Hypothyroidism alters mitochondrial morphology and induces release of apoptogenic proteins during rat cerebellar development

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Abstract

Thyroid hormone (TH) deficiency leads to extensive apoptosis during cerebellar development, but the mechanism still remains unclear. Different signals also converge on mitochondria during apoptosis to induce the release of apoptogenic proteins that activate proteolytic cascade through specific enzymes called caspases. Here we studied the effect of hypothyroidism on alterations in mitochondrial structure and translocation of apoptogenic molecules during rat cerebellar development. Structural analysis of mitochondria was studied by electron microscopy. The translocation of apoptogenic molecules was analyzed by Western blotting. TH deficiency led to vacuolization, enlargement and decrease in the number of cristae. The

majority of the proapoptotic molecule, *Bax*, was localized in mitochondria under hypothyroid conditions whereas a limited presence of *Bax* was detected in the euthyroid state. Translocation of cytochrome c, apoptosis–inducing factor (AIF) and second mitochondrial–derived activator of caspases (SMAC) from mitochondria to cytosol was detected primarily in early developmental stages in the hypothyroid condition. These experimental results demonstrate that TH maintains mitochondrial architecture and inhibits the release of apoptogenic molecules to prevent excess apoptosis during cerebellar development.

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Introduction

Thyroid hormone (TH) plays an essential role in mammalian brain development. During the critical period of brain development, insufficiency of TH results in several mental disorders (Schwartz 1983, DeLong *et al.* 1985, Porterfield & Hendrich 1993, Oppenheimer & Schwartz 1997). Hypothyroidism causes severe morphological abnormalities in the entire developing brain, albeit to different extents in different parts of the brain. The size and number of neurons, especially pyramidal neurons in the neocortex, hippocampus and the olfactory bulbs, are reduced. In the cerebellum, TH deficiency has been reported to result in the delayed proliferation and migration of the granule cells from the external to the germinal layer, stunting of the dendritic arborization of the Purkinje cells, diminished axonal myelination and extensive cell loss (Oppenheimer & Schwartz 1997 and references therein). However the exact mechanism by which hypothyroidism affects brain development remains to be elucidated.

TH is known to regulate gene expression by binding to its nuclear receptors, expressed in different parts of the brain from early to adult stages (Bernal & Pekonen 1984, Perez-Castillo *et al.* 1985, Strait *et al.* 1991, Bradley *et al.* 1992, Karmarkar *et al.* 1993, Chattopadhyay *et al.*

1995). The influence of TH on known target genes, namely *RC3*, *MBP*, *MAP* and *PCP*-2, has been demonstrated but has not increased our understanding of brain development (Silva & Rudas 1990, Farsetti *et al.* 1991, Iniguez *et al.* 1993, Zou *et al.* 1994). Therefore, several attempts have been made to isolate hitherto unknown tri-iodothyronine (T_3) regulated genes during brain development by different modern techniques and other model systems (Muñoz *et al.* 1991, Iglesias *et al.* 1995, Vega-Nunez *et al.* 1995, Denver *et al.* 1997). Using *Xenopus* as an experimental model, where true T_3 negative conditions can be created, 34 cDNAs for TH–regulated genes in the diencephalon were identified. Several of the identified genes were of mitochondrial origin, known to be involved in energy metabolism (Denver *et al.* 1997). Recently, demonstration of the TH receptor in mitochondria and its involvement in controlling gene expression in mitochondria by different groups further potentiate the role of TH in energy metabolism (Hashizume & Ichikawa 1982, Wrutnaik *et al.* 1995, Enriquez *et al.* 1999, Rochard *et al.* 2000). Establishment of the mitochondria's key role in apoptosis, a cardinal feature of neurogenesis, indicates that mitochondria may also play an important role in brain development (Oppenheim 1991, Yuan & Yanker 2000). However, no correlation has yet been established between mitochondria

and apoptosis during cerebellar development under hypothyroid conditions.

Divergent cellular stresses including DNA damage, heat shock, oxidative stress and withdrawal of growth factor converge on mitochondria during apoptosis (Adrain & Martin 2001). Mitochondria contain several apoptosis-inducing proteins in the inter-membrane space (Green & Reed 1998, Hengartner 2001). *Bax* is a pro-apoptotic gene and is an important regulator of apoptosis. *Bax* translocation from cytosol to mitochondria is considered to be an important initial event in controlling the release of different apoptogenic molecules (Jurgensmeier *et al.* 1998). Cytochrome c is the key executioner of apoptosis and recently other molecules, namely apoptosis-inducing factor (AIF) and second mitochondrial-derived activator of caspases (SMAC), also have been identified (Lorenzo *et al.* 1999, Daugas *et al.* 2000, Du *et al.* 2000, Verhagen *et al.* 2000). Efflux of cytochrome c in cytosol results in assembly of apoptosome that activates caspase-9. This leads to activation of a proteolytic cascade resulting in cellular disassembly by the effector caspase-3. The activated caspase leads to cellular disassembly and DNA fragmentation. AIF, a flavoprotein that resides in the inter-membrane space of the mitochondria, translocates to the nucleus during apoptosis. It causes partial chromatin condensation in the periphery of the nucleus by causing degradation of DNA into >50 kb fragments through a caspase-independent pathway (Zamzami & Kroemer 1999). SMAC antagonizes the function of inhibitors of apoptosis (IAPs) present in cytosol that block caspase activation (Daugas *et al.* 2000, Du *et al.* 2000, Verhagen *et al.* 2000).

In the present study, we investigated the effect of hypothyroidism on mitochondrial alterations during rat cerebellar development. Here, we report that TH deficiency leads to structural alterations in mitochondria and enhanced translocation of apoptogenic proteins to cytosol, which results in the enhanced apoptosis during cerebellar neurogenesis observed previously (Xiao & Nikodem 1998, Singh *et al.* 2003).

Materials and Methods

Experimental animals

Sprague–Dawley rats were housed with a ratio of 12 h light:12 h darkness. They had access to chow diet and tap water and were allowed to feed *ad libitum*. Pregnant rats were divided into two groups ($n=30$ in each group). Hypothyroidism was induced in rats using a previously described protocol (Calvo *et al.* 1990). Briefly, pregnant dams were given 0·025% of 2-mercapto-1-methylimidazole (MMZ) in drinking water from gestational day 8 and allowed to drink *ad libitum*. Pups from both hypothyroid and control groups ($n=15$ at each stage) were harvested at postnatal day (P)0, i.e. birth, and

thereafter on P4, P8, P12, P16, P20, P24 and P90 (adulthood). At least three pups at each developmental stage from the control and hypothyroid groups were used for all the procedures and each experiment was performed in triplicate. Pups were anesthetized with ether and de-capitated. Blood samples were collected when the animals were killed for the determination of TH. All animal procedures performed above were in accordance with the institutional guidelines for animal care and research.

Measurement of serum total T_3 (TT_3) and total thyroxine (TT_4)

Serum TT_3 and TT_4 were measured according to the manufacturer's protocol using standard RIA kits (DPC kit, New York, NY, USA).

Preparation of the cytosolic fraction

Cerebellum was collected immediately in buffer C (0·32 M sucrose, 1 mM K-EDTA, 10 mM Tris–HCl, pH 7·4), homogenized and centrifuged at 1300 *g* for 10 min. The supernatant was collected, and the pellet was re-suspended in the same buffer and centrifuged again at 1300 *g* for 10 min. The supernatant was pooled and spun at 17 000 *g* for 15 min to collect mitochondria. The assay of marker enzymes lactate dehydrogenase (LDH) and cytochrome oxidase (COX) confirmed the supernatant as the cytosolic fraction and the pellet as the mitochondrial fraction. The mitochondrial pellet re-suspended in buffer C and cytosolic fraction were aliquoted and stored at − 80 °C for further analysis (Vega-Nunez *et al.* 1997). The protein concentration was determined by a standard method (Lowry *et al.* 1951).

Electron microscopy

Ultrastructural morphology of mitochondria was determined by transmission electron microscopy. Coronal sections of cerebellum of 1 mm thickness were prepared and fixed rapidly with 4% paraformaldehyde–4% glutaraldehyde in 0·1 M phosphate buffer. After fixation, sections were osmicated in 1% osmium tetraoxide for 2 h at 4 °C and dehydrated in alcohol–acetone series. Tissues were embedded in Araldite resin, and semithin sections (2·5 μm) were removed for optical microscopy. Ultrathin sections (50 nm) were mounted on copper mesh grids and stained with uranyl acetate and lead citrate before examination with a transmission electron microscope (JEOL 1210, Peabody, MA, USA).

Western blotting

Cytosol and mitochondrial pellet equivalent to 50 μg protein having 1X sample buffer D (10% glycerol, 2% SDS, 0·0625 M Tris–HCl (pH 6·8)) were heated with

2-mercaptoethanol at 70 °C for 7 min. The samples were subjected to 0·1% SDS–15% PAGE. The gels were run in duplicate; one gel was used for transfer to nitrocellulose membrane while the other was stained with Coomassie blue to confirm equal loading of proteins. The protein was electrotransferred to nitrocellulose membrane and the filter was stained with Ponceau S to visualize protein bands, to further confirm equal transfer of the proteins.

The nonspecific binding sites were blocked with 5% BSA in TBST (20 mM Tris–HCl (pH 7·4), 137 mM NaCl, 0·1% Tween 20) for 1 h at 37 °C. The membranes were washed with 0·1% BSA in TBST three times at room temperature and were incubated with different primary antibodies (in 1% BSA in TBST) for 2 h at room temperature. After washing three times with 0·1% BSA in TBST at room temperature, the membranes were incubated with secondary antibody labeled with horseradish peroxidase for 2 h at room temperature. The filter was washed three times with 0·1% BSA in TBST (Sambrook *et al.* 1989). The color was developed by immuno–Blot Assay kits (Bio Rad, Hercules, CA, USA). Each experiment was repeated three times independently.

Statistical analysis

The relative amount of each protein was determined quantitatively. Blots from all the three replicates of control and hypothyroid groups were analyzed microdensitometrically using an 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 ANOVA and data were further analyzed for specific changes over time using Tukey's B test.

Results

Severe hypothyroidism is induced by MMZ

Hypothyroid pups showed stunted growth and sluggish behavior as compared with controls. The creeping and opening of eyes was delayed, especially from stages P4 to P16. Serum TT_4 and TT_3 levels of growing pups increased with age whereas they decreased significantly ($P<0·005$, $P<0·05$) in MMZ–treated groups and were hardly detectable in hypothyroid rats (Fig. 1).

TH deficiency alters mitochondrial structure in developing cerebellum: electron microscopic analysis

To assess whether TH deficiency during cerebellar development results in structural alteration, electron microscopy was performed at stage P12, where maximum apoptosis and caspase activity was seen previously (Singh *et al.* 2003).
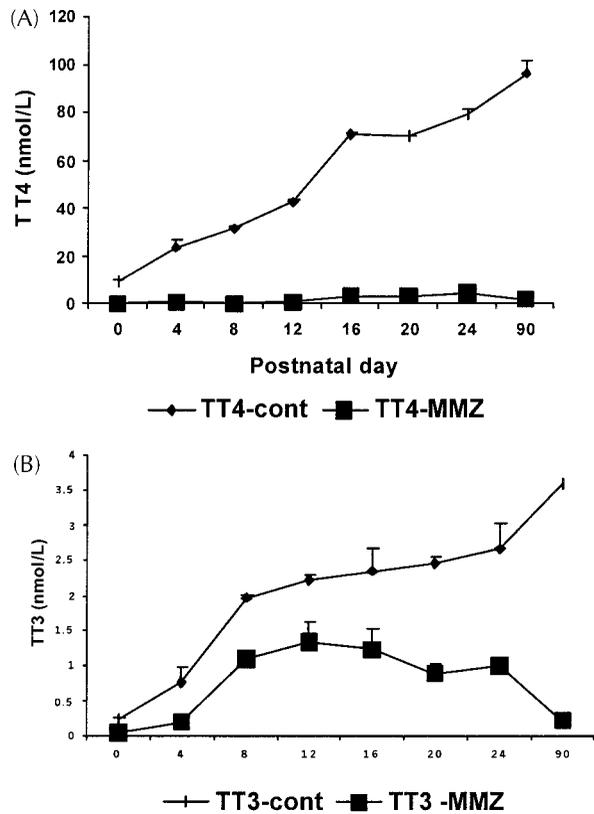

Figure 1 Serum TT_3 and TT_4 in developing pups. Serum from pups of control and hypothyroid rats were analyzed for TT_3 and TT_4 from postnatal day 0 to 90 days. The blood was collected from five of each stage and from three different groups of control and MMZ-treated pups. Serum TT_4 (A) and TT_3 (B) were determined by RIA. The results are expressed as means ± S.E.M.

It has been observed previously that the programmed cell death after birth is limited to the internal germinal layer (IGL) of cerebellum although some apoptotic cells are also observed in the external granular layer (EGL) (Xiao & Nikodem 1998). Therefore, sections from the IGL were processed for electron microscopy to see the alteration in mitochondrial morphology. The mitochondria from euthyroid cerebellum presented with highly packed inner membranes and many visible cristae characteristic of normal morphology (Fig. 2A). Altered mitochondrial morphology with enlarged size, increased vacuolization and a decrease in the number of cristae was seen under hypothyroid conditions. The number of mitochondria having altered morphology also increased under hypothyroid conditions (Fig. 2B).

Bax is translocated to mitochondria during hypothyroid conditions

Bax translocation from cytosol to mitochondria is an important event that may induce structural change in

A

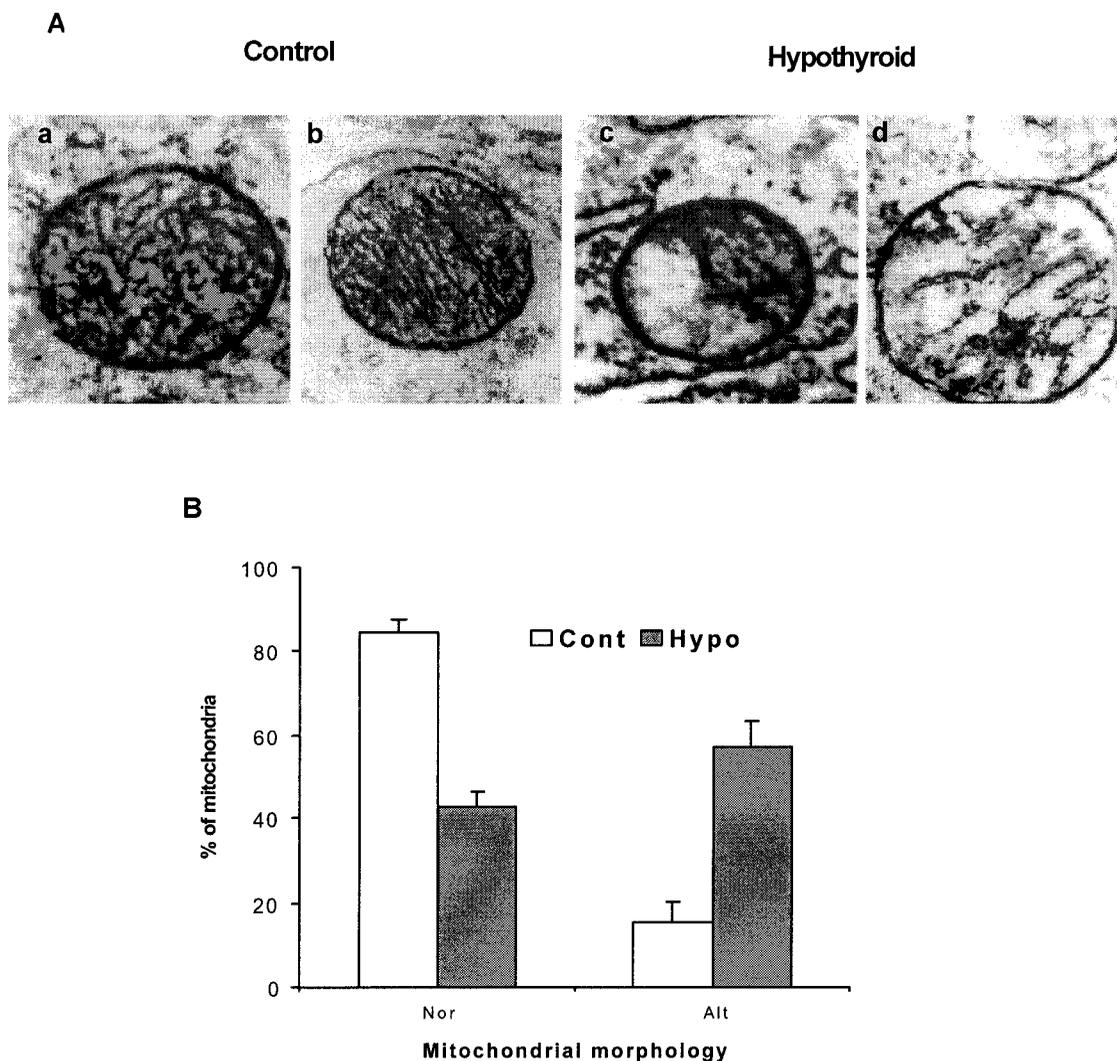**Control**   **Hypothyroid**

B

Figure 2 Structural analysis of mitochondria in developing cerebellum. (A) The coronal sections of cerebellum from the control (a, b) and hypothyroid pups (c, d) at the P12 stage were analyzed using electron microscopy for the mitochondrial morphology (20 000 ×). (B) Quantitative analysis of altered mitochondrial structure. The number of mitochondria having normal and altered structure was counted in five different views. The results were expressed from five different views from each slide of three different groups each of control and MMZ-treated pups. The results are expressed as means ± S.E.M.

mitochondria (Jurgensmeir *et al*. 1998). To see whether ultrastructural changes are due to translocation of *Bax* under hypothyroid conditions, Western blot analysis of both mitochondrial pellet and cytosol was performed. In euthyroid conditions, a 21 kDa protein was detected in mitochondria as well as in the cytosol (Fig. 3A). *Bax* was expressed constitutively from P0 to P16. The expression of *Bax* varied with age ($F_{7,24}=170$) and increased significantly at P20 to adult stage ($P<0.001$). The localization of *Bax* in the mitochondrial pellet did not show any significant variation (Fig. 3A,B). However, under hypothyroid conditions, expression of *Bax* was higher in cytosol at the P0 stage then decreased significantly ($P<0.001$) from

P4–P24 and adult, with a concomitant increase in the mitochondrial pellet. The presence of *Bax* in the mitochondrial fraction was significantly higher ($P<0.001$) as compared with the euthyroid state at all stages of development (Fig. 3C,D).

Hypothyroidism induces cytochrome c release from mitochondria to cytosol

Cytochrome c is the principal initiator molecule released from mitochondria to cytosol to initiate the apoptotic program in the cell (Green & Reed 1998). Therefore, we studied cytochrome c translocation. In the euthyroid state,
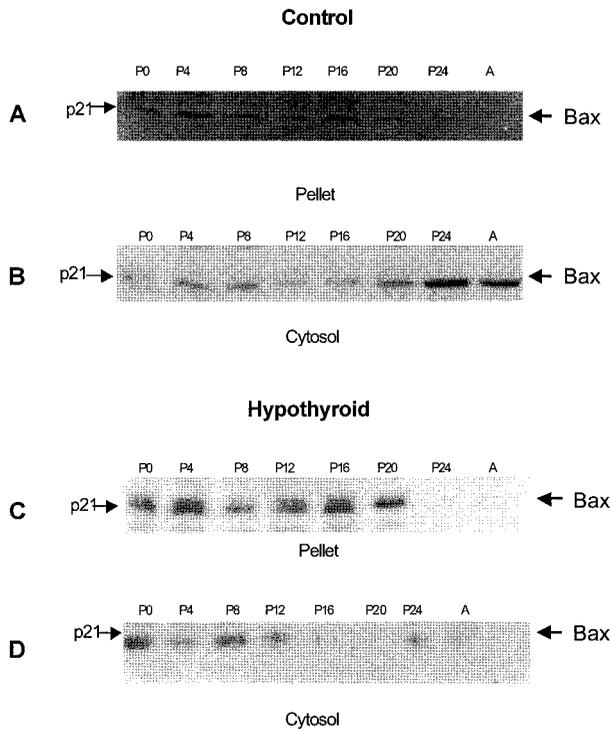
Figure 3 Analysis of translocation of *Bax* from cytosol to mitochondria. The mitochondrial pellet and cytosol were isolated from developing cerebellum (P0, P4, P8, P12, P16, P20, P24 and adult (A)) of control (A and B) and hypothyroid (C and D) pups. Cytosolic and mitochondrial proteins (50 µg) were subjected to 15% SDS–PAGE and analyzed by Western blot using rabbit polyclonal antibody. Both the experiments were performed at the same time to compare the intensity of *Bax* present in the pellet and in the cytosol. The experiments were repeated three times.

Figure 4 Analysis of cytochrome c translocation. The mitochondrial pellet and cytosol were isolated from developing cerebellum (P0, P4, P8, P12, P16, P20, P24 and adult (A)) of control (A and B) and hypothyroid (C and D) pups. Cytosolic and mitochondrial proteins (50 µg) were subjected to 15% SDS–PAGE and analyzed by Western blot. Both the experiments were performed at the same time and repeated three times.

the 20 kDa protein corresponding to cytochrome c was detected in the mitochondrial pellet (Fig. 4A) but not in the cytosolic fraction (Fig. 4B). However, the decreased intensity of the band for cytochrome c was observed in the mitochondrial pellet at P0 and P8 (Fig. 4A) but its corresponding presence was not detected in the cytosol. Conversely, in the hypothyroid condition, a significantly higher level ($P<0.001$) of cytochrome c was detected in the cytosol at the early developmental stage from P0 to P8 and the decreased intensity was observed in the mitochondrial pellet ($P<0.001$). However, at late stages of development, enhanced intensity of the cytochrome c band was observed in the mitochondrial pellet, with a concomitant decrease in the cytosol (Fig. 4C,D).

Hypothyroidism induces release of AIF from mitochondria

AIF is released from mitochondria to initiate caspase-independent apoptosis (Lorenzo *et al.* 1999). We also studied hypothyroidism-induced release of AIF from mitochondria during cerebellar development. In the
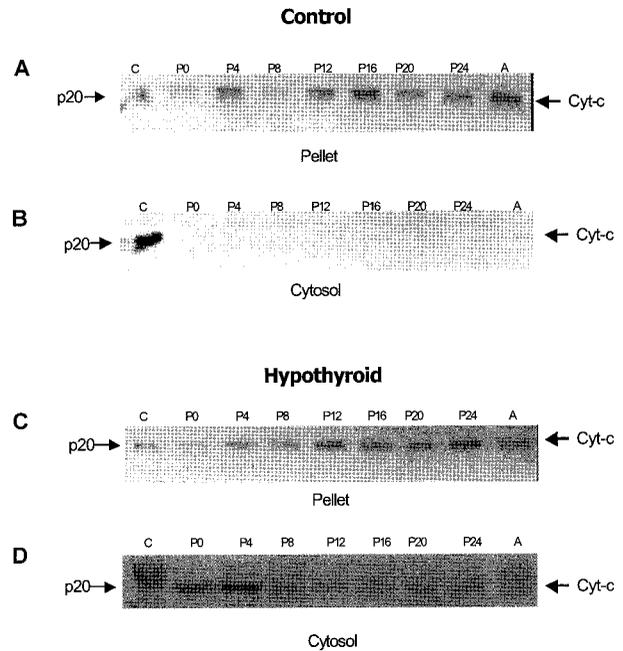
euthyroid state, Western blot analysis showed a 58 kDa protein corresponding to AIF in the mitochondrial pellet but not in cytosol (Fig. 5A,B). However, under hypothyroid conditions, low expression of AIF was detected in the mitochondrial pellet, and most of the AIF was detected in the cytosolic fraction. It was significantly ($P<0.001$) high as compared with the euthyroid condition at all the developmental stages except P20 and the adult stage (Fig. 5C,D).

SMAC translocates from mitochondria to cytosol during development

The release of SMAC from mitochondria inhibits IAPs present in cytosol, making the cell competent to undergo apoptosis (Daugas *et al.* 2000). Western blot analysis of cerebellar cellular components under euthyroid conditions revealed the presence of a ~32 kDa protein corresponding to SMAC in the cytosol from P0 to P16 that decreased ($F_{7,24}=199.0$) at later stages in euthyroid conditions (Fig. 6B). A significantly lower level of SMAC was detected in the mitochondrial pellet except at P16 and P20 where significantly higher ($P<0.001$) expression was observed (Fig. 6A). In hypothyroid conditions, the expression of SMAC was significantly higher ($P<0.001$) as compared
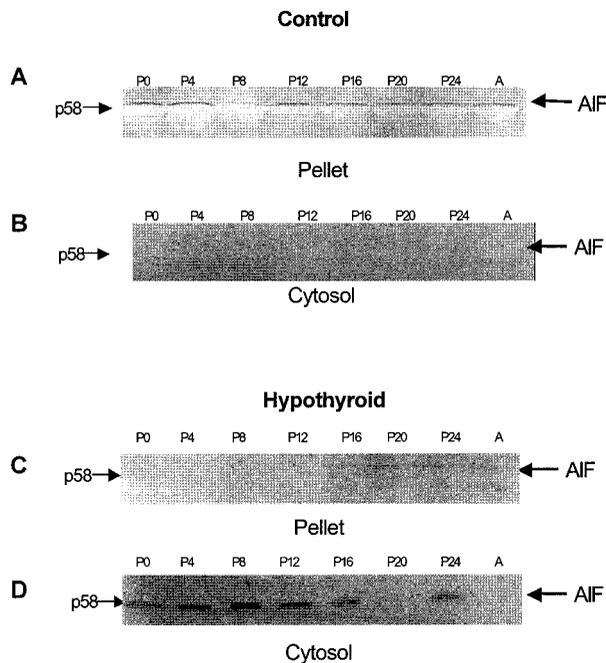
Figure 5 Analysis of AIF translocation to cytosol. The mitochondrial pellet and cytosol were isolated from developing cerebellum (P0, P4, P8, P12, P16, P20, P24 and adult (A)) of control (A and B) and hypothyroid (C and D) pups. Cytosolic and mitochondrial proteins (50 µg) were subjected to 15% SDS–PAGE and were analyzed by Western blot using goat polyclonal antibody. Both the experiments were performed at the same time and repeated three times.

Figure 6 Analysis of SMAC release from mitochondria to cytosol. The mitochondrial pellet and cytosol were isolated from developing cerebellum (P0, P4, P8, P12, P16, P20, P24 and adult (A)) of control (A, B) and hypothyroid (C, D) pups. Cytosolic and mitochondrial proteins (50 µg) were subjected to 15% SDS–PAGE and analyzed by Western blot using rabbit polyclonal antibody. Both the experiments were performed at the same time and repeated three times.

with control pups and it varied with age (Fig. 6C). Significantly ($P<0.001$) high levels of SMAC were observed in cytosol from P0 to P16 and was undetectable thereafter, with a concomitant increase in mitochondria (P20–P24 and adult) (Fig. 6D).

Discussion

Here we report that TH deficiency causes alteration in mitochondrial morphology, leading to translocation of apoptogenic molecules located in inter-membrane space to cytosol during cerebellar development. This may lead to extensive apoptosis during cerebellar neurogenesis, leading to a decrease in cell number during hypothyroid conditions, as observed previously.

We used the rat model to understand the effect of TH on mitochondrial-mediated apoptosis during cerebellar development. The cerebellar development is largely postnatal and various anatomical alterations induced by TH deficiency have been well documented and mimic the condition in humans (Koibuchi & Chin 2000). The pups of dams on MMZ were severely hypothyroid, as indicated by significantly low serum TH levels (Fig. 1).
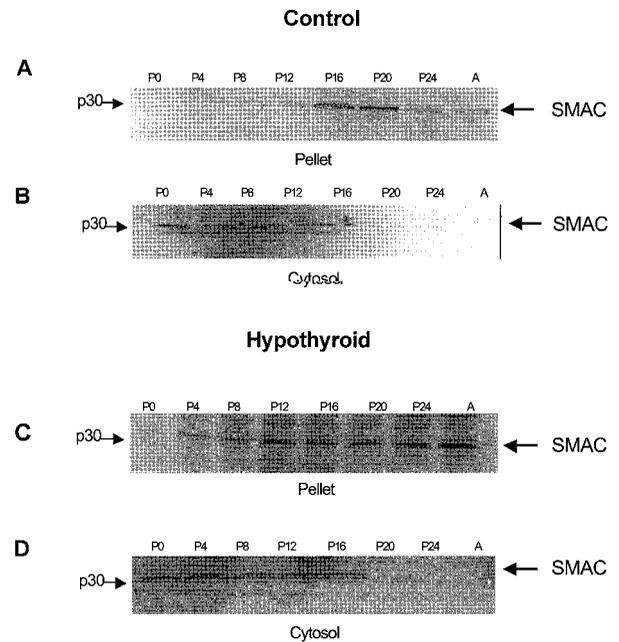
Ultrastructural analysis of mitochondria from the IGL from cerebellum revealed that under euthyroid conditions, morphological and structural integrity was maintained whereas in hypothyroid conditions, volume dysregulation, decrease in number of cristae and vacuolization was observed (Fig. 2). However, the mitochondrial membrane remained intact under both the conditions, suggesting that the alteration may be due to the loss of transmembrane potential (Green & Reed 1998). Similar findings were also reported in developing cerebral cortex under hypothyroid conditions (Vega-Nunez *et al.* 1997).

The alteration in mitochondrial morphology in hypothyroid conditions may be due to the translocation of apoptogenic molecules. Translocation of *Bax* to mitochondrial and other membrane sites and triggering of catastrophic transformation of mitochondrial function in response to death signals has been shown *in vitro* (Goping *et al.* 1998, Krajewski *et al.* 1999, Cao *et al.* 2001, Ghribi *et al.* 2001, Yamaguchi *et al.* 2001). In euthyroid conditions, we found that *Bax* was predominantly localized in mitochondria during early developmental stages and decreased with age. Previously, we observed the high expression of *Bcl-2* and *Bcl-x$_L$* under euthyroid conditions (Singh *et al.* 2003). It is reasonable to assume that

those constitutively expressed anti-apoptotic molecules, namely Bcl-2 and Bcl-x$_L$, probably heterodimerize with Bax to regulate the mitochondrial volume as proposed previously (Green & Reed 1998, Adrain & Martin 2001). Hypothyroidism leads to increased translocation of Bax from cytosol to mitochondria. The reduced expression of Bcl-2 and Bcl-x$_L$ reported previously (Singh *et al.* 2003) probably prevents the required heterodimerization necessary for maintaining the membrane potential and mitochondrial volume. To our knowledge, this is the first demonstration of translocation of *Bax* during cerebellar neurogenesis under hypothyroid conditions. Recently, immortalized neuronal precursor cells have been shown to undergo apoptosis upon serum withdrawal, where translocation of *Bax* to mitochondria plays an important role in the initiation of apoptosis (Colombaioni *et al.* 2002). Hypothyroidism is also known to reduce nerve growth factor (NGF) in all parts of the brain, including the cerebellum, specifically during the early postnatal period (P4–P8) (Figueiredo *et al.* 1993, Alvarez-Dolado *et al.* 1994). Thus, withdrawal of NGF and other neurotrophic factors during hypothyroid conditions may activate mitochondrial-mediated apoptosis through translocation of *Bax*.

Hypothyroidism-induced mitochondrial structural changes and translocation of *Bax* observed here suggest a strong possibility of the release of apoptogenic proteins from mitochondria to initiate the downstream apoptotic pathways. Mitochondria are known to harbor several molecules, namely cytochrome c, AIF and SMAC, that once released to cytosol activate the caspase-dependent and –independent pathways. Therefore we studied the translocation of apoptogenic molecules from mitochondria to cytosol. In euthyroid conditions, cytochrome c was detected in mitochondria at all the developmental stages, but was undetectable in cytosol (Fig. 4). However, hypothyroidism-induced cytochrome c release to cytosol during early development may contribute to initiation of apoptosis through formation of apoptosomes and activating a caspase cascade that may result in enhanced apoptosis (Green & Reed 1998).

Limited release of cytochrome c only partially explains the extensive apoptosis seen under hypothyroid conditions. Hence, we also investigated the translocation of other known apoptogenic molecules. AIF translocates from mitochondria to the nucleus, where it causes peripheral chromatin disintegration by a caspase-independent pathway (Zamzami & Kroemer 1999). The presence of adequate levels of TH prevent the release of AIF from mitochondria to cytosol, as AIF was not detected in the cytosol in euthyroid conditions (Fig. 5). We observed that hypothyroidism induces the translocation of AIF from mitochondria to cytosol during the early developmental period (P0–P12) to activate the caspase-independent pathway of apoptosis during cerebellar development.

In response to apoptotic stimuli, SMAC is released in cytosol and binds to the IAPs, thereby relieving the IAP inhibition of caspases (Deveraux & Reed 1999). It is known that an isoform of IAP known as NIAP (neural IAP) is confined to the neurons at high levels, preventing the apoptosis from the different physiological and pathological insults (Kuida *et al.* 1998). However during neurogenesis, due to positive selection of neurons making appropriate connections, extra-synaptic cells die by programmed cell death. The competency to undergo programmed cell death may be achieved by the release of mitochondrial SMAC during early development (P0–P16) observed here (Fig. 6). The higher levels of SMAC in cytosol under hypothyroid conditions reflect the fact that large numbers of neuronal cells achieve competency to undergo apoptosis. The absence of SMAC in adult cerebellum reported here is in agreement with earlier studies and also in consonance with steady state neurogenesis observed in adults (Du *et al.* 2000, Verhagen *et al.* 2000). The mitochondria from adult cerebellum seem to be refractory to apoptotic stimuli as limited translocation of apoptogenic molecules to cytosol is observed both in hypo- and euthyroid conditions. The recalcitrant nature of adult cerebellum has also been reported previously (Deshmukh & Jhonson 1998).

In conclusion, this study shows for the first time that the presence of adequate levels of TH maintains mitochondrial integrity and physiology to inhibit excess apoptosis by preventing the release of apoptogenic proteins during cerebellar neurogenesis. However, its deficiency leads to morphological alteration resulting in translocation of apoptogenic molecules from mitochondria. The mechanism of release of apoptogenic molecules from mitochondria, extensive apoptosis and its effect on the manifestation of neuronal disorders need further study. The present results do not undermine the nuclear action of TH but suggest that TH is an important regulator in maintaining the coordination between mitochondria and nuclei, at least in controlling the process of apoptosis, however further experimentation is needed.

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