THE EFFECTS OF EARLY HYPO- AND HYPERTHYROIDISM ON THE DEVELOPMENT OF THE RAT CEREBELLAR CORTEX. II. SYNAPTOGENESIS IN THE MOLECULAR LAYER

JEAN L. NICHOLSON AND JOSEPH ALTMAN

Laboratory of Neuropharmacology, WAW Building, St. Elizabeths Hospital, Washington, D.C. 20032 and Laboratory of Developmental Neurobiology, Department of Biological Sciences, Purdue University, Lafayette, Ind. 47907 (U.S.A.)

(Accepted March 9th, 1972)

INTRODUCTION

In addition to the effects of early hypo- and hyperthyroidism on cell proliferation and differentiation, described in the preceding paper\textsuperscript{20} there is also a profound effect on the development of the neuropil. This was indicated by the decreased cell size and increased packing density in the cerebrum and cerebellum of hypothyroid animals\textsuperscript{4,11,13,16,22} and was directly demonstrated by changes in the amount and composition of the neuropil in hypothyroid sensorimotor cortex. These changes were measured by (1) the amount of intercellular substance relative to the space occupied by pyramidal cells\textsuperscript{11}; (2) the number of nerve fibers per unit area\textsuperscript{10}; and (3) the density of axons, and length, density, and amount of branching of dendrites\textsuperscript{9}. It was estimated that such dendritic and axonal deficiencies could lead to as much as an 80% decrease in the probability of neuronal interactions in sensorimotor cortex\textsuperscript{9}. In the cerebellum, early hypothyroidism has been reported to cause delayed maturation of the Purkinje cell dendritic tree and appearance of glomeruli\textsuperscript{14,18,19}, while acceleration of Purkinje cell development and developmental increases in specific activities of AChE and ChE have been noted in hyperthyroid animals\textsuperscript{17}. Further support for the effects of thyroid hormone on maturation of the neuropil has been provided by studies on the development of metabolic compartmentation of glutamate in the rat brain following early thyroid manipulations\textsuperscript{6}. Since such development is thought to reflect maturation of dendritic processes and nerve terminals\textsuperscript{6,23}, these studies lend support to these morphological findings since development of glutamate compartmentation is retarded by thyroidectomy and accelerated by hyperthyroidism. Retardation of the development of axon terminals in rat visual cortex has also been reported in an ultrastructural study of hypothyroid neonates\textsuperscript{7}.

All of these studies indicate that early hypothyroidism has a profound influence on the development of the neuropil. It would also seem likely that hyperthyroidism
should have an effect due to decreased numbers of cells contributing processes to the neuropil. Until recently there has been no way to directly quantify these effects on the actual number of synaptic contacts among neurons. This has now been made possible by the development of the ethanolic phosphotungstic acid (E-PTA) method of Bloom and Aghajanian for the ultrastructural staining of synaptic profiles. This was combined with quantitative methods of areal measurements of cortical layers of the cerebellum to determine the effects of early hypo- and hyperthyroidism on width and area of the molecular layer, packing density of granule cells, density of synaptic profiles, total synaptic profiles, and synaptic profiles/cell in the molecular layer. By inference, changes in the shape of the dendritic domain of Purkinje cells and the density of their synaptic contacts were established, together with changes in the synaptic domain of parallel fibers.

METHODS AND MATERIALS

Animals

Newborn Long–Evans pups were pooled and 10 males (with an occasional female) were assigned to each mother. Each litter represented a given age group and experimental condition, to keep litter size constant during the experiment. Extra litters were maintained for replacement of sickly pups. Litters were injected subcutaneously daily from birth to 24 or 30 days of age with either physiological saline, propylthiouracil (PTU), or thyroxine (T4).

Treatments

The injection schedule for controls consisted of 0.05 ml physiological saline per animal on days 0–7, and 0.1 ml saline on days 8–30. Hypothyroid animals were injected with 0.05 ml 0.2% PTU in 1% carboxymethylcellulose 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. Thyroids from these animals were monitored histologically at all ages. Animals were judged to be hypothyroid on the basis of lack of colloid and hyperplastic follicular epithelium. Hyperthyroid animals were injected with 1 μg T4 in 0.1 ml saline on days 0–7; 2 μg in 0.1 ml on days 8–14; 3 μg in 0.1 ml on days 15–21 and 5 μg in 0.1 ml on days 22–30, according to the tolerance schedule devised by Hamburgh et al.

Light microscopic techniques

Animals were sacrificed by ether anesthesia and cardiac puncture at 5, 10, 15, 21, 24 and 30 days. Brains were removed and immersed in Bouin’s fixative. Brains were cut sagittally after 2–4 h and the 2 halves left in Bouin’s fixative for 24–48 h, followed by several changes of 10% neutral formalin for at least 24 h prior to embedding in Paraplast. Brains were sectioned at 6 μm, taking only near-midline sections, and stained with Harris hematoxylin and eosin.
Width and areal measurements

The width of the molecular layer was measured from tracings of the pyramis [magnified \(\times\) 200 (5–15 days) or \(\times\) 100 (21–30 days) with a Zeiss micro–macro-projector] using a Keuffel and Esser compensating polar planimeter. Measurements were made twice on the same tracing and averaged.

Cell counts

Granule cells were counted at \(\times\) 1500 magnification in the pyramis at 30 days, and expressed as granule cells/sq. mm (packing density). Total granule cells were calculated by multiplying this value by the area of the internal granular layer, obtained as for the molecular layer above. Basket cells were also counted in the molecular layer at 30 days, and combined with total granule cells to give an estimate of total interneurons contributing synapses to the molecular layer. Stellate cells were not counted due to the difficulty in identifying them. However, since granule cell parallel fiber synapses make by far the greatest contribution, this was not considered to be a major problem.

The Purkinje cell dendritic domain

Purkinje cells with nuclei were counted in the pyramis at 30 days. The area of the molecular layer was divided by the width to give the calculated length. This was divided by the average number of Purkinje cells for the 3 groups to give the length of the average Purkinje cell dendritic domain. This was plotted against the width (height) of the molecular layer to give the area of the average domain for each group (Fig. 6A). The number of synapses/domain was calculated by multiplying the density of synaptic profiles (see below) by the area of the domain to give the synaptic content of the domain (Fig. 6B). The parallel fiber synaptic domain was calculated by dividing the area of the Purkinje cell domain by the total number of granule cells. The percentage area occupied by one parallel fiber domain was calculated by dividing the parallel fiber domain by the Purkinje cell domain and multiplying by 100 (Fig. 6C).

The use of these measures presupposes the following: (1) the concentration of granule cells in the sagittal plane reflects the concentration throughout the internal granular layer, and therefore, the total number of parallel fibers crossing a Purkinje cell domain is directly proportional to the number of granule cells in the sagittal plane, (2) the lengths of the overlapping parallel fibers are constant between groups, (3) the widths of the parallel fibers are constant between groups. Although it is likely that some of these assumptions are not strictly true, they are valid for the group comparisons being made, since they are being used to express the relative areal contributions made by increased or decreased parallel fibers as a function of the granule cell population. No assumptions are necessary to arrive at the area of the Purkinje cell dendritic domain and its synaptic content.

_Brain Research, 44 (1972) 25-36_
Electron microscopic techniques

Two animals per litter were sacrificed by injection of Diabutal (0.7 mg/kg, intraperitoneally) and glutaraldehyde perfusion (6% in 0.1 M phosphate buffer, pH 7.2). Cerebellums were removed and placed in cold 6% phosphate buffered glutaraldehyde for 1–2 h, then rinsed 3 times in cold 0.1 M phosphate buffer plus 0.3 M sucrose. Tissue was either directly embedded or left in buffer at 5 °C overnight. The cerebellum was chopped sagittally at 235 μm on a Sorvall TC-2 tissue sectioner. The slices were dehydrated in ethanol and stained with E-PTA^3 followed by embedding in Araldite–Epon. Before thin-sectioning, tissue from coded blocks was trimmed to an area approximately 0.5 mm wide (in either the pyramis or hemispheres) including the pial membrane, external granular layer (where present), molecular layer, and internal granular layer. Tissue was thin-sectioned on a Sorvall MT-2 ultramicrotome and viewed with a Philips 300 electron microscope. Pictures were taken at ×6027 magnification on 70 mm film.

Counts of synaptic profiles

Samples for synaptic density counts were selected from outer, middle, and inner zones of the molecular layer, taking 4 samples from each zone, except at 5 days where only random samples were possible due to the narrow width of the molecular layer. Synaptic profiles were counted directly from the coded negatives using a photographic enlarger. Counts were expressed as the average number of synaptic profiles/96 sq. μm of the molecular layer (Fig. 1). Two counts were made for each negative and the mean computed. If the 2 counts differed by more than 5 profiles, a third count was taken. Counts for the study of synaptogenesis were taken from the hemispheres. The density of synaptic profiles in the inner and outer halves of the molecular layer were obtained by adding the middle molecular layer counts divided by 2 to the inner or outer counts and dividing by 1.5 to retain the unit area. Counts of the whole molecular layer at 30 days were taken from the pyramis (in order to correspond to other parameters which were all measured in the pyramis), and represent pooled values from the inner, middle, and outer zones.

Estimation of total synaptic profiles

Means of synaptic profiles/96 sq. μm were multiplied by the means for the areal measurements of the molecular layer (ML) to find the total number of synapses in the molecular layer (TS) according to the formula:

\[ TS = \frac{SP \times ML (sq. \mu m)}{96 \text{ sq. } \mu m} \]

Brain Research, 44 (1972) 25-36
Synaptic profiles per cell

Synaptic profiles/cell (pyramis) were calculated by dividing the estimated total synaptic profiles for a given group at 30 days by the number of granule plus basket cells for each animal in that group (n = 8).

Statistical analysis

Analyses of variance were used to obtain levels of overall significance and error terms. Duncan's multiple range test was then performed to compare means at each age using the MS error term obtained from the overall analysis.

Brain Research, 44 (1972) 25-36
RESULTS

Width and area of the molecular layer and the Purkinje cell dendritic domain

In controls, the sagittal width of the molecular layer (Fig. 2A) increased until about 24 days when it began to approach adult values. In hypothyroids, this developmental increase was significantly retarded from 10–24 days, after which control values were approached. In hyperthyroids, this increase in width was significantly accelerated from day 10 on, such that by 30 days the molecular layer was significantly wider than that of controls. The area of the molecular layer (Fig. 2B) showed a somewhat different trend for the hyperthyroid group. There was only slight acceleration at 10 days, followed by leveling off by 15 days such that thereafter the areal increment of the molecular layer was significantly decreased. Hypothyroids showed the same transitional areal retardation as was seen for the width of the molecular layer. The area of the Purkinje cell domain was similar in controls and hypothyroids, but was reduced in hyperthyroids, which exhibited taller but narrower domains (Fig. 6).
Packing density of granule cells at 30 days

The packing density of granule cells (Fig. 3) was slightly but not significantly increased in hypothyroids; in hyperthyroids it was significantly decreased, probably reflecting the increase in granule cells in hypothyroids and their decrease in hyperthyroids.

Number of Purkinje cells at 30 days

There was no significant difference in the number of Purkinje cells in the pyramis between groups at 30 days. An average was taken of the means for each group and used in the calculations of the length and area of the Purkinje cell dendritic domain.

Synaptogenesis in the outer and inner molecular layer

Synaptogenesis in the whole molecular layer is described elsewhere. In controls (Fig. 4), synaptogenesis (increase in density of synaptic profiles) in the inner half of the molecular layer exceeded that in the outer molecular layer until 24–30 days, when the density of synaptic profiles in the outer zone became greater (cross-over point on graph). Adult values in both zones were essentially reached by 30 days. Initially, in
hypothyroids there was a sharp rise in density of synaptic profiles in the inner molecular layer up to 24 days, when adult values were reached. Synaptogenesis in the outer molecular layer was retarded throughout, but continued to increase as late as 55 days and might have surpassed the inner molecular layer if older animals had been examined. In hyperthyroids, synaptogenesis in the inner molecular layer was accelerated until 21–24 days, when the outer molecular layer surpassed it. The density of synaptic profiles in the outer zone continued to increase at 55 days and was much greater than in the inner molecular layer at this time. Thus, hyperthyroidism accelerated the

**Brain Research, 44 (1972) 25-36**
synaptogenic development of the outer molecular layer, while hypothyroidism retarded it.

**Synaptic content of the molecular layer and the Purkinje cell dendritic domain at 30 days**

At 30 days, hypothyroids showed a reduction in the density of synaptic profiles (Fig. 5A), total synaptic profiles (Fig. 5B), number of synaptic profiles/cell (Fig. 5C), synaptic content of the Purkinje cell dendritic domain (Fig. 6B), and size of the parallel fiber synaptic domain (Fig. 6C), reflecting an overall retardation of synaptogenesis in the molecular layer. Hyperthyroidism caused a reduction in total synaptic profiles (Fig. 5B) and the synaptic content of the Purkinje cell domain (Fig. 6B) which was the result of decreased area of the Purkinje cell domain, since the density of synaptic profiles was not decreased (Fig. 5A), and the number of synaptic profiles/cell was actually increased (Fig. 5C), along with the size of the parallel fiber synaptic domain (Fig. 6C).

**DISCUSSION**

Hypothyroidism causes a transient retardation in the areal growth of the molecular layer, but by 30 days normal values are approached. At this age there is a reduction in the density and total number of synaptic profiles, reflecting retarded synaptogenesis from day 15 on. Whether or not this retardation is permanent has not been definitely established, but the density of synaptic profiles is still reduced at 55 days. The reduction in density and total synaptic profiles at 30 days must be reconciled with the facts that the width and area of the molecular layer are approaching control values at this time and that due to an increase in the number and packing density of granule cells, the concentration of parallel fibers must be higher, as shown by the decreased area of the parallel fiber synaptic domain. Therefore, it is postulated that hypothyroidism causes a reduction in the number of synapses formed by each individual fiber, with associated hypoplasia of Purkinje cell dendritic spines, as shown by the decreased synaptic content of the Purkinje cell dendritic domain and decreased size of the parallel fiber synaptic domain at 30 days.

In hyperthyroidism, the increase in density of synaptic profiles and width of the molecular layer are accelerated and are still higher than controls at 30 days, while the area of the molecular layer is less than controls after 15 days, indicating that the length of the molecular layer is reduced in the sagittal plane, causing a decrease in the total number of synaptic profiles. This reduction in length (area) can be indirectly related to the reduction in number of granule cells, since both are evident by 15 days. The length deficit, however, can be directly attributed to a morphogenic change produced by hyperthyroidism. The early expansion and foliation of the cortical surface of the cerebellum were attributed to the rapid proliferation of the cells of the external granular layer at a time when cell differentiation has not yet begun and there is minimal volumetric growth. The premature decline in cell proliferation caused by hyperthyroidism may therefore be expected to decrease surface growth and folia-
tion. Our observations have indeed shown that the cerebellum of hyperthyroid rats is less foliated.

Since hyperthyroidism does not reduce the number of prenatally formed Purkinje cells, the increase in width and decrease in length of the molecular layer implies that the contiguous but non-overlapping dendritic trees of Purkinje cells have become narrower and taller, as illustrated in Fig. 6A. Because of the reduction in sagittal length of the cerebellar cortex, there is obviously less room to expand laterally. The increase in height, in turn, may be due to the fact that hyperthyroidism caused a significant increase in the number of synaptic profiles/cell (essentially granule cells), and these space-occupying units (parallel fiber synaptic domains) had to be stacked upward within the narrowed confines of the Purkinje cell dendritic tree. This resulted in taller and thinner Purkinje cell domains with decreased area due to the decrease in total number of parallel fibers (reduced number of granule cells). The synaptic content of the Purkinje cell domains was also decreased due only to the decreased area since the concentration of synaptic profiles was not reduced.

The 'march' of synaptogenesis from the bottom of the molecular layer upward has been described. Synaptogenesis in the lower half of the molecular layer takes place between 12-21 days; in the upper half, between 21-30 days, after all the parallel fibers have been formed. In the present study we ignored, for the sake of simplicity, the fact that the molecular layer is not growing by 'expansion', but by 'stacking'. (That is, the true outer molecular layer is not present at 10 days, but is added later on.) Hypo- and hyperthyroidism have differentially affected the 'inner' and 'outer' molecular layer (as defined in Fig. 5 by dividing the molecular layer into two equal parts at all ages). In all animals, including controls, the density of synaptic profiles is initially higher in the inner than the outer molecular layer, reflecting this upward march. In controls, there was a crossing over by the 30th day, by which time the density of synaptic profiles in the outer molecular layer surpassed the density in the inner molecular layer. In hyperthyroids, this crossing over was seen by 24 days, by which time the increase in synaptic profiles ceased in the inner molecular layer. This accelerated crossing over could be related to the speeded up synaptogenic maturation of the cerebellar cortex following the acceleration of morphogenetic maturation (premature initiation of cell differentiation). In contrast, in hypothyroids, there was a lag in the synaptogenic maturation of the outer molecular layer and the cross-over point was approximated but not reached by 55 days, suggesting that synaptogenesis in the outer molecular layer was permanently retarded. Since there was no reduction in the number of granule cells in these animals, this analysis indicates that the number of synapses/parallel fiber (parallel fiber synaptic domain) was greatly reduced and that this was more prominent in the upper aspect of the Purkinje cell dendritic tree.

In conclusion, this analysis provides evidence that in both hypo- and hyperthyroidism, the total number of synapses is reduced at 30 days, but for different reasons. In hypothyroidism this is due to a hypoplastic neuropil (decreased density of synaptic profiles), whereas in hyperthyroidism it is due to decreased size of the unit areas (Purkinje cell dendritic domains) comprising the molecular layer. Such an analysis may be used to explain some of the behavioral and electrophysiological find-
thymus in early hypo- and hyperthyroidism. It also provides a valuable tool for the study
of the factors affecting patterns of growth of the synaptogenic components of the
cerebellar cortex.

SUMMARY

The development of the molecular layer was studied in rats made hypo- and
hyperthyroid at birth. The width and area of the molecular layer, packing density of
granule cells in the internal granular layer, and synaptogenesis in the outer and inner
halves of the molecular layer were determined. Hypothyroidism caused:

1) transient developmental retardation in width and area of the molecular
layer, with normal levels being approached by 30 days,

2) a slight, but not significant, increase in packing density of granule cells,
probably reflecting a reduction in glomeruli or synaptogenesis in the internal granular
layer, as well as crowding produced by increased numbers of granule cells20,

3) retardation of synaptogenesis in the molecular layer, especially in the outer
half, as late as 55 days. This retardation was manifested by a decrease in the synaptic
content of the Purkinje cell dendritic domain, and decreased size of the parallel fiber
synaptic domain, resulting (along with the increase in granule cells) in a greater pack-
ing density of parallel fibers in the molecular layer.

Hyperthyroidism caused:

1) an accelerated increase in width of the molecular layer from day 10 on,
such that by 30 days the molecular layer was wider than in controls. This was accom-
panied by only a small, transient increase in the area of the molecular layer and a
sharp leveling off by 15 days such that the area was greatly decreased by 30 days
compared to controls. This was interpreted to mean that the length of the molecular
layer was decreased in the sagittal plane, resulting in taller and narrower Purkinje
cell dendritic trees. Possible reasons for this are discussed,

2) a significant decrease in packing density of granule cells, probably reflecting
both the decrease in the number of granule cells20 and an accelerated development of
glomeruli,

3) an acceleration of synaptogenesis in the inner and outer halves of the molec-
ular layer and an increase in the number of synaptic profiles/cell at 30 days. This
probably is the result of accelerated growth of the Purkinje cell dendritic tree and the
deficit in granule cells and basket cells described previously20. Total synaptic profiles
were decreased, however, due to the decreased sagittal length and area of the molec-
ular layer.

Thus it was shown that early hypo- and hyperthyroidism both cause a terminal
deficit in total synaptic profiles in the rat cerebellar cortex, but for different reasons.

ACKNOWLEDGEMENT

This research was supported by the National Institute of Mental Health and
the Atomic Energy Commission.

Brain Research, 44 (1972) 25–36
REFERENCES

1 ALTMAN, J., Autoradiographic and histological studies of postnatal neurogenesis. III. Dating the
time of production and onset of differentiation of cerebellar microneurons, *J. comp. Neurol.*, 136

2 ALTMAN, J., Postnatal development of the cerebellar cortex in the rat. II. Phases in the maturation

3 ALTMAN, J., AND ANDERSON, W. J., Experimental reorganization of the cerebellar cortex. I.
Morphological effects of elimination of all microneurons with prolonged X-irradiation started at

4 BALÁZS, R., KOVÁCS, S., TEICHGRÄBER, P., COCKS, W. A., AND EAYRS, J. T., Biochemical effects

5 BLOOM, F. E., AND AGHAJANIAN, G. K., Cytochemistry of synapses: a selective staining method

6 COCKS, J. A., BALÁZS, R., JOHNSON, A. L. AND EAYRS, J. T., Effect of thyroid hormone on the

7 CRAGG, B. G., Synapses and membranous bodies in experimental hypothyroidism, *Brain Research*, 18


9 EAYRS, J. T., Protein anabolism as a factor ameliorating the effects of early thyroid deficiency,

10 EAYRS, J. T., AND HORN, G., The development of cerebral cortex in hypothyroid and starved rats,

11 EAYRS, J. T., AND TAYLOR, S. H., The effect of thyroid deficiency induced by methyl thiouracil


13 GEEL, S. E., AND TIMIRAS, P. S., The influence of neonatal hypothyroidism and of thyroid hormone on


15 HAMBURGH, M., LYNN, E., AND WEISS, E. P., Analysis of the influence of thyroid hormone on

16 KRAWIEC, L., GARCÍA ARGIZ, C. A., GÓMEZ, C. J., AND PASQUINI, J. M., Hormonal regulation of
brain development. III. Effects of triiodothyronine and growth hormone on the biochemical changes in
the cerebral cortex and cerebellum of neonatally thyroxinomasinated rats, *Brain Research*, 15

17 LEFRANC, G., GEORGE, Y., ET TUSQUES, J., Etude de l’activité acétylcholinestérásique du cortex
cérébelleux du rat nouveau-né au cours de sa maturation sous l’action de la thyroxine, *C. R. Soc. Biol.*

18 LEGRAND, J., Analyse de l’action morphogénétique des hormones thyroïdiennes sur le cervelet

19 LEGRAND, J., Comparative effects of thyroid deficiency and undernutrition on maturation of the
nervous system and particularly on myelination in the young rat. In M. HAMBURGH AND E. J. W.
BARRINGTON (Eds.), *Hormones in Development*, Appleton-Century-Crofts, New York, 1971,
pp. 381–390.

20 NICHOLSON, J. L., AND ALTMAN, J., The effects of early hypo- and hyperthyroidism on the develop-
13–23.

21 NICHOLSON, J. L., AND ALTMAN, J., Synaptogenesis in the rat cerebellum: Effects of early hypo-

22 PASQUINI, J. M., KAPLUN, B., GARCÍA ARGIZ, C. A., AND GÓMEZ, C. J., Hormonal regulation of
brain development: I. The effect of neonatal thyroidectomy upon nucleic acids, protein and two

23 PATEL, A. J., AND BALÁZS, R., Development of metabolic compartmentation in rat brains,

24 TUSQUES, J., LEFRANC, G., ET GEORGE, Y., Analyse par la technique de Golgi-Cox de la matura-
tion du cortex cérébelleux sous l’influence de la thyroxine chez le rat nouveau-né, *C. R. Soc. Biol.*