

APOPTOSIS IN THE DEVELOPING CEREBELLUM OF THE THYROID HORMONE DEFICIENT RAT

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Received 8/20/98 Accepted 9/7/98

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1. ABSTRACT

Abstract—The mechanism underlying transient reduction of cell number in the developing cerebellum have been studied for several decades. In this study we analyzed cell death by apoptosis in the developing cerebellum of euthyroid and hypothyroid rats. Results showed that in both groups the apoptotic activity is limited to the internal granular layer from postnatal (p) day 2 to day 12 in euthyroid animals, with the peak at 8 days. No apoptotic cells were detected in the cerebellum of 22 days old euthyroid rats. The level of apoptosis in the cerebellum of hypothyroid rats also reached a peak at 8 days but was four times higher than in control animals. Apoptosis in hypothyroid animals was also observed at p22 and corresponds to the value found in the time of the apoptotic peak in euthyroid cerebellum. At the age of 42 days, no apoptotic cells were found in the cerebellum of either group. Furthermore, it appears that the hormone also plays a role in the disappearance of the external germinal layer, since its presence is still apparent in 42 day old hypothyroid cerebellum. Hence, our results suggest that the deficiency of thyroid hormone (TH) not only increases, but also extends apoptosis during rat cerebellum development and affects the disappearance of the external germinal layer.

2. INTRODUCTION

Programmed cell death (PCD) plays a major role in the differentiation and development of central nervous system (CNS) (1,2,3). Cell death is often controlled by survival promoting signals from other cells, but is executed in a cell autonomous manner (4). Apoptotic cells exhibit morphological and biochemical

changes, such as, chromatin condensation, internucleosomal DNA fragmentation, cytoplasmic vacuolation, membrane blebbing and cell shrinkage (5, 6). Numerous experiments suggest that genes expression is required for neuronal death (7, 8). Most of the cell cycle regulators are involved in apoptosis, for example, cyclin D1 has been identified as an essential mediator of apoptosis of neuronal cell death (9). On the other hand, neurotrophic factors are presumably the limiting survival factors for neuronal cell types *in vivo* (10). Programmed death of supernumerary neurons occurring around the time when the neurons are making functional connections, abrogated by mRNA and protein synthesis inhibitors indicative of an activation of a specific genetic program (11).

Thyroid hormone (TH) influences gene expression, either positively or negatively, through binding to nuclear thyroid hormone receptors the members of steroid/thyroid hormone receptor superfamily (12, 13). Certain genes expressed in the brain have been shown to be under thyroid hormone control. These include myelin genes (14), the Purkinje cell specific gene, PCP2, (15) the transcription factor NGFI-A (16), and neuron specific enolase (NSE) (17). The lack of TH in early life has a marked effect on the development of the rat cerebellum (18, 19). In humans, the lack of adequate levels of TH during critical periods of development, results in cretinism, a syndrome of severe mental retardation often accompanied by growth retardation and/or neurological deficits (20).

The relative simplicity, structural homogeneity

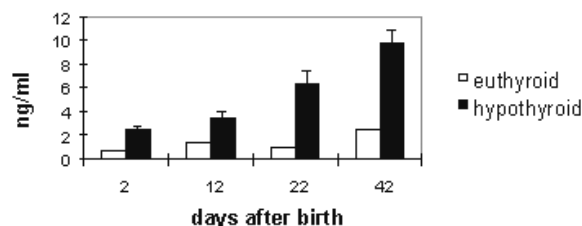


Figure 1. Serum thyroid stimulating hormone (TSH) level of the postnatal 2 days (p2), p12, P22 and p42 from the both hypothyroidism and euthyroid rats were determined by radioimmunoassay (RIA). Columns value represent the mean \pm SD (n=4).

and postnatal development of cerebellum compared with that of the cerebral cortex makes the former ideally suited for the study of TH dependent regulation of neurogenesis. Nearly all cerebellum cells are formed postnatally originating from the external germinal layer (EGL). Following the extensive mitotic activity, granular cells migrate towards the molecular layer (ML) or descend further to the internal granular layer (IGL), leaving their axons (parallel fibers) in the ML to establish connections with the Purkinje cells (21). Legrand and others studied the effect of TH on the development of the rat cerebellum evaluating mostly anatomical and histological changes in TH deficient neonatal rats (18, 22, 19). In the present study, we have applied a sensitive apoptotic detection method to compare the degree of apoptosis in the developing cerebellum of euthyroid and hypothyroid rats. We have tried to understand whether the reduction of cell number in the hypothyroid cerebellum during development is due to apoptosis and whether the deficiency of hormone may exacerbate or extend the apoptosis.

3. MATERIALS AND METHODS

3.1 Animals and tissue preparation

Hypothyroid rats (Charles River) were generated using protocol previously described (23,24). In order to induce fetal and neonatal hypothyroidism, pregnant rats were drinking 0.02% methyl mercaptoimidazol (MMI) solution (Sigma Chemical Co., St. Louis, MO) *ad libitum* from gestation day 10. This treatment continued until the day of brain samples collection. These protocol produced severe hypothyroidism in the offspring, as shown by increased serum TSH level (figure 1), reduced body growth, and the delays in developmental landmarks, such as the eye opening. Animals were handled in a humane way, following the NIH guideline for the use and care of laboratory animals. Two groups, hypothyroid and euthyroid, were raised side by side. Whole brains of rats at the postnatal (p) day 2, p8, p12, p22, and p42 were collected from both groups. The entire brains were frozen in isopentane on dry ice and stored at -80°C until sectioning. A serials coronal sections from cerebellum, 12 μm thick, were made in a cryostat at -25°C (25).

3.2 *In situ* apoptosis detection

In situ apoptosis detection was performed using

the Apop Tag TM kit (Oncor, Gaithersburg, MD) following the manufacture protocol. This procedure detects the DNA using the terminal deoxynucleotidyl transferase (TdT) mediated d-uridine 5'-triphosphate (UTP) nick end labeling (TUNEL) technique (26). Briefly, sections were fixed in 10% buffered formalin in a coplin jar for 10 min at room temperature. Washed in 2 changes of PBS for 5 min each. Post-fixed in ethanol - acetic acid (2:1) for 5 min at -20°C , washed in 2 changes of PBS for 5 min each wash. Then, the sections were quenched in 2.0% hydrogen peroxide in PBS for 5 min at room temperature and rinsed two times with PBS. Two drops of 1x equilibration buffer was applied and incubated 10 min at room temperature. The equilibration buffer was removed and 54 μl working strength TdT enzyme was added, then incubated at 37°C for 1 hour with a plastic coverslip over the specimen. The slides were washed with pre-warmed stop/wash buffer and incubated for 30 min at 37°C . Two drops Anti-Digoxigenin-Peroxidase was applied incubating 30 min at room temperature and washed again in PBS. Then, the sections were subjected to color development in 0.05% DAB (Sigma Chemical Co., St. Louis, MO) solution for 10 min. Cell death was quantified according to Neveu and Arenas (27) counting the number of brown stained nuclei in a total of 20 randomly chosen fields contained in four different sections. The values with standard deviations (SD) show the number of dying cells per square millimeter. As a negative control, adjacent sections were processed following the identical procedure, except that an equal volume of water was substituted for the omitted TdT. In these control section, no nuclei were stained, confirming the specificity of the labeling.

3.3 Detection of thyroid stimulating hormone (TSH)

TSH level in serum of euthyroid and hypothyroid rats was determined by radioimmunoassay and performed by CORNING Hazleton Inc., Vienna, VA 22182.

4. RESULTS

4.1 Evaluation of DNA fragmentation in cerebellar granular cells of developing euthyroid rats

The TUNEL technique was applied to probe the apoptotic DNA fragmentation characteristic of cell death in the developing cerebellum of euthyroid rats at the postnatal day 2, 8, 12, 22, and 42. Examination of the layers of coronal sections reveal that the programmed cell death after birth is limited to the IGL of cerebellum although scattered apoptotic cells could be seen in the EGL of p8 and p12 (figure 2A). Based on a statistical evaluation, results show that apoptotic cells are absent at 2, 22 and 42 days postnatally (figure 2A-B). The peak of apoptosis was observed in the IGL at p8. There is about 50 % reduction in apoptotic cells at p12. In summary, as a results of postnatal cerebellum development, the number of apoptotic cells observed in the IGL increased significantly from the undetectable level at p2 reaching the highest level at p8, and gradually declined being undetectable at p22. Interestingly, DNA fragmentation was not observed in Purkinje cells layers (figure 2A-B, figure 3).

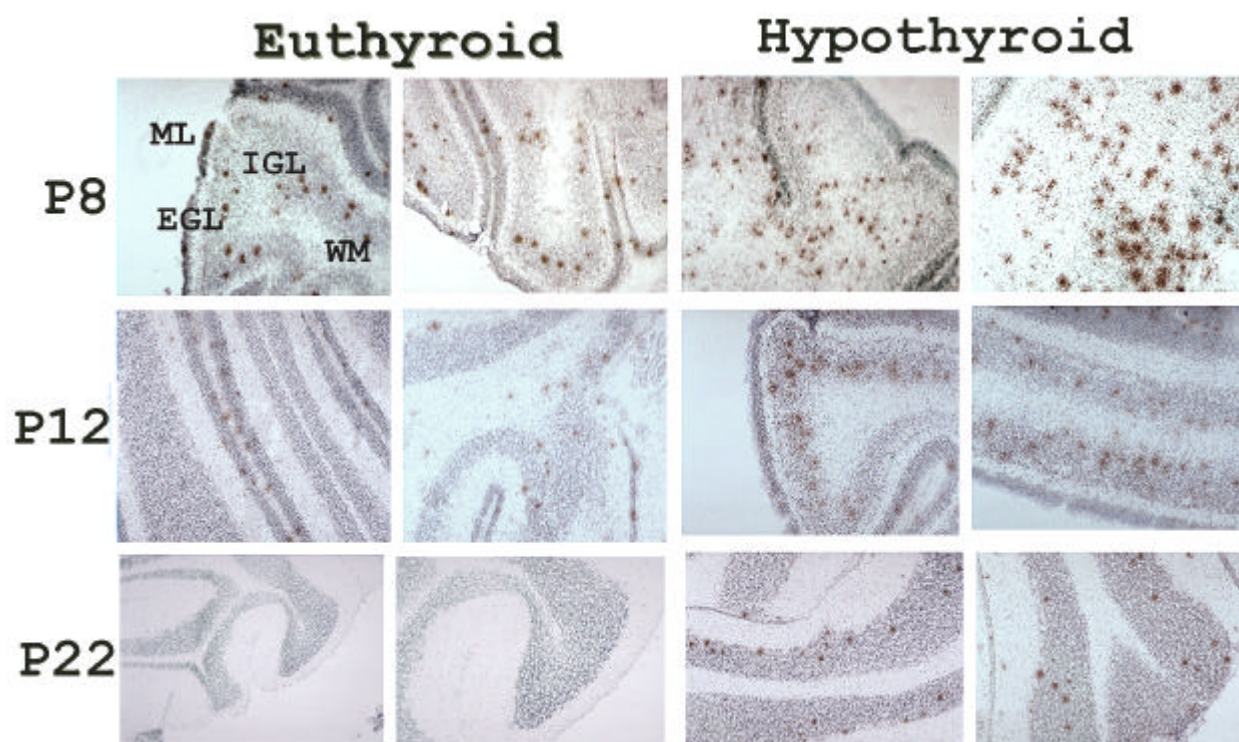


Figure 2A. Detection of DNA fragmentation of in the IGL of cerebellum of postnatal 8 days (p8), p12, and p22. Section of 12 μ m were labeled for DNA fragmentation as described in the material and methods. At the age of 8 days, some apoptotic stained cells were detected in the euthyroid rats, whereas, more apoptotic cells were detected in the 8-day hypothyroid rats. At the age of 12 days, few apoptotic cells were found in the euthyroid rats. In contrary, lots of apoptotic cells were still stained in the 12-day hypothyroid rats. At the age of 22 days, none apoptosis were happened in the euthyroid cerebellum, relatively fewer apoptotic cells were detected in the 22-day hypothyroid rats. None apoptotic cells were Purkinje cells. Magnification, 10x except the last one of left column which is 5x.

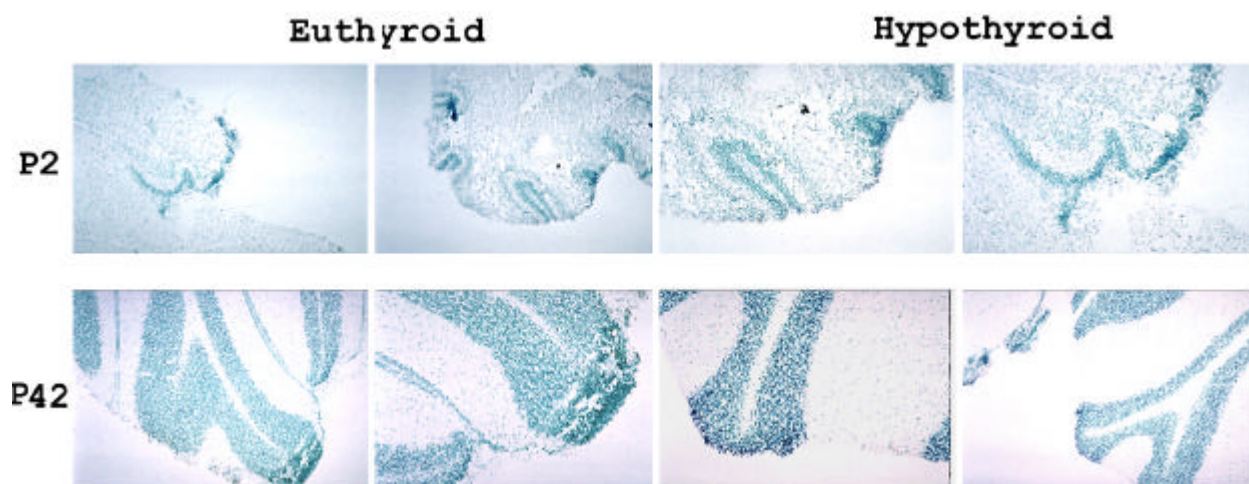


Figure 2B. Coronal sections of euthyroid and hypothyroid cerebellar at p2 and p42 stained for apoptotic cells as described in the material and methods. Magnification, 10x.

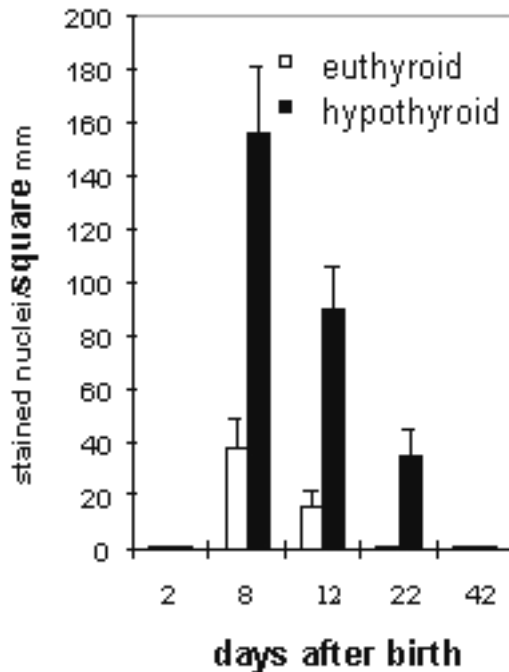


Figure 3. Statistical evaluation of a number of apoptotic cells in cerebella of euthyroid and hypothyroid rats during development. Calculation was done as described in Materials and Methods. The bars represent the \pm SD (n=20).

4.2 Evaluation of DNA fragmentation in cerebellar granular cells of developing hypothyroid rats

The cerebella of hypothyroid rats were examined at postnatal day 2, 8, 12, 22, and 42 as described for euthyroid controls. Similar to the control, no apoptotic cells were detected in the cerebellum of p2 (figure 2B). By p8, a sharp increasing number of brown stained nuclei was observed, accounting for the highest number of apoptotic cells in the cerebellum of hypothyroid rats during postnatal maturation. Based on the number of stained nuclei per square mm, this increase was about four times higher when compared with that obtained with the euthyroid rats of this same developmental stage (figure 3). Most of these apoptotic nuclei were in the IGL, a few are seen in the EGL and ML. At p12, there is still a substantial number of stained nuclei in the IGL, but the apoptotic value is about 50% of that detected at p8 (figure 3). By p22, the labeling of apoptotic nuclei was further reduced to about 25% of the p8 value, contrary to the euthyroid age matched cerebellum, where apoptosis descended. Interestingly, the level of apoptotic activity in p22 of the hypothyroid cerebellum corresponds to that detected at the peak of apoptosis in control (p8). There was no evidence for DNA fragmentation in the cerebellum of hypothyroid rats at p42 (figure 2A-B). Noteworthy is the continuing appearance of the EGL as a result of hypothyroidism, since at p42 EGL in the euthyroid cerebellum is not evident (figure 2B).

5. DISCUSSION

Although it is not arguable that TH is crucial for the cerebellum development, it has not been clearly established whether the arrested development of Purkinje cells dendrites, the primary synaptic target of granular cells, in the hormone deficient cerebellum, leads to the increased apoptotic activity of granular cells or whether the reduced number of granular cells connections with Purkinje cells affects Purkinje cell maturation. Indeed, the molecular basis for TH during brain development are poorly understood. Hormone action is mediated by thyroid hormone receptors especially the $\beta 1$, whose level reaches its highest during the first 2-3 weeks after birth. Thus, at the level of transcription, TH can regulate expression of variety of factors known to play an important role in the regulation of neuronal survival during development (28,29,30,31). Lindholm *et al* (32) reported that expression of neurotrophin 3 is under control of TH. Recently, Neveu *et al* (27) showed that neurotrophin 3 and brain derived neurotrophin factor prevent induced cell death of granular cells in hypothyroid cerebellum *in vivo*. Muller *et al* (33) reported that thyroid hormone also promotes expression of the bcl-2 protooncogene, the programmed cell death suppressor, and prevents apoptosis of early differentiating cerebellar granular neuron *in vitro*.

It is well established that programmed cell death plays an important role during maturation of the central nervous system. In this study, we have chosen to investigate the effect of the TH on the development of rat cerebellum since its maturation takes place postnatally. We have shown that the lack of TH increases and extends the apoptosis in the developing cerebellum and affects disappearance of the EGL.

In the rat, the cerebellum granular cells, a source of major excitatory afferents to the Purkinje cells, are generated from EGL (34). From p3 to p30, granular cells migrate to the IGL, by translocation of the cell body through the descending portion of the growing parallel fiber, leaving their axons (parallel fibers) in the ML forming synapse with the dendrites of Purkinje cells (21). Thus, it seems likely that growth of the descending portion of the parallel fiber is an important and perhaps rate-limiting aspect of granular cell migration and cerebellum maturation. It has been shown that the proper levels of TH are necessary to secure the growth of parallel fibers (35,36). Furthermore, hypothyroid rats show retardation in the proliferation, migration, and differentiation of cerebellar granular cells. Although a number of Purkinje cells do not appear to be affected by the lack of the hormone, there is, however, deficiency in the elaboration of Purkinje cell dendrite trees, spines and the synapses (18).

Using a sensitive method for detecting DNA fragmentation, a hall marker of apoptotic activity, our data showed that no apoptotic cells were detected in the euthyroid cerebellum at p2, 22 and p42. In agreement

with previous results reported by Wood *et al* (37), the apoptosis reach its maximal value at p7 and then declined. It is during this period that most of the histogenesis of the cerebellum occurs. Thus, granular cell death may be a key factor in regulating the final number of neurons. Indeed, in the stage where the maximal rate of migration is finished, the apoptotic activity is descended and becomes undetectable at p22. This developmental stage is also marked by the absence of the EGL. In both the euthyroid and hypothyroid rats, the p8 apoptosis reached the peak level, however, there was a greater proportion of apoptotic cells in the IGL of hypothyroid rats than that in the control. Surprisingly, the granular cells were still undergoing apoptosis at p 22 in the IGL hypothyroid cerebellum, contrary to the control. Thus, it appears that the deficiency in TH during postnatal cerebellum development promotes and also extends the event of apoptosis. The presence of the EGL in hypothyroid cerebellum at p 42, the time when brain maturation should be completed, indicates that the hormone also plays a role in morphological changes accompanying brain development. Another striking morphological change in hypothyroid cerebellum is the impaired dendritic arborization of Purkinje cell in ML. Previous results showed that in hypothyroidism, retardation in the morphological maturation of Purkinje cells is the most apparent during the second postnatal week (22,38). We have shown that this period coincides with the peak of aggressive apoptosis augmented by the absence of the hormone. It is now important to uncover the mechanism whereby TH is able to control the cell cycle so as to limit the extent and time of apoptosis in the developing cerebellum.

6. ACKNOWLEDGMENTS

We would like to express our appreciation to Drs. Jacob Robbins, J. Edward Rall, and Katherine A. Wood for critical reading of the manuscript. We thank Dr. Song Huang for the technical assistance.

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Key Words: Apoptosis, Thyroid Hormone, Developing Cerebellum, Hypothyroid Rat, Brain

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