

Hypothyroidism alters the expression of Bcl-2 family genes to induce enhanced apoptosis in the developing cerebellum

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Abstract

Thyroid hormone (TH) deficiency results in delayed proliferation and migration of cerebellar granule cells. Although extensive cell loss during the development of the cerebellum under hypothyroid conditions is known, its nature and its mechanism are poorly understood. Bcl-2 family gene expression is known to determine the fate of cells to undergo apoptosis. We evaluated the effect of hypothyroidism on Bcl-2 family gene expression in the developing rat cerebellum. Electrophoresis and Western blotting were used to analyze DNA fragmentation and expression of DNA fragmentation factor (DFF-45), Bcl-2, Bcl-x_L and Bax genes respectively. In the hypothyroid condition, extensive DNA fragmentation and enhanced

cleavage of DFF-45 were seen throughout development (postnatal day 0 to day 24) and adulthood whereas they were absent in the euthyroid state. The anti-apoptotic genes Bcl-2 and Bcl-x_L were down-regulated and the pro-apoptotic gene Bax was expressed at higher levels compared with the euthyroid state. These results suggest that normal levels of TH prevent cerebellar apoptosis to a large extent, whereas hypothyroidism not only increases the extent but also the duration of apoptosis by down-regulating the anti-apoptotic genes and maintaining a high level of the pro-apoptotic gene Bax.

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Introduction

Thyroid hormone (TH) is required for normal body functions in mammals to control a variety of physiological processes. Maternal hypothyroxenemia during early gestation results in irreversible brain damage characterized by symptoms of neurological cretinism, i.e. deaf-mutism, severe mental retardation, strabismus and lack of motor co-ordination (Schwartz 1983, DeLong *et al.* 1985). Late-gestational thyroid insufficiency or early postnatal TH deprivation leads to therapeutically reversible effects, e.g. learning deficit, low intelligence quotient level, low basal metabolic rate, reduced spatiomotor skills and reasoning etc. (Porterfield & Hendrich 1993 and references therein). TH deficiency in the cerebellum has been reported to result in delayed proliferation and migration of granule cells from the external to the germinal layer, stunting of the dendritic arborization of the Purkinje cells, diminished axonal myelination and even cell loss (Oppenheimer & Schwartz 1997). The cellular deficits caused by TH deficiency at the postnatal period recover with age and no gross abnormality in the cerebellum is observed at the

adult stage (Oppenheimer & Schwartz 1997). The prevalence of mental deficit and gait defects among hypothyroid children suggests that cellular processes yet to be defined render the cells responsive to TH deficiency.

The role of TH in cell proliferation, migration, differentiation and maturation has been investigated in detail. Programmed cell death (PCD), an important process of development, has received less attention in the context of TH and brain development. The nature of increased cell death attributed to deficiency of TH during cerebellar development is not yet understood in the context of apoptosis (Patel *et al.* 1976, Rabie *et al.* 1977, 1979, 1980, Vincent *et al.* 1982). It is not clear whether TH acts as a physiological signal to trigger PCD to adjust cell number or to prevent lethal differentiation of brain cells.

PCD resulting in apoptotic morphology involves the interplay of many pro- and anti-apoptotic genes and their activity appears to control cell death during development (Yuan & Yanker 2000). Cell cycle regulatory proteins such as p53, c-myc, Rb-1, E1A, cyclin D1, c-fos and p34^{cdc2} kinase can also modulate PCD in specific situations (Steller 1995 and references therein). A clear-cut

demonstration of their function in apoptosis is still awaited. However, several families of molecules that regulate apoptosis during brain development have now been well characterized. Bcl-2 family proteins are the key regulators of apoptosis. Proteins which are closer to Bcl-2, e.g. Bcl-x_L, promote cell survival by inhibiting adaptors needed for activation of the proteases (caspases) that dismantle the cell. More distant relatives of Bcl-2, i.e. Bax, Bcl-x_S and Bad, instead promote apoptosis probably through displacing the adaptors from the pro-survival proteins. Thus, the alteration in the ratio of pro- to anti-apoptotic molecules determines the fate of the cell to undergo apoptosis through proteolytic cascade (Adams & Cory 1998 and references therein). This cascade initiates caspase-activated DNase/DNA fragmentation factor (DFF)-40 through cleavage of the inhibitor of caspase-activated DNases/DFF-45, which act on nuclei to induce DNA fragmentation and facilitate the cell to undergo apoptosis (Liu *et al.* 1997).

The alteration of specific molecular events probably following the binding of TH to its nuclear receptor, expressed in the cerebral cortex and cerebellum, has been well reported by others and ourselves (Perez-Castillo *et al.* 1985, Karmarkar *et al.* 1993, Mellstorm *et al.* 1991, 1994, Chattopadhyay *et al.* 1995, Bradley *et al.* 1994). Ligand-bound TH receptor forms a heterodimer with other members of the steroid receptor family and subsequently binds to the thyroid response element of the target gene to alter its expression (Oppenheimer & Schwartz 1997). Whether the lack of TH in the postnatal period affects the expression of apoptotic genes to modulate cell death during cerebellar neurogenesis is not well understood. We therefore studied the effect of TH deficiency on the expression of Bcl-2, Bcl-x_L and Bax, known modulators of apoptosis during cerebellar development.

Materials and Methods

Experimental animals

Sprague–Dawley rats were housed in a 12 h light:12 h darkness cycle environment with *ad libitum* availability of chow diet and tap water. Pregnant rats were divided into two groups ($n=30$ in each group). Hypothyroidism was induced in rats using protocols described previously (Calvo *et al.* 1990). Briefly, pregnant dams were given 0.025% 2-mercapto-1-methylimidazole (MMZ) in drinking water *ad libitum* from gestational day 8. The weight, behavior and drinking water consumption in both MMZ-treated and control groups were monitored daily. Pups from both hypothyroid and control groups ($n=15$ at each stage) were harvested at birth (postnatal day 0; P0) and thereafter on P4, P8, P12, P16, P20, P24 and P90 (adulthood). At least three pups at each developmental stage from control and hypothyroid groups were used for all the procedures and each experiment was performed in triplicate. Pups were

anesthetized with ether and decapitated. The weights of pups in the control and hypothyroid groups were recorded. Blood samples were collected at the time of death for the determination of TH. Serum total tri-iodothyronine (TT3) and total thyroxine (TT4) were measured by specific and sensitive radioimmunoassays (DPC, New York, NY, USA). All the animal procedures performed above were in accordance with the institutional guidelines for animal care and research.

DNA fragmentation analysis

Cerebella collected from the pups of control and hypothyroid rats were processed simultaneously. Cerebellum samples (0.5 g) from the pups were minced in 10 vol. buffer A (5 mM Tris, pH 8.0, 5 mM EDTA and 0.5% Triton-X-100) and kept on ice for 2 h. The genomic DNA was pelleted by centrifugation at 14 000 *g* for 20 min at 4 °C. The supernatant was extracted with equal volumes of phenol/chloroform/isoamyl alcohol (25:24:1). The DNA was precipitated with two volumes of ethanol at –80 °C for 2 h and washed with 75% ethanol. The pellet, dissolved in buffer B (10 mM Tris, pH 8.0 and 1 mM EDTA) and treated with RNase A (100 µg/ml), was finally extracted with phenol/chloroform/isoamyl alcohol (25:24:1). The precipitated DNA was analyzed on a 2% agarose gel. The experiment was repeated in three different groups (Linnik *et al.* 1995).

Preparation of cytosolic fraction

Cerebella were collected immediately in prechilled buffer C (0.32 M sucrose, 1 mM K-EDTA and 10 mM Tris-HCl, pH 7.4), homogenized and centrifuged at 1300 *g* for 5 min. The supernatant was collected and the pellet resuspended in the same buffer was centrifuged at 1300 *g* for 5 min. Pooled supernatant was centrifuged at 17 000 *g* for 15 min to collect mitochondria (Vega-Nunez *et al.* 1997). Assay of lactate dehydrogenase and cytochrome oxidase confirmed the supernatant as cytosolic and the pellet as mitochondrial fractions. Cytosolic fractions were aliquoted and stored at –80 °C (Vega-Nunez *et al.* 1997) for further analysis. The protein concentration was determined by a standard method (Lowry *et al.* 1951).

Western blotting

Cytosol, equivalent to 50 µg protein suspended in 1 × buffer D (10% glycerol, 2% SDS and 0.0625 M Tris-HCl, pH 6.8) was heated with 2-mercaptoethanol at 70 °C for 7 min. These samples were subjected to 0.1% SDS–12% PAGE. The gels were run in duplicate, one gel was used for transfer to membrane while the other was stained with Coomassie blue to confirm equal loading of

proteins. The protein was electrotransferred on nitrocellulose membrane and stained with Ponceau S to confirm complete transfer.

Non-specific binding sites were blocked with 5% BSA in 20 mM Tris-HCl, pH 7.4, 137 mM NaCl and 0.1% Tween-20 (TBST) for 1 h at 37 °C. The membranes were washed with 0.1% BSA in TBST at room temperature and incubated with different primary antibodies in 1% BSA in TBST for 2 h at room temperature. After being washed three times with 0.1% BSA in TBST, membranes were incubated with a secondary antibody labeled with horseradish peroxidase in 1% BSA in TBST. The filter was washed three times with 0.1% BSA in TBST (Sambrook *et al.* 1989) and color reaction was developed according to the manufacturer's protocol (Bio-Rad, Hercules, CA, USA).

Statistical analysis

The relative amount of each protein was determined quantitatively. Blots from all three replicates of control and hypothyroid groups were analyzed microdensitometrically using Alpha Imager (Alpha Imager Corp., San Leandro, CA, USA). The standard error of the mean between the groups was analyzed by SPSS software (version 9.0) using Levene's independent sample *t*-test. Ontogenic expression of each protein was assessed by one-way analysis of variance and data were further analyzed for specific changes over time using Tukey's B test.

Results

Hypothyroidism reduces growth and weight gain in the developing rat

The uptake of drinking water containing MMZ was recorded daily and it was found that dams from both control and hypothyroid groups consumed equal amounts of feed and drinking water. The pups on the chow diet and tap water recorded normal growth and size gain during development, while those on MMZ showed retarded size and weight gain (Fig. 1A). Significantly low circulating TT4 and TT3 levels confirmed the hypothyroid status of the experimental pups ($P < 0.005$, $P < 0.05$ respectively) (Fig. 1B and C).

TH deficiency induces extensive DNA fragmentation in developing cerebellum

To check whether TH deficiency leads to apoptosis, the smaller fragments of DNA isolated from the developing cerebellum of control and hypothyroid pups were analyzed on agarose gel. In euthyroid conditions, DNA fragmentation was not observed except basal level at P8 (Fig. 2A). Cerebella from hypothyroid pups showed extensive DNA

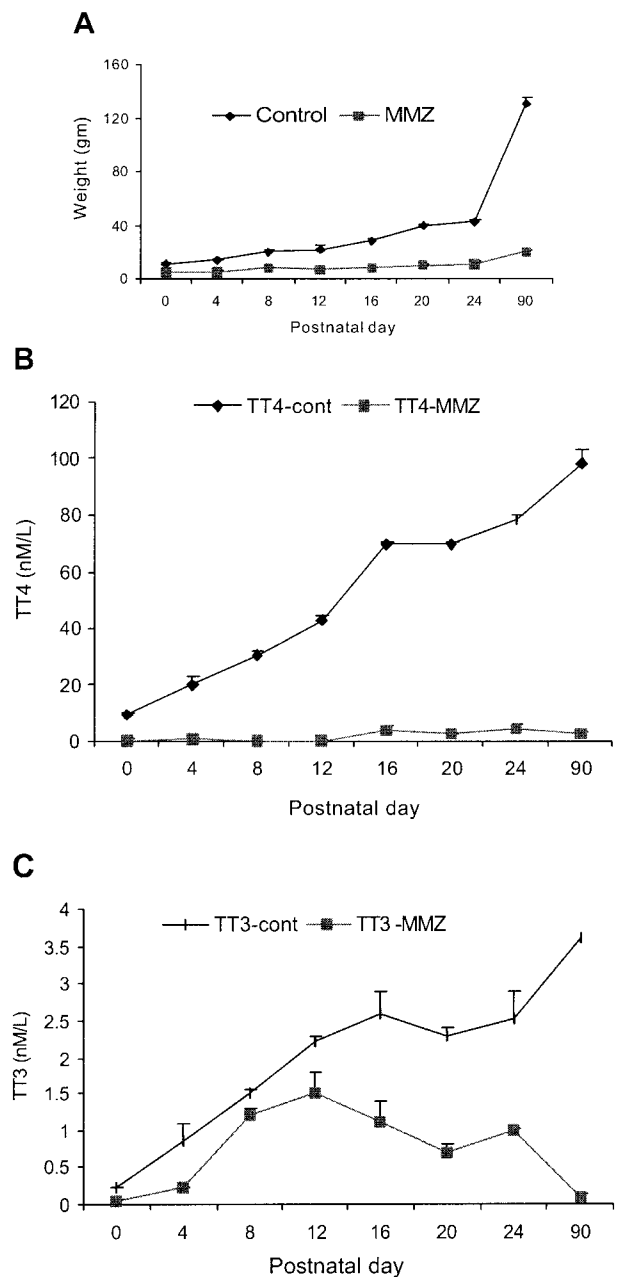


Figure 1 Weight of MMZ-treated developing pups. (A) Growth and weight of pups of control and MMZ-treated rats analyzed from P0 to P90. The results express the average weight (means \pm S.E.M.). Serum (B) TT4 and (C) TT3 in developing pups determined by radioimmunoassay. Blood was collected from pups ($n = 15$) at each developmental stage from control (cont) and hypothyroid groups. The results are expressed as the mean \pm S.E.M.

fragmentation starting from P0 to P16, this decreased thereafter and was detectable even at the adult stage of 90 days (Fig. 2B).

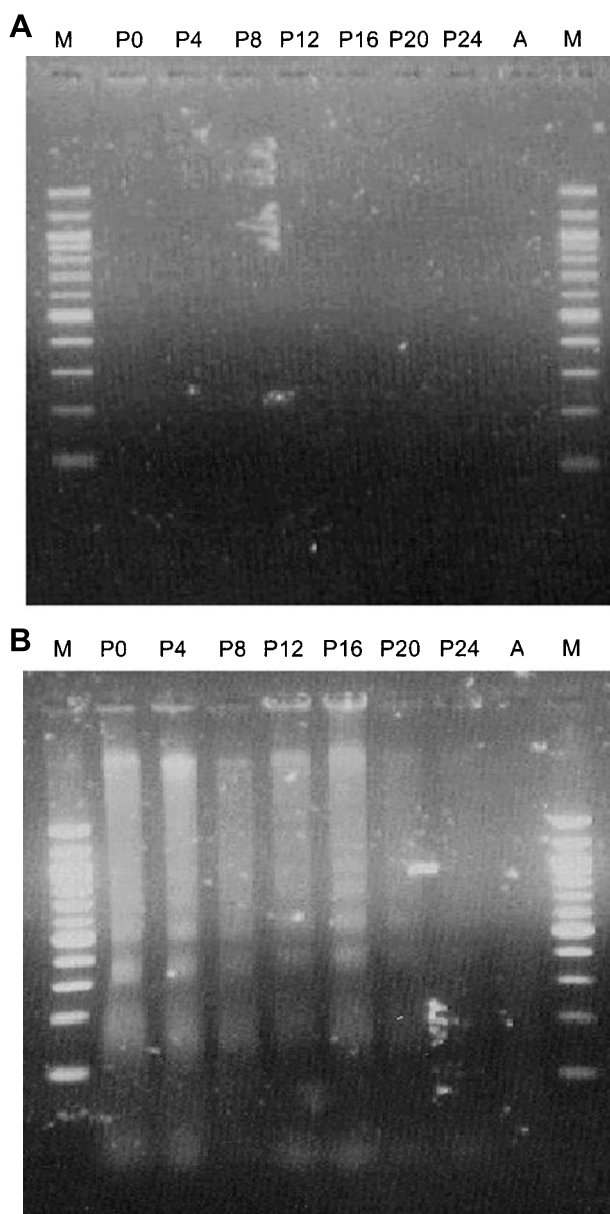


Figure 2 DNA fragmentation analysis from the cerebellum of developing pups. Smaller fragments of DNA were isolated from the cerebellum of (A) control and (B) hypothyroid pups ($n=3$) at each developmental stage (200 bp DNA ladder (M); P0; P4; P8; P12; P16; P20; P24; adult (A) and analyzed on 2% agarose gel.

Hypothyroidism leads to enhanced cleavage of DFF-45 in developing cerebellum

DFF-40, which exists in association with DFF-45 in cytosol, is known to initiate DNA fragmentation (Liu *et al.* 1997). Therefore, dissociation of DFF-40 from DFF-45 was analyzed to further confirm the apoptosis in developing cerebellum. Assay for the cleavage of DFF-45 was carried out by Western blot analysis with an antibody that

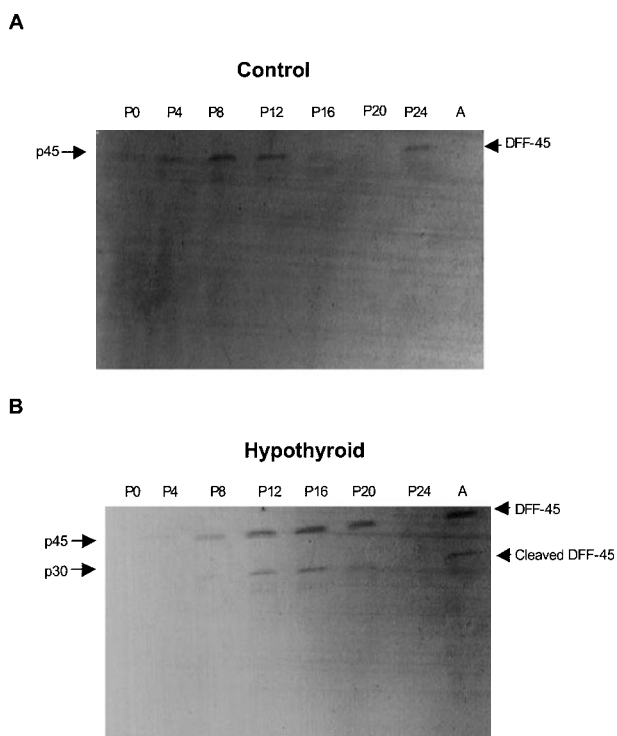


Figure 3 DFF-45 analysis in the cerebellum of developing pups. Cytosol was prepared as described in Materials and Methods from developing cerebellum of (A) control and (B) hypothyroid pups ($n=3$) at each developmental stage (P0; P4; P8; P12; P16; P20; P24; adult (A)). The cytosolic protein (50 μ g) was subjected to 12% SDS-PAGE followed by Western blotting with a rabbit polyclonal antibody recognizing the 45 kDa and 30 kDa subunits of DFF-45. To confirm the cleavage of DFF-45, the experiment was repeated independently three times in both groups.

recognizes 45 kDa and the cleaved fragment of 30 kDa (a kind gift from Dr X Wang, University of Texas, Southwestern Medical Centre, Dallas, TX, USA). In euthyroid conditions, the expression and cleavage of DFF-45 was minimal as it was barely visible in the early developmental period P0–P12 (Fig. 3A). The cleaved subunit of 30 kDa was undetectable at the late developmental stage of P20 to adult (Fig. 3A). However, under hypothyroid conditions, the proteins of molecular weight of 45 and 30 kDa were clearly visible at P8 and P12 (Fig. 3A) and their presence was detectable until late development (P16 and P24) (Fig. 3B). These observations are in conformity with DNA fragmentation pattern (Fig. 2).

TH deficiency down-regulates the expression of Bcl-2

Bcl-2, a 21 kDa membrane-bound protein, is known to prevent apoptosis in neuronal cells during development (Cleary *et al.* 1986). We analyzed the expression of Bcl-2 by Western blotting during cerebellar development. Densitometric analysis showed that the relative amount of

expression varied significantly ($P < 0.001$) with age in control pups. The level of Bcl-2 was high in developing euthyroid cerebellum from P0 to P12 and thereafter decreased significantly ($F_{7,24} = 219.8$; $P < 0.001$) at P16 and P24, and was undetectable at P20 and the adult stage (Fig. 4A). Similar age-related variation in the expression of the Bcl-2 gene was also observed in the hypothyroid group and it was more pronounced in the early stage ($F_{7,24} = 743.3$; $P < 0.001$). When the expression of Bcl-2 in developing hypothyroid cerebellum was compared with that of euthyroid cerebellum, it was significantly lower in the hypothyroid condition ($P < 0.001$) at all stages of development except the adult stage (Fig. 4).

Expression of Bcl-x_L decreases in developing hypothyroid cerebellum

Bcl-x_L, a 28 kDa Bcl-2 family anti-apoptotic protein (Chinnaiyan *et al.* 1996), is localized in mitochondria which forms a heterodimer with Bcl-2 and maintains mitochondrial integrity (Vander Heiden *et al.* 1997). Therefore, expression of Bcl-x_L was also checked in developing cerebellum. In the euthyroid condition, Bcl-x_L was expressed at a high level from P0 to P12, then decreased and expression was not observed at P20 ($F_{7,24} = 219.3$; $P < 0.001$) (Fig. 4B). However, in the hypothyroid condition, the expression was undetectable throughout development as well as in the adult cerebellum (Fig. 4B).

Hypothyroidism alters Bax expression in developing cerebellum

Bax, a member of the pro-apoptotic Bcl-2 family gene having a molecular weight of 21 kDa (Chittenden *et al.* 1995), is an important regulator of apoptosis. The relative amount of Bax in cytosol varied significantly ($P < 0.001$) with age in the euthyroid condition. Initially, low levels of Bax expression were observed from P0 to P20, thereafter they increased significantly ($F_{7,24} = 172.4$; $P < 0.001$) at P24 and the adult stage (Fig. 4C). In the hypothyroid group, expression was high at initial development from P0 to P12, then dropped significantly ($F_{7,24} = 106.3$; $P < 0.001$) at P20, increased again at P24 and then further decreased at the adult stage. In comparison with euthyroid developing cerebellum, it was significantly high ($P < 0.001$) in the hypothyroid state (Fig. 4C).

Discussion

In the present study, we observed that TH deficiency during cerebellar development leads to enhanced DNA fragmentation through activation of DFF-40 by cleavage of DFF-45. Apoptosis is not only enhanced but also extended until the late developmental stage.

Hypothyroidism down-regulates the expression of anti-apoptotic genes Bcl-2 and Bcl-x_L whereas the pro-apoptotic gene Bax is up-regulated throughout the developmental period. The alteration in the expression of Bcl-2 family genes increases the cytosolic content of pro-apoptotic molecules leading to increased apoptosis.

Severe hypothyroidism caused by the administration of MMZ results in retarded growth, poorly co-ordinated creeping and gait defects in the progeny (Fig. 1). These effects being more pronounced at P4–P16 reflect the compromised cerebellar growth and are in accordance with previous studies (Porterfield & Hendrich 1993 and references therein). The effect of TH on different cellular processes, i.e. proliferation, migration and maturation, has been well studied, but has resulted in limited progress in understanding the pathophysiology of these disorders. Apoptosis is important for normal brain development as 20–50% of neurons are known to undergo PCD. The number of cells and the location of those undergoing death in the nervous system is still a matter of conjecture (Oppenheim 1991, Yuan & Yanker 2000). Although the decrease in cell number under hypothyroid conditions is well reported, the understanding of PCD during cerebellar neurogenesis and its modulation by TH is still far from clear (Lewis *et al.* 1976, Rebiere & Dainat 1976, Rabie *et al.* 1977, 1979, 1980, Patel *et al.* 1976, Vincent *et al.* 1982).

We used a rat model to understand the effect of hypothyroidism on apoptosis during the development of the cerebellum. The rat is an altricial species where cerebellar neurogenesis occurs postnatally (Fisher & Polk 1988) and various anatomical alterations induced by hypothyroidism have been well documented and mimic the human condition (Koibuchi & Chin 2000). Hypothyroidism can be established in dam, intrauterine fetuses and neonates with relative ease (Oppenheimer & Schwartz 1997 and references therein), and therefore we used the developing rat cerebellum to understand TH-modulated apoptosis.

Recently, a decrease in cell number in the developing cerebellum has been demonstrated to be mediated through apoptosis, but the mechanism is still not clear. PCD mediated through apoptosis is initiated by the activation of cellular machinery culminating in DNA fragmentation (Yuan & Yanker 2000). To understand the mechanism of apoptosis, DNA fragmentation analysis, a typical feature of apoptosis, was therefore performed. The minimal DNA fragmentation seen only at P8–P12 under euthyroid conditions suggested that a minimal number of neuronal cells undergo apoptosis during cerebellar development. This observation, although in agreement with the report of Xiao & Nikodem (1998), is in contrast with the reported 20–50% cell death during neurogenesis (Oppenheim 1991, Yuan & Yanker 2000). The experiments were therefore repeated three times to confirm the minimal cerebellar apoptosis in three different groups of pups under

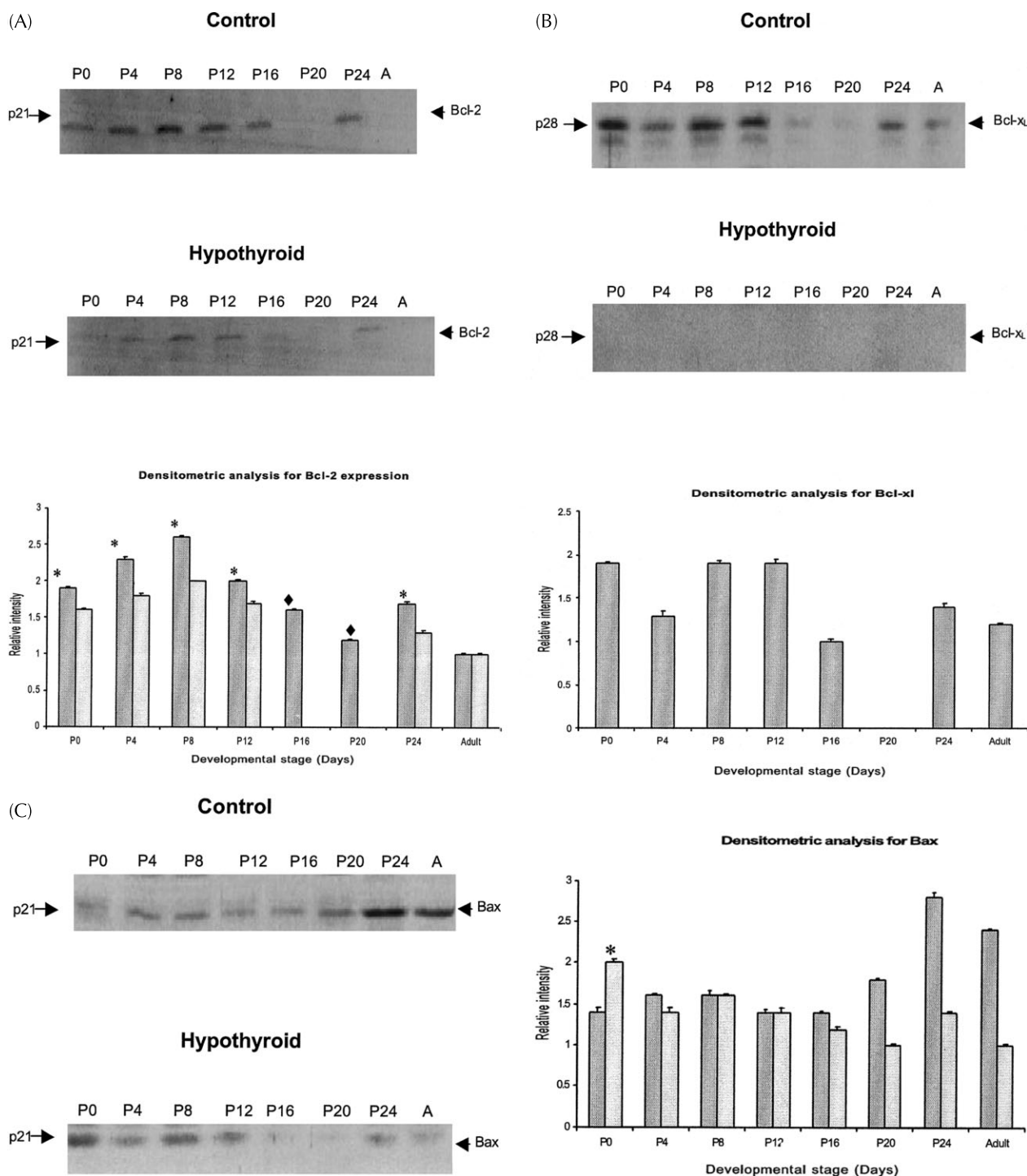


Figure 4 Expression of Bcl-2 family proteins in developing cerebellum. Cytosol from the developing cerebellum of control and hypothyroid pups ($n=3$) at each developmental stage (P0; P4; P8; P12; P16; P20; P24; adult (A)) was prepared. Cytosolic protein equivalent to 50 μ g protein was subjected to 12% SDS-PAGE followed by Western blotting with monoclonal antibody recognizing (A) Bcl-2, (B) Bcl-x_L and (C) Bax. The experiment was performed in triplicate in control and hypothyroid groups to confirm the expression. Relative intensity of controls is shown by the darker bars and that of the hypothyroid pups by the lighter bars. * $P<0.001$. ♦Bcl-2 was not detected under hypothyroid conditions.

euthyroid conditions. This was further supported by the evidence of basal expression and cleavage of DFF-45 seen under euthyroid conditions during all stages of development (Fig. 3A). However, hypothyroidism resulted in extensive and prolonged DNA fragmentation (Fig. 2B) as a consequence of enhanced cleavage of DFF-45 and activation of DFF-40 (Fig. 3B). The enhanced apoptosis at P12 observed here may explain the decrease in cell number and prominence of pyknotic nuclei in the external granular layer of the cerebellum of hypothyroid pups as reported earlier (Lewis *et al.* 1976). The effect of TH on apoptosis during development may therefore seem to be area specific rather than of an ubiquitous nature and needs to be studied further.

TH is known to regulate nuclear genes by binding to its nuclear receptor expressed widely in the cerebellum (Koibuchi & Chin 2000). The Bcl-2 family of nuclear genes constitutes the critical regulator of apoptosis (Vander Heiden *et al.* 1997, Korsmeyer *et al.* 1993 and references therein). Pro- and anti-apoptotic members of the Bcl-2 family have pleiotropic effects on the activation of caspase cascades regulated by the apoptosome (Green & Reed 1998 and references therein). Anti-apoptotic (Bcl-2 and other homolog) and pro-apoptotic (Bax and other members) family members can heterodimerize and seemingly neutralize each other's function, suggesting that their relative concentration may act as a rheostat for the suicide program. To answer whether TH, through its nuclear action, modulates the expression of these genes, expression levels of Bcl-2, Bcl-x_L and Bax were studied.

Higher expression of Bcl-2 in the euthyroid cerebellum is in consonance with earlier reports (Dubios-Dauphin *et al.* 1994, Gonzalez-Garcia *et al.* 1995, Muller *et al.* 1995, Mooney & Miller 2000). Here, we report for the first time that Bcl-x_L is expressed at a high level in developing euthyroid cerebellum (Fig. 4B). However, the pro-apoptotic gene Bax was constitutively expressed at a low level throughout postnatal development (Fig. 4C). Hypothyroidism results in lower expression of Bcl-2, undetectable levels of Bcl-x_L (Fig. 4A and B), along with a high level of expression of Bax (Fig. 4C). The lowering of Bcl-2 expression under hypothyroid conditions is in agreement with an earlier report (Muller *et al.* 1995). These results suggest that TH promotes the expression of Bcl-2 and Bcl-x_L genes along with Bax, maintaining the ratio of pro- to anti-apoptotic genes, thus preventing apoptosis of early differentiating cerebellar granular cells. However, under hypothyroid conditions, the down-regulation of the anti-apoptotic Bcl-2 and Bcl-x_L gene and the up-regulation of Bax shift the balance towards extensive apoptosis. The extent of apoptosis observed here may explain the loss of about 1% of the total cerebellar cell population within 24 h and reduction in DNA content in the germinal zone observed at P12–P16 as reported earlier, which remain unexplained (Lewis *et al.* 1976, Rebiere & Dainat 1976, Rabie *et al.* 1977, 1979, 1980, Patel *et al.*

1976, Vincent *et al.* 1982). However, it is interesting to note that the higher expression of Bax at P24 and the adult stage does not lead to a significant level of apoptosis. At the same developmental stages, the expression of Bcl-2 is also high and therefore heterodimerization with Bax may prevent apoptosis and hence minimal DNA fragmentation was observed here. The present study indicated that TH also regulates the expression of the Bcl-x_L gene which is known to maintain the mitochondrial architecture. Its loss due to hypothyroidism may also activate mitochondrial-mediated pathways during cerebellar development.

It seems that TH tightly regulates the process of apoptosis during cerebellar development and its deprivation removes the inhibitory control on this phenomenon. The extensive apoptosis observed may not only be explained by the nuclear action of TH modulating the expression of Bcl-2 family genes. The activation of DFF-40 by cleavage of DFF-45 observed here indicates the need to study the possible role of caspases during cerebellar development (Hengartner 2000 and references therein). The alteration in the levels of Bcl-2 family proteins may lead to structural changes in mitochondria, thereby suggesting the active role of mitochondrial-mediated apoptosis during cerebral neurogenesis. In light of the changes reported in mitochondrial structure and transmembrane potential in the hypothyroid cerebral cortex (Vega-Nunez *et al.* 1997), further investigations are needed to relate the changes in the whole repertoire of apoptotic molecules, their translocation and mitochondrial structural changes, if any, in developing cerebellum.

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