

Neurotrophins Promote the Survival and Development of Neurons in the Cerebellum of Hypothyroid Rats In Vivo

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Abstract. The development of cerebellar cortex is strongly impaired by thyroid hormone (T3) deficiency, leading to altered migration, differentiation, synaptogenesis, and survival of neurons. To determine whether alteration in the expression of neurotrophins and/or their receptors may contribute to these impairments, we first analyzed their expression using a sensitive RNase protection assay and in situ hybridization; second, we administered the deficient neurotrophins to hypothyroid animals. We found that early hypothyroidism disrupted the developmental pattern of expression of the four neurotrophins, leading to relatively higher levels of NGF and neurotrophin 4/5 mRNAs and to a severe deficit in NT-3 and brain-derived neurotrophic factor (BDNF) mRNA expression, without alteration in the levels of the full-length tyrosine kinase (trk)B

and trkC receptor mRNAs. Grafting of P3 hypothyroid rats with cell lines expressing high levels of neurotrophin 3 (NT-3) or BDNF prevented hypothyroidism-induced cell death in neurons of the internal granule cell layer at P15. In addition, we found that NT-3, but not BDNF, induced the differentiation and/or migration of neurons in the external granule cell layer, stimulated the elaboration of the dendritic tree by Purkinje cells, and promoted the formation of the mature pattern of synaptic afferents to Purkinje cell somas. Thus, our results indicate that both granule and Purkinje neurons require appropriate levels of NT-3 for normal development in vivo and suggest that T3 may regulate the levels of neurotrophins to promote the development of cerebellum.

DEVELOPMENT of the nervous system requires a specific sequence of events including proliferation of precursors, differentiation, survival of different cell types, and establishment of specific synaptic connections. These processes have been relatively well described, but the molecular mechanisms regulating these events are poorly understood. The cerebellum has been extensively used as a model to investigate the role of epigenetic factors during brain development because of the limited number of cell types and the detailed knowledge of the morphological transformations that occur during development. During the first two postnatal weeks, granule cells proliferate in the external granule cell layer (EGL)¹. From P3-P30, granule cells from the premigratory zone of the

EGL migrate to the internal granular layer (IGL), leaving their axons (parallel fibers) in the molecular layer (ML). These events are accompanied by a progressive differentiation of Purkinje cells, starting with the appearance of perisomatic extensions and a formation of a nest of climbing fibers (cell bodies in the inferior olive) around the soma of Purkinje cells. Then, the apical dendrites of Purkinje cells emerge and branch to form a dendritic tree in the ML where their spines receive synaptic inputs from both climbing and parallel fibers (Ramon y Cajal, 1911; Altman, 1972*a,b,c*; Mason and Gregory, 1984; Sotelo et al., 1984). Interestingly, these processes have been shown to be regulated by thyroid hormones. Indeed, hypothyroid rats exhibit retardation in the proliferation, migration, and differentiation of granule cells, an increased cell death in the IGL, and a deficiency in the elaboration of Purkinje cell dendritic trees, spines, and synapses, with no decrease in the number of Purkinje cells (Nicholson and Altman, 1972*a,b,c*; Legrand, 1979). In agreement with this, α - and β -thyroid hormone receptor subtypes are found during development in different cell types in the rat cerebellum

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1. *Abbreviations used in this paper:* BDNF, brain-derived neurotrophic factor; EGL, external granule cell layer; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; IGL, internal granule cell layer; ML, molecular layer; NSE, neuron-specific enolase; NT-3, neurotrophin 3; NT-4, neurotrophin 4/5; p75^{NTR}, low affinity neurotrophin receptor; PTU, propylthiouracyl; T3, thyroid hormone; TdT,

terminal deoxynucleotidyl transferase; Trk (trk), tyrosine kinase; TUNEL, TdT-mediated dUTP nick end labeling; UTP, uridine 5'-triphosphate.

(Bradley et al., 1989; Strait et al., 1991; Mellström et al., 1991), supporting a role for thyroid hormone in regulating gene expression in the developing cerebellar cortex.

The development of the nervous system depends on the physiological availability of survival proteins known as neurotrophic factors (Snider and Johnson, 1989). NGF (Levi-Montalcini, 1987), brain-derived neurotrophic factor (BDNF) (Barde et al., 1982; Leibrock et al., 1989), neurotrophin 3 (NT-3) (Ernfors et al., 1990; Hohn et al., 1990; Jones and Reichardt, 1990; Kaisho et al., 1990; Maisonpierre et al., 1990; Rosenthal et al., 1990) and neurotrophin-4/5 (NT-4) (Berkemeier et al., 1991; Hallböök et al., 1991; Ip et al., 1992) are four members of the neurotrophin family, which share 50–60% amino acid sequence similarities (Hallböök et al., 1991). The responsiveness of distinct neuronal populations to the different neurotrophins depends on the interaction of these ligands with their cognate tyrosine kinase (Trk) receptors, either alone or together with another cell surface receptor known as p75 or low affinity receptor (p75^{NTR}) (for review see Chao and Hempstead, 1995). Trk A is the receptor for NGF, TrkB for BDNF and NT-4, and TrkC serves as the receptor for NT-3 (for review see Barbacid, 1994). Alternative splicing within the intracellular domain of trkB and trkC has been shown to generate isoforms with a truncated kinase domain (TrkB T or TrkC T) or with an insertion in the kinase domain (TrkC KI) (Klein et al., 1990; Middlemas et al., 1991; Lamballe et al., 1993; Tsoulfas et al., 1993; Valenzuela et al., 1993; Garner and Large, 1994), which may have distinct signaling capabilities and cellular responses to the neurotrophins.

While neurotrophins are best known as regulators of neuronal survival, growing evidence suggests a role in other cellular processes including different aspects of differentiation, synaptogenesis, and regulation of axonal and dendritic growth. The expression of the four neurotrophins and their receptors during the postnatal development of the cerebellum (Large et al., 1986; Maisonpierre et al., 1990; Ernfors et al., 1992; Lindholm et al., 1993; Masana et al., 1993; Rocamora et al., 1993; Tessarollo et al., 1993; Timmusk et al., 1993a) and the regulation of NGF, NT-3, and p75^{NTR} by thyroid hormone (T3) (Figueiredo et al., 1993; Charasse et al., 1992; Lindholm et al., 1993) suggested the possibility that neurotrophins and their receptors could mediate some of the key functions of T3 in the development of cerebellum. Primary cultures of granule and Purkinje cells have shown effects of BDNF, NT-3, and NT-4 in vitro (Segal et al., 1992; Lindholm et al., 1993; Segal et al., 1995; Mount et al., 1994; Gao et al., 1995; Kubo et al., 1995). However, no function of these neurotrophins on the development of cerebellum has yet been described in vivo.

In the present study, we investigated the role of neurotrophins in the development of cerebellum in vivo, using an experimental model of hypothyroidism. The results show that T3 deficiency induced selective alterations in the developmental pattern of neurotrophins and their receptors, including a decrease in the expression of BDNF and NT-3 without impairment of trkB and trkC mRNA levels. Administration of BDNF or NT-3 to hypothyroid rats prevented the death of neurons in the IGL. Furthermore, NT-3, but not BDNF, promoted the differentiation

of both external granule neurons and Purkinje cells. These results suggest that BDNF and particularly NT-3 play a critical role in the development of cerebellum.

Materials and Methods

Animals and PTU Treatment

Sprague-Dawley rats were housed individually under a 12:12 light–dark cycle at 23°C with food and water available ad libitum. Pups were rendered hypothyroid by treating the mothers with 50 mg/d of propylthiouracil (PTU) by gastric intubation from day 17 of gestation until the age of analysis. PTU is an antithyroid drug that blocks thyroid hormone biosynthesis and that has been extensively used to induce hypothyroidism in rats (Legrand, 1967). Hypothyroidism was controlled by analyzing the levels of thyroid hormones in blood samples obtained from the pups. The average level of T4 in hypothyroid rats was 25 nM, while in age-matched controls, the level was up to 126 nM. Moreover, pups exhibited the characteristic features of hypothyroidism, including an infantile hair standing out of the body, an uncertain gait, and a delay in opening the eyes and in their behavioral development. PTU-treated rats also showed a reduction in the body weight: 33.8 g ± 2.2 in normal rats vs 19.6 g ± 2 in hypothyroid rats at P15.

Tissue Processing

For RNase protection assay and in situ hybridization analysis, pups were killed by decapitation at postnatal days 0, 5, 10, 15, 20, 25, or 30. The cerebellum was quickly dissected, frozen on dry ice, and stored at –70°C. For immunohistochemistry and in situ detection of cell death, 15-d-old rats were deeply anesthetized and perfused with 15 ml of PBS, pH 7.4, followed by 80 ml of 4% paraformaldehyde in 1 × PBS. Brains were then removed, postfixed for 2 h, and cryoprotected by immersion in 10% sucrose/1 × PBS for 2 d at 4°C. After freezing, 14- μ m sections of cerebellum were cut on a cryostat, mounted on 3-aminopropyl ethoxysilane-coated slides, and stored at –20°C.

RNA Preparation and RNase Protection Analysis

Total RNA was prepared from cerebellum using 0.3 M LiCl/6 M urea followed by phenol-chloroform extraction as described (Auffray and Rougeon, 1980). Neurotrophin mRNA levels were estimated by RNase protection assay using a RPAII Ribonuclease Protection Assay Kit (Ambion Inc., Austin, TX). Antisense cRNA probes for trkA, full-length and truncated trkB, and the four neurotrophins were prepared as previously described (Laurenzi et al., 1994; Lindefors et al., 1995). The antisense cRNA probe for full-length trkC receptor was prepared by inserting into pBSKS (Stratagene, La Jolla, CA), a 372-bp fragment spanning nucleotides 1945–2317 of the rat trkC sequence (Merlio et al., 1992) that covers the protein kinase domain of the TrkC receptor. In RNase protection assay, the probe gives a 372-bp protected band corresponding to the full-length trkC (here referred to as trkC FL) and two protected bands of 138 bp (not shown in this study) and 240 bp corresponding to all insertion forms of full-length trkC (here referred to as trkC KI) that have been previously described (Tsoulfas et al., 1993; Valenzuela et al., 1993). The antisense cRNA probe for p75^{NTR} consisted of a fragment spanning bp 810–1211 in the rat p75^{NTR} cDNA sequence (Radeke et al., 1987) cloned in pBSKS. To standardize the amount of mRNA in each lane, an antisense cRNA probe detecting the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcript was prepared using a fragment spanning bp 196–357 of a rat GAPDH cDNA clone (Fort et al., 1985). All probes were labeled by in vitro transcription using T3 or T7 RNA polymerase (Promega, Madison, WI) and [α -³²P]CTP (Amersham Intl., Little Chalfont, UK). In each reaction, total RNA were hybridized at 45°C for 16 h to one of the neurotrophin/neurotrophin receptor antisense cRNA probes together with the GAPDH cRNA probe. The assay was performed as described by the manufacturer. Protected fragments were separated on 4% polyacrylamide gels under denaturing conditions, and the gels were exposed to x-ray film at –70°C with an intensifying screen. The optical density of the autoradiograms was measured and quantified with a Quantimet 570 Image Processing and Analysis System (Cambridge Instruments Ltd., Cambridge, UK).

In Situ Hybridization

In situ hybridization was performed as described previously (Timmusk et al., 1993b). Briefly, 14- μ m sections were collected, fixed in 4% paraformaldehyde for 15 min, and rinsed once in PBS, pH 7.4, and twice in distilled water. Sections were then treated with 0.1 M HCl for 10 min, acetylated for 20 min with 0.25% acetic anhydride in 0.1 M ethanolamine, dehydrated with ethanol, and air dried. Hybridization was performed overnight at 42°C in a humidified chamber with 300 μ l of hybridization buffer containing 2.5×10^6 cpm/ml [α^{35} S]UTP-labeled cRNA probe. cRNAs were synthesized as described above, and hybridization buffer consisted of 50% formamide, 20 mM Tris-HCl, pH 7.6, 1 mM EDTA, pH 8.0, 0.3 M NaCl, 0.1 M dithiothreitol, 0.5 mg/ml yeast tRNA, 0.1 mg/ml poly-A-RNA, 1 \times Denhardt's solution, and 10% dextran sulfate. After hybridization, sections were washed once in 1 \times SSC at 48°C for 40 min, treated with RNase (10 μ g/ml) in 0.5 M NaCl, 20 mM Tris-HCl, pH 7.5, and 2 mM EDTA at 37°C for 30 min, and washed twice with 0.5 \times SSC and twice with 0.1 \times SSC for 10 min each at 60°C. Sections were finally dehydrated with ethanol and air dried. Slides were either exposed to β -max x-ray film (Amersham Intl.) for the indicated times or dipped in NTB-2 photo emulsion (diluted 1:1 in water) (Eastman-Kodak Co., Rochester, NY), exposed at 4°C for 3–5 wk, developed with D19 (Eastman-Kodak Co.), fixed with AL-4 (Agfa Gevaert, Kista, Sweden), and counterstained with cresyl violet.

Grafting of Genetically Modified Cell Lines Producing BDNF or NT-3

The BDNF cell line (designed F3N-BDNF) was generated according to the protocol used to establish the NT-3 cell line (designated F3A-NT3) (Arenas and Persson, 1994) except that the rat BDNF cDNA (Leibrock et al., 1989) was cloned into the PstI site of the plasmid MRE-(4X)-OVEC (Westin et al., 1987). For transplantation, F3A-MT, F3A-NT3, and F3N-BDNF cell lines were prepared as previously described (Arenas and Persson, 1994). Postnatal day 3 hypothyroid rats were anesthetized with ether and placed in a stereotaxic frame. The head of the pups, held by ear bars, was kept at 60° of ventral flexion. $3\text{--}6 \times 10^5$ cells were stereotaxically injected in 2 μ l at the following coordinates: AP = -5, L = +1, DV = -2.5 (in mm) relative to lambda and the skull surface. The number of cells injected was adjusted to the different growth rate of cell lines (higher in the F3N-BDNF cell line than in the F3A-NT3 or F3A-MT) and to the different levels of expression of neurotrophins so that the brains received similar doses of the factors.

Measurement of Cerebellar Layers

Transverse sections parallel to the axis of the folia in lobule X were stained with cresyl violet and used to measure the size of the ML and EGL. Analysis was performed using the Quantimet 570 Image Processing and Analysis System, at $\times 10$ for the ML and $\times 20$ for the EGL. Five randomly chosen fields were analyzed per slide in four sections per animal, in three to five animals per condition. The results are expressed as a percentage of the size determined in aged-matched control rats.

In Situ Detection of DNA Fragmentation

DNA fragmentation was histologically examined by using the terminal deoxynucleotidyl transferase (TdT)-mediated d-uridine 5'-triphosphate (UTP) nick end labeling (TUNEL) technique (Gavrieli et al., 1992) using the in situ Apoptag kit-fluorescein (Oncor, Gaithersburg, MD). Sections were stained according to the manufacturer's recommendations. Briefly, sections were postfixed in ethanol/acetic acid (2:1) for 5 min at -20°C and washed in two changes of PBS at room temperature. Sections were incubated with the equilibration buffer for 5 min and then with digoxigenin-11-dUTP and TdT. After a 30-min incubation at 37°C, the sections were washed in working strength stop/wash buffer for 30 min at 37°C and three times with PBS. The slides were then incubated with anti-digoxigenin-fluorescein antibody for 30 min at room temperature, washed three times in PBS, and mounted in PBS-glycerol containing *p*-phenylenediamine and propidium iodide. As a negative control, adjacent sections were processed following the standard procedure, except that an equal volume of water was substituted for the omitted volume of TdT. In this case, no nuclei were stained. Cell death was quantified by counting the number of fluorescein-stained nuclei within one field at $\times 125$. Only round stained nuclei were counted in a total of 20 randomly chosen fields in four different sections per cerebellum, in three to five animals per condition. The values

show the average number of dying cells per square millimeter in every condition.

Double Labeling

Tissue was prepared as previously described for detection of DNA fragmentation. Sections were incubated with digoxigenin-dUTP and TdT for 30 min at 37°C and then washed in working strength stop/wash buffer for 30 min at 37°C. After three washes with PBS, sections were incubated overnight at 4°C with antibodies against neuron-specific enolase (NSE) (Inctar, Stillwater, MN) or glial fibrillary acidic protein (1:500; Sigma Chemical Co., St. Louis, MO) in 1 \times PBS, 1% BSA, and 0.3% Triton X-100. Sections were then rinsed with PBS and incubated for 30 min at room temperature with the anti-digoxigenin-fluorescein antibody (Apop-tag-kit; Oncor) and with a rhodamine-goat anti-rabbit IgG (1:60, Jackson ImmunoResearch Laboratories, Inc.) for NSE staining or with a lissamine-rhodamine-donkey anti-mouse IgG (1:40, Jackson ImmunoResearch Laboratories, Inc.) for glial fibrillary acidic protein (GFAP) staining. Sections were washed and mounted in PBS-glycerol containing *p*-phenylenediamine. To determine the number of neurons (NSE⁺) or astrocytes (GFAP⁺) undergoing cell death in the IGL, sections were analyzed using a laser scanning confocal microscope (Wallén, 1991), and the three-dimensional reconstruction of the cells was done by performing 14 optical sections (1- μ m increment) at $\times 40$ ($\times 40/1.3$ NA immersion oil objective). Only those TUNEL-labeled (T⁺) nuclei surrounded by a completely stained cytoplasm were counted ($\sim 60\%$ of T⁺ cells). 100 T⁺ cells at four different levels in the IGL of lobule X were analyzed in every cerebellum, in three to five animals per condition. The values show the average number of NSE⁺/T⁺ and GFAP⁺/T⁺ cells per square millimeter.

Calbindin-D28k and Synaptophysin Immunohistochemistry

Sections were first incubated overnight at 4°C with antibodies against calbindin-D28k (1:500; Sigma Chemical Co.) or synaptophysin (1:50, Dako Corp., Carpinteria, CA) in 1 \times PBS, 1% BSA, and 0.3% Triton X-100. After washing, the slides were incubated for 1 h at room temperature with a lissamine-rhodamine-donkey anti-mouse IgG (1:40, Jackson ImmunoResearch Laboratories, Inc.) for the calbindin-D28k staining and with a rhodamine anti-rabbit IgG for the synaptophysin staining (1:200, Jackson ImmunoResearch Laboratories, Inc.), washed, and mounted in PBS-glycerol containing *p*-phenylenediamine. Alternatively, calbindin-D28k immunoreactivity was detected using a goat HRP-conjugated anti-mouse IgG (1:250, Dako Corp.) as secondary antibody, and slides were incubated with 0.05% DAB, 0.0066% hydrogen peroxide in 0.1 M Tris buffer, pH 7.4, dehydrated, and mounted in Pertex (Histolab Product AB, Västra Frölunda, Sweden).

The density of the dendritic tree of Purkinje cells was measured using the Quantimet 570 Image Processing and Analysis System linked to a microphot-FXA microscope (Nikon Inc., Garden City, NY) by an interfacing CCD videocamera. Analysis was performed in the center of the molecular layer on $\times 125$ magnified images. The same degree of acceptance that allows to distinguish Purkinje cell dendrites from the background was used in all measurements. For each section, the area occupied by the dendritic arborization of Purkinje cells was measured in five randomly chosen fields in the X cerebellar lobuli, and a total of four sections per animal were analyzed. The values obtained for three or five animals were averaged for each condition and expressed as percentage of control values.

The number of axo-somatic synapses on Purkinje cell somas was estimated by counting the number of immunopositive punctate structures in synaptophysin-immunostained sections. Only oval or round stained puncta of 0.5–2 μ m, observed on the surface of a Purkinje cell soma sectioned through the nucleus, were counted. 100 Purkinje cells, at four different levels in the X cerebellar lobuli, were analyzed per animal. The mean number of immunopositive punctate structures per cell were calculated from three to five cerebelli per condition.

Results

Hypothyroidism has been reported to affect several aspects of the development of the cerebellum (Nicholson and Altman, 1972a,b). To investigate whether neurotrophins could participate in the postnatal development of

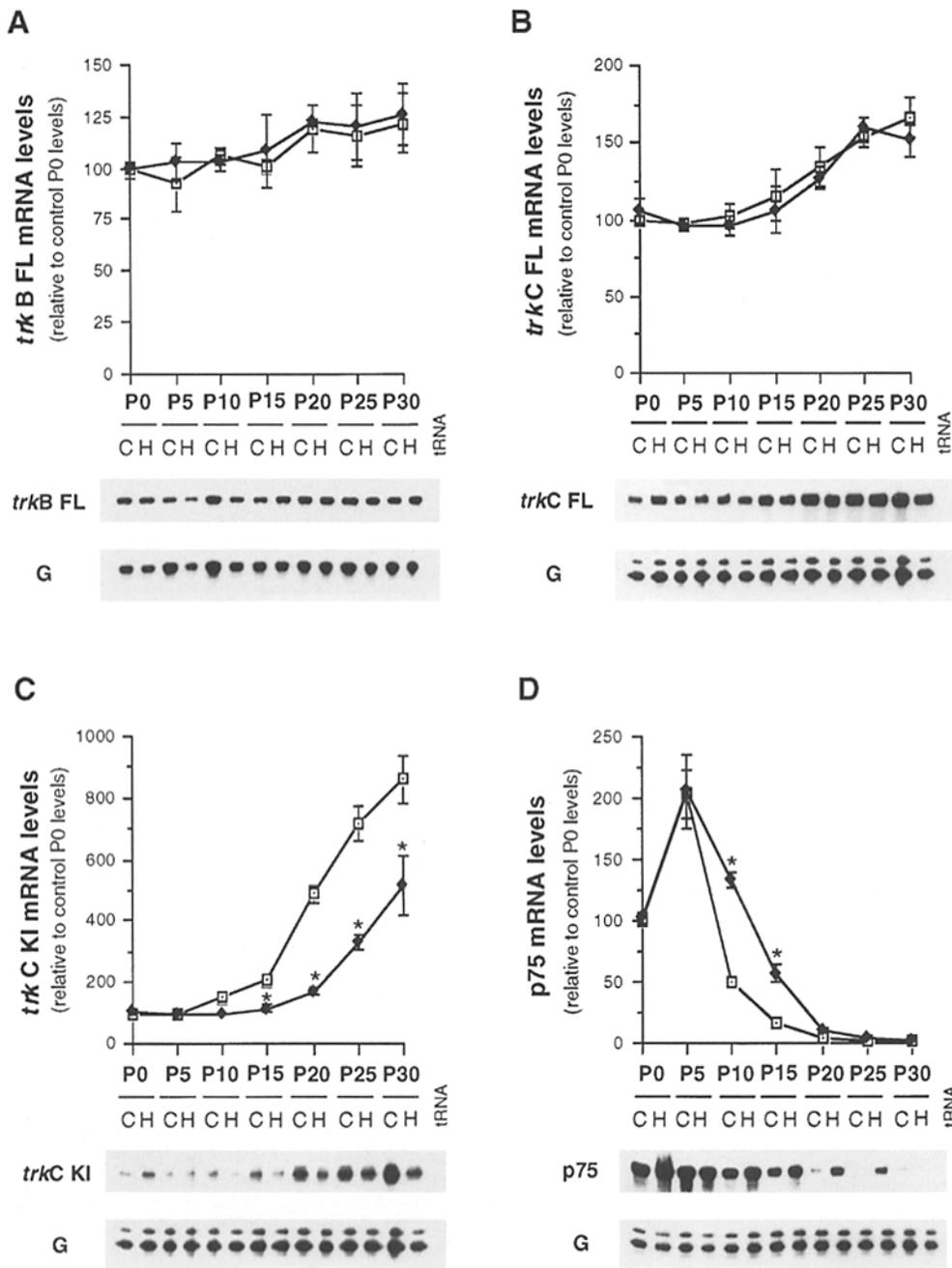


Figure 1. Expression of neurotrophin receptor mRNAs during postnatal development of the cerebellum. The levels of trk B FL (A), trk C FL (B), trk C KI (C), and p75^{NTR} (D) mRNAs were analyzed in normal (C, □) or hypothyroid (H, ●) rats by RNase protection assay. Total RNA, of the corresponding samples (10 μg for trk B FL, trk C FL, and trk C KI; 15 μg for p75^{NTR}), or yeast tRNA, as a negative control, were hybridized to specific neurotrophin receptor and GAPDH (G) riboprobes. Lanes in the lower part of the panels show autoradiograms quantified in the plot. Values of neurotrophin receptor mRNAs are expressed as percentage of their relative optical density level at P0, normalized with the corresponding optical density of GAPDH. Data represent the mean ± SD from three independent determinations. *P* < 0.05 (*t* test) compared to the corresponding control.

cerebellum, we first examined the expression of neurotrophins and their receptors both in normal and hypothyroid rats.

T3 Deficiency Delays the Developmental Pattern of Expression of trkC KI and p75^{NTR} But Not of Other trk Receptors

At all stages analyzed, trkA mRNA was below the detection limit of RNase protection assay, while high levels of trkB and trkC mRNAs were found in the cerebellum of normal rats (Fig. 1). No significant change in the expression of mRNA for the full-length (trkB FL) (Fig. 1 A) or truncated trkB (trkB T) (data not shown) was found during the postnatal development of cerebellum. However,

mRNA levels for full-length trkC (trkC FL) and particularly for trkC with kinase insertions (trkC KI) increased from P0 to P30 (Fig. 1, B and C). High levels of p75^{NTR} mRNA were found at P0 and P5 in the normal cerebellum, decreasing during the second postnatal week (Fig. 1 D). In hypothyroid rats, the levels of trkB FL, trkB T, or trkC FL mRNAs were not significantly different from the levels in normal rats. In contrast, the developmental patterns of trkC KI (Fig. 1 C) and p75^{NTR} (Fig. 1 D) mRNA expression were significantly retarded in hypothyroid rats, leading to a two- to threefold decrease in trkC KI mRNA levels, from P15 to P30, and to a three- to fourfold increase in p75^{NTR} mRNA levels at P10–15. This delay in the down-regulation of p75^{NTR} is in agreement with previous findings showing that T3 decreases p75^{NTR} mRNA in the cere-

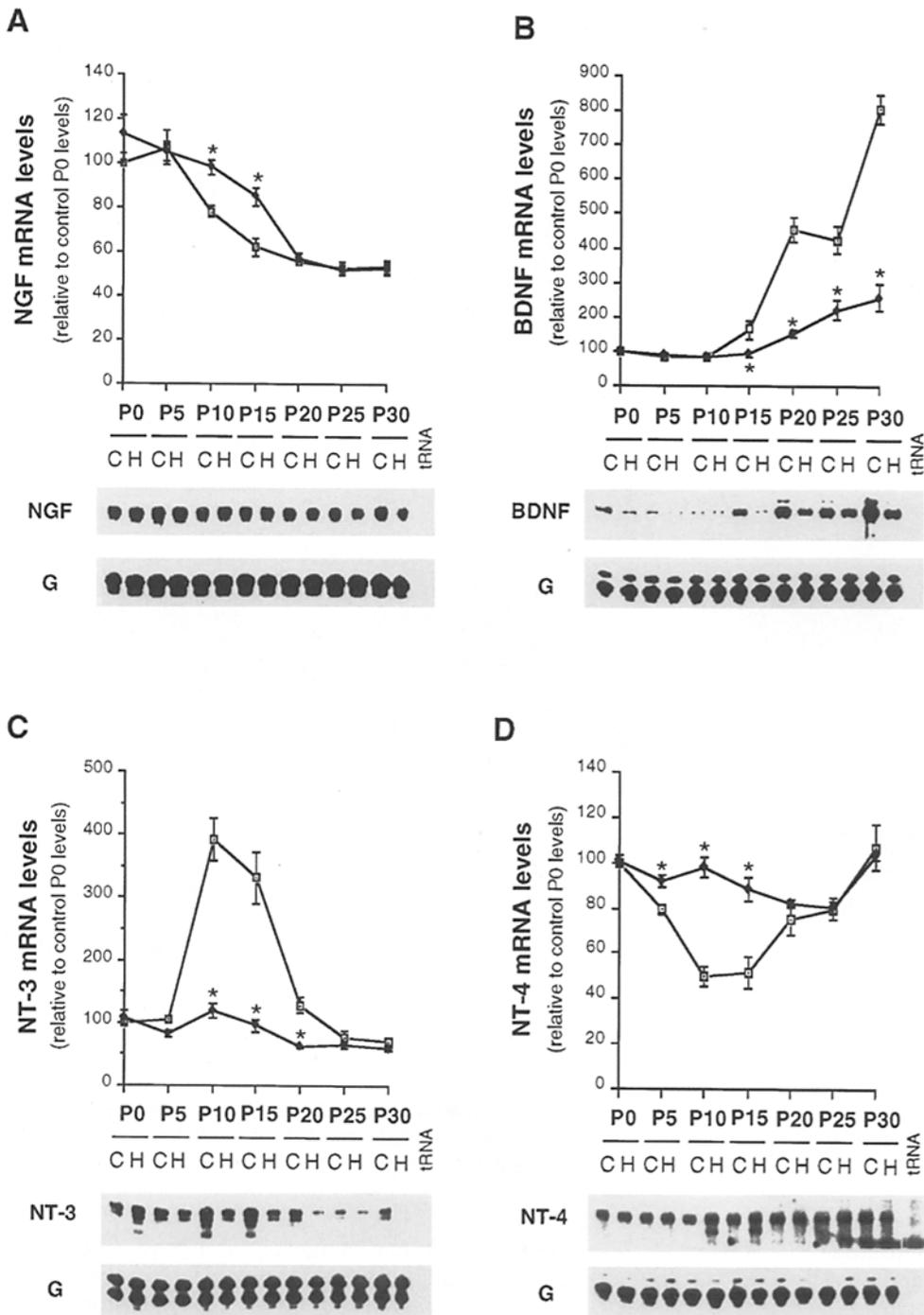


Figure 2. Expression of neurotrophin mRNAs during postnatal development of the cerebellum. The levels of NGF (A), BDNF (B), NT-3 (C), and NT-4 (D) mRNAs were analyzed in normal (C, □) or hypothyroid (H, ●) rats by RNase protection assay. Total RNA, of the corresponding samples (15 μg for BDNF and NT-3; 20 μg for NGF and NT-4), or yeast tRNA, as a negative control, were hybridized to specific neurotrophin and GAPDH (G) riboprobes. Lanes in the lower part of the panels show autoradiograms quantified in the plot. Values of neurotrophin receptor mRNAs are expressed as percentage of their relative optical density levels at P0, normalized with the corresponding optical density of GAPDH. Data represent the mean ± SD from three independent determinations. *P* < 0.05 (*t* test) compared to the corresponding control.

bellum of neonatal rats (Figueiredo et al., 1993). However, factors other than T3 seem to participate in this downregulation since p75^{NTR} mRNA levels nevertheless decreased to reach control value at P20. Thus, our results show that T3 deficiency alters the normal developmental pattern of trkB FL and trkC FL receptors in the cerebellum.

Hypothyroidism Induces Alterations in the Expression of All Neurotrophins during Cerebellar Development

The level of NGF mRNA in normal rats did not change

between P0–P5 but progressively decreased by 40–50% at P15–30 (Fig. 2 A). In hypothyroid rats, the downregulation in the expression of NGF mRNA was delayed about 5 d, started at P15, and decreased by 50% at P20 (Fig. 2 A).

Low levels of BDNF mRNA were observed in normal rats during the first 2 postnatal weeks, followed by an eightfold increase from P15 to P30 (Fig. 2 B). In hypothyroid animals, BDNF mRNA increased later, resulting in a 70% reduction in its level at P30 (Fig. 2 B). In situ hybridization showed that BDNF mRNA was expressed at a high level by granule cells in the IGL and by some Purkinje cells in the normal cerebellum at P20 (Fig. 3, A and C). In

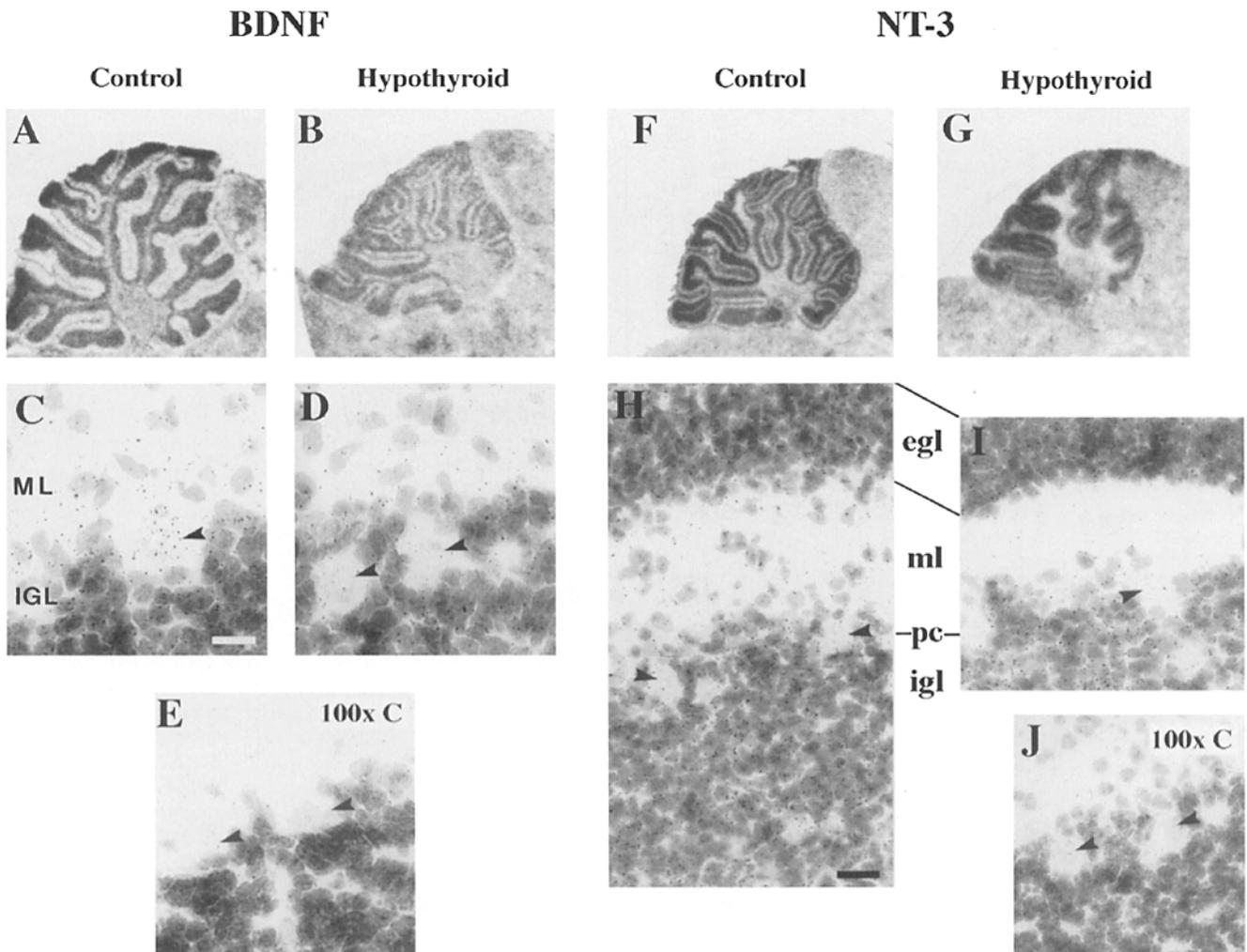


Figure 3. Expression of BDNF and NT-3 mRNA in the normal and hypothyroid cerebellum. BDNF (A–D) and NT-3 (F–I) mRNA expression was analyzed by in situ hybridization at postnatal days 20 and 10, respectively. Autoradiograms corresponding to hypothyroid rats (B and G) show a pronounced reduction of BDNF (B) and NT-3 (G) hybridization signal in the IGL, compared to control animals (A and F, respectively). Bright-field photos show a reduction in the number of neurons and in the intensity of the hybridization signal for both BDNF and NT-3 in the IGL of hypothyroid rats (D and I) compared to controls (C and H). In addition, BDNF mRNA expression by Purkinje cells (arrowheads) was reduced in hypothyroid rats (D) compared to controls (C). Hybridization signal for BDNF (E) or NT-3 (J) probes was displaced by an 100-fold excess of unlabeled probe (100 × C) to show the background hybridization level. *egl*, external granule cell layer; *ml*, molecular layer; *pc*, Purkinje cells; *igl*, internal granule cell layer. Bars: (C–E) 30 μm; (H–J) 45 μm.

hypothyroid rats, BDNF hybridization signal was decreased both in granule and Purkinje cells (Fig. 3, B and D), compared to normal development.

NT-3 mRNA expression was characterized by a three- to fourfold increase at P10–P15, followed by a marked decrease at P20 and levels below P0 at P25–30 (Fig. 2 C). In hypothyroid rats, the peak of expression between P10 and P15 was missing, and the level of NT-3 mRNA were significantly lower than in normal cerebellum from P10 to P20 (Fig. 2 C). In situ hybridization showed that NT-3 mRNA was decreased in the IGL and slightly increased in the EGL of P10 hypothyroid rats, compared to controls (Fig. 3, F and G). Emulsion autoradiograms showed a moderate decrease in the expression of NT-3 mRNA by granule cells in IGL (Fig. 3, H and I). No labeling of Purkinje neurons was found in either normal or hypothyroid animals.

NT-4 mRNA was found to be expressed at very low lev-

els in the cerebellum in agreement with previous results (Timmusk et al., 1993a). In normal rats, NT-4 mRNA decreased by twofold from P0 to P10 and progressively increased from P15 to P30 to recover P0 level. In hypothyroid rats, this downregulation was not observed, and NT-4 mRNA level only decreased by 20% from P20 to P25 (Fig. 2 D).

Thus, all neurotrophins showed specific patterns of expression during normal development and a differential regulation by T3 deficiency. The severe deficits in the expression of BDNF and NT-3 in hypothyroid rats suggested that alterations in the development of cerebellum could result from reduced levels of neurotrophins. To test this hypothesis, we investigated whether grafting of fibroblasts producing high levels of neurotrophins was able to reverse hypothyroidism-induced alterations in the developing cerebellum.

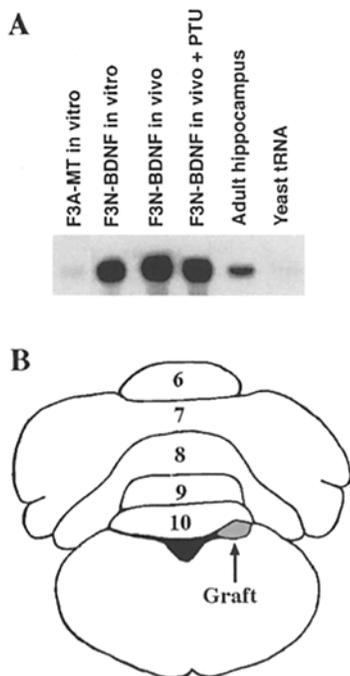


Figure 4. Characterization and grafting of a BDNF-producing cell line (F3N-BDNF). (A) Expression of BDNF mRNA in F3A-MT and F3N-BDNF cells before and after grafting. Total RNA (10 μ g) was extracted from BDNF-expressing cells (F3N-BDNF) and mock-transfected cells (F3A-MT) in culture (*in vitro*) or in the graft (*in vivo*) dissected from the cerebellum 12 d after grafting. The levels of BDNF mRNA expressed by the cell lines were analyzed by RNase protection assay and compared with that in 10 μ g of total RNA from adult hippocampus, one of the regions with the highest levels of BDNF mRNA in the rat central nervous system. Yeast tRNA was used as negative control. (B) Camera lucida drawing showing the typical position of the graft relative to the cerebellum.

Characterization and Grafting of Fibroblasts Engineered to Express High Levels of BDNF or NT-3

To assess the implication of NT-3 and BDNF in the development of the cerebellum, Fisher 344 rat 3T3 fibroblasts were genetically engineered to deliver high levels of NT-3 and BDNF. These cells, designed F3A-NT3 or F3N-BDNF, produced respectively \sim 300 ng of NT-3 (Arenas and Persson, 1994) and $>$ 100 ng of BDNF per 10^6 cells per day (data not shown), as determined in a survival bioassay performed on dissociated E9 chick sensory neurons. The mock-transfected cell line (designed F3A-MT) did not express detectable levels of NT-3 mRNA (Arenas and Persson, 1994), expressed very low levels of BDNF mRNA (Fig. 4 A), and had no survival-promoting effect on dissociated sensory neurons (data not shown). F3A-NT3 or F3N-BDNF cell lines continue to express high levels of neurotrophins after their implantation in the brain (Arenas and Persson, 1994 and Fig. 4 A, respectively). In addition, the antithyroid treatment used in the present study (propylthiouracyl) did not affect the expression of BDNF in the F3N-BDNF cells (Fig. 4 A). Therefore, we grafted the F3A-NT3 or F3N-BDNF cell lines in the cerebellum of 3-d-old PTU-treated rats, and the animals were killed at post-

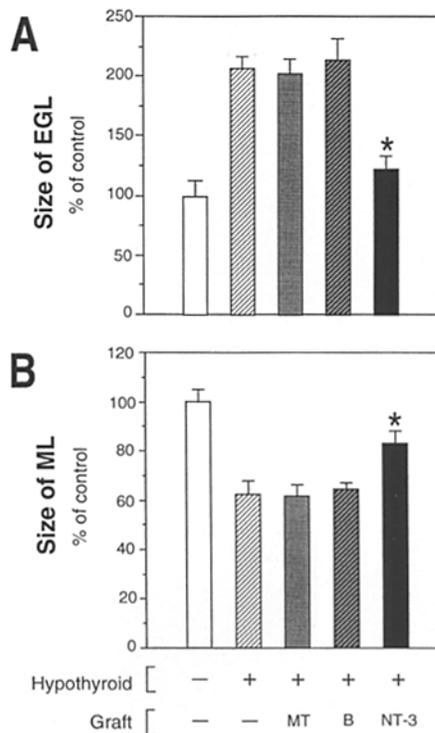


Figure 5. Size of the external granule cell layer (EGL) (A) and molecular layer (ML) (B) in the cerebellum of 15-d-old rats, expressed as percentage of control value. Hypothyroid rats were grafted with F3A-MT (MT), F3N-BDNF (B), or F3A-NT3 (NT-3) cell lines. Each column represent mean \pm SD ($n = 3-5$). $P < 0.001$ (t test) compared to hypothyroid animals (H).

natal day 15. The cells typically formed a small focal tumor protruding laterally to the fourth ventricle, just below the lobule X of the cerebellum (Fig. 4 B). Only those brains grafted in this particular location were selected for the analysis.

NT-3 Prevents Hypothyroidism-induced Deficits in the Size of the EGL and ML

Hypothyroidism leads to a delay in the disappearance of the EGL compared to age-matched control animals. This delay may be estimated by comparing the size of EGL in normal and hypothyroid rats. At P15, the EGL in the cerebellum of hypothyroid animals was two times bigger than the EGL in normal rats (Fig. 5 A). Indeed, we have found an average of two cell layers in the EGL of control animals, while four to five layers of granule cells constituted the EGL in case of hypothyroidism (data not shown). Grafting of mock-transfected cells (F3A-MT) or BDNF-transfected cells (F3N-BDNF) in the cerebellum did not modify the size of EGL compared to ungrafted hypothyroid animals (Fig. 5 A). However, grafting of NT-3-transfected cells (F3A-NT3) decreased the size of the EGL (two to three layers of granule cells), preventing by 80% the alteration induced by hypothyroidism (Fig. 5 A).

A second characteristic of the hypothyroid cerebellum is a deficient differentiation of Purkinje and granule cells, which results in a reduction in the size of the ML. Indeed, we found a 40% decrease in the size of the ML in hypothyroid rats compared to age-matched controls (Fig. 5 B). In-

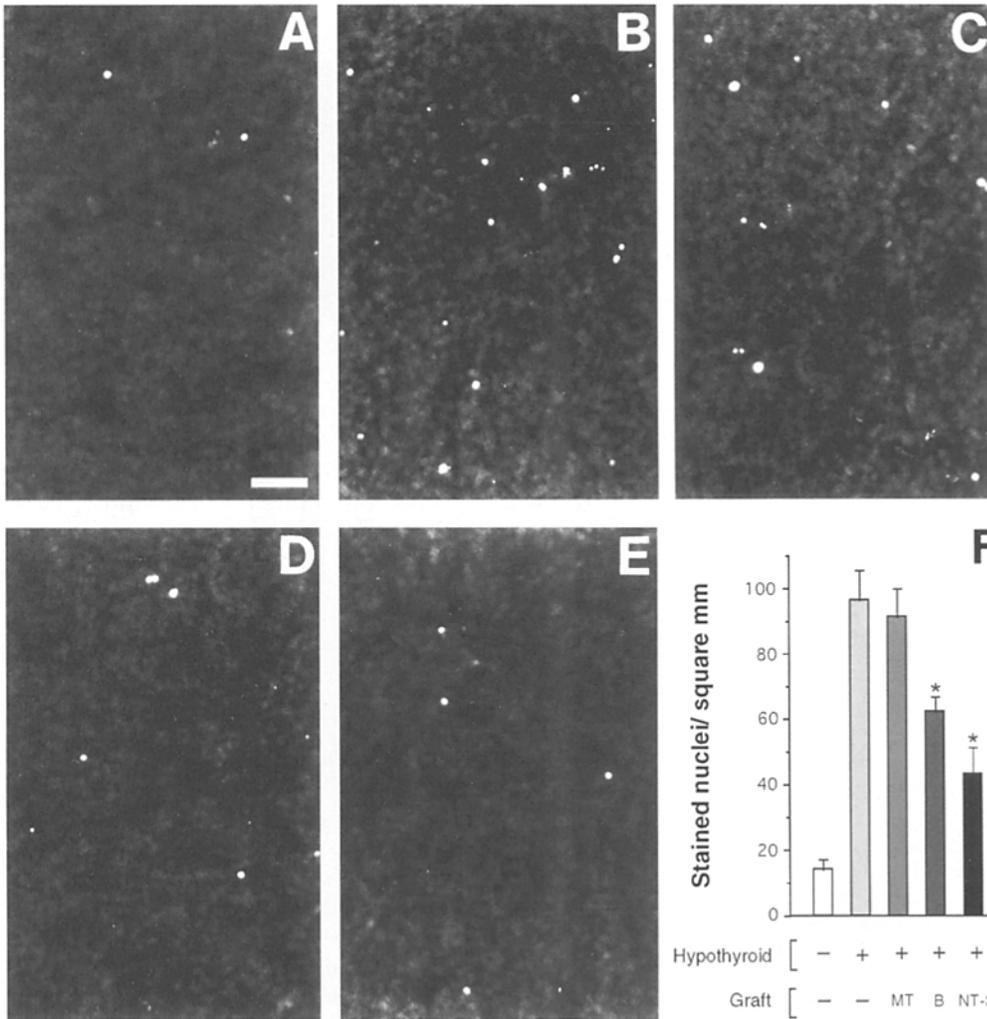


Figure 6. Detection of DNA fragmentation in the IGL of P15 cerebellum. Coronal sections (14 μm) were labeled for DNA fragmentation as described in Materials and Methods. (A) Few stained nuclei were detected in the cerebellum of normal rats. In contrast, ungrafted hypothyroid rats (B) or hypothyroid rats grafted with F3A-MT (C) showed an increased number of stained nuclei. Grafting of the F3N-BDNF (D) or F3A-NT3 (E) to hypothyroid rats reduced the number of stained nuclei. The plot in F shows the number of fluorescein-stained nuclei per square millimeter in each condition. Columns represent mean \pm SD ($n = 3-5$). $P < 0.001$ (t test) compared to hypothyroid animals (H). Bar, 40 μm .

Interestingly, grafting of the F3A-NT3 cell line to hypothyroid rats prevented the decrease in the size of the ML by 50%. No significant difference in the size of the ML of hypothyroid animals grafted with F3A-MT or F3N-BDNF cells was found.

These results suggested that granule and/or Purkinje cells may respond to NT-3 during development. Thus, we compared the effects of NT-3 and BDNF on survival of granule cells and on differentiation of both granule and Purkinje cells in hypothyroid animals *in vivo*.

BDNF and NT-3 Prevent the Death of Neurons in the IGL of Hypothyroid Rats

DNA fragmentation, a characteristic feature of cell death such as apoptosis (Arends et al., 1990), was histologically examined using the TUNEL technique. In the normal cerebellum, very few stained nuclei were detected at P15 in both EGL and IGL and to a lesser extent in the ML. In the absence of terminal transferase, no nuclei were stained, confirming the specificity of the labeling. The number of stained nuclei in the ML or in the EGL was not significantly different in the cerebellum of hypothyroid animals compared to age-matched controls (data not shown). However, in the IGL, the mean number of stained nuclei per square millimeter dramatically increased by 6.8-fold in

hypothyroid rats (Fig. 6). Grafting of the F3A-MT cell line by the lobule X of hypothyroid rats did not modify the number of stained nuclei compared to hypothyroid animals. In contrast, the number of stained nuclei in the hypothyroid cerebellum was reduced by 40 or 65% after grafting F3N-BDNF or F3A-NT3 cell lines, respectively. To determine the cell types undergoing DNA fragmentation in the cerebellum of hypothyroid rats, sections labeled with the TUNEL technique were immunostained with anti-NSE or with anti-GFAP antibodies to distinguish between neurons and astrocytes, and they were analyzed using a laser scanning confocal microscope that allows the three-dimensional reconstruction of the cells. Fig. 7 shows a typical double-stained cell, with a TUNEL⁺ nuclei (T⁺), completely surrounded by an NSE-stained (NSE⁺) cytoplasm. Similar images were obtained for T⁺ and GFAP-stained (GFAP⁺) cells (not shown). The number of NSE⁺ or GFAP⁺ cells undergoing cell death in the cerebellum of normal and hypothyroid rats was determined at P15, and only those T⁺ nuclei surrounded by a completely stained cytoplasm were scored. In the normal IGL, 62% of T⁺ nuclei were surrounded by NSE⁺ cytoplasm, while 38% corresponded to GFAP⁺ cells. In hypothyroid animals, we found a 7.5-fold increase in the number of NSE⁺/T⁺ cells (from 6.7 to 46 NSE⁺/T⁺ cells per square millimeter). In contrast, we did not observe any significant change in the

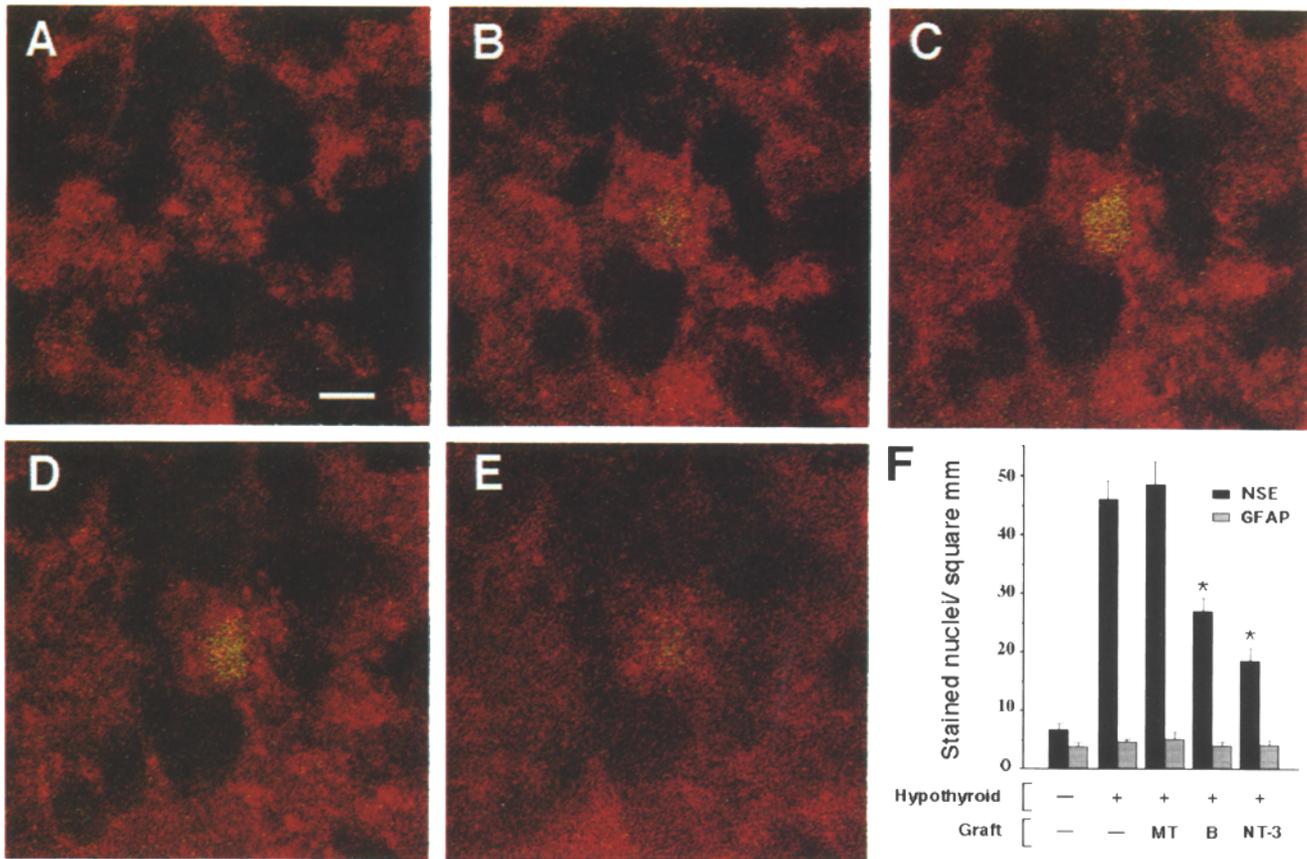


Figure 7. Identification of dying cells in the IGL of P15 cerebellum. Coronal sections through the cerebellum were double stained for DNA fragmentation and NSE or GFAP immunohistochemistry as described in Materials and Methods. (A–E) Computer-generated optical serial sections (1- μ m-thick) showing a fragmented nuclei (green pseudocolor) totally surrounded by an NSE⁺ cytoplasm (red pseudocolor). Digital images were exported to Adobe Photoshop and printed on a ColorEase PS printer (Eastman-Kodak Co.). (F) Number of TUNEL-stained nuclei in the IGL of P15 cerebellum. The plot shows the number per square millimeter of fluorescein-stained nuclei totally surrounded by NSE⁺ or GFAP⁺-labeled cytoplasm. Values represent mean \pm SD ($n = 3$ –5). $P < 0.001$ (t test) compared to hypothyroid animals (H). Bar, 2.5 μ m.

average number of GFAP⁺/T⁺ cells in hypothyroid compared to normal animals (3.7 ± 0.8 , mean \pm SD). Grafting of the F3A-MT cell line did not significantly affect the number of NSE⁺/T⁺ or GFAP⁺/T⁺ cells compared to hypothyroid rats. Grafting of F3N-BDNF or F3A-NT3 cell lines to hypothyroid animals did not modify the number of GFAP⁺/T⁺ cells but did reduce that of NSE⁺/T⁺ cells in the IGL by 45% and 65%, respectively, showing that both BDNF and NT-3 prevented the death of IGL neurons.

NT-3 Induces the Differentiation of Purkinje Cells in Hypothyroid Rats

The development of the dendritic tree of Purkinje cells has been shown to be severely impaired in hypothyroid animals (Nicholson and Altman, 1972b; Legrand, 1979). We therefore evaluated the ability of BDNF and NT-3 to prevent the hypotrophy of the dendritic tree of Purkinje cells. The density of the arborization of Purkinje cell dendrites was measured in the lower part of the vermis (lobule X), the area surrounding the grafting site. Purkinje cells were labeled with an antibody directed against calbindin-D28k, a 28-kD calcium-binding protein specifically expressed by Purkinje cells and not affected by T3 deficiency (Rabié et

al., 1986). At P15, hypothyroidism led to a sparse terminal branching and a 50% decrease in the density of the dendritic tree of Purkinje cells, compared to age-matched controls (compare Fig. 8, A and B). A similar reduction in the dendritic arborization of Purkinje cells was observed in hypothyroid rats grafted with the F3A-MT or F3N-BDNF cell lines (Fig. 8, C and D). However, grafting of the F3A-NT3 cells significantly increased the total density of the dendritic tree by 50% and enhanced the appearance of immunoreactive lateral branches from the primary dendrite as well as the total length of the dendritic tree (Fig. 8, E and F). Thus, NT-3, but not BDNF, promoted the development of the dendritic tree of Purkinje cells.

NT-3 Prevents Alterations in the Synaptogenesis of Purkinje Cells in Hypothyroid Rats

Early hypothyroidism has been previously shown to impair synaptogenesis in the cerebellum (Nicholson and Altman, 1972b,c; Crépel, 1974; Rebière and Dainat, 1976). To localize developing synaptic terminals in the hypothyroid cerebellum, we used an antibody that labels synaptophysin, an abundant glycoprotein localized in developing presynaptic terminals (Jahn et al., 1985; Wiedenmann and

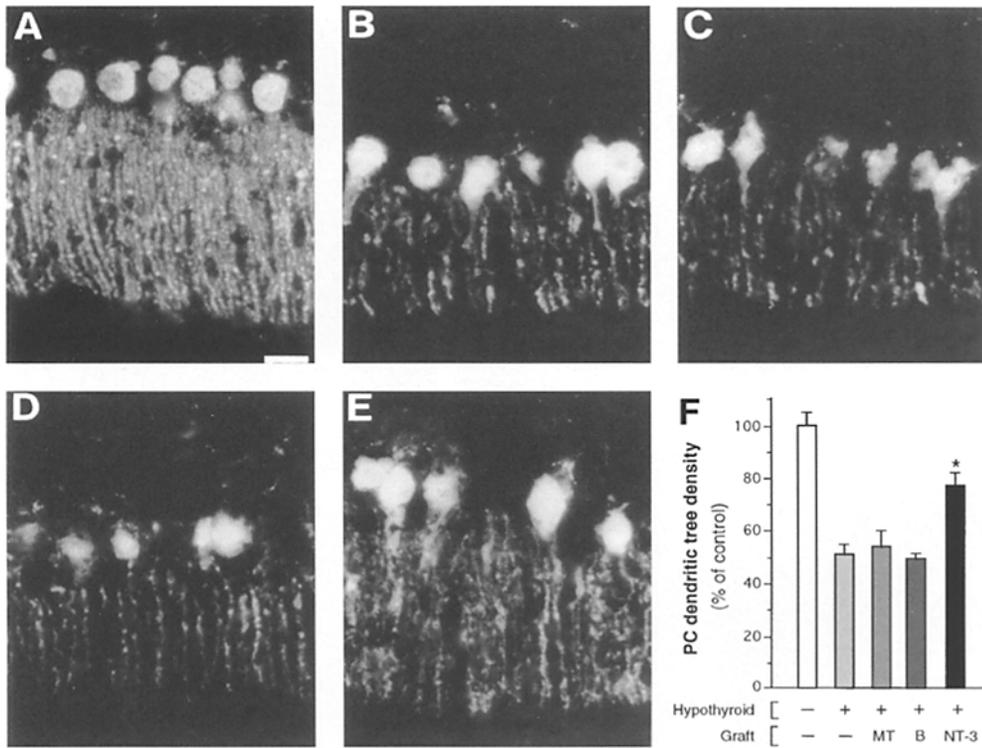


Figure 8. Staining of Purkinje cells and their dendritic tree with anti-calbindin-D28k antibody. (A) Normal rats. (B) Hypothyroid rats. (C) Hypothyroid rats grafted with the control F3A-MT, the F3N-BDNF (D), or the F3A-NT3 (E) cell lines. A significant decrease in the density of the arborization of the Purkinje cells was observed in hypothyroid (B) compared to normal (A) cerebellum. Hypothyroid animals grafted with F3A-NT3 cells showed an increase in the density of the arborization of Purkinje cells, compared to hypothyroid rats. In F the density of the dendritic tree of Purkinje cells is expressed as a percentage of the corresponding values in normal animals. Each column represents mean \pm SD ($n = 3-5$). $P < 0.001$ (t test) compared to hypothyroid rats (H). Bar, 50 μ m.

Franke, 1985). The distribution of synaptophysin immunoreactivity was analyzed in lobule X, the same area used for grafting of the cell lines. In control animals, synaptophysin immunoreactivity was mainly found in the IGL and ML of the cerebellum at P15 (Fig. 9), presumably corresponding to presynaptic terminals of mossy, climbing, and parallel fibers (Leclerc et al., 1989). The Purkinje cell layer showed low staining in the normal cerebellum; however, numerous synaptophysin-immunopositive puncta (oval or round structures of 0.5–2 μ m) were found on the soma of Purkinje cells in hypothyroid animals. The determination of the number of immunopositive puncta over each Purkinje cell body in normal rats showed an average of 1.93 ± 0.16 (mean \pm SD) puncta, localized mainly in the area of the axonic cone (Fig. 9, A and D). In contrast, in hypothyroid rats there was a striking sixfold increase in the number of immunopositive puncta per Purkinje cell body (12.08 ± 0.7) and to a lesser extent in the proximal segment of their dendritic tree (Fig. 9, B and D). In hypothyroid rats grafted with F3A-MT or F3N-BDNF cell lines, the number of the punctate structures per Purkinje cell was very similar to ungrafted hypothyroid rats (Fig. 9 D). In contrast, grafting of the F3A-NT3 cell line to hypothyroid animals significantly decreased by 40% the number of punctate structures per Purkinje cell to 7.74 ± 0.58 (Fig. 9, C and D). Thus, we found that NT-3, but not BDNF, promoted the acquisition of the mature pattern of synaptophysin-stained terminals on Purkinje cell bodies.

Discussion

The data presented in this study show that the specific pattern of expression of neurotrophins and their receptors

was developmentally regulated in the cerebellum, and that thyroid hormone deficiency differentially modified the developmental pattern of expression of all neurotrophins, resulting in a severe deficit in BDNF and NT-3. Furthermore, we have shown that administration of these neurotrophins, and particularly NT-3, reversed alterations induced by hypothyroidism in the cerebellum.

Hypothyroidism Induces Alterations in the Expression of Neurotrophin Receptors

A delay in the upregulation of trkC KI mRNA was the only significant alteration in the developmental pattern of expression of the different trk receptors in hypothyroid rats. Although the physiological roles of different TrkC isoforms still remain to be established (Lamballe et al., 1993; Valenzuela et al., 1993; Tsoulfas et al., 1993; Garner and Large, 1994), some studies have shown that TrkC FL, but not TrkC KI, stimulated process outgrowth in PC12 cells (Valenzuela et al., 1993; Tsoulfas et al., 1993). Thus, the differential regulation of trkC transcripts occurring during cerebellar development could be a potentially important mechanism, controlling specific responses to NT-3. Similarly, the precise functional role of the p75^{NTR} receptor, and of the delay in its downregulation in hypothyroid rats, remains to be determined. p75^{NTR} has been proposed either to clear or to increase the local concentration of ligands (Johnson et al., 1988; Barbacid, 1993; Barker and Shooter, 1994), to regulate signaling, ligand specificity, or discrimination of the Trk receptors (Benedetti et al., 1993; Mahadeo et al., 1994; Verdi et al., 1994), and to help to respond to limiting amounts of neurotrophins (Hantzopoulos et al., 1994). Such roles may be crucial when the levels

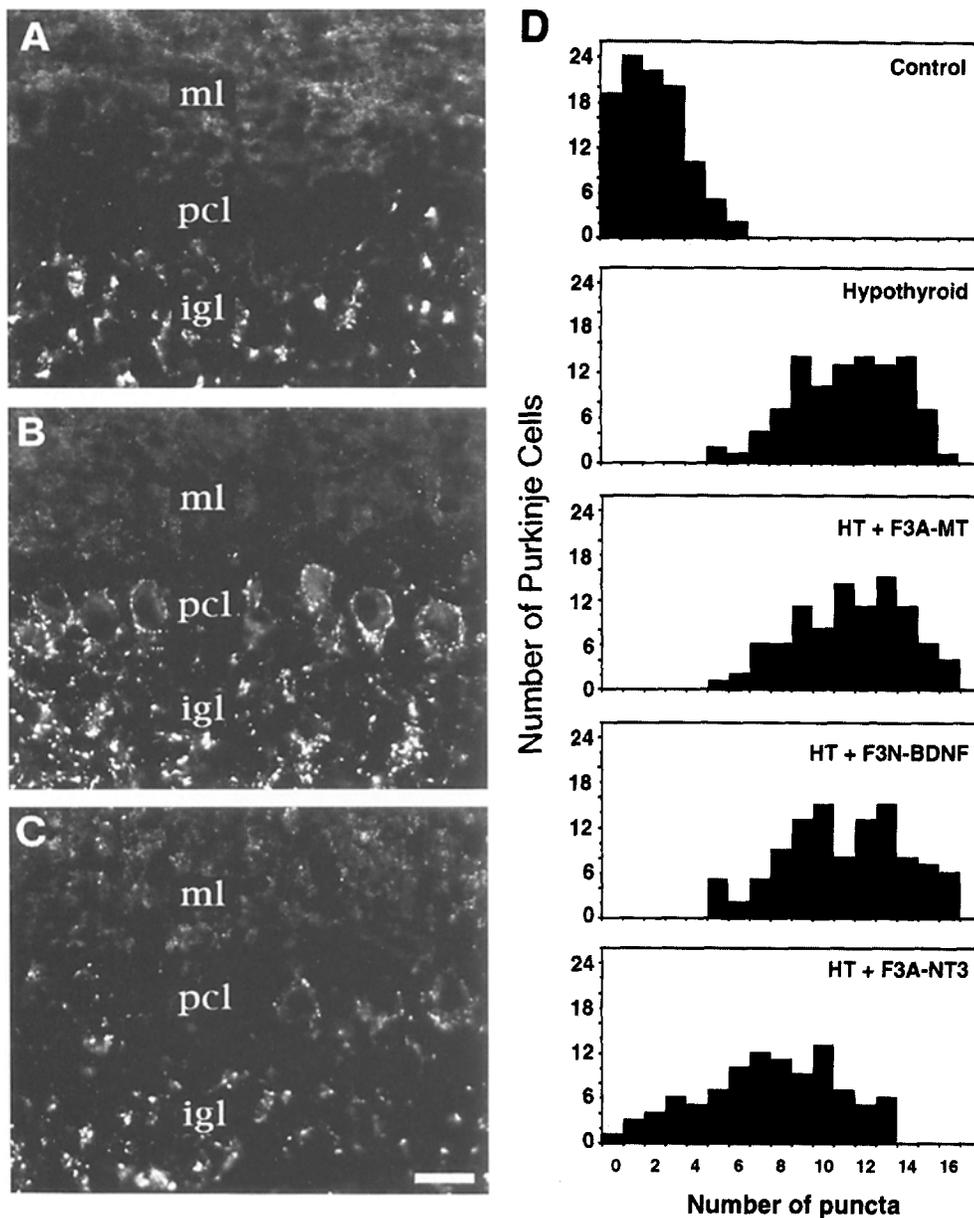


Figure 9. Distribution of synaptophysin-stained synaptic terminals on Purkinje cells. (A) A small number of immunoreactive puncta were found at P15 on the soma of normal Purkinje cells. Hypothyroid rats (B) showed an increased number of puncta over Purkinje cell soma. (C) Grafting of F3A-NT3 cells to hypothyroid animals decreased the number of fluorescent puncta. (D) Distribution of the number of fluorescent puncta per Purkinje cell soma in control animals and in hypothyroid rats ungrafted or grafted with the control F3A-MT, F3N-BDNF, or F3A-NT3 cell lines. 100 Purkinje cells were randomly chosen in four different sections in every cerebellum, in three to five animals. *ml*, molecular layer; *pcl*, Purkinje cell layer; *igl*, internal granule cell layer. Bar, 30 μm .

of neurotrophins are low in the cerebellum, including early postnatal development and hypothyroidism.

Hypothyroidism Results in Abnormally High Levels of NGF and NT-4

The developmental downregulation of NGF and NT-4 mRNA was respectively delayed or nearly absent in hypothyroid rats. This resulted in transiently higher levels of NGF (Muñoz et al., 1991; Figueiredo et al., 1993; present results) and NT-4 mRNAs (present results). While NT-4 has been reported to promote neurite extension and survival of differentiated granule cells in vitro (Gao et al., 1995), NGF has been suggested to play a role in the proliferation of immature granule neurons (Confort et al., 1991) and in the differentiation of Purkinje cells (Legrand and Clos, 1991). It should be noted, however, that the level of *trkA* mRNA was below the detection limit during the entire postnatal cerebellar development, indicating that

granule cell-derived NGF (Charrasse et al., 1992) might act as a target-derived factor for cerebellar afferents expressing *trkA* and/or signal through other neurotrophin receptor(s) expressed in the cerebellum, including $p75^{\text{NTR}}$.

BDNF Prevents Hypothyroidism-induced Granule Cell Death In Vivo

We found a relatively low level of expression of BDNF mRNA from P0 to P10, when *TrkB* FL is expressed in the EGL (Masana et al., 1993). Interestingly, BDNF has been reported to induce the expression of *c-fos* in immature granule cells of the external proliferative zone in organotypic cultures and to promote the survival of dissociated embryonic granule cells in vitro (Segal et al., 1992). Moreover, BDNF knockout mice show a delay in the maturation of the EGL (Jones et al., 1994). In the present model of hypothyroidism, these early functions of BDNF are most likely spared since both BDNF and *trkB* mRNAs are

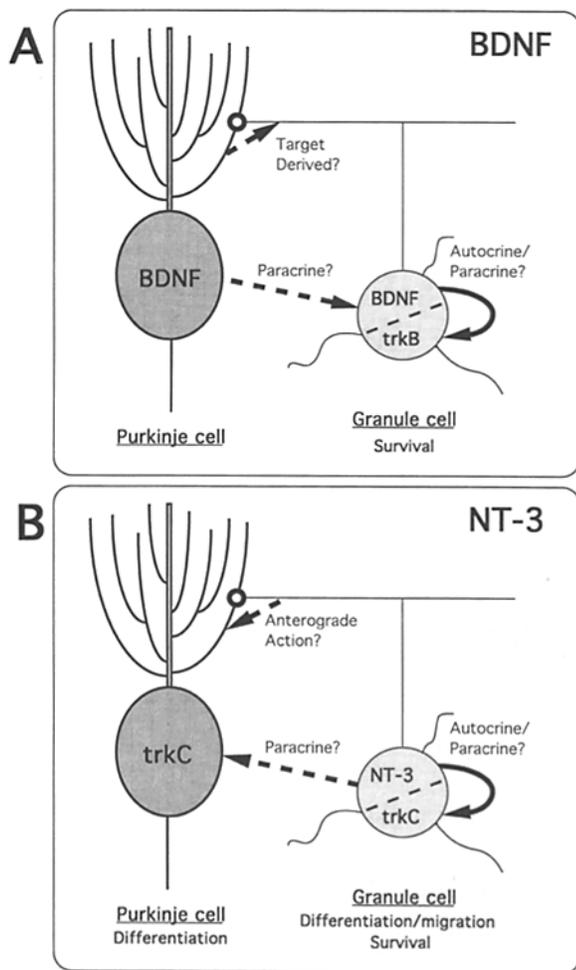


Figure 10. Possible neurotrophic interactions between granule and Purkinje cells in the cerebellar cortex during postnatal development. In the present study, we show that BDNF is expressed by Purkinje and granule cells and promotes the survival of granule cells. Thus, we speculate that Purkinje cell-derived or granule cell-derived BDNF may prevent the death of granule cells in a target-derived and/or an auto/paracrine mode of action. We also show that NT-3 is expressed by granule cells and promotes the differentiation and/or migration of premigratory granule cells and the survival of postmigratory granule cells. Thus, we suggest that granule cell-derived NT-3 may act on granule cells to promote survival and differentiation by auto/paracrine mechanisms and on Purkinje cells to induce their differentiation in a paracrine or an anterograde action.

expressed at control levels until P10. However, from P15 to P30, T3 deficiency dramatically reduced the upregulation of BDNF expression, which normally occurs in granule cells of the IGL (Rocamora et al., 1993; present results). In vitro experiments have suggested a role for BDNF in preventing the death of differentiated granule cells (Kubo et al., 1995; Gao et al., 1995). Our finding showing that BDNF partially prevented the increase in the number of neurons undergoing cell death in the IGL of hypothyroid rats reinforces that view. Furthermore, the fact that BDNF mRNA increased strongly after the peak of NT-3 mRNA expression may indicate that the survival-promoting effect of BDNF could be more important at lat-

ter developmental stages. Finally, the presence of *trkB* mRNA in the internal granule cells (Merlio et al., 1992; Gao et al., 1995) suggest that granule cell-derived, and to a lesser extent Purkinje cell-derived, BDNF may act as a survival-promoting factor for cerebellar granule neurons in an autocrine, paracrine, or target-derived fashion (Fig. 10 A). Further experiments would be required to explore these mechanisms and to determine whether the expression of BDNF by a subpopulation of Purkinje cells may have a role in the functional organization of the cerebellar cortex, as it has been recently suggested for BDNF and NT-4 in the visual cortex (Cabelli et al., 1995).

NT-3 Promotes the Differentiation and Migration of EGL Neurons In Vivo

In contrast to BDNF, NT-3 mRNA is highly expressed early in the development of cerebellum. Interestingly, both NT-3 (present results) and $\alpha 1$ thyroid hormone receptor (Mellström et al., 1991) show a prominent peak of expression between P10 and P20, when most of the cerebellar neurons are differentiating (Altman, 1972*a,b,c*). Levels of NT-3 mRNA have been found to be lower in hypothyroid rats at P10 (Lindholm et al., 1993). We show now that the developmental upregulation of NT-3 mRNA does not occur at any stage of the development of the hypothyroid cerebellum, suggesting that deficient expression of NT-3 could contribute to the alterations induced by hypothyroidism. We therefore examined if the administration of the NT-3 could prevent the alterations induced by T3 deficiency on the development of the cerebellum.

In hypothyroid animals, granule neurons from the EGL proliferate for a longer period of time and delay their migration, resulting in higher number of neurons in the EGL at P15. In this study, we show that NT-3, but not BDNF, decreases the size of the EGL compared to ungrafted hypothyroid rats, suggesting an enhancement of the differentiation and/or migration of granule cells by NT-3. In agreement with this, organotypic cultures have shown that granule neurons in the premigratory zone of the EGL respond to NT-3, but not to BDNF (Segal et al., 1992). All these data argue for the specificity of NT-3 in promoting the differentiation and/or migration of EGL neurons. In addition, the fact that both NT-3 (Ernfors et al., 1992; Rocamora et al., 1993; present results) and *trkC* (Lindholm et al., 1993; Tessarollo et al., 1993) are expressed in the EGL during cerebellar development suggests that NT-3 could be acting in an autocrine and/or paracrine fashion to induce the differentiation and/or migration of granule cells. Interestingly, similar effects have been reported for cortical and hippocampal neurons (Gosh and Greenberg, 1995; Vicario-Abejón et al., 1995), suggesting a general role of NT-3 in promoting the differentiation of early neurons in laminated cortical structures.

NT-3 Prevents the Death of IGL Neurons In Vivo

During normal development, we found that 62% of the cells undergoing cell death at P15 are neurons (NSE⁺) and 38% are astrocytes (GFAP⁺). In contrast, 5 d earlier, at P10, 50–70% of apoptotic cells were found to be astrocytes (Krueger et al., 1995), which suggests distinct developmen-

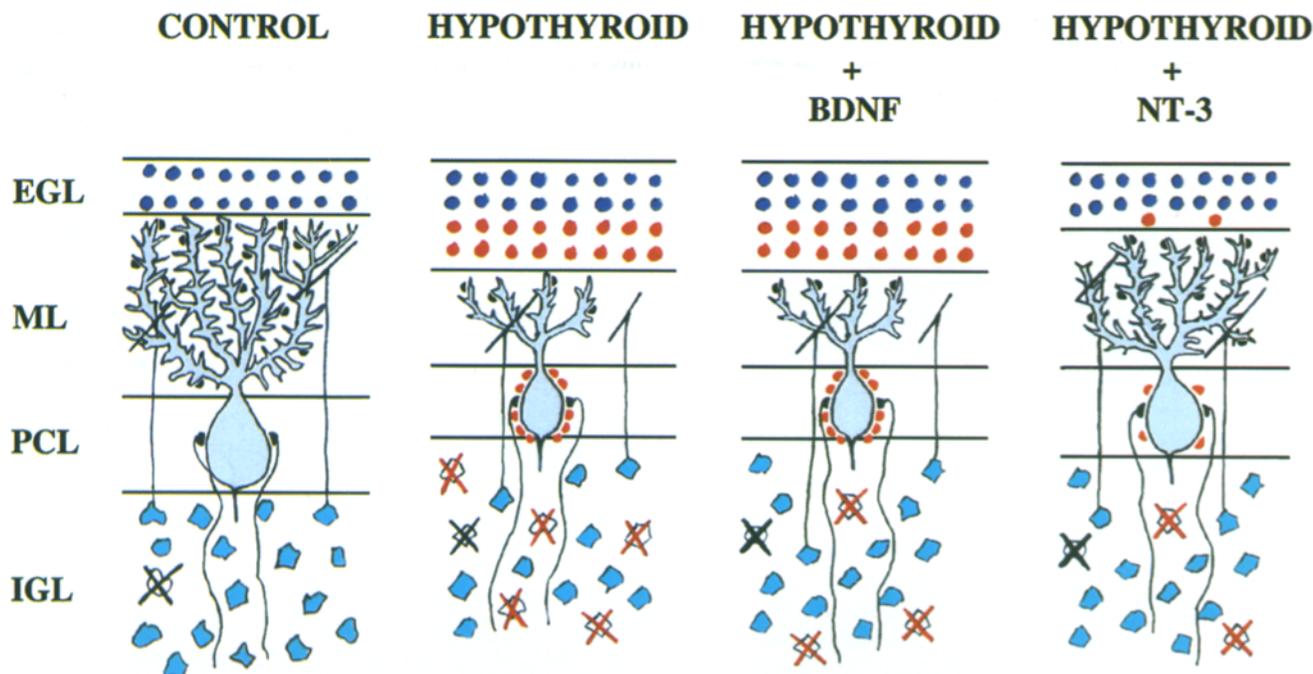


Figure 11. Summary of the effects of BDNF and NT-3 on the survival, differentiation, and maturation of granule and Purkinje cells in the developing cerebellum of hypothyroid rats. The cartoons to the left (*control, hypothyroid*) highlight the differences (*in red*) observed in the cerebellum of hypothyroid rats at P15 compared to control. T3 deficiency led to increased number of granule cells (*red circles*) in the external granule cell layer (*EGL*), an increase in the number of dying neurons (*red crosses*) in the internal granule layer (*IGL*), a hypotrophy of the dendritic arborization of the Purkinje cells in the ML, and an increase of the number of synaptophysin-immunoreactive puncta (*red semicircles*) on the Purkinje cell bodies in the Purkinje cell layer (*PCL*). As indicated in the two cartoons to the right, BDNF and NT-3 reversed some of these defects. Both BDNF and NT-3 prevented the death of neurons in the IGL (*red crosses*). NT-3, but not BDNF, promoted the differentiation/migration of granule cells as shown by their decreased numbers in the EGL (*red circles*), the development of the dendritic arborization of Purkinje cells in the ML, and the organization of a more mature pattern of synaptic afferents to Purkinje cell somas, as shown by the decrease in the number of synaptophysin-immunoreactive puncta on Purkinje cell bodies (*red semicircles*).

tal patterns of cell death for astrocytes and neurons. In agreement with the finding that hypothyroidism increased the number of pyknotic cells in the IGL (Balázs et al., 1975; Lewis et al., 1976), we have observed a selective sevenfold increase in the number of cells undergoing cell death in the IGL of hypothyroid rats at P15. Using double staining, we have found that the increase in the number of TUNEL⁺ cells in the hypothyroid IGL is due to the death of neurons, but not astrocytes, during the development of the cerebellum. In vitro studies have shown that granule cell death could be prevented by both T3 (Heisenberg et al., 1992) and NT-3 (Kubo et al., 1995), suggesting that the absence of upregulation of NT-3 in hypothyroid animals could be a factor contributing to the death of granule cells. Our results showing that NT-3 did decrease by 60% the number of neurons undergoing cell death in the IGL of hypothyroid rats at P15 strengthen this hypothesis and argue for a role of NT-3 in promoting the survival of granule cells in vivo.

The death of granule cells in hypothyroid animals has been suggested to be a consequence of their failure to make adequate synapses with hypotrophic Purkinje cells (Balázs et al., 1975; Lewis et al., 1976) and to obtain the appropriate trophic support. Our finding that NT-3 stimulates the development of the dendritic tree of Purkinje cells is compatible with this possibility, since NT-3 might have also induced Purkinje cell-derived survival factor(s)

for granule cells, including BDNF. However, the presence of both NT-3 and trkC in granule neurons of the IGL (Lindholm et al., 1993; Rocamora, 1993; Tessarollo et al., 1993; present results) suggest that NT-3 most likely exerts survival of granule cells via a direct auto/paracrine mechanism (Fig. 10 B).

NT-3 Promotes Differentiation of Purkinje Cells In Vivo

The expression of trkC by Purkinje cells in the developing rat cerebellum (Ernfors et al., 1992; Lindholm et al., 1993; Tessarollo et al., 1993), at the time when NT-3 mRNA is upregulated (present results) and Purkinje cells are differentiating, suggests that NT-3 may have direct effects on Purkinje cells. Interestingly, in hypothyroid rats, the absence of upregulation of NT-3 mRNA was accompanied by an hypotrophy of the dendritic tree of Purkinje cells and an increase in the number of synaptophysin-stained axon terminals over Purkinje cell somas. We first studied the effects of BDNF or NT-3 on the development of the dendritic tree of Purkinje cells. Our results confirm and extend previous observations on the effects of NT-3 on Purkinje cells in vitro (Lindholm et al., 1993). We show that NT-3, but not BDNF, increases the length, number of branches, and density of the dendritic arbor of Purkinje cells in hypothyroid rats in vivo. These findings suggest

that the absence of upregulation of NT-3 in hypothyroid rats contributes to the disruption of the normal development of Purkinje cells. A second implication of our findings is that endogenous NT-3, expressed by granule cells, may promote the maturation of Purkinje cells either in a paracrine fashion or in a novel mode of trophic interaction involving an anterograde transport of NT-3 from granule cell axons to Purkinje cell dendrites (Fig. 10 B). Granule neurons have been shown to supply the epigenetic factor(s) responsible for the development of mature dendrites and dendritic specializations of Purkinje cells in vivo (Baptista et al., 1994). Our results suggest that NT-3 could be one of those factors.

The second parameter in the maturation of Purkinje cells that we studied was the establishment of a mature pattern of synaptic afferents to Purkinje cell somas. Synaptogenesis in the cerebellum follows a strict developmental chronology. Between P2 and P10, climbing fibers establish transient synaptic connections with Purkinje cell somas (Altman, 1972b; Sotelo et al., 1984; Mason and Gregory, 1984). Similarly, axon collaterals from Purkinje cells may establish axosomatic synapses with neighboring Purkinje cells (Leclerc et al., 1989). As Purkinje cell dendrites grow, the climbing fibers terminals extend over the surface of the dendritic branches and the axo-somatic synapses of climbing fibers disappear. In the adult rat, the basket cell afferents and Purkinje cell collaterals are the almost exclusive axo-somatic synapses on Purkinje cells. Early hypothyroidism leads to a delay in the disappearance of the transient axo-somatic synapses and to an abnormal increase in the axonal collaterals from Purkinje cells to neighboring Purkinje cells, which is believed to be caused by a delay in the appearance of the basket cell terminals (Legrand, 1979). All these alterations result in an increased number of synapses over Purkinje cell bodies as assessed by the higher number of synaptophysin-positive puncta in presynaptic terminals over Purkinje cell somas. Grafting of an NT-3-producing cell line, but not a BDNF-producing cell line, decreased the number of synaptophysin-immunopositive puncta on Purkinje cells in hypothyroid rats, resulting in a more mature pattern of synaptic afferents to Purkinje cells, which resembled normal development. Thus, our results suggest a role of NT-3 in promoting the development and the synaptic organization of Purkinje cells.

Conclusion

We have shown that T3 deficiency leads to higher levels of p75^{NTR}, NGF, and NT-4 mRNAs and to lower levels of trkC-KI, NT-3, and BDNF mRNAs during postnatal development of the cerebellum. Moreover, replacement of the deficient neurotrophins (BDNF and NT-3) partially prevented hypothyroidism-induced deficits, including the death of neurons in the IGL. In addition, NT-3, but not BDNF, induced the differentiation and/or migration of granule cells from the EGL, stimulated the elaboration of dendritic tree of Purkinje cells, and promoted the formation of a mature pattern of synaptic afferents to Purkinje cells (Fig. 11). Thus, our findings provide evidence for multiple novel neurotrophic activities of neurotrophins in the development of the cerebellar cortex in vivo and suggest that

neurotrophins, under the control of thyroid hormone, play a key role in the development of the cerebellum.

The authors are very grateful to Drs. Peter Wallén and Russel Hill for their help in the use of the confocal microscope facility at the Department of Neurophysiology (Karolinska Institute, Stockholm). We thank Dr. Hiroshi Funakoshi for providing the p75^{NTR} and trkC probes and Dr. Philippe Naveilhan for his help and support during this work. We also thank Dr. Wilma Friedman and our colleagues at the Laboratory of Molecular Neurobiology for critical reading of the manuscript and Lotta Johansson for secretarial help.

Financial support was obtained from the Swedish Cancer Society, the Swedish Medical Research Council, and funds from the Karolinska Institute. I. Neveu was supported by a fellowship from the Human Frontier Science Program Organization and by a French-Swedish fellowship from the Institut National de la Santé et de la Recherche Médicale-Swedish Medical Research Council. E. Arenas was supported by the Swedish Medical Research Council.

Received for publication 4 December 1995 and in revised form 14 February 1996.

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