

MORPHOMETRIC ANALYSIS OF THE CEREBELLAR PURKINJE CELL IN THE DEVELOPING NORMAL AND HYPOTHYROID CHICK

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Abstract—A morphometric analysis of Purkinje cells in the developing cerebellar cortex of the chick was performed in normal animals and embryos made hypothyroid by one or two spaced injections of tetramethylthiourea. Profiles of 162 Purkinje cells, from Golgi–Cox treated sections were analysed. Soma area, perimeter and circularity index, cumulative length of the dendrites and number of dendritic bifurcations were studied. The results showed significant differences between control and hypothyroid animals. There were no important differences between birds rendered transiently hypothyroid with a single injection and those made chronically hypothyroid with dual injections. This confirms that the Purkinje cell is very dependent on thyroid hormone especially during the early phases of its morphogenesis. The development of the Purkinje cell was the most affected process of cerebellar cortex maturation in the thyroid-deficient chick. The dendritic arborization was particularly hypoplastic. Moreover, a dynamic balance appeared to exist between the development of the dendritic arborization and that of the perikaryon.

Key words: Cerebellar Purkinje cell, Development, Hypothyroidism, Morphometry.

A great number of studies have been devoted to the cerebellum: many of them deal with the mammals, but few with birds. At the beginning of the century, Ramon y Cajal (1904) expressed the view that the consistent character of the neuronal organization of the cerebellum in different species made the cerebellar circuit almost a 'biological law'. Since then, the cerebellar morphology, histogenesis and synaptogenesis^{1,3,6,8,9,14,17,18,20–22,24,25} have been studied in great detail. In birds as in mammals, a transient external germinative layer produces the granule cells, which migrate through the maturing molecular and Purkinje cell layers to reach their final location in the internal granular layer. Concomitantly, the Purkinje cell body emits thin dendritic processes towards the cerebellar surface, which are progressively arranged as a planar dendritic tree. Later stages of Purkinje cell development are characterized mainly by peripheral growth of the branches formed earlier and especially by the increase in the number of their dendritic spines.

The morphogenetic action of thyroid hormones during the CNS development is not yet sufficiently well understood in mammals: acquisition, migration and maturation of neurons are influenced by thyroid hormones, as is also glial development.

In the developing cerebellum, hypothyroidism alters severely and irreversibly the growth and branching of the Purkinje cell dendrites. By the same time, the acquisition of the granule cells is markedly delayed and the length of their axon, the parallel fiber, is irreversibly decreased. The final result is a dramatic effect on the organization and the complexity of the cerebellar neuronal networks.^{2,10,11,13–16,23}

In the present paper, we report the effects of hypothyroidism on the chick cerebellum. Development of the cerebellar cortex and especially of the Purkinje cell dendritic arborization and soma is described in experimental animals treated with tetramethylthiourea and compared to normal development.

EXPERIMENTAL PROCEDURES

Animals

All observations and measurements were made on chick cerebellar cortex (Wyandotte × Rhode Island Red). Embryos (from 16 to 21 days) and young chicks (from hatching to day 6, i.e. from 21

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to 27 days of absolute age) were decapitated and the cerebellum removed. Hatching in treated animals was delayed by 3 days.

Induction of hypothyroidism

Tetramethylthiourea (TMTU) was administered in Tyrode's solution (0.1 ml of solution containing 2 mg of TMTU) by dripping it into the chorioallantoic membrane.²⁶ Two groups of animals were examined. The first one, referred to as the transitory hypothyroid group in the following text, was given one single dose of TMTU on day 8 of incubation: this was sufficient to maintain hypothyroidism in the embryos until day 21 (analysis of Dr M. Jallageas, see Ref. 26). The second group, referred to as the chronic hypothyroid group, was given TMTU on days 8 and 19: this maintains a level of plasma thyroxine of 1.9 ± 0.2 ng/ml up to day 27 vs a level of 7.7 ± 0.4 ng/ml in controls (analysis of Dr M. Jallageas, see Ref. 27).

Histology

For the observation of the cerebellar cortex, the cerebellum was fixed in Bouin-Hollande's or Carnoy's solution and embedded in paraffin. Mid-sagittal sections (5 μ m thick) were stained with hemalun-eosine or Cresyl Violet.

The morphometric study of the Purkinje cells (PCs) was carried out on Golgi-Cox preparations of the cerebellum. Mid-sagittal sections (150 μ m thick) were used to draw the PCs.

Morphometric analysis of the Purkinje cell

PCs (162) were drawn, with a camera lucida, from the Golgi-Cox sections of the cerebellum. A computerized digitizing tablet was used for the morphometric analysis. Acquisition of the contours of the soma and the segments of the dendritic arborization permitted calculation of the following parameters: the area of the projected image of the soma, which we term here the soma surface area, the perimeter of the image of the soma projection (termed soma perimeter), the circularity index of the soma, the cumulative length of the dendrites and the number of the bifurcations of the dendritic tree. The circularity index of the soma was obtained by means of the formula: $Ci = 1000 \times 4S/P^2$, where S is the area and P the perimeter. This index expressed the complexity of the contour of the soma. This factor approaches a value of 1000 for circular contours. Its value decreases when the number and length of perisomatic processes increase.

Univariate statistics. For each parameter we calculated the arithmetic mean, standard deviation and standard error of the mean. These measures from the different experimental groups were compared using Student's *t*-test and the Fisher-Snedecor's *F*-test.

Multifactorial statistics. Principal Component Analysis (PCA) was used to give a graphic representation of the results and also to bring emphasis on the differences between the control animals and the treated animals. This permitted determination of which parameters were discriminant. The mathematical principle^{7,12} consists of searching for the eigenvalues of the space defined by the five measured parameters. The eigenvalues are linear combinations of the five parameters and define new factorial axes along which the data points are most scattered. The limits of the control and treated populations corresponding to the different stages have been represented by concentration ellipses (Fig. 5): the location of one Purkinje cell from one stage has a probability of 95% of being found in the corresponding ellipse.

RESULTS

Normal development of the cerebellar cortex

The cerebellar cortex at day 16 is composed of four layers: the external germinative layer, the molecular layer, the Purkinje cell layer and the internal granular layer, which is the final location of the cells migrating from the germinative layer. PCs are immature, with a cell body irregularly shaped and an excentric nucleus (Fig. 1A). On day 18, the molecular layer thickness is increased. The PC bodies are aligned at the surface of the internal granular layer, which contains progressively increasing number of cells (Fig. 1C). PCs have thin perisomatic processes and a growing dendrite arborization (Fig. 3A). At day 21, when the chicks hatch, the external germinative layer still exists. The PC soma has acquired its definitive structure. The migration of the cells

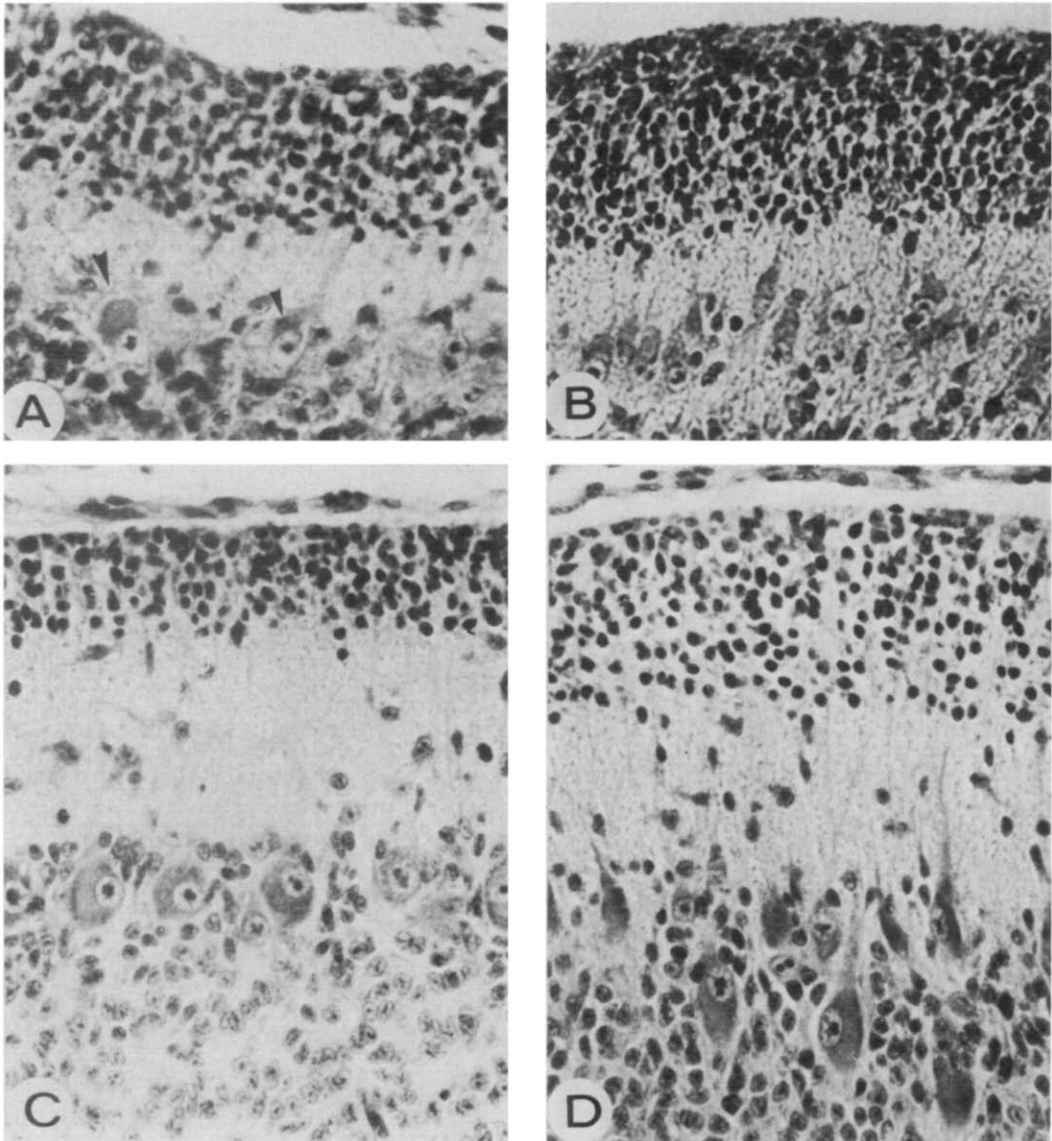


Fig. 1. Mid-sagittal sections of cerebellar cortex. (A) Control embryo at day 16, (B) hypothyroid animals. In both normal and hypothyroid animals, the differentiation of the PCs (arrows) and the migration of the internal granular cells have begun. The external germinal layer is thick. (C) Control embryo at day 18. The migration of the cells of the external granular layer is progressing, the molecular layer is thickening and the PCs are aligned at the surface of the internal granular layer. (D) Hypothyroid chick. The external germinal layer is relatively thicker. On the contrary, the molecular layer is less well developed. The PCs have not yet aligned and some appear profoundly sunken in the internal granular layer. Hemalun-eosin staining. Bar = 20 μ m.

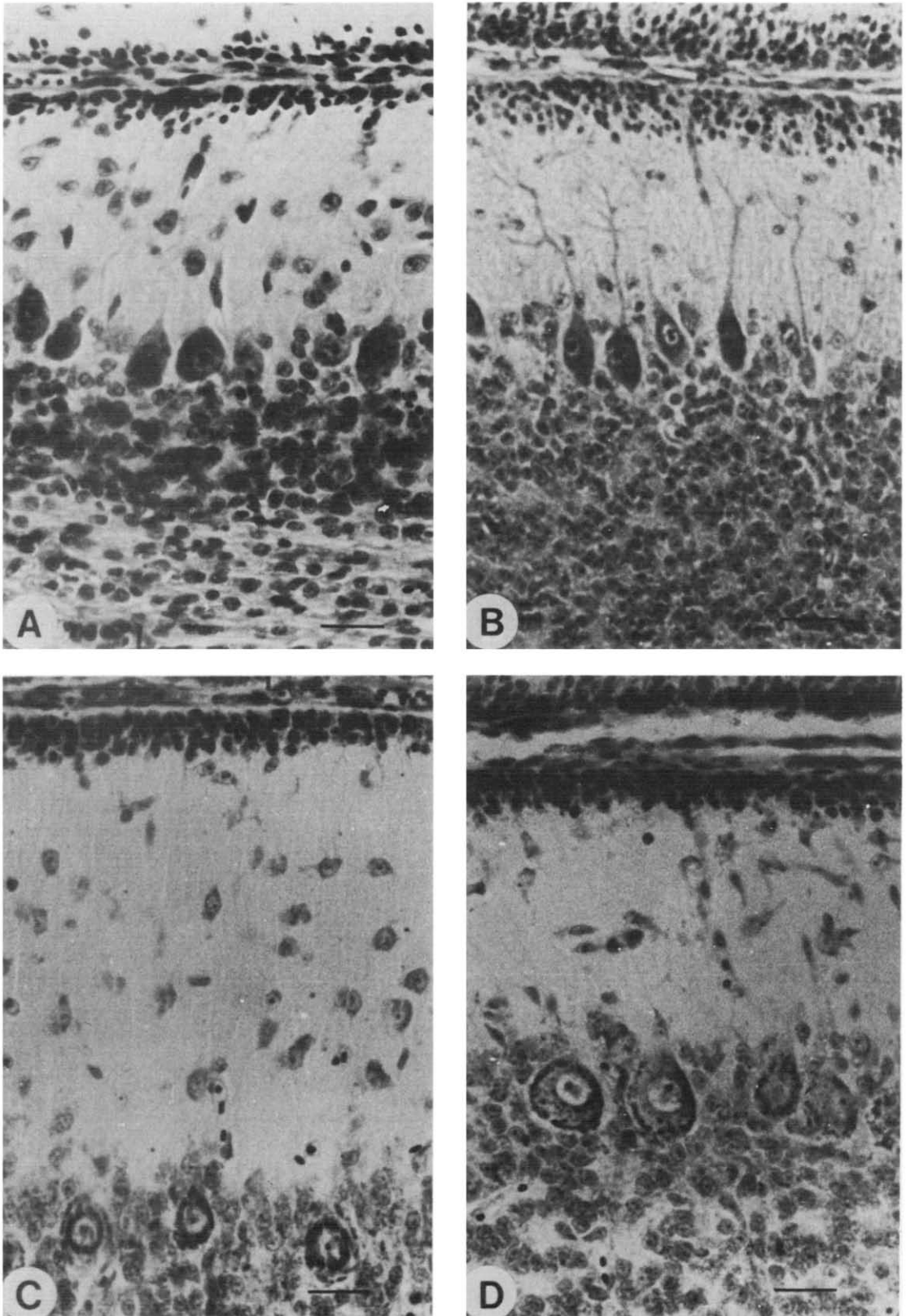


Fig. 2. Mid-sagittal sections of cerebellar cortex. (A) Control embryo at day 21. The external germinative layer is thinner than earlier. (B) Transitory hypothyroid embryo. The migration of the granular cells is less advanced, and the molecular layer is thinner. (C) Control chick at day 24. The molecular layer is very thick and the surface area of the PC soma is reduced. (D) Transitory hypothyroid animal. The molecular layer is thinner and the soma surface area is significantly larger than in controls. Hemalun-eosin (A) and (B), and Cresyl Violet (C) and (D). Bar = 20 μm .

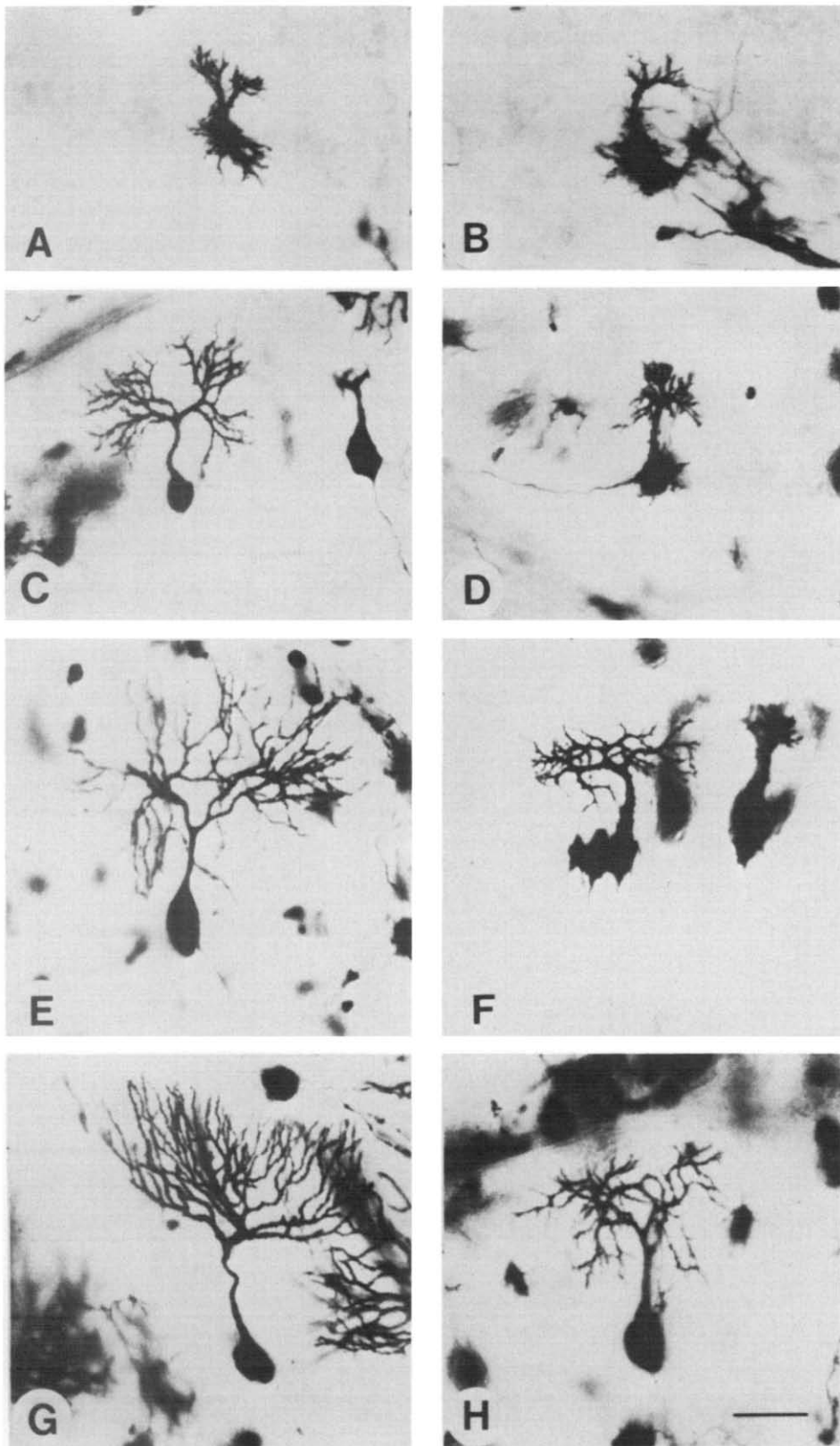


Fig. 3. Purkinje cells seen in mid-sagittal (150 μm thick) sections. Golgi-Cox impregnation. Bar = 55 μm . (A) Control chick at day 18. (B) Hypothyroid chick at day 18, dendritic arborization less developed. (C) Control chick at day 21, the soma is smooth and bears a thin descending axon on one end, a long dendrite which branches to form a fan-shaped dendritic tree at the other end. (D) Transitory hypothyroid animal at day 21: the soma still bears numerous projections and the dendritic tree is hypoplastic. (E) Control chick at day 24, the dendritic tree is large. (F) Transitory hypothyroid animal: the dendritic tree is deficient and the transient perisomatic processes are not completely retracted. (G) Control chick at day 27, numerous branches have grown in the dendritic tree. (H) Transitory hypothyroid animal: the dendritic tree is now markedly hypoplastic, the soma is smooth, as in normal chick.

of the external layer is nearing completion. The molecular layer is thickening and is invested by PC dendrites which are organized in a regular pattern. Between 18 and 21 days the soma of the PCs acquires a more mature feature (Figs 2A and 3E). Opposite the axon, a long primary dendrite emerges. This bears a large fan-shaped arborization of thorned dendrites. The length of this cell, without the axon, reaches 200 μm (Fig. 3E). Between 21 and 24 days, the structure of the cerebellar cortex is not modified: the external granular layer consists of a few persistent cells, and the PC dendrites arise in the molecular layer which is thickening (Figs 2A, C and 3C, E). At day 27 the PC has the same silhouette as before but the arborization is now more abundant because of the outgrowth of the dendrites (Fig. 3G). The external germinative layer disappears almost completely from day 34.

Development of hypothyroid cerebellar cortex and Purkinje cells

At day 16 the cerebellar cortex of hypothyroid birds presents a histological structure not very different from that of the controls (Fig. 1B). At day 18, the external granular layer is thicker than in normal animals whereas the molecular layer is thinner. The PCs are not aligned and some are deep-set in the internal granular layer (Fig. 1D). As in normal animals, they bear thin extensions all over their surfaces (Fig. 3B). The perisomatic spines persist at day 21 (Fig. 2B), the hypoplasia of the dendritic arborization becoming particularly marked, as is the delayed disappearance of the perisomatic processes (Fig. 3D). At days 24 and 27, the very strong hypoplasia of the arborization in comparison with controls persists (Fig. 3H, H). The differences between transitory and chronic hypothyroid animals are small. The morphometric analysis confirms this impression (Fig. 4).

Morphometric analysis of the Purkinje cell

Univariate statistical analysis (Fig. 4).

Soma surface area (Fig. 4A). This parameter is decreased at day 24 in both experimental and control animals but less in the latter. This is visible in the micrographs of Fig. 2A and C.

Soma perimeter (Fig. 4B). Between days 18 and 21 the length of the soma perimeter decreases rapidly by a 3/4 factor. In treated birds this process is delayed slightly until day 24, after which the values are nearly the same as in controls.

Circularity index (Fig. 4C). The value of this parameter increases rapidly between days 18 and 21, as the soma loses its thin extensions. In the treated animals this evolution is delayed, and even at day 27 the values for the treated animals are significantly lower than those of the controls.

Cumulative dendritic length (Fig. 4D). In controls, the growth of the dendritic length is dramatic, especially between days 18 and 21, while in treated birds the dendrites grow more slowly and do not reach normal values even at day 27.

Number of bifurcations (Fig. 4E). A large increase occurs between days 18 and 21. In treated animals, a delay is noticeable, but by day 27 the values are nearly the same for all groups.

Principal Components Analysis (Fig. 5). To summarize the results a multivariate statistical method was used. Principle Component Analysis (PCA) gives us a synthetic view of the relations between the control and the experimental groups, taking into account all the parameters.

The interpretation of the PCA is enhanced by using a graphical representation of the factorial planes, that is, the two-dimensional surfaces in the space defined by the parameters which provide maximum resolution of the data points. The factorial plane shown in Fig. 5a keeps 76% of the total of information contained within the initial multidimensional space defined by the five morphological parameters.

Table 1. Purkinje cells studied at the different stages of development
(drawn from 3 to 6 animals per stage)

Day of development*	18 Co	18 Hy	21 Co	21 Hy	24 Co	24 tHy	24 Hy	27 Co	27 tHy	27 Hy
Number of PCs	15	12	16	12	24	12	18	23	18	12

* Co, control; Hy, chronic hypothyroid; tHy, transitory hypothyroid.

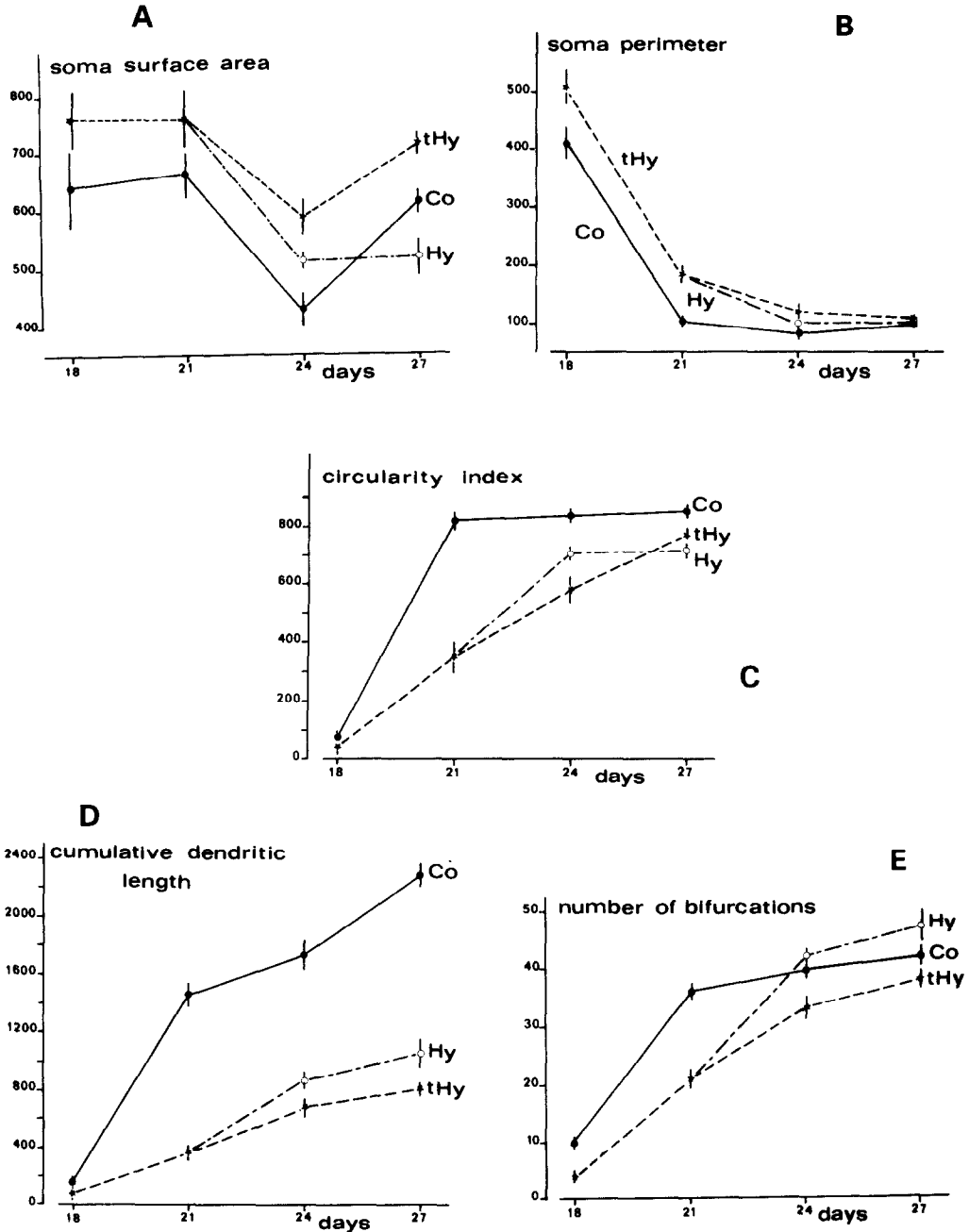


Fig. 4. Graphs of five parameters of Purkinje cell morphology during development. Each parameter is plotted from day 18 to day 27 from control (Co), transient (tHy) and chronic (Hy) hypothyroid animals. Hatching occurred at day 21 in controls and at day 24 in treated animals. (A) Surface area of the projected soma (μm^2). There are no significant differences between any of the groups on days 18, 21 and 24. On day 27 the transient hypothyroid animals have a significantly ($P < 0.01$) larger area than both the controls and the chronic hypothyroid animals. (B) Perimeter of the soma projection (μm). On days 21 and 24, the control animals have a significantly ($P < 0.01$) smaller perimeter than the treated animals. By day 27 there is no significant difference between the controls and the chronic hypothyroid chicks, but the transient hypothyroid animals still have a significantly ($P < 0.01$) larger soma perimeter. (C) Circularity index of the soma projection. Larger values indicate smoother profiles. At all stages the controls have a significantly ($P < 0.01$) higher value than the treated animals, which do not differ significantly between themselves. (D) Cumulative length of the dendrites (μm). At all stages the controls have a significantly ($P < 0.01$) greater dendritic length than the treated animals, which do not differ significantly between themselves. (E) Number of bifurcations in the dendritic tree. At days 18 and 21 the value for the control animals is significantly ($P < 0.01$) larger than for the treated birds. By day 24 there are no significant differences between any of the groups, but on day 27 the chronic animals have a significantly ($P < 0.01$) higher number of branches than the transient hypothyroid chicks. Each point represents 15–35 observations. The vertical bars show the standard error of the mean.

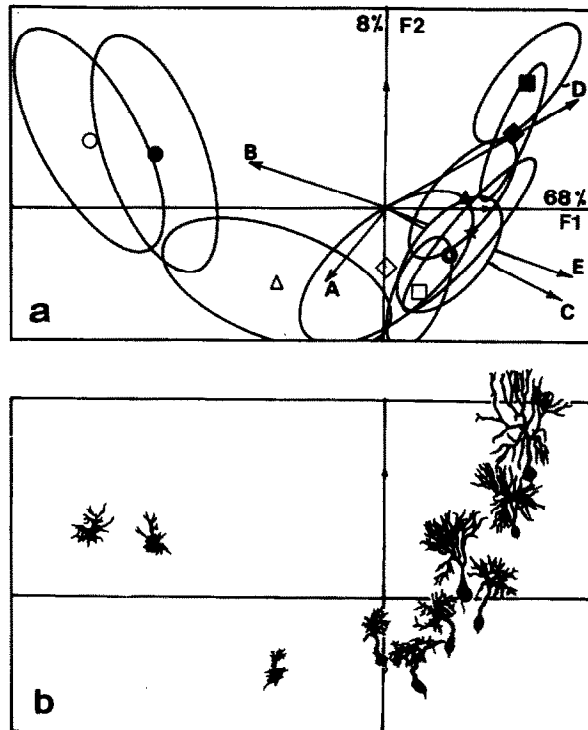


Fig. 5. Factorial plane resulting from Principal Component Analysis of the morphological features of Purkinje cells. The major (horizontal) axis of plane contains 68% of the information in the five-dimensional space defined by the parameters used. The second (vertical) axis contains 8% of the information. The parameter vectors are: A, soma surface area; B, soma perimeter; C, circularity index; D, cumulative dendritic length, and E, number of bifurcations. Graph (a) shows centers of gravity and 95% concentration ellipses for the 10 groups of animals. Symbols are: ●, ▲, ◆, ■, control animals at days 18, 21, 24 and 27, respectively; ○ and △ treated at days 18 and 21; ◇ and □ tHy at days 24 and 27; ⊙ and * Hy at days 24 and 27. In (b) we have placed the concentration ellipses of each group with a drawing of the most characteristic profile taken from the corresponding group.

This graph should be read relative to the amplitude and the direction of the parameter vectors (labelled A–E). It appears that the first factorial axis (horizontal, summarizing 68% of the information) tends to oppose the pair of parameters composed of the soma surface areas (A) and the soma perimeter (B) against the triplet of parameters composed of the circularity index (C), the cumulative dendritic length (D) and the number of bifurcations (E). The projections of the centers of gravity of each group of data points on the first factorial axis are ordered with respect to their developmental stage, independently of any experimental treatment. Accordingly, the first factorial axis can be interpreted as a maturational axis.

All of the tendencies observed in Figs 1, 2 and 3 are clearly exhibited by the PCA. In control groups between days 18 and 21 a burst of maturation occurs, during which the soma perimeter is reduced by a factor 4 and the circularity index is multiplied eight-fold. These modifications indicate that one aspect of the maturational process of the PC is the loss of roughness of the soma contour as it becomes more regular. During the same period the cumulative dendritic length and the number of bifurcations increase.

DISCUSSION

Cerebellar cortex maturation in the chick appears the same as in the rat.^{13,15,21} Thyroid hormones seem to play a similar role in both species. In its absence there is a delay in granule cell formation in the external granular cells, the molecular layer is thinner, and some PCs remain sunken in the internal granular layer.

Prior to day 18, i.e. 8 days after the onset of thyroid function in the chick, there are no significant differences between treated and control animals. The explosive differentiation of the PC in the chick takes place between days 18 and 21, i.e. during the 3 days that precede hatching. At

hatching, the PC has acquired its characteristic outline. In the rat, this morphogenesis occurs mainly during the first postnatal week, when the thyroid function grows. Although the size of these cells and their dendritic trees are the same in chick and rat, the dendritic branches appear longer in chicks while the number of bifurcations appears greater in rats. However in hypothyroid birds as in mammals, the PCs display the same developmental disturbances. The primary dendrite is long and there is a severe hypoplasia of the dendritic arborization. The number of bifurcations does not seem to be affected very much, the most important deficiency being in the length of the dendrites. As already described in the rat,^{1,8,19} the PC soma of the chick presents cytoplasmic extensions early in development which later disappear. In hypothyroid chicks as in rats,^{2,5} the retraction of these perisomatic processes is delayed, but by day 27 the soma assumes the normal form. In birds as in mammals, thyroid hormones influence both the growth and the retraction of the neurites very likely through their effects on the microtubular apparatus,^{4,15} and protein synthesis.

In both the transitory and chronic hypothyroid groups, the cumulative dendritic length is always less than the normal value, while the number of bifurcations, lowered in a first step, reached normal values by days 24 and 27. The second injection of TMTU at day 19 of incubation did not further modify the response of the PC to thyroid deficiency. This emphasizes the severity of the effects of early hypothyroidism. In birds as in mammals, the PC is more sensitive to thyroid hormone in the first steps of its development than later on.

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