td>27 504 ± 2451</td>
<td>7891 ± 1122</td>
<td>1507 ± 430</td>
<td>4719 ± 742</td>
<td>2104 ± 804</td>
</tr>
</tbody>
</table>

Values are mean ± standard error of the mean. SNpl = substantia nigra pars lateralis; SNpd = substantia nigra pars dorsalis; SNpc = substantia nigra pars compacta; N = nigrosome.

Table 2 Rostrocaudal variation in the number of dopamine-containing neurons in the substantia nigra

<table>
<thead>
<tr>
<th></th>
<th>SNpl</th>
<th>SNpd</th>
<th>Matrix</th>
<th>Nigrosomes</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rostral</td>
<td>68 ± 31</td>
<td>3673 ± 461</td>
<td>11 415 ± 2369</td>
<td>2020 ± 587</td>
<td>13</td>
</tr>
<tr>
<td>Intermediate</td>
<td>1387 ± 223</td>
<td>12 109 ± 3203</td>
<td>27 155 ± 4774</td>
<td>18 864 ± 1812</td>
<td>44</td>
</tr>
<tr>
<td>Caudal</td>
<td>674 ± 182</td>
<td>14 384 ± 5007</td>
<td>20 412 ± 2997</td>
<td>22 841 ± 3624</td>
<td>43</td>
</tr>
<tr>
<td>%</td>
<td>2</td>
<td>22</td>
<td>44</td>
<td>32</td>
<td>100</td>
</tr>
</tbody>
</table>

Values are mean ± standard error of the mean. SNpl = substantia nigra pars lateralis; SNpd = substantia nigra pars dorsalis.

separate from one another, but we could not firmly discount the presence of small connecting zones between one nigrosome and another. The remarkable consistency of these features allowed us to number them (nigrosomes 1–5) and to analyse the distribution of TH-positive neurons from pocket to pocket and within the surrounding matrix.

Numerous investigators have already attempted to subdivided the nigral complex anatomically. Hassler (Hassler, 1937) provided probably the most comprehensive study of the internal substructure of the substantia nigra. However, the 21 subdivisions that he described are difficult to identify consistently in every individual. The lack of consensus has been compounded by the fact that different planes of section have been used for analysis, from coronal, as in the original work by Hassler (Hassler, 1937, 1938), to transverse (Fearnley and Lees, 1991; Gibb and Lees, 1991; McRitchie et al., 1995). The most serious difficulty has been the absence of landmarks independent of the dopamine-containing neurons themselves. All previous subdivisions of nigral neurons have been based on a tendency for neurons in the substantia nigra pars compacta to have a clustered organization. However, because of the presence of numerous sparsely distributed neurons in the substantia nigra pars compacta in addition to the clusters, the borders of the subdivisions have not been easily defined (Fig. 3). The calbindin-based method that we describe here avoids this problem.

Nigrosomes 1–5 in relation to previous delineations of nigral subgroups

The calbindin-based delineation of the substantia nigra pars compacta allowed us to recognize several of the main subgroups already described in this region (Table 4). The ventrolateral group consistently identified in different studies (Hassler, 1937; Braak and Braak, 1986; Fearnley and Lees, 1991; van Domburg and ten Donkelaar, 1991; McRitchie et al., 1995) corresponds to the group of dopamine-containing neurons included in nigrosome 1.

The key attributes of the calbindin-based classification we introduce here are that (i) this analysis provides a method allowing definition in a reproducible way of subdivisions in the substantia nigra pars compacta, (ii) the calbindin-based landmarks can be readily recognized in different planes of section, and (iii) these landmarks are not dependent on the distribution of the dopamine-containing neurons themselves. This last point provides a new basis for analysing the midbrain in brains derived from patients who suffered from Parkinson’s disease (Damier et al., 1999).
Fig. 10 Distributions of TH-positive neurons in the different groups of the substantia nigra defined on the basis of calbindin D_{28K} immunostaining. Dots indicate the mean numbers of neurons and bars indicate the SEM calculated for the three midbrains studied in near-serial sections. Values on the abscissa indicate distance (in mm) from the rostral exit-point of the third cranial nerve fibres. Values on the ordinate indicate the number of TH-positive neurons. Areas under the curves correspond to the total number of neurons in the regions indicated; the area of the indicator box corresponds to 5500 TH-positive neurons. SNpd = substantia nigra pars dorsalis; SNpl = substantia nigra pars lateralis; N = nigrosome.

Quantitative assessment of dopamine-containing neurons in nigrosomes 1–5 and in the nigral matrix

Previous studies have provided counts of the total numbers of dopamine-containing neurons in the substantia nigra pars compacta in normal and parkinsonian brains, but to our knowledge no quantitative information has been available on dopamine-containing neurons distributed within different subgroups within the substantia nigra pars compacta. The calbindin-based method permitted us to carry out such a quantitative analysis and to estimate the variation in cell populations within each subgroup (nigrosomes 1–5 and the matrix). The results reinforced the impression of relative consistency in these subgroups from brain to brain. The total number of dopamine-containing neurons estimated for normal human midbrain by this method is in good accordance with values published previously (McGeer et al., 1977; Hirsch et al., 1988; German et al., 1989; Pakkenberg et al., 1991).

There are more dopamine-containing neurons in the matrix than in the nigrosomes, even though the nigrosomes include prominent clusters of cells.

Source of the calbindin-positive fibre template in the nigral complex

The calbindin-positive neuropil analysed here was formed by fibres running through the full rostrocaudal extent of the midbrain in an orientation roughly parallel to that of fibres in the cerebral peduncle. This pattern is similar to that of the striatonigral pathway in the monkey (Hedreen and Delong, 1991). Calbindin D_{28K} immunostaining has been observed in medium spiny projection neurons in the human striatum (Kiyama et al., 1990), and calbindin immunostaining is greatly decreased in post-mortem samples of the ventral midbrain from patients who suffered from Huntington’s disease (Kiyama et al., 1990) and striatonigral degeneration.
Fig. 11 Distributions of TH-positive neurons in the different groups of the midbrain. Filled circles indicate the mean numbers of dopamine-containing neurons and bars indicate the SEM calculated from the three midbrains studied extensively in near-serial sections. Values on the abscissa indicate distance (in mm) from rostral exit of third cranial nerve fibres. Values on the ordinate indicate the number of TH-positive neurons. Areas under the curves correspond to the total numbers of neurons in the regions indicated; the area of the indicator box corresponds to 5500 TH-positive neurons. CGS = central grey substance; M = medial group; Mv = medioventral group; A8 = dopaminergic group A8; SN = substantia nigra.

Table 3 Quantitative analysis of dopamine-containing neurons in the different midbrain groups

<table>
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<tr>
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<th>CGS</th>
<th>A8</th>
<th>M</th>
<th>Mv</th>
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<tr>
<td>Total</td>
<td>5850 ± 865</td>
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<td>9787 ± 1297</td>
<td>27 964 ± 1559</td>
<td>135 000 ± 16082</td>
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<td>Rostral</td>
<td>1052 ± 341</td>
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<td>944 ± 320</td>
<td>4113 ± 666</td>
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<td>Intermediate</td>
<td>2276 ± 504</td>
<td>4158 ± 1203</td>
<td>5301 ± 1314</td>
<td>13 669 ± 1699</td>
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<td>Caudal</td>
<td>2522 ± 470</td>
<td>16 206 ± 2164</td>
<td>3542 ± 1152</td>
<td>10 180 ± 1996</td>
<td>58 309 ± 10381</td>
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</table>

Values are mean ± standard error of the mean. CGS = central grey substance; A8 = dopaminergic group A8; M = medial group; Mv = medioventral group; SN = substantia nigra.

(Ito et al., 1992), both diseases leading to massive degeneration of these striatal projection neurons. These findings strongly suggest that the calbindin-positive neuropil in the nigral complex is mainly formed by GABAergic striatonigral fibres and terminals.

Interestingly, the intermixing between dopamine-containing neurons of the substantia nigra pars compacta and the calbindin-positive neuropil varies markedly in different species (Fig. 12). In the rat, almost all dopamine-containing neurons are located dorsal to the calbindin-positive neuropil. In the monkey, some densely packed dopamine-containing neurons invaginate the calbindin-rich region, mainly within calbindin-poor, finger-like zones. In the human, the penetration of dopamine-containing neuron clusters into the
Fig. 12 Transverse sections immunostained for calbindin D<sub>28K</sub> (A, C) and corresponding charts of TH-positive neurons (B, D) from rat (A, B) and squirrel monkey (C, D) midbrain. RN = red nucleus. Scale bar = 0.7 mm in A and B, and 1 mm in C and D.

Table 4 Correspondence between nigral subdivisions defined by calbindin patterns and other classifications

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<td>Spv (m, i, l)</td>
<td>Spcd</td>
<td>Spd (v, i, d)</td>
<td>Sal</td>
<td>Sai (m, i, l, z)</td>
<td>Spzv</td>
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<td>Fearnley and Lees, 1991</td>
<td>vl (m, i, l)</td>
<td>vm (m, i, l)</td>
<td>Pars lateralis</td>
<td>dm (?)</td>
<td>dl (?)</td>
<td>dl (?)</td>
<td>dl</td>
<td>Pars lateralis</td>
<td>am</td>
</tr>
<tr>
<td>van Domburg and ten Donkelaar, 1991</td>
<td>pm</td>
<td>pm</td>
<td>Pars lateralis</td>
<td>ps</td>
<td>al</td>
<td>al</td>
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<tr>
<td>McRitchie et al., 1995</td>
<td>Ventral intermediate/ median ventrolateral</td>
<td>Pars lateralis</td>
<td></td>
<td>Dorsolateral</td>
<td>Dorsal intermediate/ dorsolateral</td>
<td>Outside</td>
<td>Pars lateralis</td>
<td>Pars medialis</td>
<td></td>
</tr>
</tbody>
</table>

N = nigrosome; SNpd = substantia nigra pars dorsalis; SNpl = substantia nigra pars lateralis; Mv = medioventral group. Hassler: for Spe, Spv, Spcd, Spd, Sal, Spcd, Sam, Sai, Spzv, Spz, S = substantia nigra; p = posterior; a = anterior; e = external; m = medial; i = intermediate; l = lateral; v = ventral; z = central; d = dorsal; c = caudal; mH = medial horn. Olszewski and Baxter: Pn = paranigralis; Pbp = parabrachialis pigmentosum. Braak and Braak: pl = posterolateral; pm = posteromedial; m = magnocellular; ps = posterosuperior; am = anteromedial; al = anterolateral; ai = antero-intermediate, subnuclei; Di = pars diffusa. Fearnley and Lees: for vl, vm, dm, dl, v = ventral; d = dorsal; m = medial; i = intermediate; l = lateral. van Domburg and ten Donkelaar: pl = posterolateralis; pm = posteromedialis; ps = posterosuperior; al = anterolateralis; am = anteromedialis.
calbindin-positive matrix and pockets is much more pronounced, with deeply buried calbindin-poor invaginations. The reasons for the evolution of this particular nigrosomal organization are unclear, but the differences may be important to the functions of the nigral complex. Most of the GABAergic nigral pars reticulata neurons are also probably included within the calbindin-positive neuropil; these neurons are scattered among the endings of striatonigral fibres in the monkey (Francois et al., 1985). It has been found in the rat that some collaterals of projection neurons in the substantia nigra pars reticulata directly synapse on, and inhibit, dopamine-containing neurons of the substantia nigra pars compacta (Tepper et al., 1995). There are also interactions between striatonigral afferents and neurons of both the pars reticulata and pars compacta of the substantia nigra (Timmerman and Abercrombie, 1996). Such interactions may be constrained by the nigrosomal organization described here for the substantia nigra in the human.

Given the relationship between the calbindin immunostained neuropil and the concentration of TH-positive neurons in the calbindin-poor pockets, it seems likely that most striatonigral afferents project more strongly to the dopamine-containing neurons included in the matrix than to those belonging to nigrosomes. There is as yet no evidence to support such differential connectivity. Similarly, the projection sites of the dopamine-containing neurons of these different substantia nigra pars compacta compartments are still not known. One might expect, however, that these are different, and that the different compartments might project differentially to the putamen or caudate nucleus, or to the different histochemical compartments of the striatum, i.e. the striosomes and matrix (Graybiel and Ragsdale, 1978). Such differential projection patterns have been suggested in the cat and monkey (Szabo, 1980; Parent et al., 1983; Jiménez-Castellanos and Graybiel, 1987; Langer and Graybiel, 1989), but this issue will not be easy to resolve in the human brain. What the compartmental patterns of calbindin immunohistochemistry do provide, for the first time, is a reliable and easily reproducible method for analysing the human substantia nigra complex independent of its dopamine-containing neurons. As we show in the accompanying paper, this compartmental analysis allows the substantia nigra to be studied with great accuracy in brains from patients who suffered from Parkinson’s disease.

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