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Estimates of volumes and pyramidal cell numbers in the prelimbic subarea of the prefrontal cortex in experimental hypothyroid rats

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INTRODUCTION

Thyroid hormone deficiency exerts an influence of paramount importance on the development and maturation of numerous cell types and different body structures (for review see Hamburgh, Lynn & Weiss, 1968; Gilbert & Frieden, 1981). The nervous tissue has been found to be highly vulnerable to this condition, as it can be ascertained from the abundant literature on the subject. The study of the structural alterations induced by thyroid hormone manipulations during brain development provides an additional tool for the understanding of brain neurogenesis (Lauder, 1979) as well as for the establishment of functional correlations with the morphological findings that result from this process (Eayrs, 1971; Hamburgh *et al.* 1977). In fact, it has been demonstrated that any disturbance of the normal balance of thyroid hormone levels interferes with the entire process of neuronal formation, involving therefore cell proliferation, migration and maturation; moreover, the resulting structural changes can easily be discerned (for review see Eayrs, 1971; Bass & Young, 1973; Legrand, 1982–3; Legrand, 1984).

From a quantitative morphological point of view, the process of acquisition of the cerebellar granule cells of hypothyroid rats has attracted the attention of numerous research workers (Nicholson & Altman, 1972; Lewis, Patel, Johnson & Balázs, 1976; Lauder, 1977). The peculiar mode of neurogenesis displayed by cerebellar granule cells makes them a convenient model for postnatal developmental studies as they are formed during the first three postnatal weeks from a secondary germinative matrix and then migrate to their final location where the complex maturation process occurs (Altman, 1969). Although the study of the cerebellar granule cell neurogenesis in hypothyroid rats has made it possible to evaluate the effects of thyroid hormone deficiency at all stages of neurogenesis, there still remains some controversy over the end result of this deficiency upon the number of these cells (for review see Legrand, 1982–3). By applying morphometric procedures to 30 days old hypothyroid rats we have been able to clarify this subject. An unequivocal decrease in the volume of the cerebellar granule cell layer as well as of the total number of granule cells was found (Madeira, Paula-Barbosa, Cadete-Leite & Tavares, 1988*a*).

It is almost forty years since Eayrs and his co-workers (Eayrs & Taylor, 1951; Eayrs, 1955; Eayrs & Horn, 1955; Horn, 1955), in an attempt to determine the morphological background for the marked behavioural changes displayed by hypothyroid animals, pioneered the histological study of the developing cerebral neocortex in this condition.

However, they restricted their observations to the sensorimotor and visual cortices, whose neuropil changes reflect the changes in the neuronal processes. As regards the number of neurons, Horn (1955) evaluated the number of pyramidal cells per unit surface area of visual cortex and found that in cortical layers III–V the neurons from hypothyroid rats displayed a greater packing density when compared to controls.

In spite of these encouraging results, which were supported at the biochemical level (Patel, Rabié, Lewis & Balázs, 1976), and of the advances in morphometric techniques, as far as we know detailed determinations of the cerebral cortex volumes and concomitant estimations of the total number of cortical neurons have not been performed in hypothyroid rats. However, such studies, if carried out in the same cortical area, could contribute to a better understanding of the structural organisation of the cerebral cortex and, in the present conditions, could provide an additional clue to the explanation of the functional and behavioural alterations observed in cretinism.

Taking all this into account we decided to address this issue and, to do so, we studied the prelimbic subdivision of the medial prefrontal cortex (Krettek & Price, 1977; Van Eden & Uylings, 1985*a*) from 30 days old hypothyroid rats using recent stereological techniques and compared the results with those obtained in age- and sex-matched controls. Several factors determined the selection of this cortical area: (*a*) its involvement in cognitive tasks, which are deeply affected in hypothyroid states (Kolb, 1984); (*b*) its sharp boundaries and easily-recognisable layers, which permit a precise volumetric determination (Krettek & Price, 1977; Van Eden & Uylings, 1985*a*); (*c*) the existence of sound previous studies of its volumetric development, which enable us to verify the consistency of our data (Van Eden & Uylings, 1985*b*); (*d*) the evidence of sexual dimorphism in some subareas of the prefrontal cortex (Van Eden, Uylings & Van Pelt, 1984). The latter was taken into consideration as, in our previous studies in the cerebellum and hippocampus (Madeira *et al.* 1988*a*, *b*), we found sex-related dimorphic differences in control rats that were undetectable in the hypothyroid groups.

MATERIALS AND METHODS

Animals and treatment

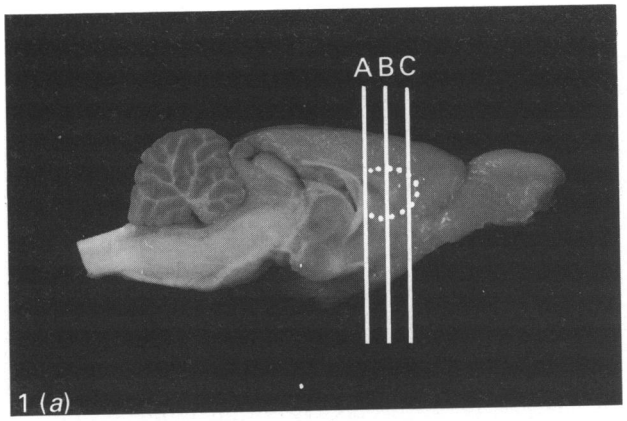
Sprague–Dawley rats from the colony of the Gulbenkian Institute of Science (Oeiras) were used. On the day of birth (Day 0) the litters were standardised to eight (4 males and 4 females); the groups studied were made up of 6 animals from 5 different litters. Rats were rendered hypothyroid by a subcutaneous daily injection of propylthiouracil (PTU) from Day 0 up to Day 30: 0·05 ml 0·2% PTU on Days 0–10, 0·1 ml 0·2% PTU on Days 11–20 and 0·1 ml 0·4% PTU on Days 21–30 (Nicholson & Altman, 1972). Animals were considered to be hypothyroid as a result of histological characteristics of the thyroid gland and of blood assays of T4 (Pharmacia 100).

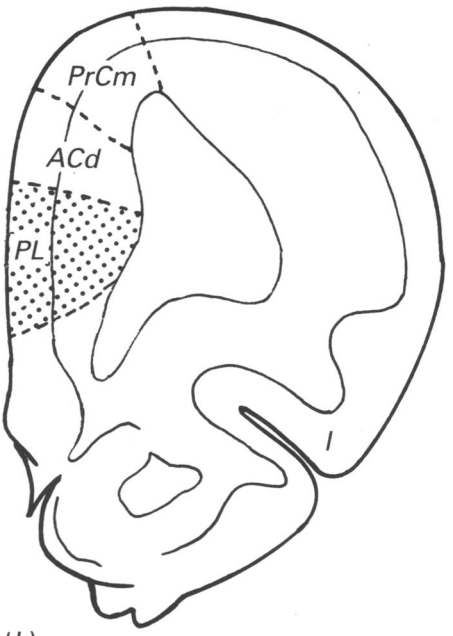
Sex- and age-matched control animals were also obtained from 5 different litters of the same size and injected daily with 0·1 ml of physiological saline.

General procedures

At Day 30 the animals were anaesthetised with ether. After collecting 1 ml of blood from the heart for T4 determination, they were transcardially perfused with a solution of 1% paraformaldehyde/1% glutaraldehyde in 0·12 M phosphate buffer at pH 7·2. The brains were removed, weighed and immersed for 2 hours in the perfusion solution.

Cell counts, volume estimations and shrinkage factor determinations were made in each group of male and female hypothyroid and control rats.





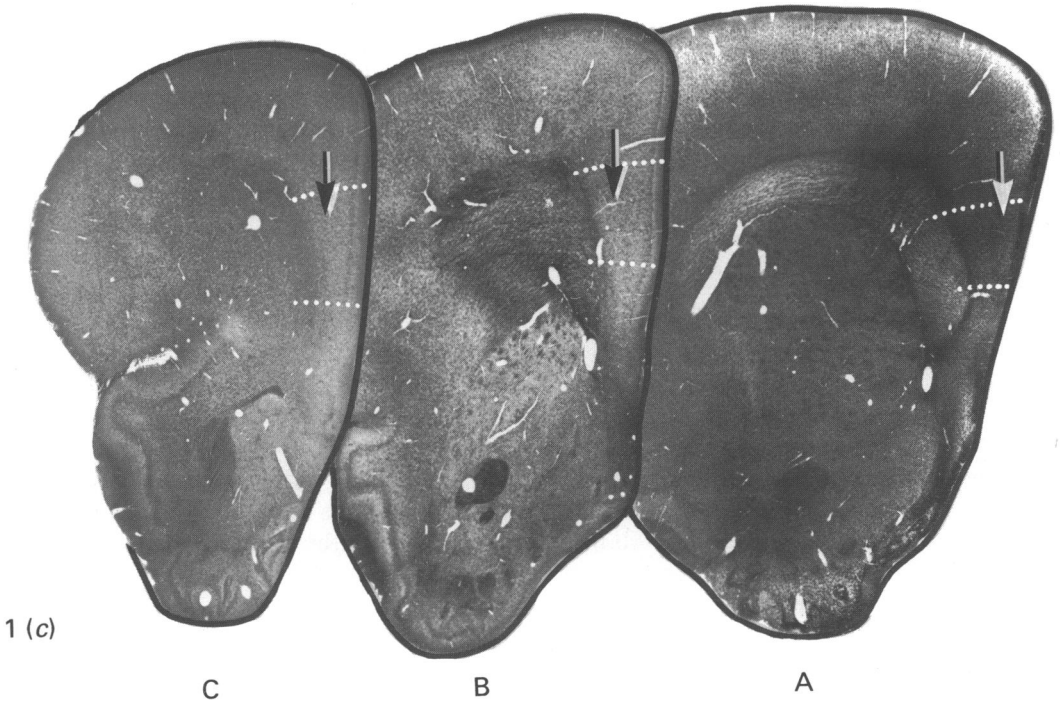


Fig. 1 (*a–c*). (*a*) Photograph of the medial surface of the left hemisphere of the rat brain. The prelimbic subarea of the medial prefrontal cortex is delineated by a dotted semicircle. Vertical lines (A, B, C) indicate the location from which the sections present in (*c*) were obtained. ×2·5. (*b*) Schematic drawing of a rat brain coronal section through the frontal part of its right hemisphere, from a section close to line B. The medial precentral area (*PrCm*), the dorsal anterior cingulate area (*ACd*) and the prelimbic area (*PL*) of the medial prefrontal cortex are diagrammatically represented. (*c*) Photographic montage of three coronal sections of the rat brain right hemisphere, whose location is indicated by lines drawn in (*a*). The structural rostrocaudal features of the PL cortex can be seen. The boundaries of the PL cortex are shown; the arrows indicate its Layer III. Cresyl violet. ×14.

Characterisation of the prelimbic cortex and its Layer III

Our studies were performed in a specific subarea of the agranular medial prefrontal cortex – the prelimbic area (Fig. 1*a*). According to Krettek & Price (1977) and Van Eden & Uylings (1985*a*) three subareas can be identified in the medial prefrontal cortex: the medial precentral area, the dorsal anterior cingulate area and the prelimbic area (Fig. 1*b*). These areas can be discriminated on the basis of the connections established with the subnuclei of the mediodorsal nucleus of the thalamus and also from their cytoarchitectonic organisation. In the prelimbic area (Fig. 1*c*) the cellular lamination is more marked than in the two adjacent areas, the dorsal anterior cingulate and the infralimbic. Layer I has virtually no cells; Layer II consists of densely-packed neurons; Layer III is large and contains loosely-packed, lightly stained cells; Layer V is composed of three sublayers, the middle one containing large round cells; Layer VI is bilaminar and contains tightly-packed cells with their long axes arranged tangentially to the pial surface (Fig. 1*c*). Since the prelimbic cortex is of the agranular type, i.e. it lacks Layer IV, most of the fibres of the thalamic projection end in Layer III. Thus, we centred our observations on this layer. As Layer III is not clearly delimited, it is possible that small parts of the adjacent Layers II and V may have been included in some delineations. However, as this occurs both in controls and in hypothyroid animals (Fig. 2*a*, *c*), there is no reason to assume that this might have interfered with our results. Yet, it must be recognised that this handicap is a hindrance to the unbiased estimation of cortical layer volume.

Correction for tissue shrinkage

After immersion of the brains in the perfusion solution for two hours, the right hemispheres were coronally sectioned just rostral to the optic chiasma. The first slice of the caudal part of the hemisphere was first obtained on a Vibratome and photographed. The rostral part of the same hemisphere was embedded in celloidin as for volume determinations; the first cross-sectional celloidin section obtained on a sliding microtome was stained with cresyl violet and photographed. In these photographs the entire surface areas (A_v – vibratome section area and A_c – celloidin section area) were determined with the aid of a MOP-Videoplan.

The shrinkage factor (SF_v) was calculated according to Van Eden & Uylings (1985*b*) by applying the formula,

$$SF_v = \frac{cA_v^{\frac{3}{2}}}{cA_c^{\frac{3}{2}}} = \frac{V_v}{V_c},$$

in which c is the shape-dependent constant and V_v and V_c are the forebrain volumes in Vibratome and celloidin sections, respectively. For an unbiased determination of the cortical volume it would be necessary to calculate the cortical shrinkage factor instead of that of the forebrain, as it was made. This would also render the formula employed completely adequate, as it was developed with the assumption that the shrinkage of the tissue under evaluation is isometric and homogeneous. For technical reasons the determination of the SF_v of the prelimbic cortex was a task impossible to achieve. Thus, the methodology advanced by Van Eden & Uylings (1985*b*) was followed.

Volume of the prelimbic area and its Layer III

For volumetric determinations, the right hemispheres of 6 animals per group were celloidin-embedded; 54 μm serial celloidin sections were obtained with a sliding

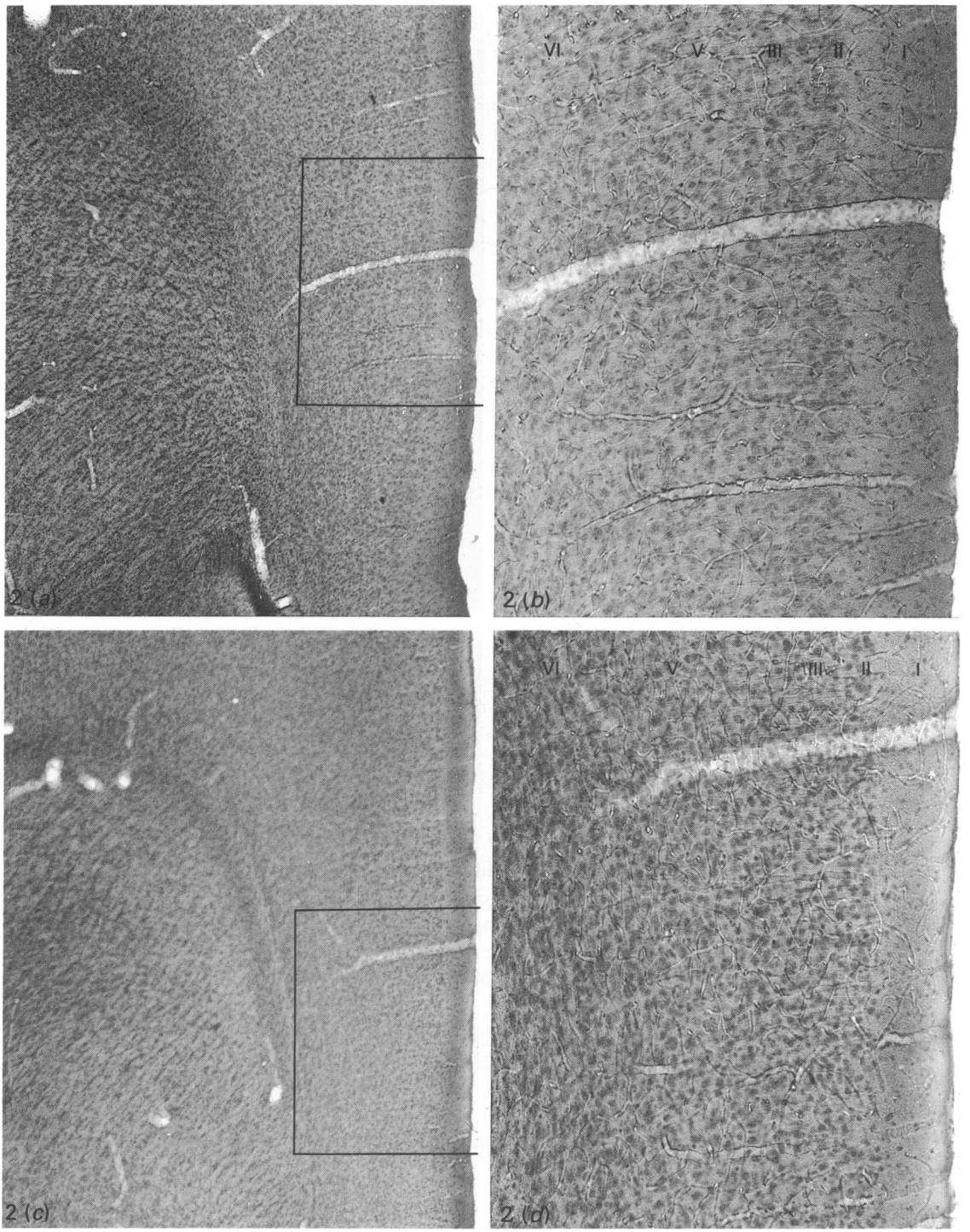


Fig. 2 (*a–d*). Photographs of the prelimbic cortices from a control (*a*) and a hypothyroid rat (*c*). Cresyl violet. × 52. On the right side of the Figure the photographs (*b*) and (*d*) represent higher magnifications of the insets. × 130. No striking differences can be detected between controls and hypothyroids. In both cases Layer III is easily recognised in spite of the lack of sharp boundaries with the adjacent layers.

microtome and stained with cresyl violet. The average thickness of the sections was calculated with an oil-immersion objective (40×). As it is difficult to determine the boundaries of the rostral part of the different subareas of the medial prefrontal cortex on the basis of their cytoarchitecture (Van Eden & Uylings, 1985*a*), volumetric determinations were restricted to the medial prefrontal cortex caudal to the rostral part of the forceps minor of the corpus callosum. For that reason, the choice of the first rostral section for volumetric measurements was based on the characteristics of the underlying white matter (Fig. 1*c*). The selection was blindly made by two researchers. The opinion of a third was requested when the choice made by the first two did not fit. It must be stressed, however, that according to the Cavalieri principle (Gundersen, 1986), of which the present formula is a modification, the choice of the referred section should have been made at random. Every second section was drawn with the aid of a camera lucida at ×30 magnification. At the level of the corpus callosum all the sections were drawn, as the shape of the subareas differ considerably from section to section. In each section, the boundaries of the prelimbic cortex and limits of its layers were determined by microscopic observation, using the criteria described by Krettek & Price (1977) and Van Eden & Uylings (1985*a*).

The areas of the prelimbic cortex and its Layer III were measured with the aid of a MOP-Videoplan. Volumes were obtained according to the formula described by Van Eden & Uylings (1985*b*),

$$V = SF_v \sum_{i=1}^{n-1} \frac{A_i + A_{i+1}}{2} d_i,$$

in which A_i is the surface area of the ith section, n is the number of sections measured and d_i is the distance between surface areas A_i and A_{i+1}. This distance is calculated from the mean thickness of the sections and the number of the sections skipped between successively measured surfaces. Volumes were subsequently corrected for tissue shrinkage.

Numerical density of Layer III pyramidal cells

The numerical density of the pyramidal cells was calculated in the right hemisphere and the disector method applied (Sterio, 1984). As the cerebral cortex is, for morphometric purposes, an inhomogeneous specimen, small blocks from the entire prefrontal cortex including its prelimbic area were obtained (Gundersen, 1986). The blocks, after being osmicated in a solution of 2% osmium tetroxide in 0·12 M phosphate buffer for two hours, were stained with uranyl acetate, dehydrated in ethanols and Epon-embedded. For quantification, three blocks were chosen at random. After trimming the blocks containing the prelimbic cortex, semithin sections were obtained perpendicularly to the pial surface and stained with toluidine blue. In this way, serial sections were obtained uniformly random in the tissue volume, which is necessary to estimate the numerical density of pyramidal cells using the disector (Sterio, 1984; Gundersen, 1986). From each block, 4 groups of 3 consecutive 2 μm thick semithin sections were obtained. The exact section thickness was ascertained after re-embedding 10 similar sections from different blocks and cutting 5 cross-sections of each at the same thickness (Lima & Coimbra, 1983). Three measurements were made in each cross-sectioned section and the mean thickness of the 150 measurements was found to be 2·03 ± 0·17 μm. Photographs of the same area were taken of the sections at a magnification of ×345. A total of 16 disectors per animal was made.

Diameter of pyramidal cell nuclear profiles

For the determination of the mean diameter of the nuclear profiles of Layer III pyramidal cells of the prelimbic area, an average of 240 nuclei per animal was drawn at a magnification of ×345 and the diameter calculated with the aid of a MOP-Videoplan.

Total number of pyramidal cells from Layer III prelimbic area

The total number of pyramidal cells was obtained by multiplying their numerical density by the volume of the prelimbic cortex Layer III. It must be stressed, however, that an unbiased estimation would only be achieved if the referred parameters could have been calculated in the same hemisphere and processed in the same way. For technical reasons this was not practicable. For comparative purposes, the numbers obtained can be considered reliable and informative.

Statistical analysis

A two-way analysis of variance (ANOVA) interacting the effects between PTU treatment and the sex of the animals was carried out on data from control and treated rats, with repeated measurements. The remainder mean square was used as the error term.

The non-parametric two-tailed Mann–Whitney U-test for two independent samples was applied.

Differences were considered significant if $P < 0{\cdot}05$.

RESULTS

Animal weights and hormonal determinations

At Day 30 the body weights of the hypothyroid groups were significantly reduced when compared with the respective controls. A significant difference was found between the body weights of male and female control groups (Fig. 3). The ANOVA showed significant differences in the body weight dependent on both the sex ($F_{1,89} = 5{\cdot}44$, $P < 0{\cdot}05$) and the thyroid hormone levels ($F_{1,89} = 483{\cdot}5$, $P < 0{\cdot}001$).

The brain weights were significantly reduced in hypothyroid groups. In the female control group the brain weight was significantly smaller when compared with that of the male control group (Fig. 4). The ANOVA showed significant differences in the brain weight dependent on both the sex ($F_{1,89} = 6{\cdot}97$, $P < 0{\cdot}001$), and the thyroid hormone levels ($F_{1,89} = 148{\cdot}7$, $P < 0{\cdot}001$).

Serum T4 concentrations were markedly reduced in hypothyroid rats (Fig. 5).

Qualitative observations

No changes could be detected in the prelimbic area of the medial prefrontal cortex of hypothyroid rats (Figs. 1*c*, 2*a–d*).

Volumes of the prelimbic area and its Layer III

The volume of the entire prelimbic area was significantly reduced in both male and female hypothyroid groups when compared with the respective controls (Fig. 6). The volume of the prelimbic area Layer III was smaller in both hypothyroid groups when compared with the respective controls, although no significant differences could be found (Fig. 7).

In males, the shrinkage factor calculated was 1·7 for controls and 1·9 for hypothyroid rats; in females, 1·7 for both control and hypothyroid rats.

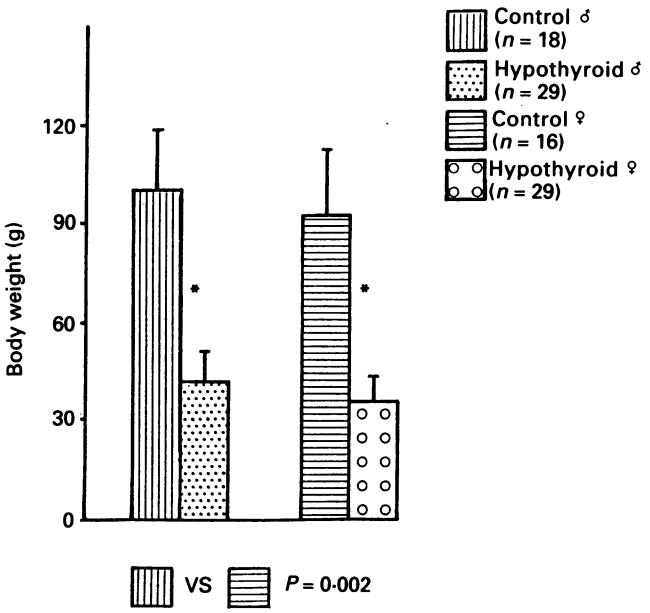


Fig. 3. Graphic representation of the mean body weights at the end of the experiment – Day 30. Columns represent means and vertical bars one standard deviation (S.D.). n, number of animals studied. * $P = 0{\cdot}002$.

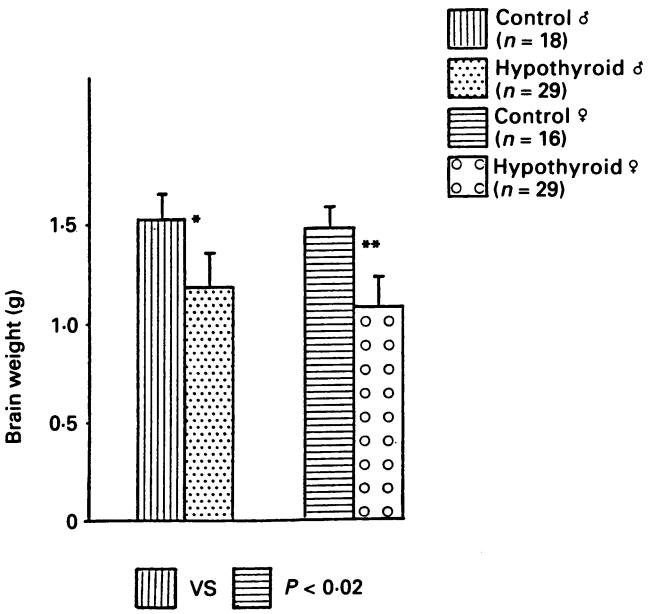


Fig. 4. Graphic representation of the mean brain weights at Day 30. Columns represent means and vertical bars one S.D. * $P < 0{\cdot}02$; ** $P = 0{\cdot}002$.

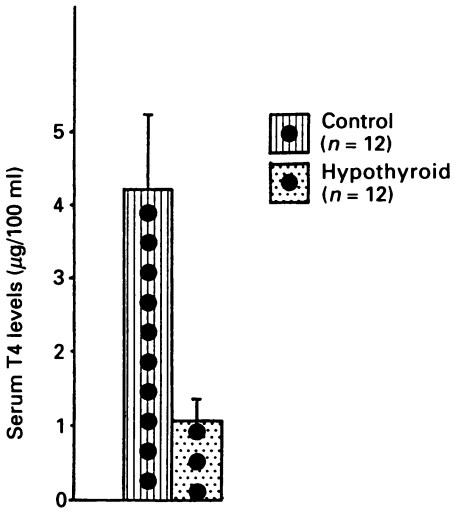


Fig. 5. Graphic representation of the mean serum T4 concentrations at Day 30. Columns represent means and vertical bars one S.D. Reference value: T4 – 5·0/9·4 μg/100 ml.

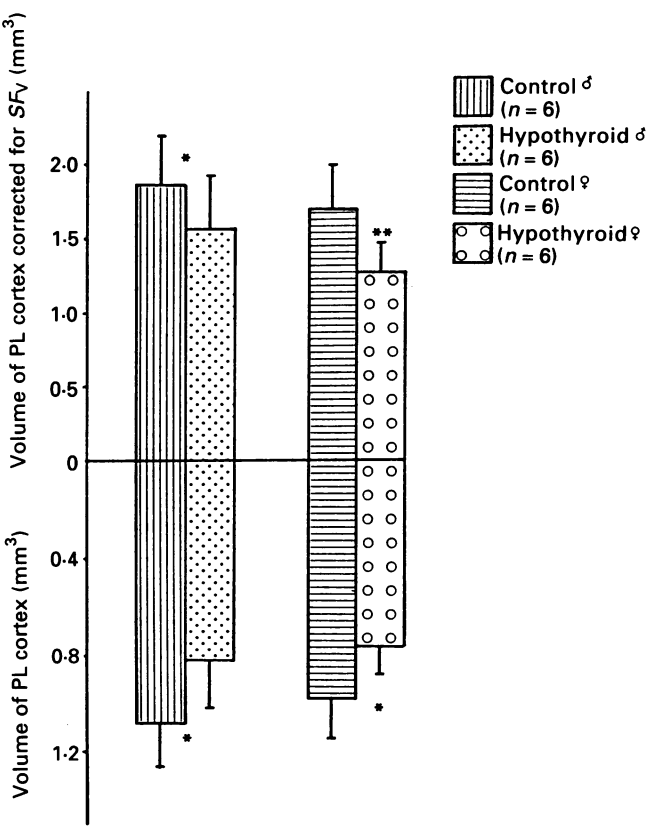


Fig. 6. Graphic representation of the mean volumes of the prelimbic area (PL) of the medial prefrontal cortex, before and after correction for the shrinkage factor (SF_v). Columns represent means and vertical bars one S.D. * $P = 0{\cdot}05$; ** $P < 0{\cdot}02$.

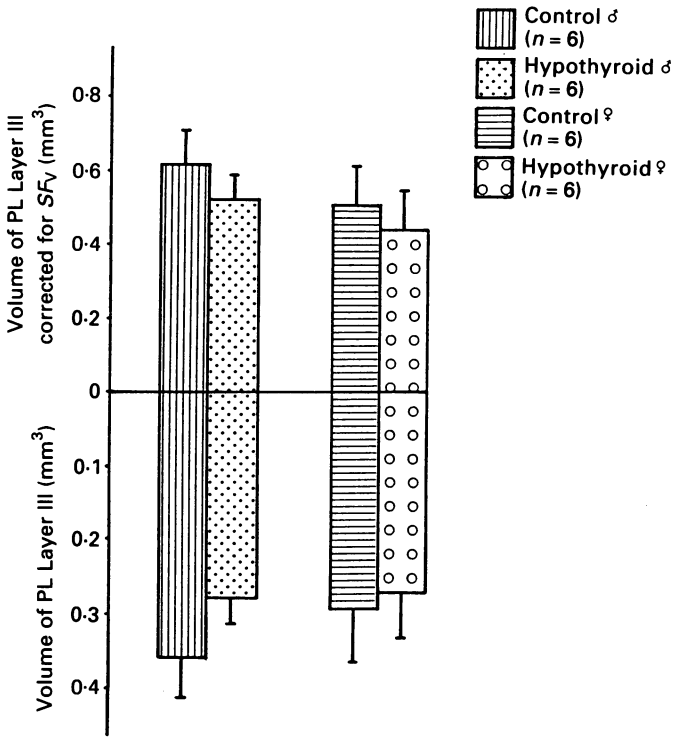


Fig. 7. Graphic representation of the mean volumes of the prelimbic area Layer III, before and after correction for the shrinkage factor (SF_v). Columns represent means and vertical bars one S.D.

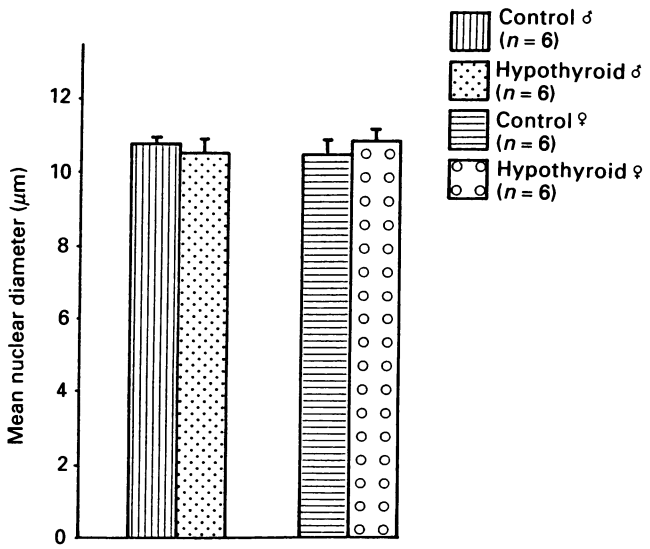


Fig. 8. Graphic representation of the mean nuclear diameters of the pyramidal cells. Columns represent means and vertical bars one S.D.

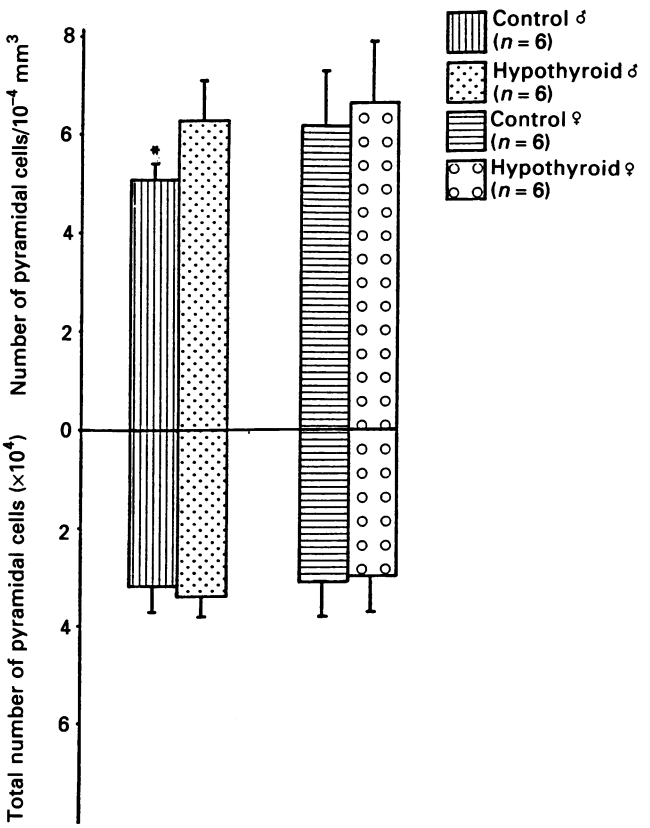


Fig. 9. Graphic representation of the number of pyramidal cells per unit volume of the prelimbic area Layer III and its total number. Columns represent means and vertical bars one S.D. * $P = 0{\cdot}002$.

The ANOVA showed significant effect of hypothyroidism in the volumes of the entire prelimbic cortex ($F_{1,21} = 7{\cdot}63$, $P < 0{\cdot}05$), whereas with regard to Layer III volume no such effect could be found ($F_{1,21} = 3{\cdot}37$, P not significant).

Nuclear diameter of pyramidal cells

No differences were found in the mean nuclear diameters of the Layer III pyramidal cells (Fig. 8).

Numerical density of pyramidal cells

A significant increase in the number of pyramidal cells per unit volume was found in the male hypothyroid group when compared with the respective control group. In females, the numerical density of pyramidal cells was also increased in the hypothyroid group when compared with the respective control group, but this difference was not significant (Fig. 9).

The ANOVA showed significant effect of hypothyroidism in the numerical density of pyramidal cells ($F_{1,21} = 4{\cdot}48$, $P < 0{\cdot}05$). No effect of the sex was found ($F_{1,21} = 3{\cdot}38$, P not significant).

Total number of Layer III pyramidal cells

No significant differences in the total number of pyramidal cells were detected between hypothyroid rats and controls in either male or female groups (Fig. 9).

Unlike the conditions that have been found in the cerebellum and hippocampus (Madeira *et al.* 1988*a*, *b*) no sexual dimorphic differences were found either between male and female controls or male and female hypothyroid animals.

DISCUSSION

It is commonly accepted that hypothyroid-induced cerebral cortex structural disturbances and the inherent behavioural alterations are dependent on the impoverishment of the neuronal processes rather than on a numerical decrease in the neuronal population (Eayrs, 1955, 1961; Horn, 1955; Lewis *et al.* 1976). This indicates that for the neurons of the prelimbic area, as in other zones of the cerebral cortex, the neurogenic phase that is mainly affected by the lack of thyroid hormones is the process of maturation (Eayrs, 1955; Horn, 1955), as opposed to the neurogenesis of cerebellar and hippocampal granule cells where neuronal proliferation and migration are also deeply disturbed (Lauder, 1977; Rami, Rabié & Patel, 1986; Rami, Patel & Rabié, 1986; Madeira *et al.* 1988*a*, *b*).

The cortical dendritic and axonal alterations observed in hypothyroid rats were brilliantly described by Eayrs and his collaborators (Eayrs & Taylor, 1951; Eayrs, 1955; Eayrs & Horn, 1955) in a series of investigations mainly performed in the sensorimotor area. By applying quantitative methods to the study of neuronal processes, including a modification of the Sholl method for dendrites, it was found that the axonal plexus and dendritic trees of hypothyroid rats were less exuberant than those of euthyroids, and it was suggested that thyroid hormones play an important role in cortical maturation (Eayrs, 1955). This assessment was later corroborated by other workers in different cortical areas: marked dendritic changes in the auditory and visual cortices were described in detail by Ruiz-Marcos *et al.* (1979, 1983), whereas Cragg (1970) had previously reported, in the latter cortical area, a reduction of the number of synapses per neuron.

Conversely to the results obtained with the quantitative analysis of neuronal processes, these authors did not pay a great deal of attention to the quantification of neurons themselves; it was found however, that in hypothyroid animals there were more pyramidal cells per unit surface area of cerebral cortex than in respective controls (Eayrs & Taylor, 1951; Horn, 1955). This finding was linked with a neuropil impoverishment and interpreted as a change in neuronal packing density. However, it was suggested by Horn (1955) that numerical differences between control and hypothyroid rats were unlikely to occur. This assumption was corroborated by Patel *et al.* (1976) through the evaluation of the DNA content in the forebrain of hypothyroid and control rats which was found to be similar in both groups.

The estimation of the number of cells on the basis either of the DNA content of a given area or on the evaluation of the number of neurons per unit surface can lend a bias to the interpretation of the results, as these methods are not very informative (Rami, Rabié & Patel, 1986; Swaab & Uylings, 1987). This is because of the presence of polyploidy and nuclear abnormalities in the CNS of hypothyroid rats in the former method (Balázs *et al.* 1971) and of the importance of the orientation, size, distribution and variation in shape of particles under study in the latter (Uylings, Van Eden & Verwer, 1984). To overcome these handicaps it is advisable to determine the total number of cells of a reference area. This is a time-consuming process which requires the determination of the volume of the area under investigation, its correction for the tissue shrinkage factor and the estimation of the number of cells per unit volume of the same zone (see Materials and Methods).

The employment of this methodology in the cerebellum and hippocampal formation of hypothyroid animals allowed us (Madeira *et al.* 1988*a*, *b*) to obtain detailed quantitative information regarding the total number of the respective granule cells. These results encouraged us to extend these investigations to the prelimbic subarea of the medial prefrontal cortex, closely related from a functional point of view, to the limbic structures (Kolb, 1984). As this cortical area is of the agranular type, cell counts were made in its Layer III, where the thalamic afferents end; these are known to be markedly reduced in hypothyroid animals (Eayrs, 1955).

For the selection of this cortical area we also took into consideration the well-established roles that it displays in behavioural tasks, known to be deeply affected in hypothyroids (Kolb & Nonneman, 1978; Kolb, 1984), and its delayed neurogenesis when compared with the rest of the cerebral cortex (Van Eden & Uylings, 1985*a*, *b*). In fact, its Layers II and III start forming by postnatal Day 6 and only reach a full differentiation by Day 18 (Van Eden & Uylings, 1985*b*). In addition, it is known that it is also only by Day 6 that fibres coming from the mediodorsal nucleus (which form the main afferent system to this area) reach the cortical upper plate, in which the prelimbic cortex Layer III is then developing (Van Eden, 1986). Consequently, the effects of hypothyroidism upon cell migration and maturation are obviously felt for longer periods in this cortical area than in the remaining neocortex.

As it is commonly accepted that disturbance of the maturation process mainly affects the growth capability of the neurites (Lauder, 1978), it is likely that in hypothyroid rats the dendritic arborisations of the prelimbic area Layer III will be still more affected than those in other cortical areas; likewise, under these circumstances, the axonal ending of the thalamic afferents in Layer III will probably be severely delayed. These assumptions allow one to infer that both the processes of cortical lamination which depend on the arrival of the cortical afferents (Lund & Mustari, 1977; Van Eden, 1986) as well as the formation of the cortical axonal networks (Van Eden, 1986) can be deeply affected in the prelimbic area of hypothyroid rats.

The volume of the prelimbic cortex observed in euthyroid groups, which is in agreement with that obtained by Van Eden & Uylings (1985*b*) in normal rats, is greater than that found in hypothyroids. It must be noted, however, that the Layer III volumetric reduction was found to be smaller than that observed for the entire prelimbic cortex. This is difficult to explain, given that there is abundant neuropil in this layer and, as previously mentioned, the neuronal processes are the structures particularly affected in hypothyroidism.

As opposed to the volumetric determinations, the number of the Layer III neurons per unit volume in hypothyroid rats is greater than that of the respective controls. As the total number of cells of Layer III is obtained multiplying the volume by the respective numerical density, it was not surprising to find that the total number of neurons did not differ when hypothyroid rats were compared to controls. By applying stereological methods we were able to confirm the existence of a greater packing density in the prelimbic cortex, similar to that described in the earlier investigations using coarser quantitative methods for the sensorimotor (Eayrs & Taylor, 1951) and visual cortices (Horn, 1955). Besides, it is most likely that the increased packing density may depend almost exclusively on the reduced amount of the neuropil, rather than on changes in cell size, as is strongly suggested by the absence of karyometric differences between control and hypothyroid rats.

The maintenance, within normal values, of the number of neurons in different areas of the cortex of hypothyroid rats, as opposed to that which happens in the cerebellum (Nicholson & Altman, 1972; Madeira *et al.* 1988*a*), hippocampus (Rami *et al.* 1986;

Madeira *et al.* 1988*b*) and olfactory bulb (Legrand, 1982–3), leads us to admit that there are regional variations in neuronal vulnerability due to the lack of thyroid hormones. This is most probably related to the neurogenic pattern displayed by different cell populations. In the cerebral neocortex, maturation is likely to be affected by the lack of thyroid hormones, as opposed to what happens with the replication and migration that occur before birth or early postnatally. It is now established that changes in the neuritic maturation observed in hypothyroid rats are basically dependent on cytoskeletal alterations, namely of the neuronal filaments and microtubules (Faivre, Legrand & Rabié, 1985; Faivre-Sarrailh & Rabié, 1988). Such changes could be explained either by a reduction in the polymerisation rate of the actin (Faivre-Sarrailh & Rabié, 1988) or by alterations in the microtubular-associated proteins (Benjamin, Cambray-Deakin & Burgoyne, 1988). Conversely, the components of the extracellular matrix, which could also interfere with neuritic growth, seem to be unaffected (Normand, Clos, Vitiello & Gombos, 1989; Normand, Vitiello, Clos & Gombos, 1989).

Under normal conditions, some subareas of the prefrontal cortex, namely its orbital part, display sexual dimorphism during their development (Van Eden *et al.* 1984). In previous studies we have shown that dimorphic changes observed in the number of granule cells of the cerebellum and hippocampus of euthyroid animals could no longer be found in the hypothyroid groups (Madeira *et al.* 1988*a*, *b*). Thus we carried out this investigation separately for males and females in an attempt to obtain more information on this matter. Sex differences were not found in the prelimbic cortex either between controls or hypothyroids.

A final remark is advisable regarding the experimental model used. The study of the interrelationship between thyroid deficiency and brain development requires one to take into consideration at least two uncontrolled variables, which must be faced as associated factors of thyroid deficiency-induced alterations. The first is the undernourishment displayed by hypothyroid rats which, by itself, induces neurogenic changes and subsequent structural alterations that we still cannot differentiate from those seen in hypothyroid rats (Eayrs & Horn, 1955; Paula-Barbosa, Madeira, Porto-Carrero & Camões, 1989). Furthermore, close attention must be given to the effects of hypothyroidism upon the morphology and function of several endocrine glands (Dumm, Cortizo & Gagliardino, 1985), as low levels of insulin, prolactin and adrenal steroids, also known to interfere with brain development, have been described in hypothyroid rats. It would thus be wiser to admit that the changes observed in the brain of hypothyroid rats are most probably induced by the lack of thyroid hormones and subsequently aggravated by related neonatal abnormalities.

SUMMARY

In previous quantitative studies we demonstrated that the volumes of the cerebellar and hippocampal granular layers, as well as their total number of cells, were reduced in 30 days old hypothyroid rats. We decided to extend these studies to the prelimbic subarea of the medial prefrontal cortex using the same morphometric procedures. The cortical volume and the total number of neurons of its Layer III were determined. After correcting for the tissue shrinkage factor, it was found that the volume of the entire prelimbic cortex and that of its Layer III was smaller in hypothyroid rats than in controls. Conversely, the number of neurons per unit volume of cortical Layer III was greater in hypothyroids. This indicates that there is a markedly increased cell packing density, probably related to the neuropil impoverishment, as was described by

other authors almost forty years ago for the sensorimotor and visual cortices, applying inaccurate quantitative methods.

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REFERENCES

ALTMAN, J. (1969). Autoradiographic and histological studies of postnatal neurogenesis. III. Dating the time of production and onset of differentiation of cerebellar microneurons in the rat. *Journal of Comparative Neurology* **136**, 269–294.

BALÁZS, R., KOVÁCS, S., COCKS, W. A., JOHNSON, A. L. & EAYRS, J. T. (1971). Effect of thyroid hormone on the biochemical maturation of rat brain: postnatal cell formation. *Brain Research* **25**, 555–570.

BASS, N. H. & YOUNG, E. (1973). Effects of hypothyroidism on the differentiation of neurons and glia in developing rat cerebrum. *Journal of the Neurological Sciences* **18**, 155–173.

BENJAMIN, S., CAMBRAY-DEAKIN, M. A. & BURGOYNE, R. D. (1988). Effect of hypothyroidism on the expression of three microtubule-associated proteins (1A, 1B and 2) in developing rat cerebellum. *Neuroscience* **27**, 931–939.

CRAGG, B. G. (1970). Synapses and membranous bodies in experimental hypothyroidism. *Brain Research* **18**, 297–305.

DUMM, C. L. A. G., CORTIZO, A. M. & GAGLIARDINO, J. J. (1985). Morphological and functional changes in several endocrine glands induced by hypothyroidism in the rat. *Acta anatomica* **124**, 81–87.

EAYRS, J. T. (1955). The cerebral cortex of normal and hypothyroid rats. *Acta anatomica* **25**, 160–183.

EAYRS, J. T. (1961). Age as a factor determining the severity and reversibility of the effects of thyroid deprivation in the rat. *Journal of Endocrinology* **22**, 409–419.

EAYRS, J. T. (1971). Thyroid and developing brain: anatomical and behavioral effects. In *Hormones in Development* (ed. M. Hamburgh & E. J. Barrington), pp. 345–355. New York: Appleton Century Crofts.

EAYRS, J. T. & HORN, G. (1955). The development of cerebral cortex in hypothyroid and starved rats. *Anatomical Record* **121**, 53–61.

EAYRS, J. T. & TAYLOR, S. H. (1951). The effect of thyroid deficiency induced by methyl thiouracil on the maturation of the central nervous system. *Journal of Anatomy* **85**, 350–358.

FAIVRE, C., LEGRAND, CH. & RABIÉ, A. (1985). The microtubular apparatus of cerebellar Purkinje cell dendrites during postnatal development of the rat: the density and cold-stability of microtubules increase with age and are sensitive to thyroid hormone deficiency. *International Journal of Developmental Neuroscience* **3**, 559–565.

FAIVRE-SARRAILH, C. & RABIÉ, A. (1988). A lower proportion of filamentous to monomeric actin in the developing cerebellum of thyroid-deficient rats. *Developmental Brain Research* **41**, 293–297.

GILBERT, P. L. & FRIEDEN, E. (1981). *Metamorphosis. A Problem in Developmental Biology*. New York: Plenum Press.

GUNDERSEN, H. J. G. (1986). Stereology of arbitrary particles: a review of unbiased number and size estimators and the presentation of some new ones. In memory of William R. Thompson. *Journal of Microscopy* **143**, 3–45.

HAMBURGH, M., LYNN, E. & WEISS, E. P. (1968). Analysis of the influence of the thyroid hormone on prenatal and postnatal maturation of the rat. *Anatomical Record* **150**, 147–162.

HAMBURGH, M., MENDOZA, L. A., BENNETT, I., KRUPA, P., KIM, Y. S., KAHN, R., HOGREFF, K. & FRANKFORT, H. (1977). Some unresolved questions of brain-thyroid relationships. In *Thyroid Hormones and Brain Development* (ed. G. D. Grave), pp. 49–72. New York: Raven Press.

HORN, G. (1955). Thyroid deficiency and inanition: the effects of replacement therapy on the development of the cerebral cortex of young albino rats. *Anatomical Record* **121**, 63–79.

KOLB, B. (1984). Functions of the frontal cortex of the rat: a comparative review. *Brain Research Reviews* **8**, 65–98.

KOLB, B. & NONNEMAN, A. J. (1978). Sparing of function in rats with early prefrontal cortex lesions. *Brain Research* **151**, 135–148.

KRETTEK, J. E. & PRICE, J. L. (1977). The cortical projections of the mediodorsal nucleus and adjacent thalamic nuclei in the rat. *Journal of Comparative Neurology* **171**, 157–192.

LAUDER, J. M. (1977). Effects of thyroid state on development of the rat cerebellar cortex. In *Thyroid Hormones and Brain Development* (ed. G. D. Grave), pp. 235–254. New York: Raven Press.

LAUDER, J. M. (1978). Effects of early hypo- and hyperthyroidism on development of rat cerebellar cortex. IV. The parallel fibers. *Brain Research* **142**, 25–39.

LAUDER, J. M. (1979). Granule cell migration in developing cerebellum. Influence of neonatal hypo- and hyperthyroidism. *Developmental Biology* **70**, 105–113.

LEGRAND, J. (1982–3). Hormones thyroïdiennes et maturation du système nerveux. *Journal de Physiologie* **78**, 603–652.

LEGRAND, J. (1984). Effects of thyroid hormones on central nervous system development. In *Neurobehavioural Teratology* (ed. J. Yanay), pp. 331–363. Amsterdam: Elsevier.

LEWIS, P. D., PATEL, A. J., JOHNSON, A. L. & BALÁZS, R. (1976). Effect of thyroid deficiency on cell acquisition in the postnatal rat brain: a quantitative histological study. *Brain Research* **104**, 49–62.

LIMA, D. & COIMBRA, A. (1983). The neuronal population of the marginal zone (lamina I) of the rat spinal cord. A study based on reconstructions of serially sectioned cells. *Anatomy and Embryology* **167**, 273–288.

LUND, R. D. & MUSTARI, M. J. (1977). Development of the geniculocortical pathway in rats. *Journal of Comparative Neurology* **173**, 289–306.

MADEIRA, M. D., PAULA-BARBOSA, M. M., CADETE-LEITE, A. & TAVARES, M. A. (1988*a*). Unbiased estimate of cerebellar granule cell numbers in hypothyroid and in sex-age-matched control rats. *Journal für Hirnforschung* **29**, 587–594.

MADEIRA, M. D., PAULA-BARBOSA, M. M., CADETE-LEITE, A. & TAVARES, M. A. (1988*b*). Unbiased estimate of hippocampal granule cell numbers in hypothyroid and in sex-age-matched control rats. *Journal für Hirnforschung* **29**, 643–650.

NICHOLSON, J. L. & ALTMAN, J. (1972). The effects of early hypo- and hyperthyroidism on the development of the rat cerebellar cortex. I. Cell proliferation and differentiation. *Brain Research* **44**, 13–23.

NORMAND, G., CLOS, J., VITIELLO, F. & GOMBOS, G. (1989). Developing rat cerebellum. I. Effects of abnormal thyroid states and undernutrition on sulfated glycosaminoglycans. *International Journal of Developmental Neuroscience* **7**, 323–328.

NORMAND, G., VITIELLO, F., CLOS, J. & GOMBOS, G. (1989). Developing rat cerebellum. II. Effects of abnormal thyroid states and undernutrition on hyaluronic acid. *International Journal of Developmental Neuroscience* **7**, 329–334.

PATEL, A. J., RABIÉ, A., LEWIS, P. D. & BALÁZS, R. (1976). Effect of thyroid deficiency on postnatal cell formation in the rat brain: a biochemical investigation. *Brain Research* **104**, 33–48.

PAULA-BARBOSA, M. M., MADEIRA, M. D., PORTO-CARRERO, M. C. & CAMÕES, I. (1989). Stereological study of the postnatal effects of hypothyroidism and undernutrition on the supraoptic nucleus (SON) of the rat. *Journal of Endocrinological Investigation, Suppl.* **12**, 122.

RAMI, A., PATEL, A. J. & RABIÉ, A. (1986). Thyroid hormone and development of the rat hippocampus: morphological alterations in granule and pyramidal cells. *Neuroscience* **19**, 1217–1226.

RAMI, A., RABIÉ, A. & PATEL, A. J. (1986). Thyroid hormone and development of the rat hippocampus: cell acquisition in the dentate gyrus. *Neuroscience* **19**, 1207–1216.

RUIZ-MARCOS, A., SALAS, J., SANCHEZ-TOSCANO, F., ESCOBAR DEL REY, F. & MORREALE DE ESCOBAR, G. (1983). Effect of neonatal and adult onset hypothyroidism on pyramidal cells of the rat auditory cortex. *Developmental Brain Research* **9**, 205–213.

RUIZ-MARCOS, A., SANCHEZ-TOSCANO, F., ESCOBAR DEL REY, F. & MORREALE DE ESCOBAR, G. (1979). Severe hypothyroidism and the maturation of the rat cerebral cortex. *Brain Research* **162**, 315–329.

STERIO, D. C. (1984). The unbiased estimation of number and sizes of arbitrary particles using the disector. *Journal of Microscopy* **134**, 127–136.

SWAAB, D. F. & UYLINGS, H. B. M. (1987). Comments on review by Coleman and Flood 'Neuron numbers and dendritic extent in normal aging and Alzheimer's disease". Density measures: parameters to avoid. *Neurobiology of Aging* **8**, 574–576.

UYLINGS, H. B. M., VAN EDEN, C. G. & VERWER, R. W. H. (1984). Morphometric methods in sexual dimorphism research on the central nervous system. In *Sex Differences in the Brain. The Relation Between Structure and Function* (ed. G. F. de Vries, J. P. C. de Bruin, H. B. M. Uylings & M. A. Corner). *Progress in Brain Research*, Vol. 61, pp. 215–222. Amsterdam: Elsevier Science Publishers, B.V.

VAN EDEN, C. G. (1986). Development of connections between the mediodorsal nucleus of the thalamus and the prefrontal cortex in the rat. *Journal of Comparative Neurology* **244**, 349–359.

VAN EDEN, C. G. & UYLINGS, H. B. M. (1985*a*). Cytoarchitectonic development of the prefrontal cortex in the rat. *Journal of Comparative Neurology* **241**, 253–267.

VAN EDEN, C. G. & UYLINGS, H. B. M. (1985*b*). Postnatal volumetric development of the prefrontal cortex in the rat. *Journal of Comparative Neurology* **241**, 268–274.

VAN EDEN, C. G., UYLINGS, H. B. M. & VAN PELT, J. (1984). Sex-difference and left–right asymmetries in the prefrontal cortex during postnatal development in the rat. *Developmental Brain Research* **12**, 146–153.