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Birth and migration of neurons in the central posterior/prepacemaker nucleus during adulthood in weakly electric knifefish (*Eigenmannia* sp.)

(postnatal neurogenesis/neuronal plasticity/synaptogenesis/electrocommunication/Gymnotiformes)

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ABSTRACT **In contrast to mammals, fish maintain their capacity to generate neurons in the central nervous system even during adulthood for prolonged periods of life. By employing immunohistochemical, autoradiographic, and electron microscopic techniques, we studied such a postnatal neurogenesis within the complex of the central posterior/prepacemaker nucleus (CP/PPn) in knifefish (*Eigenmannia* sp.), a weakly electric teleost. The CP/PPn is a bilateral cluster of neurons in the thalamus. It controls frequency modulations of the electric organ discharge as they are used during social interactions. In the CP/PPn region adjacent to the wall of the third ventricle ("ventricular zone"), cells are born continuously and at high rates. They undergo multiple cell divisions before differentiating into neurons. Concomitant with this development, the newborn neurons migrate toward lateral regions of the CP/PPn. In the course of this lateral migration, they appear to acquire immunological and morphological characteristics that are typical for mature CP/PPn neurons. We hypothesize that at least some of the newly generated cells develop finally into functional CP/PPn neurons.**

While postnatal neurogenesis in mammals is either limited to a short period after birth or to a very few brain regions (see ref. 1), in nonmammalian vertebrates the generation of new neurons during adulthood may persist over long periods of life. In fish, the capability for postembryonic neurogenesis appears to be present in many regions of the central nervous system (2–8) but has been well documented only in the retina and tectum opticum where neuronal elements continue to be added to already existing ones throughout life (9, 10). This proliferative capacity parallels the enormous ability of fish to regenerate nervous tissue after injuries or experimentally induced lesions (11–13).

In the present study, we examine the postembryonic generation of neurons and their subsequent fate within the complex of the central posterior nucleus/prepacemaker nucleus (CP/PPn) of weakly electric knifefish (*Eigenmannia* sp.). The CP/PPn is a bilateral cluster of neurons in the thalamus; it stretches away from the wall of the third ventricle, ≈500 μm ventro-laterally. Traditionally, the medial part of this complex has been termed "CP" and the lateral portion has been termed "PPn" (14). However, according to our present investigation, it is likely that at least a certain portion of the CP consists just of immature cells that migrate away from the ventricle and differentiate into neurons of the PPn, thus suggesting that the CP is actually part of the PPn. This notion is in agreement with recent light microscopic and ultrastructural studies that failed to find differences between the two nuclei (15).

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The neurons of the PPn are identified by retrograde labeling from their projection site, the medullary pacemaker nucleus. The PPn controls frequency modulations of the otherwise extremely constant electric organ discharge (16). Since stimulation of its dorso-medial portion leads to *gradual* frequency rises, as they are observed during the jamming avoidance response (17), this subnucleus is called "PPn-G." By stimulating the ventro-lateral part, abrupt frequency modulations, often followed by brief interruptions ("chirps"), are elicited. Such modulations occur in the context of courtship and aggression and play a major role for electrocommunication (18, 19). The *chirp*-controlling subnucleus is referred to as "PPn-C." Seasonally induced variations in the propensity to chirp are accompanied by drastic changes in the morphological and synaptic structure of the PPn-C (20, 21). The discovery of postnatal neurogenesis in the complex of the CP/PPn, as described in the present paper, may add a new dimension to the perception of a dynamic neuronal structure underlying a social behavior in these fish.

MATERIALS AND METHODS

Animals. Experiments were conducted with knifefish (*Eigenmannia* sp.) obtained from fish importers or raised in our laboratory. Thirteen individuals were used for immunohistochemistry, 29 were used for autoradiography, and 6 were used for electron microscopy.

BrdUrd Immunohistochemistry. Incorporation of the dThd analogue BrdUrd into DNA was used for the detection of mitotic active cells in S phase. Fish were anesthetized in 2% urethane (Sigma) and injected intraperitoneally with ≈100 μl of labeling reagent per g of body weight [labeling reagent = BrdUrd and 5-fluoro-2′-deoxyuridine (10:1) and is from the cell proliferation kit (Amersham)]. After a survival time of 12 hr, the fish were deeply anesthetized with MS-222 (Sigma) and intracardially perfused with 4% freshly depolymerized paraformaldehyde (Sigma) in 0.1 M phosphate buffer (pH 7.3). BrdUrd-containing cells were detected by a monoclonal anti-BrdUrd antibody from the cell proliferation kit and visualized either with a rabbit anti-mouse IgG (Chemicon) and a rhodamine-conjugated goat anti-rabbit IgG (Chemicon) or with a sheep peroxidase anti-mouse immunoglobulin (Amersham), which was allowed to react with 0.05% 3,3′-diaminobenzidine tetrahydrochloride (Sigma).

[^{3}H]dThd Autoradiography. While labeling with BrdUrd decreases after a survival time of ≈12 hr, incorporation of [*methyl*-^{3}H]dThd into replicating DNA is very stable, thus

Abbreviations: CP, central posterior nucleus; PPn, prepacemaker nucleus; PPn-C, chirp-controlling subnucleus of the PPn; PPn-G, subnucleus of the PPn controlling gradual frequency rises.

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allowing time-lapse experiments. Fish were anesthetized in 2% urethane and injected intraperitoneally with ≈100 μCi of [^{3}H]dThd (Amersham; 1 Ci = 37 GBq) per g of body weight. After variable survival times, the fish were given a lethal dose of MS-222 and intracardially perfused with a solution of 2% freshly depolymerized paraformaldehyde (Sigma)/2.5% glutaraldehyde (Tousimis Research, Rockville, MD)/0.1 M phosphate buffer, overall pH 7.3.

Transverse sections of the brain were cut on a vibratome at 500 μm, dehydrated in a graded ethanol series, and embedded in Historesin (LKB). Thin sections were taken at 3 μm and every other section was mounted on chrome alum/gelatin-coated slides, which were dipped in autoradiographic emulsion, type NTB2 (Kodak). After an exposure time of 2 weeks, the slides were developed and counterstained in methylene blue.

Electron Microscopy. Ultrathin transverse sections of the CP/PPn region were prepared as described (21, 22). They were observed under Hitachi H-500, JEOL 100 CX, and JEOL 2000 FX II electron microscopes.

Morphometric Analysis. Light microscopic sections were examined under a Zeiss microscope by using an eyepiece reticle and a camera lucida. Morphometric analysis of electron micrographs and statistical evaluation were performed as described (21).

RESULTS

For the following anatomical description, the traditional nomenclature and cytoarchitectonic delineation of Maler *et al.* (14) will be employed. In addition, we will distinguish the "ventricular zone" and "subventricular zone" of the CP from lateral areas (Fig. 1*A*). This could easily be done in Nissl-stained sections. Whereas CP neurons were round to oval with a lightly stained nucleus but relatively dense perikaryon, the ventricular cells displayed a slender shape with a dark nuclear mass. The ventricular zone extended for 10–20 μm laterally away from the ventricle. Adjacently, a band of densely clustered cells stretched for another 50–80 μm into the CP. Following the terminology of the Boulder Committee (23), we call this region subventricular zone. At its lateral edge, the subventricular zone fused with the lateral portion of the CP.

BrdUrd Immunohistochemistry. Sections processed for BrdUrd immunohistochemistry revealed that labeling was predominant in the ventricular zone and restricted to an area stretching 50 μm laterally from the wall of the third ventricle. Twelve hours after the injection of BrdUrd ≈10–30% of all ventricular cells were labeled, thus indicating a high mitotic activity in this region.

Autoradiography. In fish that were sacrificed 12 hr after the injection of [^{3}H]dThd, labeling resembled the pattern observed when employing BrdUrd immunohistochemistry. In one fish killed at this time, the location and morphology of 91% of 135 cells labeled and identified corresponded to those of ventricular cells (Fig. 2).

After longer periods of survival, the number of cells labeled in the ventricular zone relative to the total number of labeled cells found in the CP/PPn decreased. This was paralleled by an increase of labeled cells found in the adjacent subventricular zone, until the ratio of labeled cells in the ventricular and subventricular zones reached a constant value after ≈5 days of survival (Fig. 2). The percentage of labeled cells in lateral regions of the CP/PPn (100–500 μm from the ventricle; Fig. 1 *B* and *C*) relative to the total number of labeled cells in this nucleus also showed a survival time-dependent pattern: within the first 24 hr of survival only ≈10% of all labeled cells were found in the lateral CP/PPn. This percentage constantly increased until it reached its maximum, with 36% at 7 days after the injection of [^{3}H]dThd. Even longer periods of survival led to a drop in the relative number of labeled cells in the lateral CP/PPn (28 days, 27%; 49 days, 19%; 60 days, 17%). In a fish sacrificed after 126 days of survival, no ventricular cells were labeled any more, although one labeled glial cell and two labeled neurons were found in lateral regions of the CP/PPn.

The decrease in the relative number of labeled cells in the ventricular zone within the first few days of survival is paralleled by a "dilution" of grains associated with labeled cells. While the number of grains over individual cells after 12 hr of survival was on the order of 100, this number was cut roughly in half every 2 additional days of survival (number of fish examined within the first 7 days, 8), thus suggesting that a "typical" ventricular cell divides, on the average, once within 2 days. This estimate is in agreement with the results achieved by BrdUrd immunohistochemistry and [^{3}H]dThd autoradiography, which indicated that ≈10–30% of all ventricular cells undergo mitosis within 12 hr. A few ventricular cells, however, were quite heavily labeled even after longer periods of survival. This points to the insertion of "wait states" in some cells.

Whereas in fish killed 12 hr after the injection of [^{3}H]dThd only 4% of all labeled cells displayed the morphology of neurons, this percentage gradually increased with longer survival time and reached its maximum, with 58% and 60% in two fish killed 28 days after the injection (Fig. 3). Within these first 28 days, the relative number of neurons among labeled cells in the CP/PPn was positively correlated with survival time (Spearman rank correlation coefficient, r_s = 0.982, $P < 0.001$, two-tailed). The percentage of labeled glial cells, on the other hand, remained rather constant with increasing survival time and was, on the average, 11% among all labeled cells in the CP/PPn. This result, together with the concomitant decrease in the relative number of ventricular cells and the shift of the median distance of labeled cells away from the ventricle toward lateral regions, suggests that the newborn neurons differentiate from other cell types, presumably from ventricular cells. The drop in the relative number

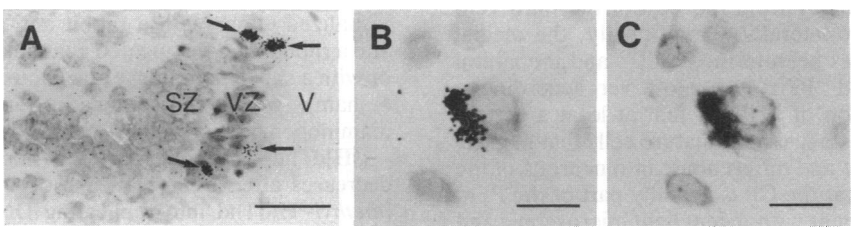

FIG. 1. Labeling in the CP/PPn revealed by [^{3}H]dThd autoradiography. After a survival time of 2 days (*A*), all four labeled cells (arrows) are located in the ventricular zone, next to the ventricle (V). Note morphological differences between cells in the ventricular zone (VZ) and subventricular zone (SZ). Whereas ventricular cells display a slender shape with dark nuclear mass, the subventricular cells are round to oval with light nuclear staining and a high degree of clustering. Forty-nine days after the injection [^{3}H]dThd, a cell located 440 μm laterally from the wall of the ventricle in the CP/PPn is heavily labeled (*B*, focus on grains; *C*, focus on cell). The labeled cell resembles in its appearance the morphology of the large multipolar neurons of the PPn-C. (Bars = 30 μm in *A* and 10 μm in *B* and *C*.)

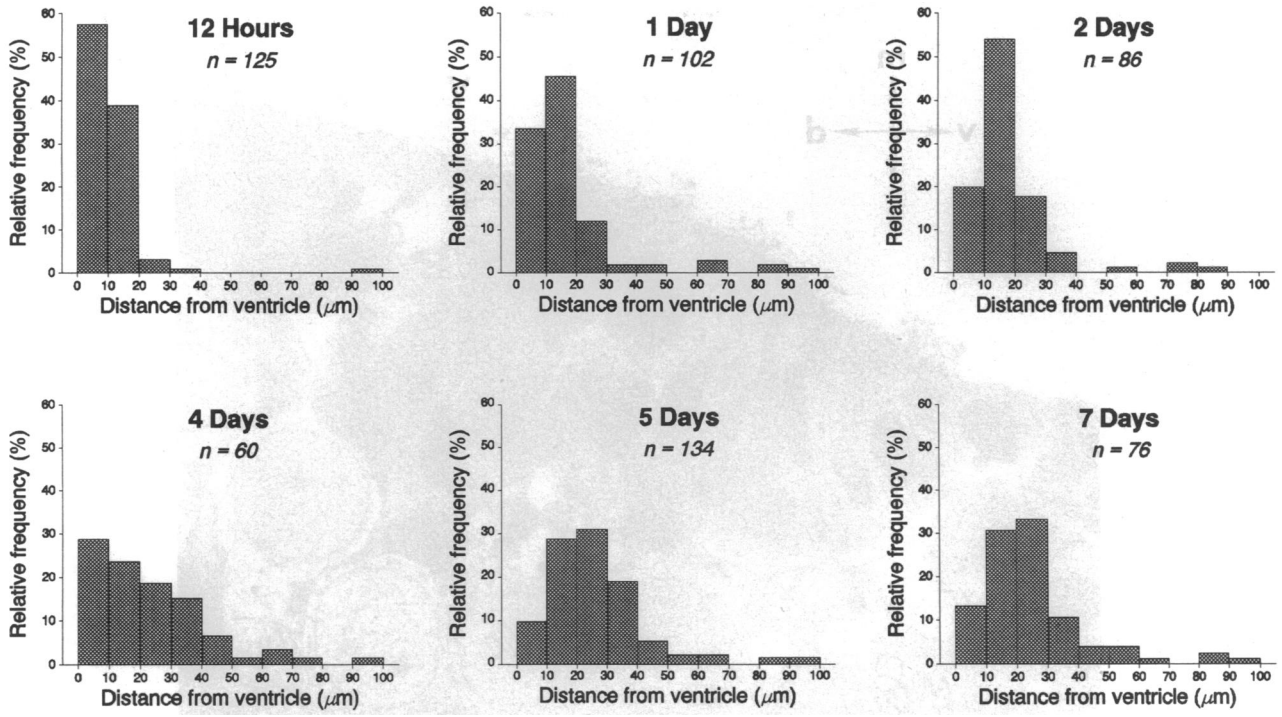

FIG. 2. Distances of [^{3}H]dThd-labeled cells in the medial CP/PPn from the wall of the third ventricle after various survival times. Although the relative number of labeled cells in the ventricular zone (which stretches 10–20 μm away from the ventricle) decreases with longer survival time, the percentage of cells labeled in the adjacent subventricular zone steadily increases. *n*, Total number of ventricular cells, glial cells, and neurons found bilaterally in the region of the CP/PPn.

of neurons by 49 days of survival might indicate that the newly differentiated neurons were either dying or migrating away from the CP/PPn.

Ultrastructure. Also at the electron microscopic level, the ventricular zone could easily be distinguished from the subventricular zone (Fig. 4). The cells in the ventricular zone were densely aggregated. Their cytoplasm displayed a moderately electron-dense matrix with dark mitochondria, and the karyoplasm showed fine grains of high electron density. A characteristic feature was prominent invaginations of the nucleus, which might reflect the high mitotic activity of these cells. In contrast, the nuclei of the cells in the subventricular zone were oval with either no invaginations at all or only little "notches." Karyoplasm and perikaryal cytoplasm were light. Table 1 summarizes some morphological characteristics of the subventricular cells as obtained by a quantitative analysis in three fish and compares these results with the data on laterally located CP/PPn-G neurons from a previous study (15).

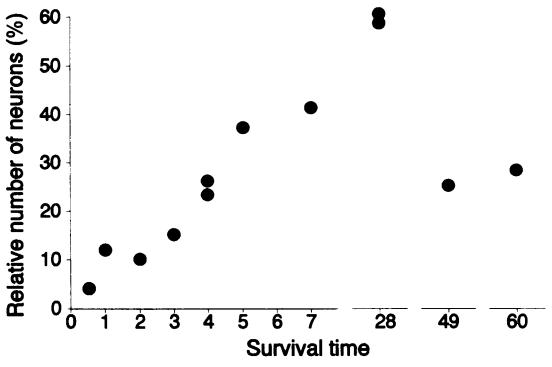

FIG. 3. Relative number of neurons in the CP/PPn, labeled with [^{3}H]dThd after various survival times. The type of cell (ventricular cell, glial cell, or neuron) was identified according to its light microscopic morphology.

Two remarkable characteristics of the cells in the subventricular zone were their dense clustering and the elongated shape of the soma. The elongation was expressed by values between 1.31 and 1.91 of the ratio of major vs. minor axis of the nucleus of these cells (Table 1). The major axes of the cell bodies were aligned in parallel to the line connecting the ventricular zone with the PPn. The elongated shape and the directional arrangement made possible the definition of a medial pole (toward the ventricular zone) and a lateral pole (toward lateral regions of the CP) of the cell bodies. Neurites, if visible, usually originated from the lateral pole of the soma. Organelles were highly concentrated in the lateral half of the cell body, whereas the nucleus was shifted toward the medial pole. Notches, if present, occurred predominantly at the lateral pole of the nucleus.

A few cells in the subventricular zone exhibited signs of mitotic activity. Fig. 5 shows a profile of such a cell, apparently in late telophase. Although the two nuclei are clearly separated, the division of the cytoplasm is incomplete. In one part of the soma the cell membranes have already separated the perikaryal spaces, whereas the cytoplasm in the remaining portion of the cell forms a continuous matrix without any indications of membranous division.

Some cells, located typically near the boundary of ventricular and subventricular zone, were difficult to classify as one of the two cell types described above. Their nuclei, although not perfectly round or oval, displayed much smoother contours than did ventricular cells (Fig. 4). Invaginations were not visible. The density of the karyoplasm was between that of the ventricular and subventricular cells. The cytoplasm was lighter than that of the ventricular cells. We refer to this cell type as "intermediate cell."

A striking feature of the subventricular cells was the complete absence of synaptic input. Although we examined 147 somata with a total profile length of 3257 μm in three fish, we could not find a single chemical synapse making contact with a cell body (Table 1).

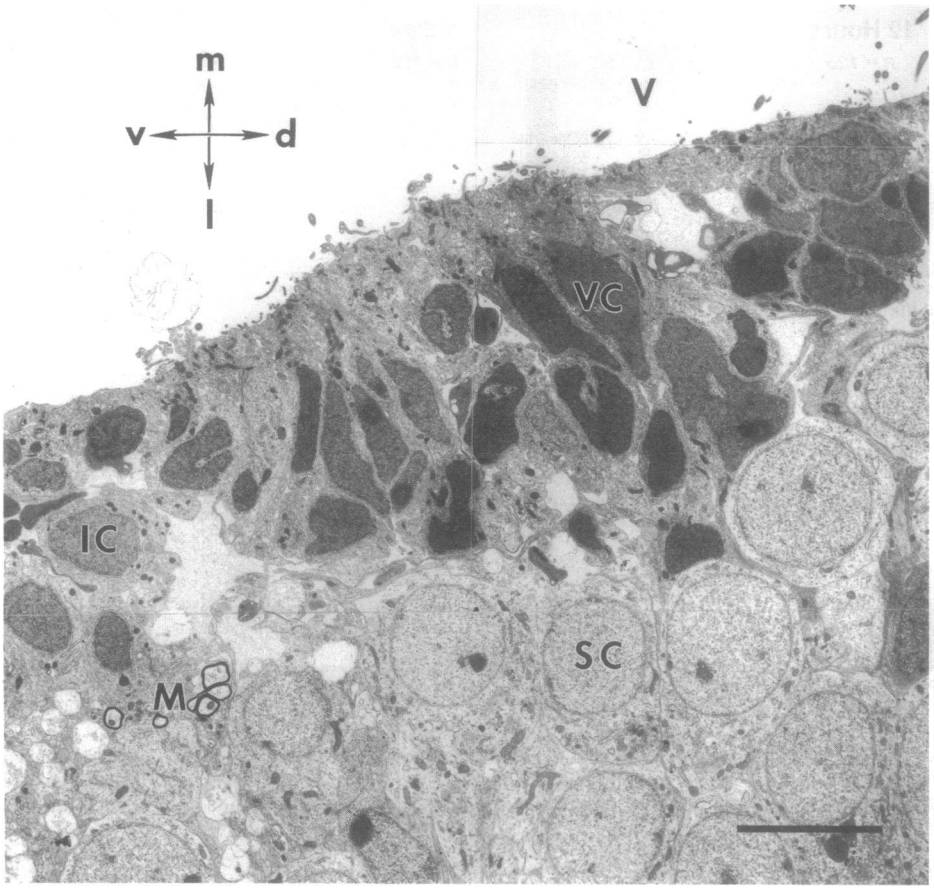

FIG. 4. Electron micrograph of ventricular and subventricular zone of the CP/PPn. Ventricular cells (VC), located at the wall of the third ventricle (V), are characterized by their dark karyoplasm displaying fine granularity and prominent invaginations. In contrast, subventricular cells (SC) have round to oval nuclei with light karyoplasm of high granularity. The vast majority of organelles in the subventricular cells is concentrated at the lateral pole of the perikaryon. Some cells share morphological characteristics of the ventricular and subventricular cells and are, therefore, called "intermediate cells" (IC). Note complete absence of chemical synapses and the low number of myelinated fibers (M) in the ventricular and subventricular zone. d, Dorsal; m, medial; l, lateral; v, ventral. (Bar = 5 μm.)

DISCUSSION

The results of our immunohistochemical and autoradiographic experiments have demonstrated a high degree of labeling in the ventricular zone of the CP/PPn, thus suggesting an enormous mitotic activity of these cells (Figs. 1*A* and 2). This is also indicated by the pronounced invaginations of their nuclei observed in electron micrographs (Fig. 4). Before differentiating, the ventricular cells appear to undergo a number of cell divisions, as demonstrated by the dilution of silver grains associated with [^{3}H]dThd-labeled cells in the time-lapse experiments.

The shift of the median distance of labeled cells into regions situated laterally to the ventricular zone of the CP/PPn demonstrates migration in this direction (Fig. 2). This is paralleled by a change of the morphology of labeled cells from that of ventricular cells to the one found in neurons of the subventricular zone and of the lateral CP/PPn (Figs. 1 *B* and *C* and 3). The intermediate cells (Fig. 4) may represent transitional stages of this development. Although the subventricular cells completely lack synaptic input, their light microscopic and ultrastructural appearance clearly corresponds to the morphology of neurons. This notion is supported by our failure to find morphological differences be-

Table 1. Morphological characteristics of cells in the subventricular zone of the CP

	Eigenmannia no.					
	100		147		185	
Morphological parameter	SV cells (n = 54)	CP/PPn-G cells (n = 36)	SV cells (n = 29)	CP/PPn-G cells (n = 39)	SV cells (n = 64)	CP/PPn-G cells (n = 50)
Profile area of soma, μm^2	29	34	21	39	29	30
Ratio of perikaryal vs. nuclear profile area	1.35	1.17	1.11	1.13	1.06	0.92
Ratio of lengths of major vs. minor axis of nucleus	1.31	1.43	1.43	1.31	1.91	1.85
Number of synapses found (total length of somatic profile evaluated in μm)	0 (1183)	2 (858)	0 (525)	3 (970)	0 (1549)	0 (1171)

Results were obtained by evaluating electron micrographs of ultrathin transverse sections through the CP. The values are expressed as mean for n somatic profiles in each fish. Values of CP/PPn-G cells are included for comparison and were taken from ref. 15. The difference in profile area of soma between subventricular (SV) cells and cells of the CP/PPn-G in *Eigenmannia* no. 147 is significant at $P < 0.001$ (Mann–Whitney U test, two-tailed). All other differences within individual pairs are not significant at $P < 0.05$.

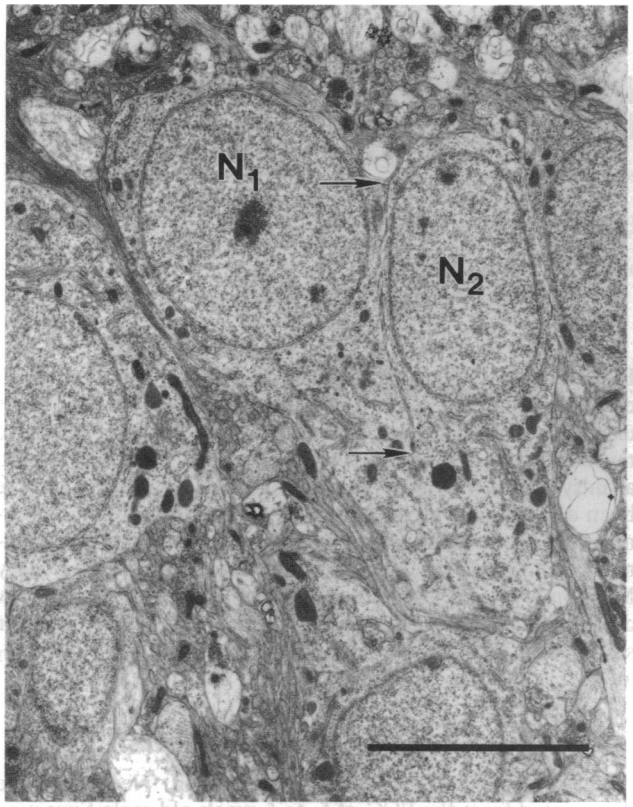

FIG. 5. Subventricular cell, apparently in late telophase. The cellular lumen is clearly separated into two parts by the cell membranes (arrows) in the region of the nuclei (N_1 and N_2), but in the remainder of the soma the cytoplasm forms a continuous matrix. (Bar = 5 μm.)

tween subventricular cells and neurons of the lateral CP and PPn-G (see Table 1).

The hypothesis of migration of the young neurons from the subventricular zone toward lateral regions of the CP/PPn also receives strong support from the ultrastructural data. The elongated shape of the cell bodies in the CP, their alignment in medio-lateral direction (i.e., the direction in which our autoradiographic results indicate migration of cells), the polarized distribution of organelles in the perikaryon and of notches on the nucleus, and the origination of neurites from the lateral pole are typical features of migrating neurons, as observed during embryonic development by *in vivo* and *in vitro* studies (24–26). The modest elongation of the CP cell bodies compared to migrating cells in other systems during embryogenesis suggests, however, a rather low speed of migration. Chemical synapses, which are absent on cell bodies in the subventricular zone, may be added in the course of migration. This is suggested by a comparison of the synaptic densities in different regions of the CP/PPn: whereas somata of neurons in the lateral CP and medial portion of the PPn, the PPn-G, have on the average <1 synapse per 100-μm profile length, cell bodies of the lateral part, the PPn-C, are contacted by ≈10 synapses per 100-μm profile length (15, 21).

The maturation of the newborn neurons in the course of their lateral migration may also include the acquisition of specific immunological characteristics. Part of the CP/PPn cells reacts immunopositive for the neuropeptide somatostatin (27). Although somatostatin immunoreactivity can be found in cells throughout the CP/PPn (except in the ventricular zone), labeling for somatostatin mRNA is present only in the lateral region of this complex (28). This discrepancy may be explained by a possible maturation of the labeled cells: in medially located cells, the amount of mRNA is still too low to be detected, although the presence of the peptide indicates the existence of gene expression for somatostatin. From medial to lateral cells, however, the number of mRNA copies in a cell gradually increases, so that, finally, somatostatin mRNA *and* the corresponding peptide can be found.

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