THE EFFECTS OF EARLY HYPO- AND HYPERTHYROIDISM ON THE DEVELOPMENT OF RAT CEREBELLAR CORTEX. I. CELL PROLIFERATION AND DIFFERENTIATION

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INTRODUCTION

The effects of hypo- and hyperthyroidism on the development of the brain have been widely studied. Both conditions produce decreases in adult cerebral and cerebellar weights[6–8,10–12,16,21,24], but for apparently different reasons. In hypothyroidism, the cerebral weight deficit is partly the result of decreased cell size[7,12–14,24] with a concomitant increase in packing density[7,12,13,21,24]. In the cerebellum there is also a transient decrease in cell number as well as a more permanent reduction in cell size and increase in packing density[7]. This transient decrease in cell number is presumably due to a reduced rate of cell proliferation in the external granular layer. In hyperthyroidism the cerebral and cerebellar weight deficits are attributed to a permanent decrease in total cell number[6,29] with no concomitant change in packing density or cell size[5], perhaps reflecting premature termination of cell proliferation due to early initiation of cell differentiation[6,18,29]. It appears, therefore, that thyroid hormone plays a key role in the process of cell differentiation, since hypothyroidism produces smaller, more tightly packed (less differentiated) cells, while hyperthyroidism produces fewer cells due to early differentiation and associated termination of cell proliferation. The purpose of the present study was to specify the effects of hypo- and hyperthyroidism on cell proliferation and differentiation in the rat cerebellar cortex by means of quantitative histological methods and to correlate these effects with changes in specific cell populations. A subsequent study[23] explores the effects of these treatments on synaptogenesis in the molecular layer.

METHODS

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 (T_4).

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 T_4 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.*[19].

Brain and cerebellar weights

Brain weights were pooled from radioactive and non-radioactive litters ($n = 8$) for each age and experimental group. Cerebellar weights were taken from non-radioactive litters ($n = 6$). Tissue was weighed on a Mettler analytical balance immediately after removal from the animals which were sacrificed by ether anesthesia and cardiac puncture.

Short-survival autoradiography (cell proliferation)

Two animals per experimental group were injected with [^{3}H]thymidine (10 μCi/g body weight) at 5, 10, 15, 21 and 24 days and sacrificed by ether anesthesia and cardiac puncture after a 2 h survival period. Whole brains were removed and immersed in 10% neutral formalin. Brains were cut sagittally after 2–4 h and the 2 halves left in formalin for at least 24 h before being embedded in Paraplast. Brains were sectioned at 6 μm, taking only near-midline vermal sections. Slides were deparaffinized and coated in the dark with Kodak NTB-3 nuclear emulsion, dried, and exposed with a desiccant at 5 °C for 90 days, according to procedures already described[1]. Slides were then developed and stained with cresyl violet. Labeled cells were counted in the pyramis (lobule VIII) at × 1500 magnification in 10 grid areas of the subpial proliferative zone of the external granular layer. Since the external granular layer consists only of cell bodies, with no intervening neuropil, labeled cells/sq. mm is considered a valid measure of the proportion of labeled cells/unlabeled cells (expressed as 'proportion of labeled cells'). Ten measurements of the width of the proliferative zone were made for each animal using the above slides and an ocular grid. The area of the proliferative zone was measured from tracings of the cerebellar pyramis (magnified × 300 with a Zeiss micro-macroprojector) using a Keuffel and Esser compensating polar planimeter. Measurements were made twice on the same tracings and averaged.

Long-survival autoradiography (cell differentiation)

Four animals per experimental group were injected with [^{3}H]thymidine (10 μCi/g body weight) at 5, 10, 15, 21 and 24 days and allowed to survive for 60 days. Animals were sacrificed by cardiac perfusion with 10% neutral formalin, followed by immersion of the removed brains in formalin for at least 24 h prior to embedding in Paraplast. Brains were sectioned at 6 μm and prepared for autoradiography as described above. Heavily labeled cells were counted at $\times$ 675 magnification in 10 samples taken from each of the 2 halves of the molecular layer which was divided into equal parts with an ocular grid. Total heavily labeled cells per whole molecular layer were calculated by multiplying the totalled cells/sq. mm for both zones by the area of the molecular layer for that animal. Areal measurements were made from tracings of the cerebellar pyramis [magnified $\times$ 200 (5–15 days) or $\times$ 100 (21–30 days)].

Non-radioactive cell counts (basket cells, granule cells and astrocytes)

Animals were sacrificed by ether anesthesia and cardiac puncture. Brains were removed, weighed, 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 as described and stained with Harris hematoxylin and eosin. Granule cells were counted at $\times$ 1500 magnification in the pyramis of 10-, 15-, 21-, 24- and 30-day-old animals (n = 8). Ten samples/animal were taken from the middle of the internal granular layer, while 6 samples were taken from the margins. Total granule cells were estimated by multiplying granule cells/sq. mm $\times$ the area of the internal granular layer. Total basket cells and astrocytes were counted directly in the molecular layers of the same cerebellums at 30 days only. Basket cells were identified as cells with relatively light nuclei and dark staining cytoplasm which were larger than either stellate cells or glia and lying mainly in the inner half of the molecular layer. Astrocytes were identified as having large light nuclei, little cytoplasm and being located throughout the molecular layer.

Statistical analysis

Analyses of variance were used to obtain levels of overall significance and error terms. Duncan's multiple range test[9] was then performed to compare means at each age using the MS error term obtained from the overall analysis.

RESULTS

Body growth

Hypo- and hyperthyroid animals showed typical reductions in body weight[5–7, 17,25]. Retardation became more drastic after 15 days with hypothyroids showing minimal growth thereafter.

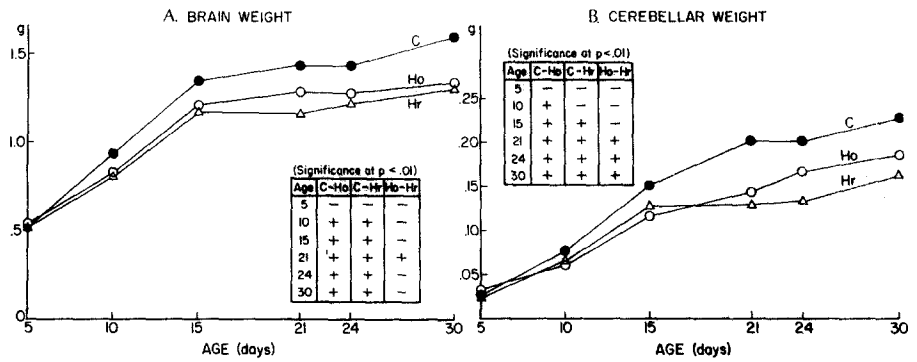

Fig. 1. A, Brain weight. B, Cerebellar weight. Statistical significance given in insert. C, controls; Ho, hypothyroids; Hr, hyperthyroids.

Brain and cerebellar growth

Growth of the brain (Fig. 1A) and cerebellum (Fig. 1B) was retarded in both experimental groups by 10 days. Terminal retardation (30 days) was somewhat greater in hyper- than hypothyroids.

Cell proliferation in the external granular layer (short-survival autoradiography)

In normal animals, the external granular layer disappeared by 24 days of age (Fig. 2B). In hypothyroid animals, however, it was still present at 24 days (as previously reported[22]), whereas in hyperthyroid animals it had disappeared by 21 days. A similar pattern was seen with regard to the proportion of labeled cells in the proliferative zone (Fig. 2A), which was used as an index of the number of cells preparing to divide at the time of [^{3}H]thymidine injection. In hypothyroids this was increased

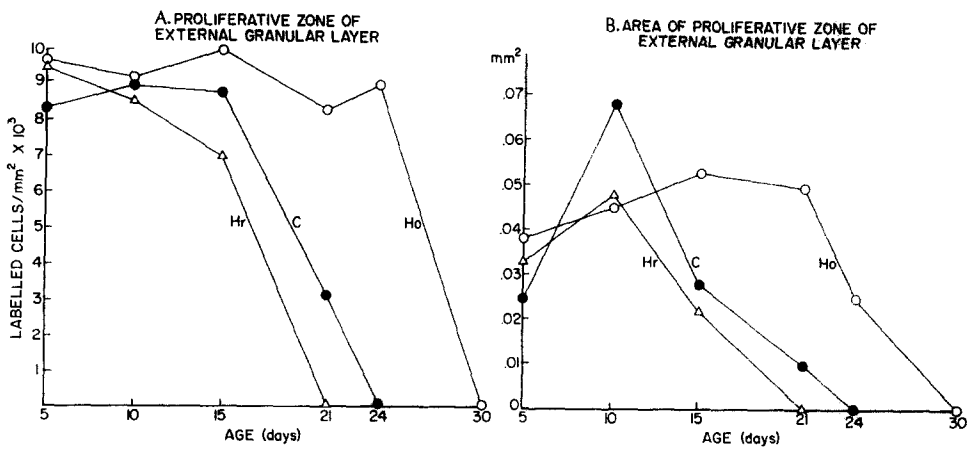

Fig. 2. A, Cell proliferation in the external granular layer (proportion of labeled cells in proliferative zone). B, Area of the proliferative zone of the external granular layer. Age at injection (2 h survival) indicated. C, controls; Ho, hypothyroids; Hr, hyperthyroids.

as compared to controls until 30 days when the proliferative zone disappeared (Fig. 2B), indicating that these cells remained in the proliferative phase longer than in normals (also reported by Hamburgh *et al.*[20]). In hyperthyroids, however, the proportion of labeled cells was increased only at 5 days, after which a sharp decline occurred such that by 21 days the proliferative zone had entirely disappeared, indicating that these cells migrated early.

Cell differentiation in the molecular layer (long-survival autoradiography)

The total number of heavily labeled cells in the molecular layer represents those cells that ceased to multiply soon after [^{3}H]thymidine injection, since cells continuing to divide will dilute label concentration with each subsequent division. Because the termination of cell proliferation signals the initiation of differentiation[6,18], the timing

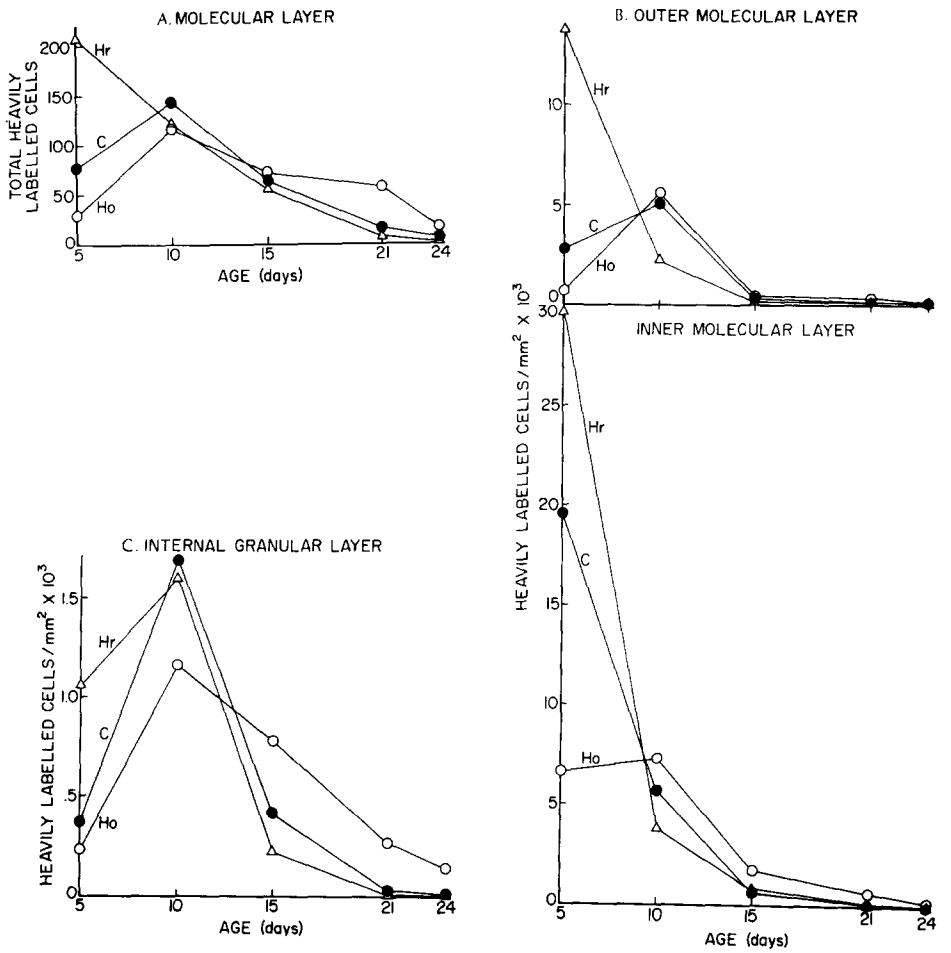

Fig. 3. A, Cell differentiation in the whole molecular layer. B, Cell differentiation in the inner and outer halves of the molecular layer. C, Cell differentiation in the internal granular layer. All animals sacrificed at 60 days of age. C, controls; Ho, hypothyroids; Hr, hyperthyroids.

of cell differentiation was studied with this method in the molecular layer. In hyperthyroid animals there was a 5 day displacement of the peak of cell differentiation in the molecular layer (Fig. 3A), which was correlated with the previously described early termination of cell proliferation in the external granular layer (Fig. 2A). In hypothyroid animals there was a decreased rate and prolonged period of cell differentiation in the molecular layer (Fig. 3A), which was correlated with prolonged cell proliferation in the external granular layer (Fig. 2A).

Cell differentiation was also studied in the inner and outer halves of the molecular layer (Fig. 3B). In controls, the concentration of heavily labeled cells in rats injected at 5 days was higher in the inner than the outer molecular layer, while in those injected at 10 days it was highest in the outer zone, indicating that cell differentiation occurs earliest in the inner molecular layer (also shown by Altman[3]). Hypothyroidism caused a displacement of the peak of differentiation from 5 to 10 days in the inner zone, while peaking was normal in the outer zone, such that both zones peaked at 10 days. If the area under the curve is used as an index of the total differentiated cells in each zone, it can be seen that the inner zone was more severely affected than the outer zone. Hyperthyroidism caused an acceleration of differentiation in both zones, leading to early peaking in the outer zone, so that the peak of differentiation occurred at 5 days or less in both zones.

Cell differentiation in the internal granular layer (long-survival autoradiography)

In the internal granular layer, no displacement of peaks of cell differentiation occurred in either experimental group (Fig. 3C), but differentiation was initiated earlier in hyperthyroids (increased concentration of heavily labeled cells in animals injected at 5 days) and was retarded and prolonged in hypothyroids (decreased concentration in animals injected at 5 days, and persistence of heavily labeled cells in animals injected at 21 and 24 days).

Non-radioactive cell counts (granule cells, basket cells and astrocytes)

An attempt was made to correlate these changes in cell proliferation and differentiation with developmental increases in granule cells and terminal numbers of basket cells and astrocytes. In controls, granule cells (Fig. 4A) increased in number until 24 days, when adult values were reached. In hypothyroids, increases in granule cells were initially retarded, but continued to increase longer, resulting in a significantly greater number of granule cells at 30 days. In hyperthyroidism, however, early differentiation of granule cells caused the attainment of adult values by 15 days, combined with terminal reduction in total granule cells at 30 days, indicating that premature termination of cell proliferation caused a significant decrease in total cells produced (as previously predicted[6]). This was accompanied by a significant decrease in basket cells at 30 days (Fig. 4B).

In hypothyroidism, a slight decrease in basket cells was also seen at 30 days. Since this was combined with an increase in granule cells (Fig. 4A) and retardation

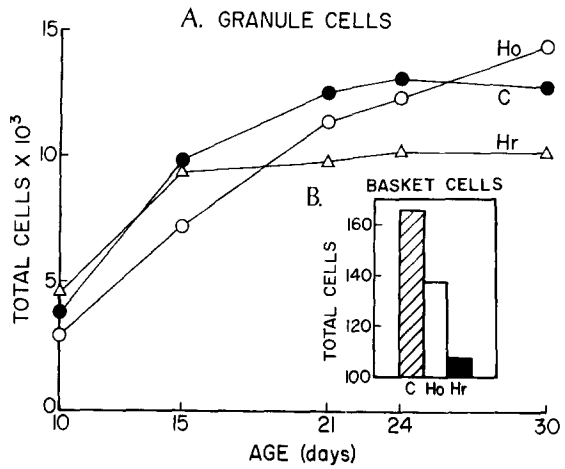

Fig. 4. A, Developmental increases in granule cells. Statistical significance: controls *vs.* hypothyroids: (10 d) not significant, (15 d) $P < 0.01$, (21 d) $P < 0.05$, (30 d) $P < 0.01$; controls *vs.* hyperthyroids: (10, 15 d) not significant, (21–30 d) $P < 0.01$; hypothyroids *vs.* hyperthyroids: (10–30 d) $P < 0.01$. B, Total basket cells at 30 days. Statistical significance: controls *vs.* hypothyroids, not significant; controls *vs.* hyperthyroids, $P < 0.01$; hypothyroids *vs.* hyperthyroids, not significant. C, controls; Ho, hypothyroids; Hr, hyperthyroids.

of cell differentiation in the inner molecular layer (Fig. 3B), it appears that retarded differentiation of external granular layer stem cells may have caused a reduction in the number of early differentiating basket cells while more cells differentiated as later forming granule cells. A second specific effect of hypothyroidism involved the formation of astrocytes (primarily Bergmann glia) in the molecular layer, as indicated by a significant increase in astrocytes at 30 days (Fig. 5B) which may have been due to prolonged local proliferation of cells around Purkinje cell bodies (Fig. 5A).

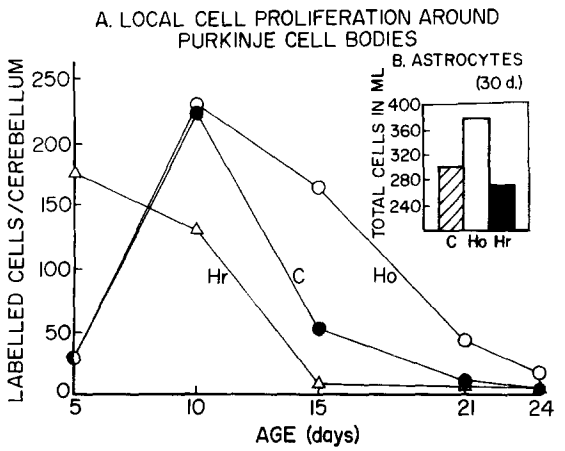

Fig. 5. A, Proliferation of cells around Purkinje cell bodies. B, Total astrocytes in the molecular layer at 30 days. Statistical significance (B): controls *vs.* hypothyroids, $P < 0.01$; controls *vs.* hyperthyroids, not significant; hypothyroids *vs.* hyperthyroids, $P < 0.01$. C, controls; Ho, hypothyroids; Hr, hyperthyroids.

DISCUSSION

Early hyperthyroidism causes terminal reduction in cell number in the cerebellum[6]. It has been postulated that one of the actions of thyroid hormone is the initiation of cell differentiation[6,18,20]. According to this idea excess thyroid hormone causes early termination of cell proliferation in the external granular layer and premature initiation of cell migration and differentiation, resulting in the reduction in the total number of cells formed[6]. Our results are consistent with this concept since the premature acceleration of cell differentiation in the molecular and internal granular layers produced by hyperthyroidism was associated with early termination of cell proliferation in the external granular layer. This resulted in the previously reported reduction in total cells[6] which were identified as granule and basket cells that arise from the external granular layer.

Acceleration of cell differentiation may be related to the well-documented stimulation of RNA and protein synthesis by thyroid hormone[26,27] since the onset of cell differentiation is thought to be associated with new patterns of protein synthesis[28]. Knowledge presently available[3] about events associated with cell differentiation in the external granular layer, suggests that thyroid hormone causes early differentiation of cells in the basal aspect of the proliferative zone, resulting in accelerated accumulation of bipolar cells in the premigratory zone and more rapid depletion of the proliferative zone. The retarded and prolonged differentiation associated with hypothyroidism may likewise be related to decreased protein synthesis[15], allowing cells to proliferate longer, with decreased accumulation of cells in the premigratory zone and a decreased rate of disappearance of the proliferative zone.

This study also indicated that hypothyroidism differentially affected the type of cells formed in the cerebellar cortex, resulting at 30 days in a reduced number of basket cells and increased number of granule cells. In normative studies it was established that most basket cells are formed on days 6–7, stellate cells on days 8–11, and the majority of granule cells after the 10th day[2]. Since hypothyroidism retards the peak of cell differentiation in the inner molecular layer by 5 days, the slight reduction in basket cells may indicate that sequential order is less important than absolute chronology in the differentiation of cells in the cerebellar cortex, and that fewer basket cells are formed because less cells are differentiating in the hypothyroid animals at 6–7 days. An additional factor may be a correlated strict chronology in the synaptogenic and gliogenic maturation of the domain of the Purkinje cell somata, which normally occurs between 7–12 days[4]. The differentiation of basket cells presumes the formation of axosomatic synapses with Purkinje cells. However, if the differentiation of basket cells is delayed and the Purkinje cell soma loses its synaptogenic competence, there will be a reduction in the number of cells that can differentiate as basket cells. The excess cells formed over a prolonged period in hypothyroidism must then differentiate as later forming cells, namely granule cells, resulting in an increase in the ultimate number of these cells. The prolonged proliferation of cells around the Purkinje cell bodies and ultimate increase in astrocytes shown in the present study in hypothyroid animals may also be related to this basket cell deficit,

since Bergmann glia are thought to sheath the Purkinje cell soma and dendrites in those areas where synaptic contacts are not made. If fewer basket cells are making synaptic contacts with the soma, it is possible that more astrocytes are needed to sheath the Purkinje cell. The other possibility is that prolonged proliferation of astrocytes prior to differentiation causes this increase.

This study not only provides evidence that thyroid hormone plays a key role in the initiation of cell differentiation, but also introduces a valuable tool for studying the patterns and mechanisms of cell differentiation in the laminated maturational zones (proliferative, premigratory, migratory and terminal zones) of the cerebellar cortex.

SUMMARY

Cell proliferation and differentiation were studied in the cerebellar cortex of rats made hypo- and hyperthyroid from birth. Hyperthyroidism caused:

(1) decreased body, brain and cerebellar weight,

(2) early termination of cell proliferation in the external granular layer accompanied by early disappearance of this layer,

(3) early cell differentiation in the molecular and internal granular layers,

(4) terminal decrease in granule cells, basket cells, and astrocytes.

Hypothyroidism caused:

(1) decreased body, brain and cerebellar weight,

(2) prolonged cell proliferation in the external granular layer and retarded disappearance of this layer,

(3) retarded cell differentiation in the molecular and internal granular layers,

(4) terminal increase in granule cells and astrocytes, and decrease in basket cells.

Hypo- and hyperthyroidism had different effects on the inner and outer halves of the molecular layer, which differentiate sequentially. Hypothyroidism caused a retardation of the peak of cell differentiation in the inner half from 5 to 10 days whereas hyperthyroidism caused an overall acceleration of differentiation in both zones, with an early peak of differentiation in the outer zone at 5 days.

These results are discussed with regard to mechanisms of differentiation of cells derived from the external granular layer, and the role played by thyroid hormone in the initiation of cell differentiation in the central nervous system.

ACKNOWLEDGEMENT

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