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The human locus coeruleus complex: an immunohistochemical and three dimensional reconstruction study

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Summary. The CA (catecholamine/catecholaminergic) cell populations of the locus coeruleus (LC) and subcoeruleus (SubC) were studied using serial sections of the human brainstem immunostained with an antibody against tyrosine hydroxylase. The tyrosine hydroxylase-immunoreactive (TH-IR) neurons were plotted in a computer reconstruction system and their number and soma size determined. Serial section computer analysis was then used to create a three dimensional reconstruction of the LC complex. The number of cells containing neuromelanin pigment was also determined and compared with the number of TH-IR cells. In our sample there were 53,900 TH-IR cells in the LC and a further 6260 cells in the SubC. These numbers were very similar to our estimates of the number of cells containing neuromelanin pigment and we concluded that virtually all of these cells were also tyrosine hydroxylase positive. The average soma size of the TH-IR cells of the LC was 37 μm and in the SubC 34 μm. In addition to these quantitative observations the morphology of the TH-IR and the Nissl stained cells is described in some detail. We also compared the groups of immunoreactive cells in the human pons with the noradrenergic groups A5–A7 described in the rat. Although in the human these groups are contiguous, A5 is not part of the LC complex. However we did find that the A7 group is equivalent to the rostroventral part of SubC while the remainder of SubC is formed by ventral A6.

Key words: Tyrosine hydroxylase – Nucleus subcoeruleus – Quantitative analysis – Pontine tegmentum – Morphology

Introduction

Although the LC was originally discovered in the human brain (Reil 1809), the anatomical, histochemical and pharmacological analysis of its neurons is largely found in reports on animal experiments. There was little interest in this relatively small structure of the brain until Dahlström and Fuxe (1964) discovered that the nucleus is an aggregate of CA neurons. Large numbers of animal studies followed regarding the LC and the widespread projections, physiological characteristics and the possible functions of its noradrenergic neurons were described (for reviews see Foote et al. 1983; Loughlin and Fallon 1985). The locus coeruleus became known as the primary source of dense networks of noradrenergic axons which innervate large regions of the forebrain, in particular the cerebral cortex, the limbic system and hypothalamus (Morrison et al. 1982; Palkovits et al. 1980). The human locus coeruleus attracted special interest when it was reported that in certain degenerative diseases (particularly in Alzheimer's disease) it suffers a specific loss of its neurons (Iversen et al. 1983).

The neurons of the human locus coeruleus are characterized by the presence of neuromelanin pigment and several studies have studied the disposition and number of pigmented neurons in the LC and adjacent pontine tegmentum. Olszewski and Baxter (1954) delineated the LC and the region of SubC, which they subdivided into ventral and dorsal parts, on the basis of the presence of pigmented neurons in them. A description of the topography of pigmented cells was given by Saper and Petito (1982), and the number of pigmented cells in the human LC was counted by Vijayashankar and Brody (1979), Tomonaga (1983), Walker et al. (1985), Yoshinaga (1986), and German et al. (1988). Based on plots of pigmented cells a reconstruction of the human LC was created by German et al. (1985; 1988). A detailed immunohistochemical study of the human LC and SubC, combined with distribution and cell size analyses, and three-dimensional reconstruction is still lacking. Our work aims to remedy this situation not only by

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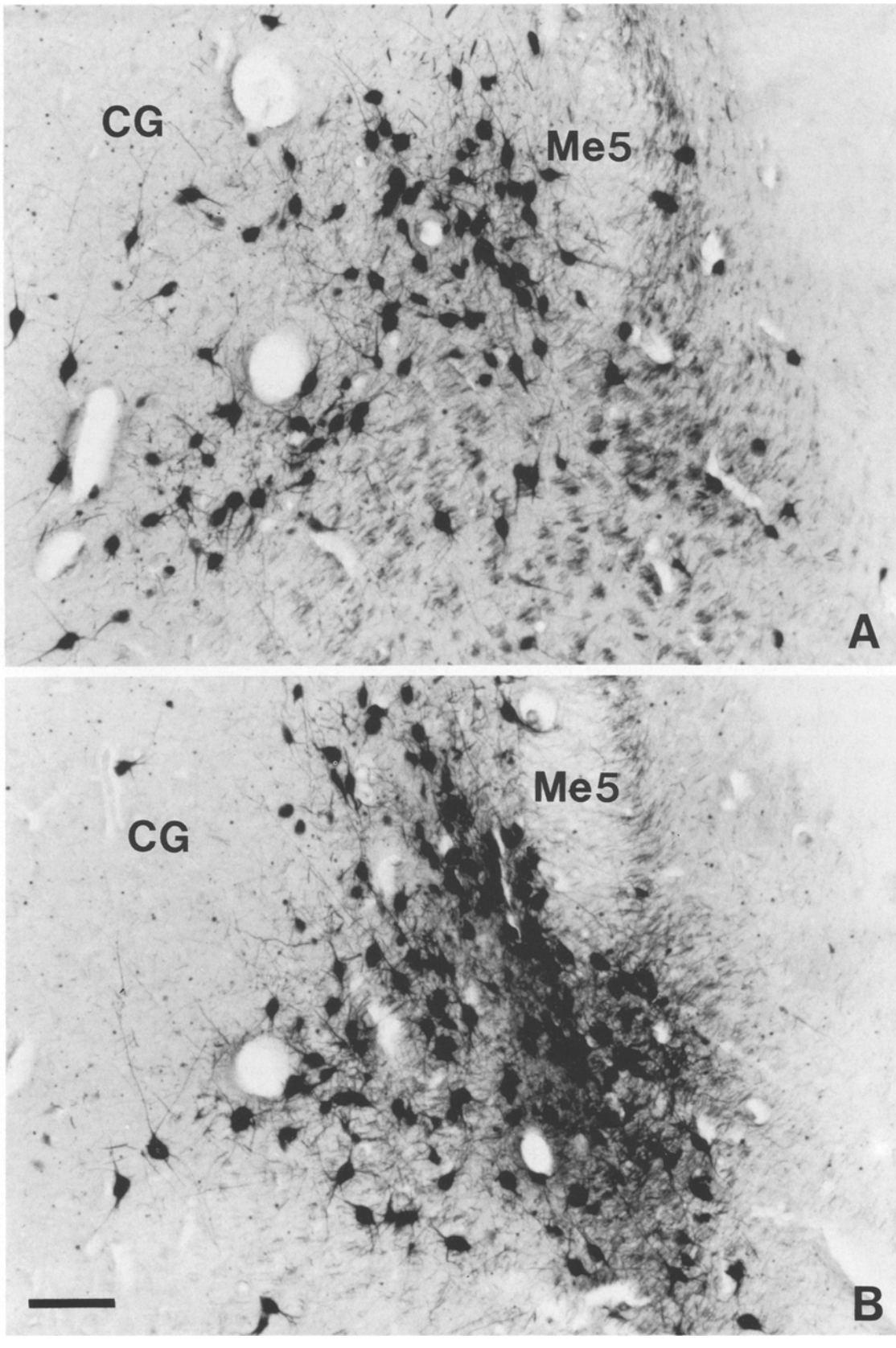
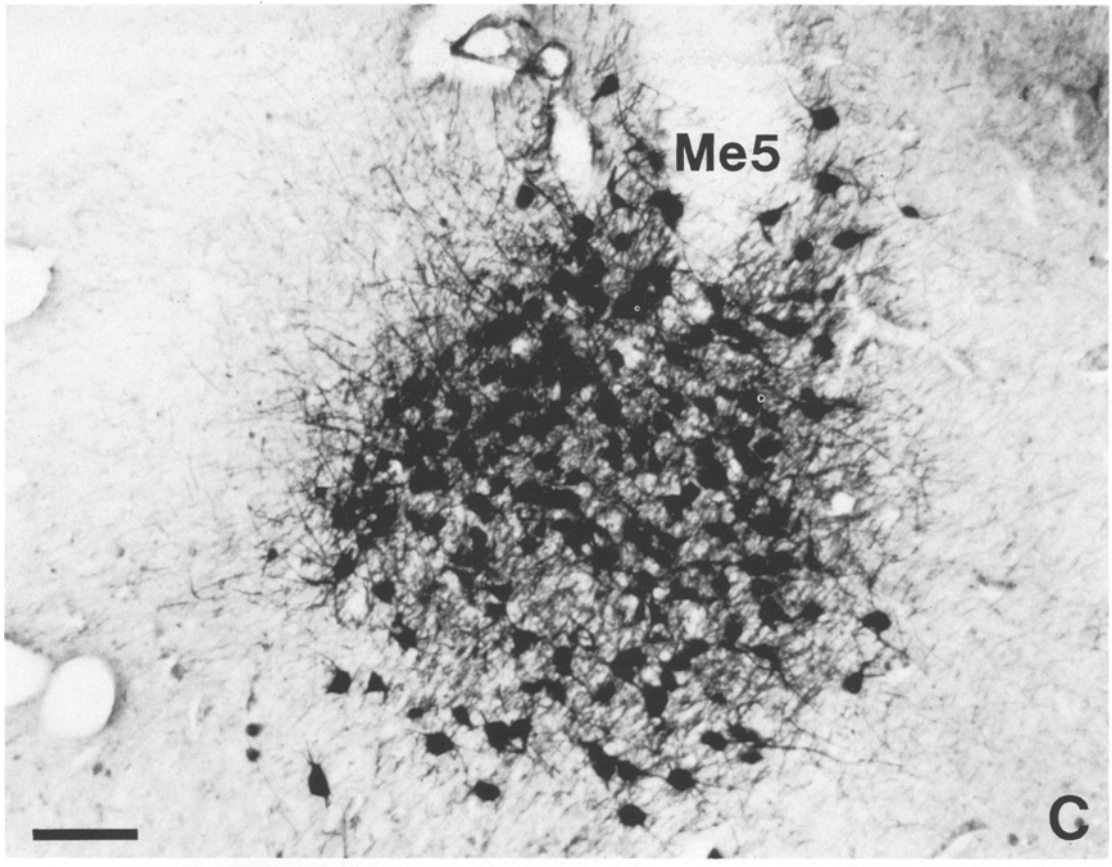
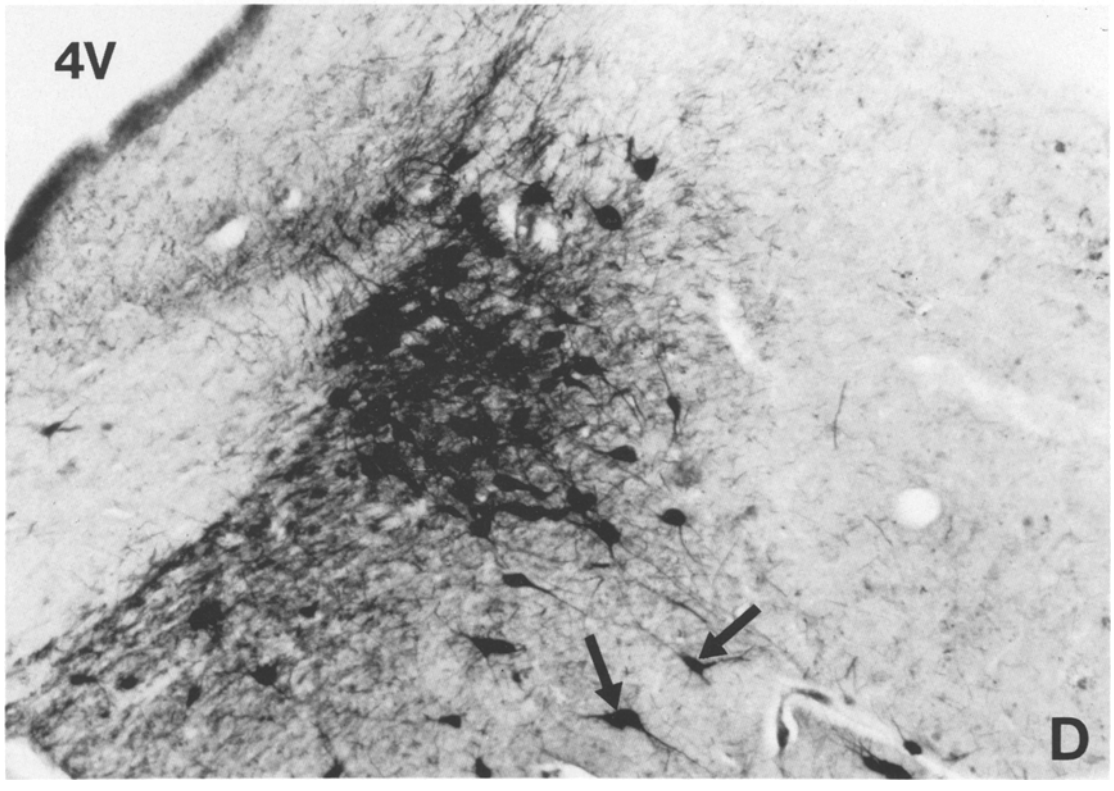


Fig. 1A–D. Transverse sections of the human LC at regular intervals throughout the rostrocaudal extent of the nucleus. Bars = 200 μm. **A** Photomicrograph of the LC approximately 1 mm caudal to its rostral end. The TH-IR neurons are dispersed mostly in the central grey matter (CG) medial to the mesencephalic tract of the trigeminal nerve (Me5). Relatively few cells are found in the rostral part of the nucleus and this area contains large numbers of TH-IR axons. **B** Cells of the LC at the junction of its rostral and middle thirds. The TH-IR cells form a compact cluster and are located in a more lateral position than in **A**, ventral and ventromedial to Me5. The neuropile of the nucleus is darkly stained as it contains a dense network of TH-IR





dendrites and axons. **C** The LC at the junction of its middle and caudal thirds. This is the region of the LC which has the greatest number of immunoreactive cells per section. The LC is in a position similar to that in **B** but acquires a cylindrical shape. **D** Photomicrograph of the LC approximately 1 mm from its caudal end. The nucleus is reduced to a small, compact, cylindrical cluster at the junction of the central grey matter and the pontine reticular formation. Ventrolaterally the TH-IR cells of the SubC are visible (arrows). Note that the TH-IR axons are mainly concentrated in the ventromedial aspect of the LC

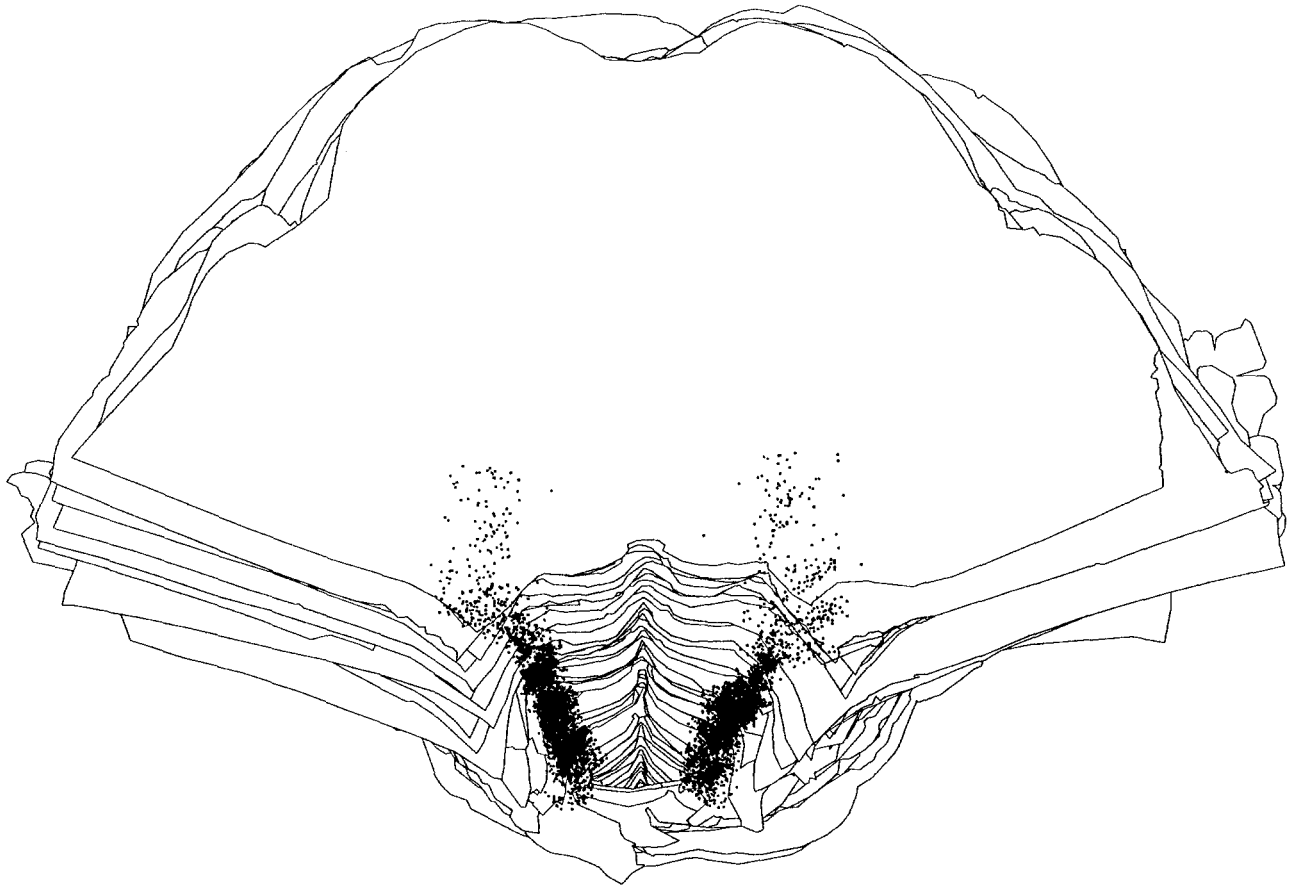


Fig. 2. Three dimensional computer reconstruction of a series of spaced sections from the human LC and SubC seen in a caudorostral direction. Each dot represents one cell. As only every eighth section was used the displayed dots represent only one eighth of the TH-IR population of the LC. The total length of the LC was calculated to be 12 mm. The scattered dots in the foreground (caudal pons) represent cells of the SubC in the pontine tegmentum, whereas the dense accumulation of cells in the pons and isthmus belong to the LC proper. Bar = 5 mm

describing the neurons of LC and SubC in the human on the basis of tyrosine hydroxylase immunohistochemistry, but also by correlating the groups of immunoreactive neurons in the human pons with the noradrenergic groups A5–A7 described in the pontine tegmentum of the rat.

Material and methods

The human brainstems used for this study were obtained at the postmortem examination from cases without any evidence of neurological disease. The observations described herein were obtained from four such cases, and the postmortem examinations for each were performed within 12 h of death. The cell counts of TH-IR and pigmented neurons, and the computer reconstructions of the LC complex were based on a complete series of sections through the same brainstem. This brain was that of a 53 year old motor vehicle accident victim, with no evidence for brain damage, and the fixation of the brain began 5 h and 30 min after death.

The brainstems were fixed by immersion in a solution containing 4% paraformaldehyde in 0.1 M phosphate buffer. The fixation was performed at room temperature and the specimens were kept in this fixative for several months. Following fixation the brainstems were cut into several blocks, each about 10 mm high, with the cutting plane being at a right angle to their longitudinal axis. The blocks were then immersed in a 30% sucrose solution in 0.1 M phosphate buffer, for a period of up to one week, but at least until they sank.

The brainstems were blocked using a device fitted with a protractor to ensure that the cutting plane would subtend a 90 degree angle to the sagittal plane. This was necessary to achieve symmetrical cutting of the blocks. Serial sections were then cut on a freezing microtome at a thickness of 50 µm. Once cutting was completed every fourth section was selected for the demonstration of tyrosine hydroxylase-like immunoreactivity. Parallel with each section a neighbouring section was stained with cresyl violet to reveal the cytological details of nuclei within the brainstem. These sections were also used for the counting of pigmented neurons within the LC and SubC.

Immunohistochemical procedures

The immunohistochemical reaction used for the demonstration of TH in the tissue was a modification of the ABC-reaction of Hsu et al. (1981). The immunohistochemical reaction was commenced with a pretreatment of the sections with 1% normal goat serum in phosphate-buffered saline, pH 7.4, containing

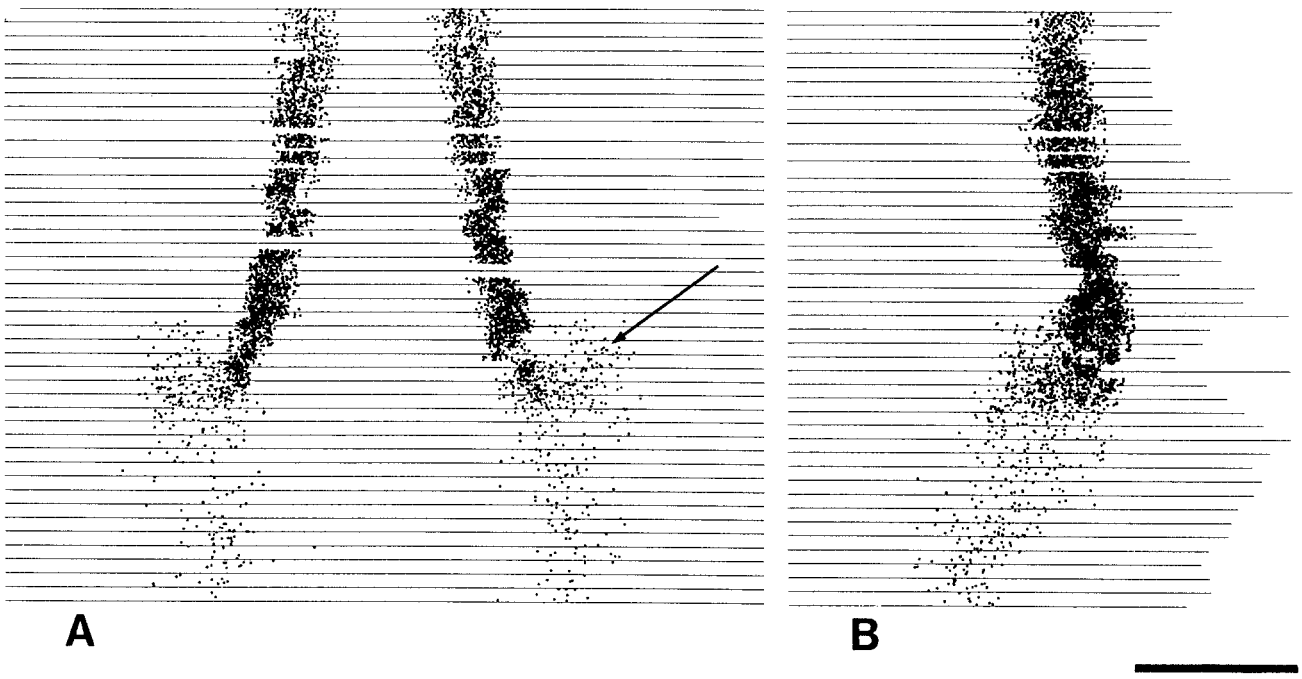


Fig. 3A, B. Computer generated dorsal (**A**) and lateral (**B**) views of the LC and SubC. The parallel lines represent the sections which contain the cells that are displayed. As the cells are within the sections it was necessary to randomly scatter the dots along their z (rostrocaudal) coordinate through a distance equivalent to the spacing of the sections (400 μm) in order to make them visible when viewed from the angle shown (90°). The rostral, dense region of the LC-SubC complex corresponds to cluster A6 (LC proper) of Dahlström and Fuxe (1964) while most of the caudal, loosely arranged dots represent the SubC group equivalent to A6v. The rostrolateral part of the SubC group (at arrow) is group A7. Bar = 5 mm

0.1% Triton X-100 (PBS Triton). Following this, the sections were incubated with an antibody against tyrosine hydroxylase at a dilution of 1:1500 in PBS-Triton at 4° C for 48 h. The antibody was a gift from the Department of Pharmacology, University of Oxford (Prof. David Smith) and has been fully characterized (Van den Pol et al. 1984).

Following treatment with the first antibody the sections were rinsed with PBS-Triton (3 × 10 min), and then reacted for 1 h, at room temperature, with the second antibody, a biotinylated goat anti-rabbit serum (Vector laboratories, Burlinghame, California), at a concentration of 1:100. This was followed by 3 rinses in PBS-Triton and then treated with the ABC reagent at a concentration of 1:100. This treatment was also for 1 h and was followed by two rinses in PBS, each for 10 min.

The diaminobenzidine (DAB) reaction was performed in tris-HCl buffered saline (pH 7.4) containing 0.04% nickel ammonium sulphate (Nickel-TPS). The sections were pretreated in DAB (Sigma, St. Louis, MO.) solution, at a concentration of 0.5 mg/ml, for 10 min. Following the preatment the section trays were removed from the solution and to each 100 ml of DAB solution 18 μl of concentrated hydrogen peroxide was added, then the sections were placed back into the solution and incubated for 5 min. Following this the sections were washed in TPS and mounted on gelatinized slides. Finally the sections were mounted in DPX.

Study of pigmented cells

The principal noradrenaline-containing neurons of LC contain characteristic neuromelanin pigment. The bluish-black colour of this pigment is easily recognized in bright-field microscopy. Some difficulty arises, however, when only a small amount of pigment is present in a cell and differentation from the common lipofuscin pigment becomes uncertain. We have used a Zeiss Axiophot fluorescence microscope with a filter set for the 450–490 nm range to demonstrate the two different pigments; the strongly autofluorescent lipofuscin was then readily distinguished from the non-fluorescent neuromelanin.

Computer reconstruction of the LC complex and quantitative analysis

A computer-driven plotting microscope was used with the application of the Magellan programme (Halasz and Martin 1985) for the plotting and quantitative analysis of LC neurons in both the cresyl violet and immunostained preparations. The graphics display of a microcomputer was mixed optically through a drawing tube with the microscopic image of the specimen. It was then possible to plot the labelled cells in a complete series of sections together with the outline of each section. The data from each section were saved as individual files. Using an NEC-APC IV computer these files were then used to create a three-dimensional reconstruction of the nucleus and illustrate its topography, changing shape and location in the pons. For cell size measurements the outline of the somata of the neurons was entered, the area of each such profile automatically calculated and the diameter of a circle of the same area was taken as the average diameter of the cell body.

The motorized microscope stage used for plotting the labelled or pigmented cells was accurate to within one micrometre and the Magellan programme had the facility to account for both section thickness and the distance between sections. Consequently the dimensions of the reconstruction precisely corresponded to those of the specimen.

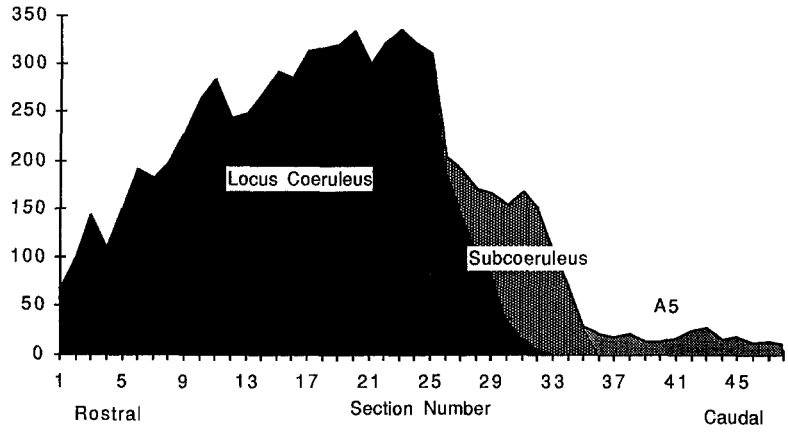


Fig. 4. Number of TH-IR neurons in a series of 50 μm thick sections spaced at 400 μm throughout the rostrocaudal extent of the human LC and SubC. The rostral part of A5 is also visible. The far greater size of LC is apparent and the bulk of SubC can be seen to overlap the caudal end of LC. The total number of neurons in both sides of the human LC was calculated to be 53,900 and there were an additional 6,260 SubC neurons (as only one section in every eight was counted this graph was based on one eighth the total number of cells in both sides of the LC complex)

The TH-IR cells were counted in every eighth section of the series, thus the total cell numbers were estimated from a section taken at every 400 μm of the entire length of the LC and SubC complex. Because of the solid black immunohistochemical staining of the TH-IR cells not only the somata but also a substantial part of the dendritic tree of the cells was demonstrated. This enabled us to count only those cells which had their complete soma visible, and smaller fragments of cells were not counted. This procedure eliminated the need to make corrections for double counting of neurons. The application of Abercrombie's (1946) formula would not have been practical because the dense immunoreactive product obscured the nucleus and nucleolus of the cells and the average diameter of the cells (37.4 μm, see below) was too close to the average section thickness (50 μm). Considering the fact that we have not counted fragmented cells at all and that some loss of the immunoreactive cells must have occurred during the postmortem delay our counts probably underestimate the total number of TH-IR cells in the LC and SubC complex.

Results

Tyrosine hydroxylase immunohistochemical results

The LC is readily apparent in sections of the rostral half of the pons owing to the intense staining of its TH-IR cells. Viewed at intervals through its length the LC is seen to vary in size and shape (Fig. 1 A–D). In the rostral part of the nucleus the TH-IR cells are dispersed in the ventrolateral region of the central grey matter, medial to the mesencephalic tract of the trigeminal nerve but they already indicate coherence (Fig. 1 A). At this level very few immunoreactive cells appear in the reticular formation outside the boundaries of the central gray matter, although many TH-IR axons can be seen emerging from the nucleus, aligned along the longitudinal axis of the brainstem. In subsequent more caudal sections the increase in the number of labelled cells is accompanied by the appearance of TH-IR cells outside the boundaries of the central gray matter. Figure 1 C shows that at this level the LC can be divided into at least two distinct regions. Dorsomedially the neurons are packed closely together. Ventrolaterally, the cells are more dispersed and are randomly aligned. Further caudally the nucleus becomes very compact and most of its cells are ventral to the central gray matter (Fig. 1 D). From this level caudally TH-IR cells are seen streaming away from the nucleus in a ventrolateral direction, to enter the region of the SubC (Fig. 5 D).

The topography of the TH-IR neurons in LC and SubC is graphically illustrated in the three-dimensional reconstruction (Fig. 2). While Fig. 2 shows the reconstruction of these nuclei in relation to the outline of the pons, Fig. 3 gives more precise views (a dorsal and a lateral) of the relationship of the cell clusters in the LC and SubC to each other. Figure 3 A also illustrates that the subcoeruleus cluster commences about 2 mm rostral to the caudal tip of LC with more laterally positioned cells. Further caudally, SubC moves medially and ventrally.

The numerical distribution of TH-IR cells at 400 μm intervals through LC and SubC is illustrated in Fig. 4. The number of TH-IR neurons in LC increases steadily from the rostral pole of the nucleus to about three quarters of its rostrocaudal extent. In the last quarter the cell numbers decrease rapidly, while the number of cells in the SubC increases such that the combined number of labelled cells in the two nuclei will remain about 150/section for about 2.5 mm. Caudal to this, the number of cells in the SubC region decreases rapidly and levels out to a mean of 17 cells/section. The point of transition to this maintained low number of cells is approximately at the level of the oral pole of the superior olivary complex. Olszewski and Baxter (1954) described this region as the caudal end of SubC. In LC, counting of every eighth section produced totals of 3328 and 3410 TH-IR cells in the left and right sides of the nucleus, respective-

ly. Multiplication of the sum of these numbers by a factor of eight produced a total of 53,904 TH-IR cells in the human LC. Counting every eighth section of SubC (left and right sides), the total number of TH-IR cells was 783. The total number of TH-IR neurons in SubC was therefore calculated to be 6,264.

The morphological description of TH-IR cells of LC is based on the examination of the less densely packed rostral part of the nucleus where individual neurons are more easily seen. The TH-IR cells of the LC are multipolar and tend to have round or oval somata (Fig. 5A, B). Each cell usually has three or four long, thin, cylindrical dendrites which branch once or twice a short distance from the cell body. The dendrites are up to a millimetre in length and may extend beyond the LC into the periventricular grey matter or underlying reticular formation. Although a number of spindle shaped neurons with two tufts of dendrites are found within the LC (Fig. 2A) cells of this type are much more likely to be found in the subcoeruleus nucleus (Fig. 6A). The axons of the TH-IR neurons arise from the cell body as thin curved processes which quickly join the noradrenergic bundles just lateral and ventral to LC (Fig. 1A). The axons of SubC accumulate dorsally forming a dense cluster in the region ventral to the lateral angle of the fourth ventricle occupied more rostrally by LC. This dorsal bundle is a continuation of the LC noradrenergic bundle.

In the rostral end of the SubC cell cluster there were large multipolar TH-IR neurons with no preferential orientation. More caudally neurons occupying a similar position were clearly oriented in a dorsomedial to ventrolateral direction. Examination of serial sections extending into the caudal midbrain failed to reveal other large TH-IR cells in the ventromedial aspect of the superior cerebellar peduncles. The large non-oriented multipolar TH-IR cells were therefore assumed to correspond to the A7 cell group. These TH-IR cells were also visible as a distinct protuberance of SubC when this nucleus was reconstructed three-dimensionally (Fig. 3A).

Owing to the dense black staining of the TH-IR cells it was not possible to see intracellular structures such as the cell nucleus or cellular organelles. The Nissl substance, the nucleus and nucleolus were clearly visible using a Nissl stain (cresyl violet) and these features are described below.

The mean soma size of the TH-IR neurons in the LC was 37.4 μm and in SubC 34.3 μm. Figure 7 shows a normal distribution curve for the size distribution of neurons within the LC and SubC (about 95% of these cells fall within plus or minus two standard deviations). The measurement of all TH-IR cells in LC from ten evenly spaced sections throughout the length of the nucleus failed to show any significant rostrocaudal variation in mean soma size.

Pigmented and non-pigmented cells of LC

The LC contains large numbers of pigmented and non-pigmented cells. Primarily on the basis of size and pigmentation the cells of the LC can be divided into four categories: (1) Medium-sized pigmented cells (35–45 μm), (2) Small pigmented neurons (15–25 μm), (3) Small unpigmented neurons (10–25 μm), (4) Glial cells. Apart from noting that large numbers of glial cells are present both inside and outside the boundaries of LC, and distinguishing them from small neurons, these cells have little relevance to this study.

Medium-sized pigmented neurons. These cells are the most characteristic of LC and have been studied in detail by many authors. They form a strikingly uniform population of cells with characteristically round or oval-shaped somata (Fig. 5C). Almost all medium sized neurons in the LC are pigmented. Although the amount of pigment in each cell varies, most of these cells have at least half of their cytoplasm obscured by small black spherical granules of pigment. The nucleus is visible in most cells and is dark, spherical and eccentrically placed. The violet coloured Nissl substance (rough endoplasmic reticulum) is visible throughout the cytoplasm but extends into only the proximal part of the dendrites (Fig. 6B). There was no peripheral concentration of Nissl substance or chromatolysis indicative of fatigue or neuronal damage.

Counting medium-sized pigmented neurons in both sides of every eighth section in a series of 50 μm sections throughout the nucleus produced a total of 6862 neurons. Multiplication by a factor of eight produced a total of 54,896 medium-sized pigmented cells in the human LC.

Careful examination of the medium-sized pigmented neurons reveals that they have the same shape and similar size as the TH-IR neurons. Indeed in some TH-IR neurons the immunostaining is weak enough to allow the pigment granules in the cytoplasm to be seen.

Small pigmented neurons. These small neurons tend to be either triangular or ellipsoidal although thinner and longer than the oval, medium-sized cells. The percentage of pigment in these cells is signifi-

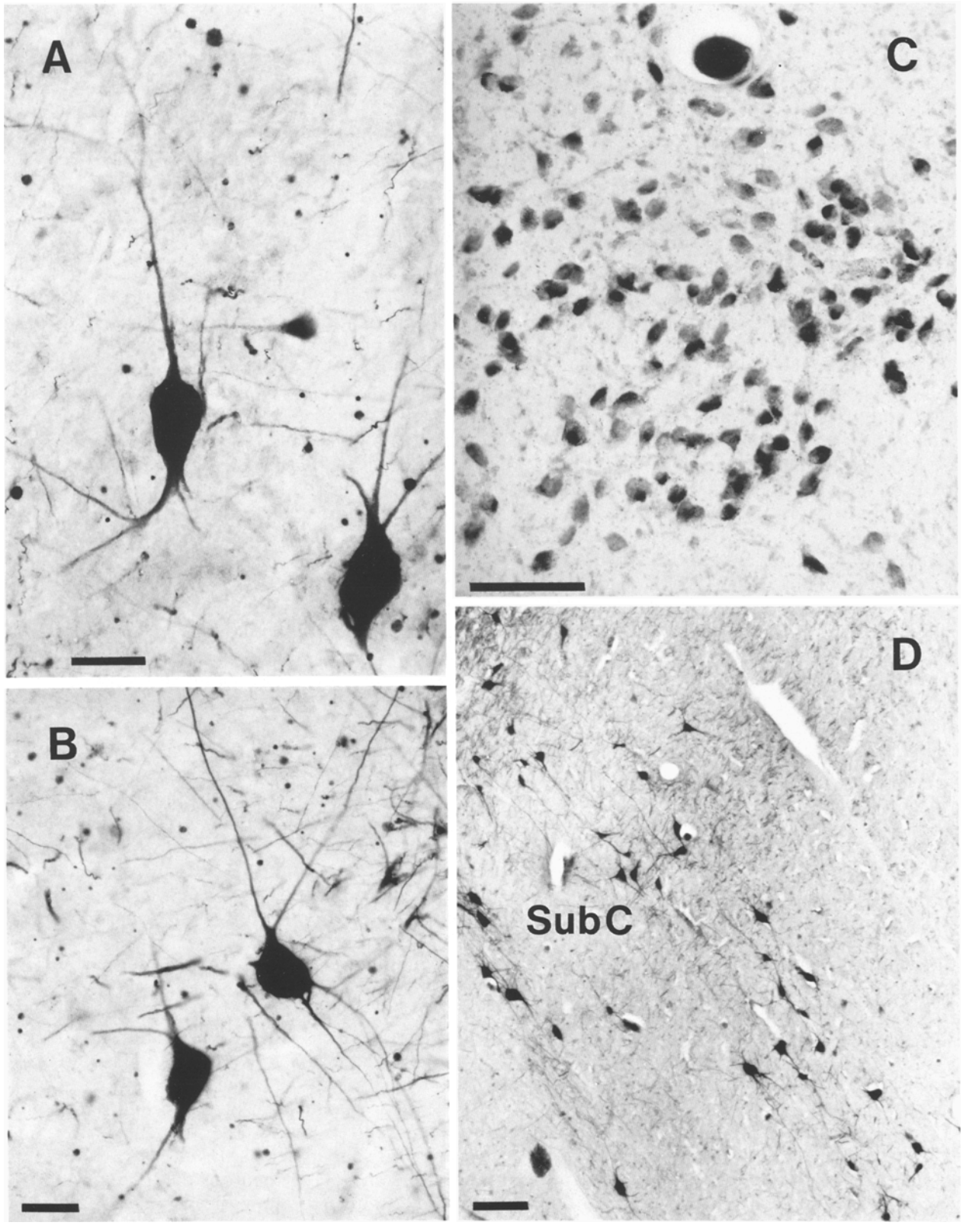


Fig. 5A–D. Photomicrographs of the neurons of the LC and SubC demonstrated with TH (**A, B, D**) and with cresyl violet (**C**). **A** Large fusiform cells from the rostral region of the LC. The dendrites arise from the cell body in two clusters. Although thick close to the cell body, they gradually become thinner and extend for long distances. Bar = 50 μm. **B** Spherical TH-IR cells. Several thin, non-varicose dendrites of uniform thickness can be seen arising from the cell body. Bar = 50 μm. **C** Pigmented cells of the LC. The majority of the medium-size neurons can be seen to contain neuromelanin pigment which occupies most of their cytoplasm. Bar = 200 μm. **D** Low-power photomicrograph of the TH-IR cells of the human SubC. These cells are oriented in a dorsomedial ventrolateral direction (from the upper left to the lower right of the picture) and extend into the ventrolateral tegmentum. Bar = 200 μm

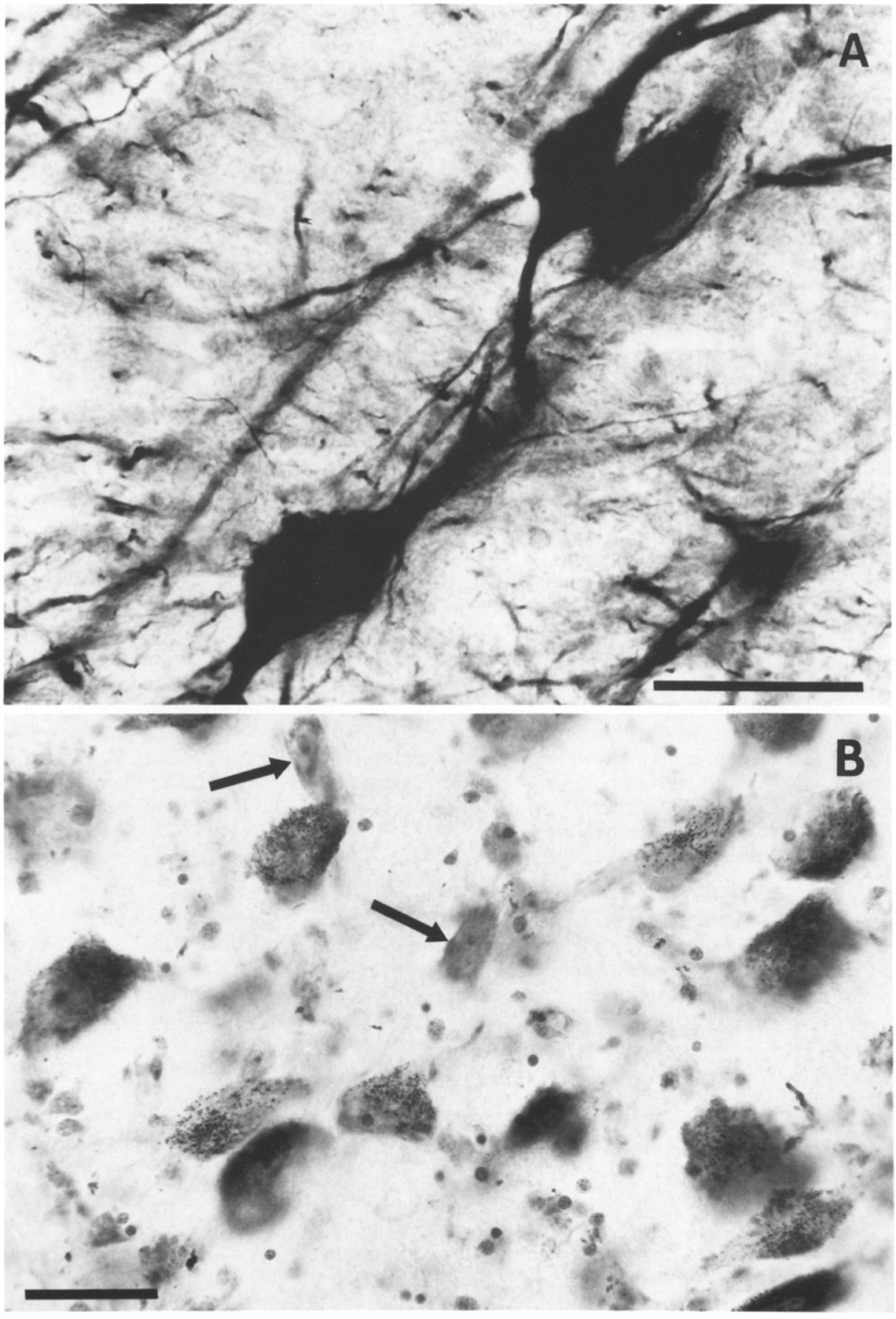


Fig. 6 A, B. High-power photomicrograph of cells of SubC (**A**) and LC (**B**). Bars = 50 μm. **A** Several typical fusiform SubC neurons demonstrated with TH. Tufts of dendrites can be seen emerging from both ends of these cells and their uniform dorsomedial-ventrolateral orientation (upper right to lower left of the picture) is also apparent. The dense immunoreactive product obscures the nucleus and nucleolus and extends into the dendrites demonstrating their morphology. **B** Cells of the LC demonstrated with cresyl violet. Granules of dense black melanin pigment are seen to at least partially obscure the Nissl substance of the medium-sized neurons. Small non-pigmented neurons are also seen (arrows)

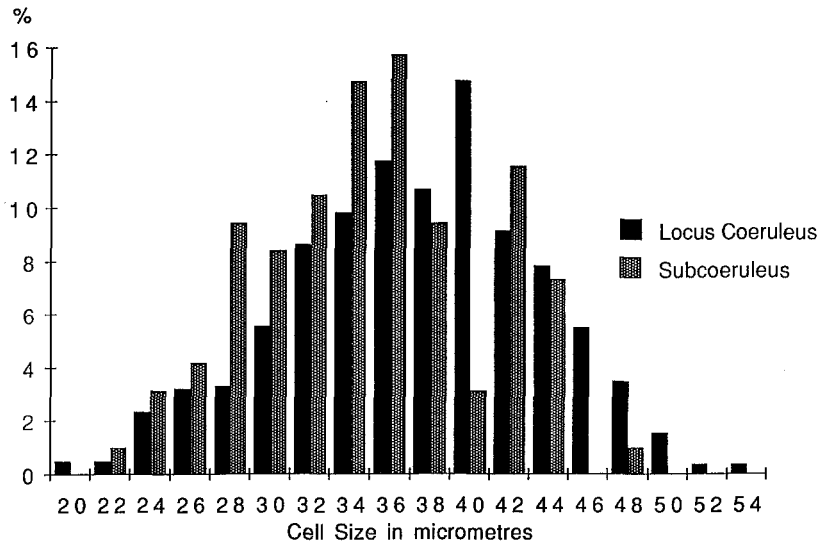


Fig. 7. Size distribution histogram of TH-IR cells in the human LC complex. The mean cell size of the LC neurons (37.4 μm) is slightly greater than that of the SubC neurons (34.3 μm)

cantly less than that of the medium sized neurons (Fig. 6B). Consequently the pigment can be difficult to see when it is present in very small quantities making differentiation between the pigmented and unpigmented cells difficult. The following evidence suggests that this pigment is lipofuscin rather than neuromelanin. As this pigment is relatively less concentrated it does not have the dense black appearance frequently seen in the pigment of the larger medium-sized neurons. With fluorescence microscopy the lipofuscin pigment was easily detected because of its strong autofluorescence. Such autofluorescence only occurred in the small pigmented neurons while non-fluorescent neuromelanin was only present in the medium-sized neurons of LC.

Small unpigmented neurons. These cells were identical to the majority of the background cells surrounding the LC. Because of this fact attempts to count these neurons within an approximate and arbitrary boundary proved unreliable. However the results did indicate that there are many times more unpigmented than pigmented small neurons. This discrepancy is particularly evident at the rostral end of the nucleus. It was not difficult to distinguish these small neurons from glial cells because of the presence of Nissl granules in the neurons while only the nuclei of the glial cells were visible (Fig. 6B).

Pigmented and non-pigmented cells in SubC

The dorsal and ventral regions of what is called the nucleus subcoeruleus contain a diverse population of neurons, a population which is characteristic for the reticular formation of the pons and medulla. The cell types described for the LC were also observed in the region of SubC, but in addition medium-sized and large non-pigmented cells are also present. In Nissl preparations, however, the most prominent cells were those that contained neuromelanin pigment and the area containing these neurons was originally delineated by Olszewski and Baxter (1954) to define the region of SubC. In a series of cresyl violet stained sections, parallel with those immunostained for tyrosine hydroxylase, we have counted the number of pigmented cells. Counting every eighth section there were 736 cells, i.e. we estimate that the total population of pigmented cells in the SubC is 5,888.

Discussion

This study presents a qualitative and quantitative morphological analysis of the human locus coeruleus complex based on tyrosine hydroxylase immunohistochemistry. We describe the morphological features and localization of TH-IR neurons in the locus coeruleus and subcoeruleus nucleus. The labelled cells, together with the outline of the brainstem, were plotted in a computerized microscope system and the data used for a three-dimensional reconstruction of these nuclei. In addition to estimating the total number of TH-IR neurons in the human LC and SubC, we have also correlated their number with the number of neuromelanin pigmented cells.

Morphology and topography of TH-IR neurons in LC and SubC

One of the aims of this study was to describe the TH-IR neurons in the different regions of the LC. Prior to examining divisions within the LC it is

necessary to determine what is meant by the LC complex. Swanson (1976) suggested that the variation in the interpretation as to what constitutes the boundaries of the LC is partly due to interspecies variation. Unfortunately there is also a lack of agreement regarding the components of the complex within individual species. Although most authors refer to the original classification of catecholaminergic nuclei in the rat brain (Dahlström and Fuxe 1964) and specify group A6 as being equivalent to the LC proper, at various times one or more of the areas designated as A4, A5 and A7 have been included as components of the LC complex in the rat (Grzanna and Molliver 1980; Olson and Fuxe 1972; Swanson 1976), cat (Jones and Moore 1974; Wiklund et al. 1981) and monkey (Felten and Sladek 1983; Hubbard and DiCarlo 1973). On the other hand, Hökfelt et al. (1984) have basically adhered to the original classification of Dahlström and Fuxe (1964), and have excluded A4, A5 and A7 from their definition of the LC complex. These authors considered that the LC complex consisted of three components: the A6r (rostral), A6 and A6v (subcoeruleus). However, the distribution of the TH-IR neurons in the human brainstem is more widespread than in the rat, which results in an almost continuous stream of immunolabelled cells appearing in the lateral regions of the pontine reticular formation, connecting the ventral and caudal aspects of LC with the region of the caudal ventrolateral pons, where the catecholaminergic neurons of group A5 are found. We also have to consider the definition of the subcoeruleus nucleus, as described by Olszewski and Baxter (1954).

The existence of A6r in the human, as proposed by Hökfelt et al. (1984) in the rat, is confirmed by our studies. This loose cluster of cells occupies about the rostral quarter of the human LC, and the most rostrally located cells extend well into the mesencephalon. The compact part of the LC (A6) is what most textbooks and atlases define as the locus coeruleus. The relationship of this part of LC to the TH-IR neurons of the subcoeruleus is supported by several observations. First, the size and the morphology of the cells in them is very similar; most of them having round or fusiform somata and relatively few, straight dendrites. Second, the subcoeruleus cluster (A6v) is clearly connected to the the ventral aspect of LC and some of the most dorsal cells could easily be assigned to either regions. Third, the axons of the subcoeruleus cells rise to the dorsal region of the pontine reticular formation and join the axons of LC cells. A fourth observation is that, in the rat, SubC has been found to share several projection sites, namely the cerebellum and spinal cord, with much of LC proper (Loughlin et al. 1986).

Kemper et al. (1987) believed that in man the ventral part of subcoeruleus was equivalent to A5 caudally and A7 rostrally. Our results agree with this to the extent that the group A7 in the human clearly falls within a region that was described by Olszewski and Baxter (1954) as the nucleus subcoeruleus ventralis. Although the A7 cell cluster is continuous with the more caudal subcoeruleus cells in the pontine tegmentum, the lack of dorsoventral orientation of the cells makes them relatively easy to distinguish. Although located more medially than in the rat the relationships of this cluster are the same as that of group A7 in that species, i.e. they are found rostral to the motor nucleus of the trigeminal nerve and immediately medial to the lateral lemniscus. Although Saper and Petito (1982) concluded that the classification of Dalhström and Fuxe (1964) was valid for man and group A7 was distinct from SubC, our computer reconstruction clearly indicated that the original delineation of the nucleus subcoeruleus by Olszewski and Baxter (1954) should be maintained. Loughlin and Fallon (1985) also considered that A7 was part of SubC. For these reasons we have included the cells of A7 in our cell counts of SubC. On the other hand, the charting of the cell numbers in a series of sections through the LC complex clearly indicated the boundary between the caudal extent of SubC and the rostral limit of the A5 group. We suggest that the A5 group begins at the point where the number of TH-IR cells stabilizes to about 17 per section (Fig. 4). This level is just caudal to the trigeminal motor nucleus and is co-existent with the abducens nucleus. Thus we suggest that the TH-IR cells of SubC in the human brain appear at the rostral, medial and caudal aspects of the motor nucleus of the trigeminal nerve and include both groups A7 and A6v but not A5.

Dopamine-β-hydroxylase studies in both the rat (Loughlin and Fallon 1985) and human (Kemper et al. 1987) indicated that the cells in the rostral part of the LC were significantly larger than in the caudal parts. We have measured the average diameter of TH-IR cells in ten evenly spaced rostrocaudal levels of the LC and could not reveal any significant difference in cell size throughout the length of the nucleus.

Number of TH-IR and pigmented cells in the LC complex

As individual section counts of the right and left sides of LC did not differ by more than 2.4%

it is evident that the sectioning of the brainstem was symmetrical about the midline.

It is important to appreciate that in comparison to pigmented-cell studies the TH-IR method enables a more accurate estimation of the number of catecholaminergic cells in the human LC. Furthermore it is possible to examine a number of features, such as dendritic morphology, that are not visible using most traditional stains. Normal quantitative studies are important to establish base line data necessary for quantitative investigations of the effect of aging and disease on the human brain.

Although it has been possible for some time to map catecholaminergic neurons in the adult human brain using immunohistochemistry (German et al. 1985; Kitahama et al. 1988; Pearson et al. 1979, 1983), all but one of the previous quantitative studies of the human LC have relied on counting only the neuromelanin pigmented neurons to determine the number of catecholaminergic neurons within this nucleus (Bondareff et al. 1982; Tomonaga 1983; Vijayashankar and Brody 1979; Walker et al. 1985; Yoshinaga 1986).

Vijayashankar and Brody (1979), who counted only one side of LC and also counted SubC, and Yoshinaga (1986) found a very similar number of pigmented neurons in adults under 60 years of age; a mean of 16,840 cells and 16,984[1] cells respectively. A similar estimated cell number was reported by Bondareff et al. (1982) as being in the range 9551–16,427[1]. These numbers are difficult to reconcile with our data. On the other hand Walker et al. (1985) and German et al. (1988) estimated that there were 60,032 and 45,562[1] pigmented cells in both sides of the human LC respectively. Although these authors used Schmorl's ferricyanide method to specifically stain neuromelanin, the findings of German et al. (1988) suggest that this is not the reason their results differed from those of other authors. German et al. (1988) also used cresyl violet to stain another series of sections for Nissl substance and found that cell counts were not significantly different from those obtained using Schmorl's ferricyanide method.

The total number of catecholaminergic neurons, as established in our experiments agrees well with the cell counts of Iversen et al. (1983) who have found in the LC an average of 9426 dopamine-β-hydroxylase-IR neurons, counting every fifth section. This cell count corresponds to a total number of 47,130 cells. This average number was obtained from 5 cases, with an average age of 84 years. Although these cases were all neurologically negative it might be expected that there was a minimal loss of LC neurons beyond the age of 60. It is therefore likely that the normal number of catecholaminergic neurons in the human LC would be in the range of 50–60,000.

An important aim of our study was to compare TH-IR counts and pigmented-cell counts in the same brain. By confining our cell counts to parallel series cut from the same brain it was possible to eliminate some of the variables affecting the comparisons of quantitative data, such as age and individual variation. Although the number of pigmented cells was not significantly higher than the number of TH-IR cells, the question of whether enzyme degradation was responsible for the lower TH-IR total must be addressed. As the brains were fixed within several hours of death, and it is known that sufficient TH remains in the cells to react immunologically up to at least 36 h after death (Beach et al. 1987), loss of enzyme was an unlikely explanation for the discrepancy in totals.

The accuracy and reliability of our counting procedure was tested when all TH-IR cells in every eighth section throughout the nucleus were recounted. The difference between the two counts was only 50 cells, indicating that the lower number of TH-IR cells as against the number of pigmented cells was unlikely to be due to counting error. However, the medium-sized pigmented cells were more difficult to count and the possibility of error cannot be entirely discounted.

A further source of error in morphometric studies is the overestimation of cell populations owing to overprojection and truncation of individual cells (Abercrombie 1946). There are a number of formulae available for the correction of split cell counting error (Braendgaard and Gundersen 1986; Haug 1986). However as cell nuclei and nucleoli are not visible in TH-IR material it was not practicable to apply formulae such as that of Abercrombie to correct for overcounting. As no correction was made for the double counting of cells in the TH-IR series, and a comparison was made with the number of medium-sized pigmented cells, the pigmented-cell count was also not corrected.

Because of the relationship between neuromelanin and CA synthesis, it is unlikely that the small difference between the TH-IR total and the number of medium-sized pigmented cells is due to the presence of non-CA pigmented neurons within the LC. The presence of serotonergic cells within the LC has been noted in the cat (Wiklund et al. 1981) and the monkey (Felten and Sladek 1983) and we have also observed that a small number

[1] Abercrombie's (or other) split cell correction factor applied

of presumed serotonergic neurons (cells with tryptophan-hydroxylase-like immunoreactivity; Törk and Hornung 1989) are present in the rostral half of LC, but these do not contain neuromelanin pigment. The small discrepancy is possibly due to our inability to distinguish some weakly pigmented neuromelanin containing cells from cells containing larger amounts of lipofuscin pigment. Therefore it is possible to conclude that virtually all medium-sized pigmented cells in the LC are CA.

It has become evident that there are two types of pigment in the human LC. The medium-sized pigmented neurons almost certainly contain neuromelanin and our results suggest that the small pigmented neurons contain lipofuscin. Lipofuscin is believed to be a breakdown product resulting from the oxidation of lipids and lipoproteins (Stevens 1982). Neuromelanin and lipofuscin have a number of common properties and are thought to be related (Barden 1969; Barden 1979; Mann 1983). Neuromelanin is believed to be a melanised form of lipofuscin and to result from the deposition of melanin on lysosomes or on lipofuscin derived from lysosomes (Marsden 1983). It is also possible to synthesize melanin by oxidation and further polymerization of several catechol rings. A leucocompound can then be formed by cyclization of the lateral chain which forms an indolequinone derivative. This derivative can then be polymerized into different species of melanic compound (Murray et al. 1988).

The distribution of these two pigments in the substantia nigra of the rhesus monkey (Barden 1969) was found to parallel the arrangement we noted in the human LC. Moderate sized polygonal neurons were found to contain neuromelanin whereas the small fusiform cells contained lipofuscin.

In conclusion, evidence is presented here that counting of neuromelanin pigmented cells in the human LC and SubC complex accurately reflects the number of CA cells in these nuclei. Consequently a direct comparison can be made between studies in which TH-IR cells were counted and those utilizing counts of pigmented cells. However, the immunohistochemical staining revealed new information regarding the morphology of the CA cells in the different regions of the LC-SubC complex. On this basis and also aided by the computer reconstructions we have identified distinct subregions of the LC-SubC complex. From experiments in animals it is known that the subregions of LC and SubC have different projections and this might have different functional significance. We suggest that in the future pathological studies of the LC-SubC complex could map the specific cell losses and precisely localize the morphological changes affecting the CA cells. This should significantly help establish the clinical significance of the individual cell groups in the human. Recently Marcyniuk et al. (1986) have indeed reported that only specific areas of LC are affected by pathological conditions such as Alzheimer's disease and it may well be that other conditions have similar, differential impact on the subgroups of cells in the LC-SubC complex.

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