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The Effects of Thyroid Hormone on Growth Factor Expression in Developing Cerebella of Mice: In Vivo and in Vitro Studies

by

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A dissertation submitted to the Graduate Faculty in Psychology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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Abstract

The Effect of Thyroid Hormone on Growth Factor Expression in the Developing Cerebellum of the Mouse: In Vivo and In Vitro Studies

by

Emilce Carrasco

Advisor: Professor Victoria Luine

In this study, I investigated the effects of thyroid hormone on cerebellar development and growth factor expression, specifically the epidermal growth factor receptor (EGFR), its ligand transforming growth factor-alpha (TGF-α), and basic fibroblast growth factor (bFGF) *in vivo* and *in vitro*.

In vivo, profiles of thyroid hormone expression were measured in postnatal animals and were found to peak at postnatal day 15 (P15). These levels dropped below detectable levels when mice were made hypothyroid with propylthiouracil (PTU). TGF-α and EGFR expression were maximal at P6 in normal animals, but remained low in hypothyroid animals, suggesting that thyroid hormone was responsible for their induction. Immunohistochemical analysis of EGFR protein expression revealed that this receptor was expressed on granule cells within the inner zone of the external granule cell layer (EGL), where post-mitotic granule cells are found. The persistence of EGFR expression on migrating granule cells and subsequent down-regulation of expression in the internal granule cell layer (IGL) implicates a role for EGFR-ligands in migration and/or differentiation. In hypothyroid animals, I observed a delayed progression of granule cell migration, consistent with the persistence of EGFR

labeling in the EGL, and in the "pile-up" of labeled cells at the interface between the molecular layer and the IGL.

In vitro, I examined the effects of thyroid hormone on mixed cultures of cerebellar granule neurons and several types of glia, thus permitting biologically relevant neuronal-glial interactions. I found that L-triiodothyronine (T_3) had differential effects on neurons and glia, depending on cell density. At low plating densities, T_3 accelerated neuronal and glial differentiation and the switch in astrocyte morphology from radial to velate. At high densities, T_3 decreased proliferation of non-neuronal cells. Western blotting of cell lysates showed that T_3 treatment regulated levels of epidermal growth factor receptor (EGFR), and basic fibroblast growth factor (bFGF) proteins in opposite directions, attenuating bFGF, but inducing EGFR.

Taken together, these results implicate thyroid hormone in the coordinated expression of TGF-α and EGFR, which appear to play a role in post-mitotic developmental events, and bFGF, which appears to be involved in earlier mitotic events.

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for my mother and father

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Chapter 1

General Introduction

Thyroid hormone is essential for normal brain development. Hypothyroidism during late gestation and postnatal development causes morphological and biochemical alterations in the central nervous system (CNS) that can lead to severe and irreversible mental retardation in humans (Dussault and Ruel, 1987; Timiras and Nzekwe, 1989). The main mechanism of action of thyroid hormone is genomic, but it has nongenomic actions as well (Williams, 1994; Davis and Davis, 1996). Growth factors are also important for normal development of neurons and glia. These polypeptides, such as the epidermal growth factor (EGF) family, fibroblast growth factor (FGF) family, and neurotrophins have been shown to have diverse effects on cells of the CNS, such as promoting proliferation, neuronal survival, neurite outgrowth, and migration. It is hypothesized that a possible mechanism of action of thyroid hormone may be to regulate, directly or indirectly, the availability of growth factors. Most studies that have tested this hypothesis have focused on gene and protein expression of neurotrophins.

The epidermal growth factor family is widely expressed and developmentally regulated in the CNS. Thyroid hormone has been shown to have significant effects on the expression of epidermal growth factor (EGF) and the epidermal growth factor receptor (EGFR) in cells outside the CNS (Gresik, 1981; Kesavan et al., 1991; Hume et al., 1992; North et al., 1992; Fujieda, 1993; Sheflin et al., 1993; Rogers et al., 1995; Rogers et al., 1996). In the brain, however, reports on the effect of thyroid hormone on these polypeptides are limited to EGF mRNA expression and receptor binding activity in whole brain homogenates (Sadiq et al., 1985; Stein et al., 1989). A recent study shows that thyroid hormone administration upregulates

transforming-growth factor-alpha (TGF-α, a member of the EGF family) gene expression in the striatum of mice (Blum et al., 1999).

The objective of this thesis was to determine if thyroid hormone regulates TGF-α and epidermal growth factor receptor (EGFR) gene expression. To elucidate on the functions of these factors, I characterized the cell localization and distribution of EGFR immunoreactivity. The results of this study are presented in Chapter 2. Many studies that have investigated the role of thyroid hormone on cerebellar cell development have used either pure neuronal or pure glial cultures. Therefore, I characterized the developmental effects of thyroid hormone on neurons, glia and bFGF (also a potent mitogen) and EGFR in mixed cerebellar cell culture system that best simulates the in vivo condition. The results of this study are presented and discussed in Chapter 3. In the remainder of this chapter I discuss the background, significance, and specific aims of this thesis.

Background and Significance:

Thyroid Hormone Synthesis, Regulation, and Developmental Expression

The thyroid gland produces two hormones, thyroxine (T_4) and triiodothyroinine (T_3). These are small lipophilic/hydrophobic molecules that require carrier proteins for their transport in the blood and extracellular fluid to their target cell. Thyroxine (T_4) is the main circulating hormone but is converted by deiodinases intracellularly to triiodothyronine (T_3), the active hormone. The deiodianting enzymes are found in both neurons and glia (St. Germain, 1994). Briefly, the onset of thyroid gland function (production and secretion of thyroid hormone) in rodents is around embryonic day (E) 17 (Fisher et al., 1977). Serum

T_4 (thyroxine) levels rats are low at birth but then begin to rise around postanatal day (P) 6 to reach peak levels between P14 and P15 (Walker et al., 1979). Thereafter, T_4 declines to adult levels. In humans, the initiation of fetal thyroid hormone synthesis is between 10-12 weeks of gestation. Serum T_4 levels are first detectable during the second trimester and progressively increase thereafter (Fisher et al., 1977). Since thyroxine and T_3 are small lipophilic/hydrophobic molecules that are able to cross the blood-brain barrier, suggesting that capillary transport is their main route of entry into the brain (Robbins et al., 1991).

Thyroid-stimulating hormone (TSH) secreted by the adenohypophysis regulates the synthesis and the release of thyroid hormone from the thyroid gland. Thyrotropin (TRH) released by the hypothalamus controls the secretion of TSH. Pituitary and hypothalamic cells have thyroid hormone receptors that mediate the negative feedback action of thyroid hormones on the pituitary and hypothalamus. These events are illustrated in Figure 1.

The biological activities of thyroid hormone are largely mediated by nuclear receptors that as ligand-dependent transcription factors (Figure 2). The functional role of thyroid hormone during brain development has been difficult to elucidate due to the complex spatial and temporal expression of these receptors. This complexity, nevertheless, is believed to be necessary for fine tuning the biological responses to thyroid hormone. Moreover, thyroid hormone also has non-genomic actions that are independent of nuclear receptors. These actions have been described at the level of the plasma membrane, cytoskeleton, and cytoplasm. They include alterations in calcium,

sodium and glucose transport, changes in the activities of kinases, efficiency in mRNA translation, and mRNA half life (Davis and Davis, 1996).

Molecular Mechanisms of Thyroid Hormone Action in the CNS

Thyroid hormone receptors (TRs)

Thyroid hormone receptors belong to the steroid/thyroid hormone nuclear receptor superfamily. These receptors have three chief domains: An N-terminus that is the transactivation region (the mechanism by which these receptors induce or repress transcription of target genes), DNA binding, and ligand binding or C-terminus (Fig. 2). Thyroid hormone receptors bind DNA response elements in the absence of thyroid hormone and repress transcription. Gene transcription is initiated when thyroid hormone binds to the receptors. There are two genes for thyroid hormone receptors (TR), α and β. Alternate splicing of their transcripts generates multiple isoforms of each receptor (α1, α2, 3α, and β1, β2) (Glass and Holloway, 1990; Lazar, 1993; Williams, 1994). Thyroid hormone receptor α2 is identical to α1 in 370 amino acids, including the DNA binding domain, but lacks the α2 C-terminal form found in α1. Instead, TR-α2 contains 122 amino acids, fused to the receptor at amino acid 370 (Williams, 1994). This difference in the C-terminus is believed to be the reason that α2 and does not bind T_3 and is unable to induce transcription. Thyroid hormone receptor β1 and β2 differ in their N-terminus. Thyroid hormone receptor β1 has 106 amino acids and β2 has 159 in the N-terminus (Lazar, 1993; Williams, 1994). Thyroid hormone receptors bind DNA in three different ways: monomers, homodimers, and heterodimers (Lazar, 1993; Glass, 1994). Additionally, TRs require heterodimerization with retinoid X receptors (RXR) for maximum DNA binding affinity (Glass, 1994).

Numerous studies, using *in situ* RNA hybridization and immunocytochemistry, show that TR isoforms have different expression patterns in developing neurons and glia (Strait et al., 1990; Mellstrom et al., 1991; Strait et al., 1991; Wills et al., 1991; Bradley et al., 1993; Lebel et al., 1993; Carlson et al., 1994; Carlson et al., 1996; Carre et al., 1998). In the embryonic rat brain, TR-α1 mRNA has been localized in the neocortical plate, the site of neuronal differentiation. TR-β1 mRNA has been localized in the germinal trigone, the site of neuroblast proliferation, suggesting that these receptors (α1 and β1) may have different functions during embryonic development (Bradley et al., 1993). Additionally, during postnatal brain development TR-α1 mRNA appears to be developmentally regulated. Peak expression of this receptor occurs during the first three postnatal weeks in the forebrain and cerebellum, coinciding with the postnatal increase in serum thyroxine levels. In the cerebellum TRβ1 immunoreactivity is expressed only in Purkinje cells, whereas TRα2 is expressed in the nuclei of granule cells of the cerebellum, suggesting that Purkinje cells but not granule cells are direct targets for T_3 (Strait et al., 1991). Finally, the overall expression of TR-α and TR-β mRNAs are reported to be much lower in the brains of adult rats compared to that of postnatal rats (Sherer et al., 1993). These data are consistent with the hypothesis that the developing brain is more sensitive to thyroid hormone than the mature brain (Legrand, 1979).

The functions of TR isoforms during development of the CNS are largely unknown. Some of the functions of TR isoforms are just beginning to be elucidated using knockout mice. For example, TR-β is essential for the regulation of the hypothalamic-pituitary axis and auditory function, whereas knockout mice for both TR-α and β exhibit an extremely hyperactive pituitary-thyroid axis, poor female fertility, retarded growth, and retarded bone maturation (Forrest et al., 1996; Forrest et al., 1996; Abel et al., 1999; Gothe et al., 1999).

Influence of thyroid hormone on gene expression

It is believed that thyroid hormone affects brain development by initiating the transcription of a finely tuned program of genes. Genes that respond to hormones are categorized as either primary (direct) or secondary (indirect) response genes. Primary response genes respond rapidly (< 4 hours) without the need for protein synthesis, indicating that they are direct targets of regulation by a particular transcriptional activator; however, the induction of secondary genes is easily blocked by protein synthesis inhibitors. Two primary response genes to thyroid hormone are *hr* and *Srg,;* they are both induced in less than 4 hours, suggesting they are regulated directly by thyroid hormone (Thompson, 1996). These genes have been identified in the developing cerebellum, and are specifically expressed in the IGL where differentiated neurons are found; *hr* encodes a zinc finger protein (related to the product of the mouse gene *hairless*) many of which are involved in transcriptional regulation (Thompson, 1996). *Srg 1* encodes a protein related to synaptotagmin, known to be involved in regulating neurotransmitter release. Since they are expressed in the IGL they are hypothesized to play a role in granule cell maintenance and synaptognesis (Thompson, 1996).

Numerous other genes are known to be affected by thyroid hormone status; but whether their regulation by thyroid hormone is direct or indirect is not clear. Thyroid hormone appears to regulate the expression of myelin-associated genes, neurotrophins, neurofilament-associated genes, RC3 (neurogranin), MT-3 (metallothionein-3), *reelin*. and thyroid hormone receptor genes (Farsetti et al., 1991; Munoz et al., 1991; Figueiredo et al., 1993; Iniguez et al., 1993; Lebel et al., 1993; Rodriguez-Pena et al., 1993; Piosik et al., 1996; Ibarrola and Rodriguez-Pena, 1997; Pombo et al., 1998; Alvarez-Dolado et al., 1999; Gosh et al., 1999; Pombo et al., 1999; Yeiser et al., 1999). The RC 3 gene codes for a

protein kinase C substrate, speculated to be involved in synaptic enhancement, since phosphorylation of protein kinase C substrates have been implicated with this phenomenon (Munoz et al., 1991; Iniguez et al., 1993). The *reelin* gene is necessary for appropriate neuronal migration and lamination during development of the cerebral cortex, hippocampus and cerebellum (Alvarez-Dolado et al., 1999). MT-3 is speculated to act as a neuronal growth inhibitory factor (Yeiser et al., 1999). Thyroid hormone status during early postnatal development has also been found to affect the mRNA abundance of genes found exclusively in Purkinje cells, including ROR alpha (an orphan nuclear hormone receptor) and Purkinje cell protein-2 - Pcp-2 (Zou et al., 1994; Koibuchi et al., 1999). ROR alpha is speculated to augment thyroid hormone receptor-mediated transcriptional activation, whereas the function of Pcp-2 is unknown (Zou et al., 1994; Koibuchi et al., 1999).

Normal Development of the Rodent Cerebellum

Organization of the cerebellar cortex

The cerebellar cortex consists of five neuronal phenotypes; Purkinje, basket, Golgi II, stellate and granule neurons (Figure 3). These cells are arranged in layers- in the following order from the pial (outer) surface: the molecular layer, the Purkinje cell layer, and granule cell layer. The molecular cell layer has a very low cell density. It contains two types of interneurons: basket cells in the inner portion and stellate cells in the outer portions. The dendrites and axons of these interneurons are confined to the molecular layer. The axons of stellate cells make synaptic contacts with Purkinje cells and dendrites. The axons of basket cells wrap around the soma of Purkinje cells, forming a basket-like meshwork. Basket cells and stellate cells are believed to release gamma-aminobutyric acid (GABA) (Batini, 1990).

Purkinje cells form a monocellular layer. Purkinje cells are among the largest GABAergic cells the CNS. Each cell has an elaborate dendritic tree that arises from the neck of the cells as 2-3 large dendrites. The axons of Purkinje cells are myelinated and pass through the granule cell layer and white matter. Cells in the IGL are densely and tightly packed. Cerebellar granule neurons are glutaminergic with 4-5 short dendrites with claw like endings. The axons of granule cells are unmyelinated and ascend vertically into the molecular layer where they bifurcate into two branches known as the parallel fibers. The parallel fibers are found throughout the molecular layer, synapsing on dendrites of Purkinje cells, stellate, basket, and Golgi II cells. Golgi II cells are also GABAergic neurons that are found mainly in upper parts of the granule cell layer. The axon terminals of these cells synapse with granule cell dendrites.

Development of the cerebellum

The cerebellum is derived from the alar plate of the neural tube. Neurons of the cerebellum arise from two different germinal zones; the neuroepithelial ventricular zone and the caudal germinal trigone, also known as the rhombic lip (Adler et al., 1996). The ventricular epithelium zone gives rise to the deep nuclear cells, Purkinje, basket, stellate and Golgi II cells (Hallonet et al., 1990; Hallonet and Alvarado-Mallart, 1997). In mice, the birth and exit of nulclear neurons and Purkinje cells from the ventricular zone occurs between E10 and E13. Purkinje cells and nuclear neurons stop dividing by E13-E14. The formation of the secondary germinal matrix-the external granule cell layer (EGL) in mice-occurs at about this time when nuclear and Purkinje cells have stopped dividing (Miale and Sidman, 1961). The initial migration of presumptive granule cells from the rhombic lip is about E13 in the mouse and the EGL is observed as early as E 14-15. These stem cells migrate over the surface of the cerebellum from the rostral

part of the rhombic lip and continue to proliferate until the entire surface of the cerebellum is covered (Miale and Sidman, 1961). The EGL is thin on P1 and is mostly composed of densely packed proliferating cells. The EGL reaches its maximal thickness between P5 and P10 in the rat (Altman, 1972; Altman and Bayer, 1978). This increase in thickness is partly due to the expansion of the inner zone (where postmitotic cells are found), also known as the subproliferative or premigratory zone). Migration of granule neurons out of the EGL towards the internal granule cell layer (IGL), their final destination, begins around P3 in mice (Fujita, 1967; Fujita et al., 1967). Before they migrate, however, the granule cells extend axonal processes. These eventually become the parallel fibers that make synaptic connections with Purkinje cell dendrites. Additionally, a recent study (chick cerebellum) shows that before postmitotic granule cells migrate out of the premigratory zone of the EGL they migrate medially and laterally (Ryder and Cepko, 1994). Processes extending from the granule cell soma associate with the radial fibers of the Bergmann glia (Rakic, 1971; Altman and Bayer, 1997). At this point, granule cells begin to migrate along these fibers toward the IGL. During this process the Purkinje cell dendrites begin to extend and branch extensively into the molecular layer and finally obtain the highly dendritic trees that reach the pial surface of the cerebellum (Altman, 1972; Altman and Bayer, 1997). By P24, the EGL has virtually disappeared in the rat (Altman, 1972). The Purkinje cell layer is reduced in thickness between P1 and P10 because it is transformed from a multicellular sheet to a monocellular sheet of Purkinje cells (Altman, 1972). The molecular layer is thin on P1 but increases in thickness on the days that follow due to the increase in parallel fibers, dendritic outgrowth of Purkinje cells and production and differentiation of basket and stellate cells (Altman, 1972). Peak production and differentiation of basket cells occur between P6-P11 and peak

production and differentiation of stellate cells occurs between P8-P30 in rats (Altman and Bayer, 1978).

Influence of Thyroid Hormone on Cerebellar Development, In Vivo and In Vitro Studies

In vivo studies

Thyroid hormone receptor subtypes are found during development of different cell types in the rat cerebellum, supporting the role for thyroid hormone in regulating gene expression in the developing cerebellar cortex. Classic studies on the effects of thyroid hormone on development of the cerebellum of the rat have focused largely on the EGL and Purkinje cell layer (Nicholson and Altman, 1972; Nicholson and Altman, 1972; Lauder, 1977; Patel et al., 1979; Rabie et al., 1979). Briefly, the onset of thyroid gland function (production and secretion of thyroid hormone) in rats is around embryonic day 17 (E17) (Fisher et al., 1977) this is when the germinal cells that eventually make up the EGL begin to migrate. Serum T_4 (thyroxine) levels in the mouse are low at birth but then begin to rise around P6 to reach peak levels between P14 and P15, and decline to adult levels thereafter. This period overlaps with the peak of presumptive granule cell proliferation, postmitotic granule cell migration, Purkinje cell dendritic outgrowth, and synapse formation.

It is hypothesized that thyroid hormone is the signal that terminates proliferation and stimulates differentiation (Hamburg et al., 1971). In support of this hypothesis it is reported that in altered thyroid status the above time course is changed (Nicholson and Altman, 1972; Lauder, 1977; Patel et al., 1979; Rabie et al., 1979). These reports, based on ^{3}H-thymidine incorporation (an indicator of DNA synthesis), show that hypothyroidism decreases the rate of cell proliferation, delaying the disappearance of the EGL from P24 to P30.

Conversely, hyperthyroidism accelerates the disappearance of the EGL from P24 to P21. Exposure to hyperthyroid levels of T_4 increases the rate of cellular proliferation in the cerebellum (Lauder, 1977). This apparently results from shortening of the G1 phase of mitosis, producing a phasic increase in total cell number, ultimately resulting in the premature termination of cell proliferation (Lauder, 1977).

Thyroid hormone may also play a role in the rate of migration of differentiating cells out of the EGL. In hypothyroid rats the rate of migration of granule cells and the outgrowth of parallel fibers are slowed, resulting in a permanent deficit in their length (Lauder, 1977; Lauder, 1979). Delayed migration of granule cells is suggested to be related to retarded differentiation of Bergmann glia, which are required for granule cell migration (Clos et al., 1973; Clos et al., 1980). Additionally, death of granule cells in the IGL is suggested to result from the failure of their axons to make adequate connection with Purkinje cells.

The number of Purkinje cells is not affected in hypothyroidism, but maturation is impaired, as reflected by their abnormal arborization and decrease in dendritic spines. This may be because hypothyroidism delays the disappearance of the EGL and decreases the number and density of synaptic contacts with already defective Purkinje cells. Consequently, a permanent impairment of neuronal connectivity and signaling occurs (Legrand, 1979).

In vitro studies

In vitro systems enable the removal of cells from their complex environment and the manipulation of molecular and cellular events under controlled conditions. The disadvantage of these systems is that they are non-physiological. Developmental events are initiated by genomic events that depend in turn on epigenetic influences in vivo. Thus, genes influence the developmental

environment - and the developmental environment influences genes. Moreover, temporal development within the CNS is heterogeneous. It is well established that the response of neurons and glia to exogenous signals, such as T_3 or growth factors, are dependent on the brain structure the cells are obtained from, developmental stage, and culture conditions (Hatten et al., 1988; Gao et al., 1991; Engele and Bohn, 1992; Segal et al., 1992; Andres-Barquin et al., 1994). For example, granule cells of the cerebellum change their responsiveness to neurotrophins as a function of developmental stage (Segal et al., 1992). Also, thyroid hormone treatment causes a decrease in glial fibrillary acidic protein (GFAP) and its mRNA in cultures of astroglia derived from postanal rats but not adult rats (Andres-Barquin et al., 1994). Conversely, in vivo, GFAP mRNA concentrations were shown to be higher than normal in postnatal cerebella of hypothyroid rats (Faivre-Sarrailh et al., 1991).

The influence of thyroid hormone on astroglial morphology, differentiation, and proliferation has been investigated in several in vitro studies. T_3 treatment induces morphological changes in primary cultured astrocytes from cerebral hemispheres, and proliferation in astrocytes from the cerebellum (Gavaret et al., 1991; Trentin et al., 1995; Paul et al., 1996; Lima et al., 1997; Trentin, 1997; Lima et al., 1998). Thyroid hormone is also suggested to participate in the development of oligodendrocytes, the myelin producing cells of the CNS, since myelin synthesis is known affected by thyroid hormone status (Wysocki and Segal, 1972; Bhat et al., 1979; Shanker et al., 1984; Shanker et al., 1985; Yusta et al., 1988; Farsetti et al., 1991; Rodriguez-Pena et al., 1993; Ibarrola and Rodriguez-Pena, 1997; Pombo et al., 1998; Pombo et al., 1999). In support of this hypothesis, thyroid hormone receptor isoforms are expressed in both mature oligodendrocytes and their bipotential progenitor O-2A cells in vitro (Yusta et al., 1988; Baas et al., 1998; Carre et al., 1998). In cultures of dissociated brain cells

from embryonic mice, thyroid hormone appears to regulate the synthesis of myelin constituent glycolipids and enzymes related to myelination, including myelin basic protein methyltransferase, sialyltransferase, and 5'-nucleotidase (Shanker and Pieringer, 1983; Amur et al., 1984; Shanker et al., 1984; Shanker et al., 1985; Pombo et al., 1998).

The timing of oligodendrocyte differentiation is believed to depend on an intrinsic program in the progenitor cells and environmental factors. When purified O-2A cells are cultured in serum-free medium in the presence of mitogens, proliferation increases and differentiation is delayed. When T_3 is added to these cultures, proliferation decreases and differentiation into oligodendrocytes increases (Barres et al., 1994). Nevertheless, if O-2A cells are cultured without mitogens, they stop dividing and differentiate prematurely into oligodendrocytes without T_4 indicating that this hormone is not required for differentiation (Barres et al., 1994). Therefore, it hypothesized that the role of thyroid hormone is to control the timing of the proliferative phase of O-2A progenitors in the developing brain (Barres et al., 1994).

A number of <u>in vitro</u> studies have investigated the role of thyroid hormone on granule cell development, but their results have disagreed regarding whether granule cells are a direct target for T_3 action (Balazs et al., 1985; Messer et al., 1985; Heisenberg et al., 1992). These studies used purified neuronal cultures plated at similar densities in serum free medium and treated with similar concentrations of T_3. Perhaps the variable factor among them was the schedule of T_3 treatment; a significant increase in granule cell survival and differentiation was observed only in cultures treated with a single dose of T_3 (Heisenberg et al., 1992).

Thyroid hormone has been shown to increase the expression of neurotrophin -3 (NT-3), nerve growth factor (NGF), and bcl-2 (a proto-oncogene

known to prevent cell death) in cultured granule cells (Charrasse et al., 1992; Lindholm et al., 1993; Muller et al., 1995). Additionally, conditioned medium obtained from T_3 treated astrocytes is reported to increase proliferation of granule cell neuroblasts, indicating that T_3 stimulates astrocytes to secrete factors that influence proliferation. Using neutralizing antibodies two of these factors have been identified to be transforming neurotrophic factor- beta (TNF-β) and EGF (Gomes et al., 1999).

Thyroid Hormone and Growth Factors in the Developing CNS

The possible interaction between thyroid hormones and growth factors in brain development and maintenance are implied by several in vivo and in vitro studies. Because most of these studies focused on the neurotrophin family, a brief review is provided below.

Neurotrophins

The neurotrophins are structurally related molecules that regulate developmental processes of numerous populations of neurons. These include cell survival, axon arborization, synaptic transmission, plasticity, and synaptogenesis (Cohen-Corey et al., 1991; Segal et al., 1992; Gao et al., 1995; Levine et al., 1995; Kang and Schuman, 1996; Neveu and Arenas, 1996; Zirrgiebel and Lindholm, 1996; Morrison and Mason, 1998; Shimada et al., 1998). Neurotrophins bind to a low affinity receptor (p75) and to a family of high affinity receptors with tyrosine kinase activities (trk A, B, and C). The neurotrophin family includes nerve growth factor brain derived neurotrophic factor (BDNF), NT-3, and neurotrophin 4/5 (NT-4/5) (Hallbrook et al., 1991). The ligand-receptor interaction in target neurons is highly specific among the neurotrophins; NGF binds trk A, BDNF and NT-4/5 bind trk B, and NT-3 binds trk C.

Thyroid hormone status is known to affect NGF and its low affinity receptor mRNA and immunoreactivity in the developing and adult brain of rats (Charrasse et al., 1992; Giordano et al., 1992; Figueiredo et al., 1993; Figueiredo et al., 1993; Calza et al., 1996; Neveu and Arenas, 1996; Calza et al., 1997). Also, the effects of thyroid hormone on NGF expression appear to be age-dependent. For example, NGF immunoreactivity in the cerebellum of hyperthyroid rats was reported to be significantly higher only at P2, whereas in hypothyroid rats NGF values were similar to age-matched controls (Figueiredo et al., 1993). Hypothyroidism, however, in adult rats lead to an increase in both NGF and p75 NGFR (low affinity nerve growth factor receptor) in the basal forebrain (Calza et al., 1997).

Since neurotrophins and their receptors are highly expressed throughout the development of the cerebellum, it is speculated that these polypeptides may mediate some of the effects of thyroid hormone on this brain structure. Early hypothyroidism has been shown to disrupt the developmental pattern of expression of the neurotrophins, leading to relatively higher levels of NGF, and NT 4/5 mRNAs and to a deficit in NT-3 and BDNF mRNAs in the cerebellum (Neveu and Arenas, 1996). When hypothyroid rats were grafted with cell lines expressing high levels of NT-3 or BDNF, cell death was attenuated in the IGL (Neveu and Arenas, 1996). Thyroid hormone administration also increased NT-3 mRNA levels in the cerebellum and in cerebellar granule neurons in vitro. When NT-3 was added to cerebellar cultures, neurite outgrowth was induced in Purkinje cells, which express trk C receptors, suggesting that T_3 may affect the development of these cells by regulating the expression of NT-3 in granule cells (Lindholm et al., 1993). Moreover, postnatal animals treated with anti-NGF antibodies exhibit the same alterations in the cerebellum seen in hypothyroid

animals, including a delay in the disappearance of the EGL, reduction in the size of the Purkinje cell bodies and dendritic trees (Legrand and Clos, 1991).

EGF family

Although the epidermal growth factor family and its receptor are widely expressed in the CNS, including the cerebellum, studies of the possible interaction between thyroid hormone and this family of growth factors have focused largely on cells outside the CNS. Thyroid hormone has been shown to regulate the mRNA expression and immunoreactivity of EGF, TGF-α, and EGFR in somatic tissue and breast carcinoma A 431 cells in culture and (Gresik, 1981; Kesavan et al., 1991; Hume et al., 1992; North et al., 1992; Fujieda, 1993; Rogers et al., 1996). In the CNS, however, the effects of thyroid hormone on these polypeptides are limited to EGF mRNA expression and receptor binding activity in whole brain homogenates (Sadiq et al., 1985; Stein et al., 1989). Recently, thyroid hormone administration has been shown to increase TGF-α mRNA levels in the striatum of mice (Blum et al., 1999).

Epidermal growth factor and TGF–α are members of the epidermal growth factor family, and bind to the EGFR with similar or equal affinity (Massague, 1983; Ebner and Derynck, 1991; French et al., 1995). The mature or secreted form of EGF is a 53 amino acid polypeptide chain with a molecular weight (MW) of 6,053 or 6 kilodaltons- kDa (Carpenter and Cohen, 1979; Carpenter and Cohen, 1990). This polypeptide is generated from preproEGF, a precursor protein composed of 1217 amino acids a molecular weight of 128-133 kDa, (Lee et al., 1995). The mature form of EGF is released from the membrane-anchored precursor by proteolytic cleavage. The secreted form of TGF-α consists of 50 amino acids (MW 6 kDa) and is generated from proTGF–α, a transmembrane protein of 159-160 amino acids. The mature TGF–α is released

by proteolytic cleavage at the cell surface (Lee et al., 1995). Cleavage of proTGF-α is often incomplete, however, giving rise to the secretion of larger peptides as well as the accumulation of proTGF-α at the cell surface (Lee et al., 1995). Mature TGF–α shares 30-35% amino acid sequence with EGF, including the conservation of the 6 cysteine residues important for disulfide bond formation and its tertiary structure that renders it a ligand for the EGFR (Derynck, 1988). Both EGF and TGF-a exert their biological activity upon binding to EGFR. The EGF receptor family includes EGFR, erbB 2, erbB3, and erbB4. They are tyrosine kinase receptors and products of the cellular proto-oncogene c-erbB (Thompson and Gill, 1985; Martin, 1986). EGFR is a 170 kDa transmembrane glycoprotein composed of a protein core of 1,186 amino acids (MW kDa) and N-linked carbohydrates (MW 40 kDA) which are essential for EGF binding (Carpenter, 1984; Schlessinger, 1986). The binding of a ligand to the receptor induces the activation of the protein tyrosine kinase, which phosphorylates various cellular proteins as well as the EGF receptor itself (Schlessinger, 1986). In addition to TGF-α and EGF, EGFR binds and is activated by, amphiregulin, heparin-binding EGF (HB-EGF), betacellulin and epiregulin (Shoyab et al., 1989; Higashiyama et al., 1991; Shing et al., 1993). The specificity of the cellular response is thought to be determined by the nature of the various signaling molecules recruited to the phosphorylated receptor. Dimerization of EGFR can occur between two identical receptors (homodimerization) or with any of the three members of the erbB family of oncogenes (heterodimerization), depending on which receptor proteins are present in a given cell (Spivak-Kroizman et al., 1992; Sibilia et al., 1998; Wells, 1999). This increases the number of signaling pathways that can be activated after EGFR stimulation, thereby augmenting the signaling complexity and function of this receptor (Spivak-Kroizman et al., 1992; Sibilia et al., 1998; Wells, 1999).

Localization and function of EGF : <u>In vivo</u> and <u>in vitro</u>

In the mouse brain, EGF mRNA is expressed throughout development (Lazar, 1991; Lazar and Blum, 1992). The concentration of EGF mRNA in the CNS remains relatively constant until after birth, and decreases thereafter to reach normal adult levels by P10 (Lazar, 1991; Lazar and Blum, 1992). EGF mRNA has been detected in the brain stem cerebral cortex, olfactory bulb, basal hypothalamus, striatum, thalamus and cerebellum (Lazar, 1991; Lazar and Blum, 1992). Studies in culture have shown that EGF is a potent mitogen for glia derived from the cerebellum, cerebral cortex, mesencephalon, bipotential neuronal/astroglial and unipotential neuronal progenitors derived from developing and adult rodents (Leutz and Schachner, 1981; Honegger and Guentert-Lauber, 1983; Huff et al., 1990; Casper et al., 1991). Epidermal growth factor is reported to increase differentiation and survival of mesencephalic dopaminergic neurons and cerebellar granule cells (Abe et al., 1991; Casper et al., 1991; Abe and Saito, 1992; Casper et al., 1994).

Localization and function of TGF-α: In vivo and in vitro

TGF-α is ubiqitous throughout embryonic and postnatal development in the CNS of rodents (Fallon et al., 1984; Lazar and Blum, 1992; Seroogy et al., 1994; Seroogy et al., 1995; Kornblum et al., 1997). Additionally, it is developmentally regulated in the CNS (Lazar, 1991; Lazar and Blum, 1992; Ma et al., 1992). TGF-α mRNA levels rise steadily from embryonic day 14, peak during the first postnatal week and then fall to adult levels by P10 (Lazar, 1991; Weickert and Blum, 1995). TGF-α mRNA has been localized by in situ hybridization in neurons and glia across numerous forebrain regions, including the hippocampus and hypothalamus (Seroogy et al., 1993). Moreover, TGF-α mRNA synthesis has been shown to increase in motor neurons of mice in response to injury (Lisovoski et al., 1997).

In culture, TGF-α is a mitogen for bipotential neuronal and glial precursors cells derived from the embryonic retina, striatum and mesencephalon (Anchan et al., 1991; Reynolds et al., 1992; Santa-Olalla and Covarrubias, 1995). Furthermore, it can induce astroglial proliferation in mesencephalic cultures. TGF-α also appears to affect neuronal cell expression, enhancing dopamine uptake, survival and neurite length of embryonic dopaminergic neurons (Casper et al., 1991; Alexi and Hefti, 1993). A recent study also showed that TGF–α partially protects against phenotypic degeneration of a subpopulation of striatal cells after lesioning with quinolinic acid (Alexi et al., 1997).

In vivo, TGF-α is implicated in mitogenesis in the subventricular zone, since the ventricular zones and mitotic index during development are significantly reduced in TGF–α deficient mice (Weickert and Blum, 1995). It also appears to regulate hypothalamic LHRH neuronal secretion in female mammals, particularly during puberty (Ma et al., 1992). Furthermore, TGF-α knockout mice contain 50% fewer dopaminergic neurons in the substantia nigra, indicating that this factor is needed for proliferation and/or differentiation (Blum, 1998).

Localization and function of EGFR in the CNS

EGFR is widely distributed in the CNS, localized to both neurons and astrocytes. In the human and rodent nervous system, EGFR immunoreactivity (EGFR-IR) has been detected during embryonic development through late age in many types of cells, including cerebral cortical pyramidal cells, hippocampal pyramidal cells, Purkinje cells, anterior horn cells, and dorsal root ganglion cells (Gomez-Pinilla et al., 1988; Werner et al., 1988; Birecree et al., 1991). In the embryonic CNS of rats, EGFR mRNA is expressed in germinal zones during late stages of neurogenesis and early stages of gliogenesis (Kornblum et al., 1997; Misumi and Kawano, 1998). In the postnatal brain of rats EGFR mRNA is present

in the subventricular zones of the neostriatum, which primarily produces glia and the EGL of the cerebellum, which produces neurons exclusively (Seroogy et al., 1995).

Recently, EGFR null mice have been generated to assess the function of EGFR in the developing brain. Mice lacking EGFR exhibit strain-dependent phenotypes ranging from placental to postnatal skin, lung and brain defects (Threadgill et al., 1995). Nonetheless, all mutant mice have been found have massive degeneration (partly due to apoptosis) of the frontal cortex, olfactory bulb and thalamus during the second postnatal week. In addition, these mice show a delay in GFAP expression (Kornblum et al., 1998; Sibilia et al., 1998). Apparently, EGFR is critical for the survival of postmitotic neurons in the forebrain and normal development of astrocytes.

FGF family

The fibroblast growth factor is composed of nine members (Unsicker et al., 1992). The family members most abundantly distributed and well characterized in the CNS are acidic and basic FGF (aFGF and bFGF) (Unsicker et al., 1992). Both are synthesized and cleaved from protein precursors. Multiple weight forms of bFGF have been identified in the CNS of rats and humans, ranging from 18 kDa to 22 kDa (Thomas, 1993). The expressions of different molecular weight forms of the protein are developmentally regulated in different brain regions (Giordano et al., 1992). For example, in the embryonic cerebellum the 18-21 kDa forms predominate, whereas, during postnatal maturation the 18 kDa form increases and the 21 kDa form decreases (Giordano et al., 1992). It has been suggested that the variation of weight forms may represent a functional difference for these proteins that may account for the varied reported effects of bFGF in the CNS (Giordano et al., 1992). In addition, bFGF is internalized by

neurons and astrocytes and processed into three peptides of different molecular weights (15.5 kD, 9kD, 4 kD) molecular weights (Walicke and Baird, 1991). Basic FGF internalizes into vesicles in the cytoplasm, localizes to the perinuclear cytoplasm, and is translocated to the nucleus, suggeting they function as intranuclear as well as intercellular signals -through cell surface receptors (Walicke and Baird, 1991). Thus, the actions of bFGF can be indirect for if bFGF isoforms are located in the cell nucleus it can affect a program of gene expression that might induce the expression of other growth factors and /or their receptors (Giordano et al., 1992).

FGF receptors

The FGF receptors are transmembrane proteins that belong to the family of tyrosine kinases and consists of four members (FGFR-1, FGFR-2, FGFR-3 and FGFR-4) (Wanaka et al., 1991). FGF receptor mRNAs, except for FGFR-4, are expressed widely but differentially in the adult rat brain. For example, FGFR-1 is preferentially expressed in neurons, whereas FGFR-2 and FGFR-3 are preferentially expressed in glia (El-Husseini et al., 1994). Expression of all FGFR mRNAs, except FGFR-4, have been shown to be expressed at high levels during development. FGFR-4 is expressed in the embryonic brain and binds only FGF-6 (Miyake et al., 1995; Ozawa et al., 1996). FGFR-4 is also temporarily expressed in the developing cerebellum between P7 and P15, primarily in the EGL, suggesting an important role for EGF-6 and its receptor in proliferation, differentiation and/or migration of granule cells (Miyake et al., 1995).

bFGF function

In vitro, bFGF functions as a neurotrophic, mitotic or differential factor depending on the biological, spatial and temporal context in which it is placed. For example, bFGF has been shown to promote survival of cerebellar neurons

cultured from neonatal rats but not fetal or late postnatal rats (Morrison et al., 1988; Matsuda et al., 1990; Abe et al., 1991). Additionally, bFGF is reported to function as a survival, mitogenic and differentiation factor for granule cells depending on the culture conditions (Hatten et al., 1988; Morrison et al., 1988; Abe et al., 1991; Gao et al., 1991; Trenkner et al., 1996; Luo et al., 1997; El Idrissi et al., 1998). In vivo, bFGF and its receptor mRNA have peak expression at P1, coinciding with the beginning of granule cell neurogenesis (Tao et al., 1996; Tao et al., 1997). During the first week of postnatal develpment Purkinje cells express bFGF like immunoreactivity in the apical cytoplasm and proximal dendrites, but by the third week immunoreactivity is detected only in the cytoplasm and nucleus (Matsuda et al., 1992; Matsuda et al., 1994). Peripheral injections of bFGF increases the proportion of mitotic granule cell precursors in the cerebellum, conversely intraventricular treatment with anti-bFGF antibodies decreases DNA synthesis (Tao et al., 1997). Thus, bFGF plays a significant role in neurogenesis of the cerebellum. To date the interaction of thyroid hormone and bFGF, if any, in the cerebellum remains to be investigated.

Rationale for Study and Specific Aims

In summary, the literature indicates that the main mechanism of action of thyroid hormone during brain development, is transcription of target genes through nuclear receptors that are widely distributed throughout embryonic and postnatal development in the CNS of mice and rats. Only a few genes are known to be, directly and indirectly, regulated by thyroid hormone during CNS development. Growth factors are also known to be necessary for the development and survival of neurons and glia. It is hypothesized that some of the developmental effects of thyroid hormone on the CNS are mediated by growth factors. Numerous studies on the effects of thyroid hormone on the

cerebellum have provided significant support for this hypothesis. Thyroid hormone status has been shown to significantly alter the cytoarchitecture and the expression of neurotrophins in developing cerebella of rodents. Studies on the interaction between the EGF family and thyroid hormone, in vivo and in vitro, are limited to cells outside the nervous system, whole brain homogenates, and striatum. Moreover, less is known about the potential interaction between thyroid hormone and members and the FGF family. These growth factors are widely distributed in the developing and the adult CNS, suggesting they play a significant role in development and maintenance of this system.

To reiterate, the main objective of this thesis is to elucidate the interaction between thyroid hormone and members of the EGF and FGF family during developmental events. First, I focused on the effect of thyroid hormone on TGF–α and EGFR gene expression in the developing cerebella of mice in vivo. Epidermal growth factor and TGF-α bind to the EGFR, a tyrosine kinase, with equal or similar affinity (Massague, 1983; Ebner and Derynck, 1991; French et al., 1995). Therefore, I measured TGF-α because the mRNA levels of this factor are 15-170 higher than those of EGF in the CNS (Lazar and Blum, 1992), suggesting that TGF-α may be the major physiological ligand for EGFR in the brain (Seroogy et al., 1991; Lazar and Blum, 1992; Seroogy et al., 1993; Seroogy et al., 1994; Weickert and Blum, 1995).

I chose the cerebellum of the mouse because the majority of cells develop during the first three postnatal weeks; this is also the period when the cerebellum is most sensitive to thyroid hormone (Legrand, 1979). Additionally, the developmental profiles of EGF and TGF–α mRNAs in normal cerebella of the mice have been characterized (Lazar, 1991; Lazar and Blum, 1992). The mRNAs encoding these two homologs are quite different, but the profiles are similar, with levels of expression being highest between P6-P10 (Lazar, 1991;

Lazar and Blum, 1992). This period overlaps with the peak of presumptive granule cell proliferation, postmitotic granule cell migration, and Purkinje cell dendritic outgrowth and synapse formation. Purkinje cells are reported to express EGFR and it has been shown that EGF can enhance the survival and process outgrowth of Purkinje cells in vitro. Thyroid gland function begins approximately at E17 in the rodent, but thyroid hormone levels are baseline until P6; the time when they begin to rise until reaching peak levels at P15 before they decline to adult levels. These data suggest that thyroid hormone may be implicated in the timely expression of EGF, TGF-α and/or EGFR. These growth factors may be involved in the proliferation, differentiation, and/or migration of granule cells, and process outgrowth and synaptogenesis of Purkinje cells.

Since interpretation of studies in vivo are difficult because of the complexity of the environment, I also investigated the effect of thyroid hormone on the expression of EGFR and bFGF in cerebellar mixed cells in vitro to manipulate thyroid hormone under controlled conditions. Cerebellar mixed cell cultures are characterized by the presence of granule neurons, glial cells (predominantly astroglia) and fibroblasts. This system resembles the in vivo environment most closely, regarding neuron -glia interaction, in comparison to pure neuronal or pure glial cultures. The EGFR and bFGF appear to promote a range of developmental functions, depending on the biological, spatial, and temporal context in which they are placed. Since EGF and TGF–α and their receptor are usually associated with proliferation, I hypothesized that one way thyroid hormone might terminate proliferation of presumptive granule cells would be by down regulating TGF-α and/or EGFR gene expression. In support of this hypothesis thyroid hormone has been shown to down-regulate the expression of these factors in tissue outside the CNS. Thus, inhibiting thyroid hormone production should maintain the mRNA levels of these polypeptides

elevated past P10 in vivo. Conversely, adding T_3 to cell cultures should down-regulate the expression of EGFR. Moreover, EGFR expression should be densest in the outer (proliferative zone) segment of the EGL. To test this hypothesis the specific aims of this thesis are as follows:

Specific aim 1. To determine if hypothyroidism affects EGFR and TGF-α gene expression in developing cerebella of mice by measuring mRNA levels using a quantitative nuclease protection assay.

Specific aim 2. To determine if hypothyroidism affects the expression and localization of EGFR in the developing cerebella of mice using histochemistry.

Specific aim 3. To establish an optimal culture system, using cerebellar mixed cells, that is quantitative regarding the effects of thyroid hormone on proliferation, differentiation, and survival of neurons and glia.

Specific aim 4. To determine the effect of thyroid hormone on EGFR and bFGF expression in mixed cerebellar cell cultures using Western blotting and densitometry.

Figure 1: The hypothalamic-pituitary-axis Neurosecretory cells of the paraventricular nucleus of the hypothalamus release thyrotropin releasing hormone (TRH) to the median eminence of the hypothalamus. Pulsatile secretion of TRH from the median eminence results in stimulation of thyroid stimulating hormone (TSH) secretion from anterior pituitary thyrotrophs. TSH stimulates the synthesis and secretion of T_3 and T_4 from the thyroid gland. Circulating T_4 is converted to T_3 in target cells by deiodinases (Williams, 1994).

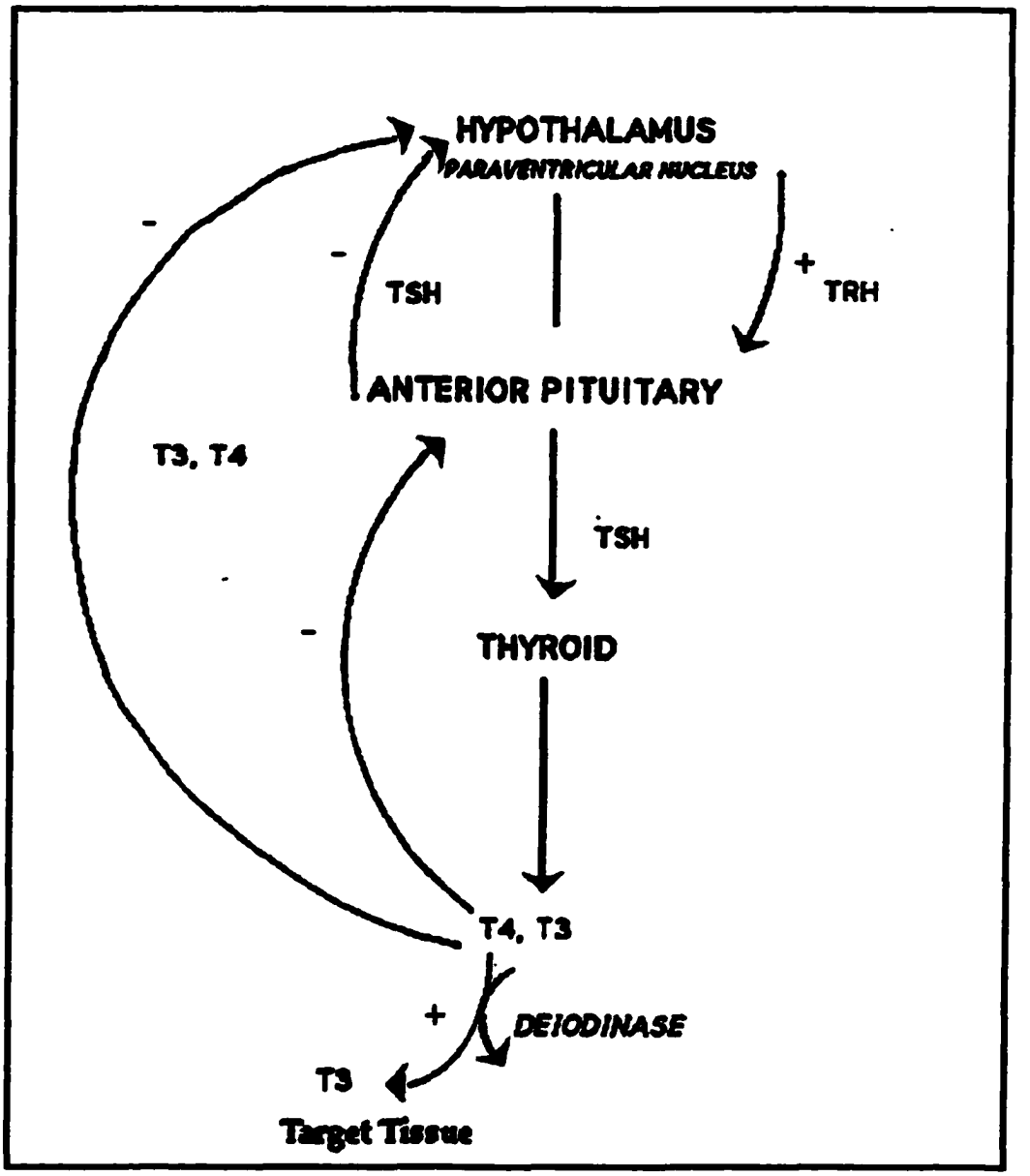


Figure 2: Thyroid hormone's mechanism of action T_4 enters the cell by passive and facilitated diffusion. A specific deiodinase converts T_4 into the more biologically active T_3. Thyroid hormone receptors located on response elements of target genes are activated upon binding of T_3. This leads to an increase or decrease in mRNA synthesis and final protein product (Glass and Holloway, 1990)

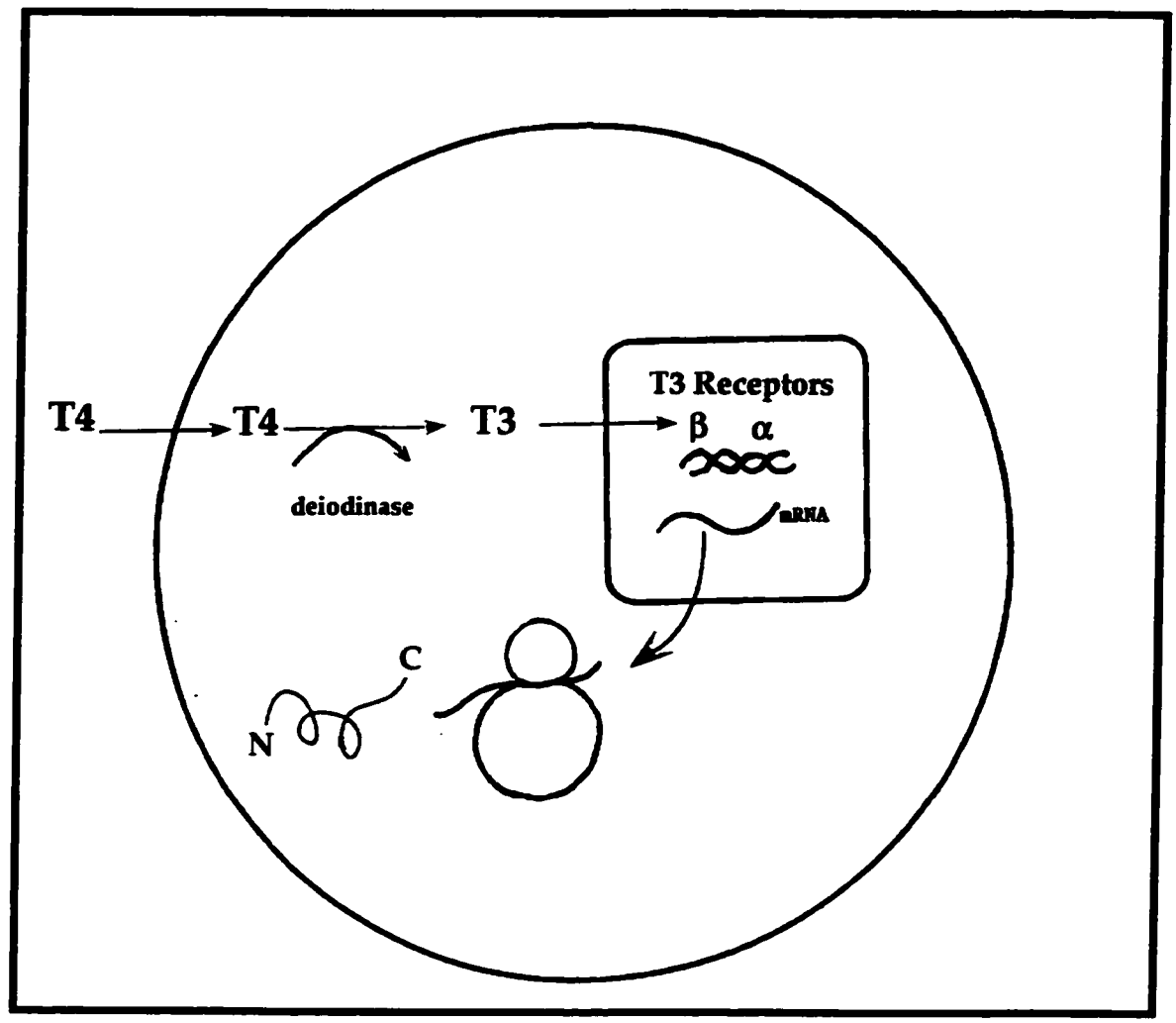
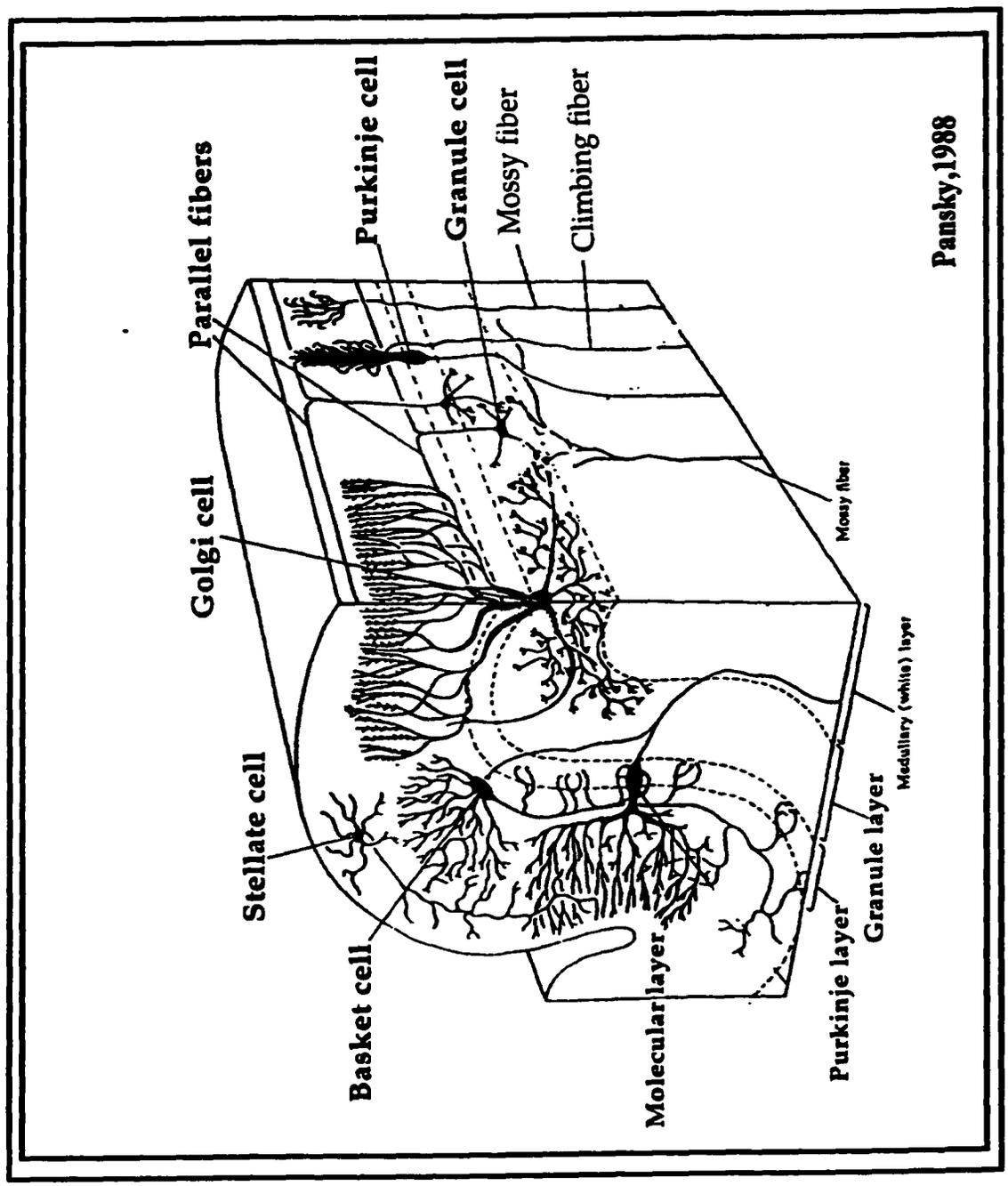


Figure 3: Organization of the cerebellar cortex . The cerebellar cortex consists of three layers that contain five types of neurons: Purkinje cells, Golgi II, granule, basket, and stellate cells. The granule cells, located in the inner most layer of the cerebellum, form synapses with the terminals of Mossy fibers. The molecular layer, the outer layer, consists mostly of parallel fibers (granule cell axons) synapsing on Purkinje cell dendrites. The local interneurons, basket and stellate cells, are also found scattered in the molecular layer (Pansky, 1988).



Chapter 2

Thyroid Hormone Regulates Cerebellar Development: The Roles of the Epidermal Growth Factor Family in Post-mitotic Events *In Vivo*

Abstract

In this study, I investigated the effects of thyroid hormone on cerebellar development and the coincident expression of the epidermal growth factor receptor (EGFR) and transforming growth factor-alpha (TGF-α), a ligand in the epidermal growth factor (EGF) family. Profiles of thyroid hormone expression were measured in postnatal animals and were found to peak at postnatal day 15 (P15). These levels dropped below detectable levels when mice were made hypothyroid with propylthiouracil (PTU). TGF-α and EGFR expression was maximal at P6 in normal animals, but remained low in hypothyroid animals, suggesting that thyroid hormone was responsible for their induction. Immunohistochemical analysis of EGFR protein expression revealed that this receptor was expressed on granule cells within the inner zone, of the external granule cell layer (EGL), where post-mitotic and pre-migratory granule cells are located, suggesting that EGFR-ligands were not inducing granule cell proliferation. The persistence of EGFR expression on migrating granule cells and subsequent down-regulation of expression in the internal granule cell layer (IGL) implicates a role for EGFR-ligands in migration and/or differentiation. In hypothyroid animals, I observed a delayed progression of granule cell migration, consistent with the persistence of EGFR labeling in the EGL, and in the "pile-up" of labeled cells at the interface between the molecular layer and the IGL. Taken together, these results implicate thyroid hormone in the coordinated expression of TGF-α and EGFR, which appear to play a role in post-mitotic developmental events in the cerebellum.

Introduction

Thyroid hormone is essential for normal brain development. Consequently, hypothyroidism during late gestation and early postnatal development results in morphological and biochemical alterations in the brains of experimental animals (Dussault and Ruel, 1987) and in humans can lead to severe mental retardation (Frost, 1986; Delange, 2000). The actions of thyroid hormone are mediated by nuclear receptors that act as ligand-dependent transcription factors (Williams, 1994). Messenger RNA encoding thyroid hormone receptor subtypes have been localized throughout the developing CNS, including the cerebellum (Mellstrom et al., 1991; Bradley et al., 1993). Thyroid hormone levels dramatically alter the rate of proliferation and differentiation of developing granule cells in the EGL of the cerebellum (Nicholson and Altman, 1972). Since many developmental events in the CNS are believed to be mediated by growth factors (see (Fisher et al., 1982)for review), I hypothesized that some of thyroid hormone's effects on cerebellar development may be mediated indirectly by regulating growth factor levels. Outside the CNS, thyroid hormone has been reported to regulate the expression of members of the epidermal growth factor (EGF) family, particularly TGF-α and EGFR, in somatic tissues and in breast carcinoma cells in culture (Kesavan et al., 1991; Fujieda, 1993; Rogers et al., 1995; Rogers et al., 1996). These proteins have been localized to the CNS, where they are developmentally regulated (Lazar, 1991; Ma et al., 1992; Seroogy et al., 1995; Kornblum et al., 1997; Weickert and Blum, 1995). I focused on TGF-α because mRNA levels of this factor are 15-170 times higher in brain than those of EGF itself (Lazar and Blum, 1992). It is therefore believed that TGF-α may be the major physiological ligand for EGFR (Lazar and Blum, 1992; Seroogy et al., 1993). One group has demonstrated that *weaver* mice are hypothyroid, and in these

mice, TGF-α mRNA levels were significantly lower than their wild-type counterparts, moreover, they found that thyroid hormone administration increased TGF-α mRNA expression in the striatum of P26 mice (Blum et al., 1999). Other studies have shown that thyroid hormone regulates EGF mRNA expression and receptor binding activity in whole brain homogenates (Sadiq et al., 1985; Stein et al., 1989).

EGF family members have been shown to induce proliferation of both neuronal and glial precursors in vitro and to be expressed in germinal zones in the developing brain of rats (Leutz and Schachner, 1981; Reynolds et al., 1992; Seroogy et al., 1995). Since the EGL of the postnatal murine cerebellum contains abundant proliferating granule precursor cells and EGFR mRNA has been localized to this germinal zone, I hypothesized that endogenous EGF family members may influence the proliferation and/or differentiation of these mitotic precursors as previously suggested by Seroogy et al., and Kornblum et al., (Seroogy et al., 1995; Kornblum et al., 1997). Moreover, there is also evidence that members of the EGF family may mediate post-mitotic events (Casper et al., 1991; Alexi and Hefti, 1993; Alexi et al., 1997; Kornblum et al., 1997; Kuhn et al., 1997; Weickert et al., 1999). In this study, I examined the effects of thyroid hormone on growth factor expression and developmental events by comparing normal mice with those made hypothyroid with PTU. PTU passes through the milk of lactating mothers and is the most effective way to inhibit production of thyroid hormone in neonatal rodents (Wysocki and Segal, 1972). I focused on the cerebellum, since the majority of granule cell development occurs during the first three postnatal weeks (while pups are nursing) and the effects of thyroid hormone on rodent cerebellar cytoarchitecture have been previously described (Legrand, 1979).

I compared the postnatal developmental profiles of TGF-α and EGFR mRNA expression in normal and hypothyroid mice using a quantitative nuclease protection assay, because differences in mRNA levels reflect differences in the synthetic potential of these proteins. I found that TGF–α and EGFR mRNA expression and immunoreactivity changed dramatically during the early postnatal period, and were significantly, but transiently affected by thyroid hormone deficiency. I also performed EGFR immunohistochemistry to localize this receptor to specific regions and cell types within the cerebellum. Surprisingly, EGFR was expressed in the inner zone (also called the pre-migratory zone) of the EGL where post-mitotic granule cells accumulate, extend processes, and cell bodies begin to migrate toward the IGL. EGFR has been localized to glia in some CNS regions (Nieto-Sampedro et al., 1988; Junier et al., 1993), however, my morphological examination of immunohistochemical studies suggested that granule cells, but not surrounding Bergmann glia or astrocytes, were responsible for EGFR synthesis. Immunohistochemical staining of Bergmann glia with antibodies to glial fibrillary acidic protein (GFAP), present in Bergmann glia, and in developing and mature astrocytes (Bignami and Dahl, 1974; Bignami and Dahl, 1974) was performed, confirming that the pattern of EGFR immunoreactivity was distinct from that of GFAP. Hypothyroid animals demonstrated an appropriate, but delayed progression of EGFR mRNA expression during the first and second postnatal weeks, implicating thyroid hormone's role in the timing of these developmental events.

Overall, my results suggest that thyroid hormone regulates granule cell development by regulating the expression of the EGF-family ligands and receptors, which directly influence post mitotic granule cell differentiation and/or migration.

Materials and Methods

Animals

C57Bl6/J female and agouti CBA male mouse breeding pairs were purchased from Jackson Laboratories (Bar Harbor, ME) and Charles River (Wilmington, MA). Breeding colonies were established and were checked daily for birth of new pups. Approximately equal numbers of male and female pups were used in this study. Animals were cared for and sacrificed in accordance with guidelines established by National Institutes of Health.

Hormone manipulation

Experimental hypothyroidism was induced in developing pups beginning at P1 by treating the lactating mothers with PTU (Sigma, St. Louis, MO) at a concentration of 0.1% (wt/wt) in the food, and 0.001% (wt/vol) in the drinking water. This dose insured 75% survival of nursing pups. As noted before, PTU passes through the milk of lactating mothers and is the most effective way to inhibit production of thyroid hormone in postnatal rats (Wysocki and Segal, 1972).

Tissue and fluid collection

To monitor serum thyroid hormone levels during development of both normal and PTU-treated animals, cardiac blood was collected from 2-3 pups per litter of F1 pups at P6, P10, P12, P13, P14, P15, P18, P22, P38. Mice were decapitated and cardiac blood was quickly collected into microcentrifuge tubes, allowed to clot at 4°C, and centrifuged at 10,000 X g for 20 minutes at 4°C. Brains were removed from the skulls, the cerebella were separated by severing the peduncles, and these were snap-frozen on dry ice for subsequent mRNA analysis.

For immunohistochemical staining experiments, two pups per litter from C57Bl6/J mice were killed by CO_2 asphyxiation at P6, P7, P9, P12, P14, and P22. Whole brains were dissected and fixed in 4% paraformaldehyde in phosphate buffer saline (PBS; 10 mM sodium phosphate, 140 mM sodium chloride, pH 7.4). After fixation, the brains were cryoprotected in 30% sucrose, paraffin embedded, and sagital 5 μm sections were generated using a microtome.

T_4 Radioimmunoassay

Hypothyroidism in experimental animals was confirmed by collecting cardiac blood at day of sacrifice and measuring total thyroxine (T_4) in the serum using a commercially available radioimmunoassay kit (Coat-a-Count, Diagnostics Products Corporation, Los Angeles, Ca.). Total T_4 was measured according to manufacture's recommendations. Briefly, 25 μl aliquots of serum were mixed with Total T_4 antibody-coated tubes and 1.0 ml ^{125}I-T_4 (0.05 μCi/ml). Samples were incubated for 1 hour at 37°C. Subsequently, the supernatant of each sample was aspirated and the antibody-antigen complex was counted in a gamma counter. Total T_4 standards ranged from 0- 24 μg/dL.

Isolation of cytoplasmic RNA

Total cytoplasmic RNA was prepared from dissected cerebella as previously described (Lazar and Blum, 1992). Briefly, tissue was removed from -80°C storage, placed into the barrel of a tuberculin syringe, and thawed for about 1 minute. Tissue was homogenized in ice cold ribonuclease-free lysis buffer (10 mM Tris-HCL, pH 7.4, 1.5 mM $MgCl_2$, 5% Nonidet P-40, 0.25% sodium deoxycholic acid, 0.3 M sucrose). Lysates were layered over a sucrose cushion (10 mM Tris-HCl, pH 7.4, 1.5 mM MgCl2, 0.4 M sucrose) and centrifuged at ~ 5000 x g for 10 minutes at 4°C. Cytoplasmic fractions in the supernatant were recovered

and treated with 0.2 mg/ml proteinase K, 0.03 vol. 5 M NaCl, and 0.1 vol. 10 x SET buffer (1 X SET: 1% SDS, 5 mM EDTA, 10 mM Tris-HCl, pH 8.0) for 1 hour at 45°C. Phenol-chloroform and chloroform extractions were then performed. The cytoplasmic RNA fractions were precipitated at -20° C with 2 vol. 4.5 M sodium acetate and the RNA samples to be assayed were recovered by centrifugation at ~ 16, 000 x g for 20 minutes at 4°C. The pellets were washed with 70% ice-cold ethanol, dried, and resuspended in DEPC-treated water. RNA concentrations were determined by a spectrophotometer using OD 260 readings. Agarose gel electrophoresis was performed to confirm that the RNA was intact and free of DNA. Aliquots of RNA samples were dried and resuspended in 30 μl hybridization buffer (80% formamide, 40 mM PIPES, pH 6.7, 1 mM EDTA, pH 8.0, 0.4 M NaCl), and stored at -80°C until use in RNAse protection assays.

Preparation of DNA templates for in vitro transcription of RNA standards and ^{32}P-labeled antinsense riboprobes

The plasmid pmTGF-α 373, constructed with the Bluescript II/KS (+) vector (Stratagene), contained 373 bp *BAMHI/ AVAI* fragment obtained from the 5' end of a 1400 bp TGF-α genomic *Sau* 3AI sequence, generously provide by Dr. R Derynck (Wilcox and Derynck, 1988). The preparation of DNA templates for in vitro transcription, the in vitro transcription reaction, the purification of the probe and RNA standards were performed as previously described in detail (Blum, 1989; Lazar and Blum 1991).

The plasmid *SpeI / ApaI* mouse EGFR cDNA constructed with Stratagene Bluescript KS (+) vector containing a *Afl* III/*Afl II* 624 bp fragment was generouly provided by Dr. S.A. Orellana (Orellana et al., 1995). A shorter, .235 kd fragment was produced by enzymatically restricting the *Afl III/Afl II* fragment at the 5' end for the antisense riboprobe and at the 3' end for

the sense RNA standards. The in vitro transcription reactions, the purification of the probe and RNA standards were performed as previously described (Blum, 1989; Sambrook et al., 1989; Lazar and Blum, 1992).

TGF-α and EGFR solution hybridization ribonuclease protection assays

Solution hybridization assays were performed as described in detail previously (Sambrook et al., 1989; Lazar and Blum, 1992). RNA standards were prepared with increasing quantities of in vitro synthesized sense-strand RNA. Aliquots of total yeast RNA were added to all tubes of a given assay to equalize the total quantity of RNA. TGF-α standards ranged from 0.312-10 pg and EGFR standards ranged from 0.5-20.0 pg. All standards were in a final volume of 30 μl hybridization buffer (see above). Samples contained 5-20 μg of RNA and were processed in a similar fashion. Samples without RNA sense strand controlled for degradation of the probe itself. Excess radiolabeled riboprobe (1 μl of 100-200 pg/μl) was added to each tube. Samples were heat denatured at 85°C for 10 minutes and transferred to a 45°C water bath for solution hybridization overnight (16-18 hours). Samples were then incubated with 300 μl RNAse digestion mixture (40 μg/ml RNAse A, 2 μg/ml RNAse T1, 10 mM Tris-HCl, pH 7.5, 5 mM EDTA, pH 8.0, 300 mM NaCl) for 1 hour at 37°C, treated with 0.1 mg/ml proteinase K (15 minutes at 37°C in the presence of 1% SDS). The RNA was then extracted with phenol-chloroform and precipitated with ethanol with 15 μg of total yeast RNA added. Pellets containing RNA:RNA hybrids were recovered by centrifugation, washed with ethanol, dried and resuspended in 7 μl gel-loading buffer, and electrophoresed on 4% nondenaturing acrylamide gels. Dried gels were exposed to X-ray film. Protected bands of predicted size were quantified by densitometry using a Molecular Dynamics phosphor-imager. A regression line was generated by plotting the amount of RNA of the standards

against the measured density of the protected bands. Sample RNA concentrations were calculated by interpolation from the regression line and dividing by the total RNA in each sample.

EGFR Immunohistochemistry

Tissue slides were deparaffinized in xyline and rehydrated in progressive ethanol/water solutions. Antigen unmasking was performed with heat treatment by placing slides in a container with 10 mM citrate buffer (pH 6.0), and heating in a microwave oven for 5 minutes twice, each time with fresh buffer. To visualize EGFR, the tissue was incubated with anti- EGFR at 4°C overnight (2 μg/ml, rabbit polyclonal; Santa Cruz, Santa Cruz, CA), HRP anti-rabbit secondary antibody for 3 hours at room temperature (1:200 Amersham, Arlington Heights, IL), and the DAB staining kit (Vector Laboratories, Burlingame, CA). A western immunoblot analysis of cerebellar homogenates was performed, as described in detail in Weickert et al., 2000, to confirm the specificity of the antibody. I detected one major band at 170 kDa (see Figure 3I). Astrocytes were identified by a rabbit polyclonal antibody to GFAP (1:50; Biomeda Corp., Foster City, CA) and visualized as described for EGFR. Sections were counter-stained with toluidine blue (to visualize the nuclei of cells) before mounting. No immunostaining was observed in tissues incubated without primary antibodies (not shown).

Statistical Analysis

Statistical analysis was done using Statview (Abacus Concepts, Berkeley, Ca). A factorial (two- way) ANOVA with a post hoc Fisher *F* test was performed for all analyses. Five to 15 samples per time point were analyzed for the thyroid hormone profile and 3 to 4 samples per time point were collected for

the TGF-α and EGFR mRNA profiles. The EGFR immunoreactivity profile (Figure 5) represents the means of 3-4 fields per sagital sections from two separate experiments (n= 2 per time point). Data are presented as the mean ± standard error (S.E.M ±) of measurement. Significance was set at $p < 0.05$.

Results

Postnatal thyroid hormone profiles of normal and PTU-treated mice

I assessed the levels of total thyroxine (T_4) in the serum of both normal and PTU-treated mice at specific time points from P6 to P38 (Figure 4). In normal mice, notal T_4 levels increased from 0.43 μg/dL at P6 to a maximum of 8.2 μg/dL at P15. After P15 T_4 levels decreased (4.0-5.0 μg/dL) starting at P22 through P38 where they remained through puberty and young adulthood (data not shown). In contrast, serum levels of PTU-treated mice were below the minimum detectable dose of the assay (< 1 μg/dL), indicating that they were hypothyroid.

TGF-α expression in the developing cerebellum of normal and hypothyroid mice

To determine whether experimental manipulation of T_4 levels could alter levels of TGF-α and EGFR mRNA expression in the cerebellum, tissues were collected from normal and hypothyroid mice and mRNA's were quantified by a nuclease protection assay. Figure 5 (panel A) shows the developmental profile of TGF–α mRNA expression in the cerebella of both treatment groups. In normal mice, TGF-α mRNA expression was highest at P6 (0.16 ± 0.031 pg/μg RNA), the earliest time point measured, and decreased more than 50% by P10 (0.07 + 0.015 pg/μg RNA) and P14 (0.07 ± 0.013 pg/μg RNA). In contrast to normal mice, levels of TGF–α mRNA in hypothyroid mice showed an increase,

rather than a decrease over the first two weeks of development. In hypothyroid mice, TGF-α mRNA expression was ten-fold lower than control levels at P6 (0.016 ± 0.003 pg/μg RNA), but increased to normal levels by P10 (0.053 ± 0.033 pg/μg RNA), and P14 (0.12 ± 0.038 pg/μg RNA), and decreased by P22 (0.055 ± 0.0060 pg/μg RNA).

EGFR mRNA expression in the developing cerebellum of normal and hypothyroid mice.

Figure 5 (panel B) shows the developmental profile of EGFR mRNA expression of normal and hypothyroid mice. EGFR mRNA levels of normal mice were highest at P6 (1.0 ± 0.17 pg/μg RNA) followed by a sharp 75% decrease at P10 (0.25 ± 0.012 pg/μg RNA), that leveled off by P14 (0.38 ± 0.017 pg/μg/RNA) and decreased three-fold at P22 (0.16 ± 0.017). In hypothyroid mice, EGFR mRNA levels were 5% and less of control values at P6 (0.05 ±0.012 pg/μg RNA) and P10 (0.023 ± 0.002 pg/μg RNA), but increased to normal levels by P14 (0.32 ±0.089 pg/μg RNA) and P22 (0.13 ± 0.01 pg /μg RNA).

EGFR immunoreactivity (EGFR-IR): developmental expression profiles in the cerebella of normal and hypothyroid mice

The previous experiment suggested that thyroid hormone up-regulated TGF-α and EGFR mRNA levels in the developing cerebellum. Since neurotrophic factors affect both neurons and glia, I wanted to determine the cell types that had the potential to respond directly to EGFR-ligands. In addition, I wanted to determine the possible effects of hypothyroidism on the timing of developmental events in the cerebellum since I demonstrated alterations in the timecourses TGF-α and EGFR expression. Therefore, I performed immunohistochemical staining experiments to determine the regions within the cerebellum where EGFR was

localized during postnatal development in normal mice, and whether the lack of thyroid hormone affected EGFR expression, localization, and/or the kinetics of postnatal development.

Figure 6 illustrates the distribution of EGFR-immunoreactivity (EGFR-IR) in the cerebellum of normal (panels A, C, E, and G) and hypothyroid (panels B, D, F, and H) mice. Panel I shows a western blot with a signal at 170 kDa representing EGFR in cerebellar homogenates and 431 cell lysates where the EGFR gene is amplified. At P6 (panels A and B), the most intense EGFR-IR was localized to the inner segment of the EGL, also known as the pre-migratory zone, of both treatment groups. Additionally, in normal mice (panel A) EGFR-IR was detected in the leading processes of "spindle- shaped" cells next to the pre-migratory zone (panel A inset), suggestive of migrating granule cells (Altman, 1972; Altman and Bayer, 1997). At P6, more of these cells were observed in normal cerebella than in hypothyroid cerebella.

At P9, intense EGFR-IR was observed in the pre-migratory zone of the EGL and the cell bodies of Purkinje cells of both treatment groups (arrows; panels C and D). While in normal mice, EGFR-IR was dense in the IGL region in apposition to the Purkinje cell layer; EGFR-IR was lighter and more sparsely distributed in cerebellar IGL of hypothyroid mice (arrowheads). In normal cerebella, the cells of the inner zone of the EGL were loosely packed, indicative of their recent migration through the molecular layer; but in hypothyroid cerebella, cells of this inner zone were more densely packed. At P9 in normal mice, EGFR-IR was also observed in cells within the molecular layer. These cells may could either be migrating granule cells, or basket cells, known to be located in the molecular layer in the vicinity of Purkinje cells, since there is an overlap of granule cell migration with the peak of production and differentiation of basket cells at this postnatal age.

At P14 (panels E and F), more dense EGFR-IR was observed in the EGL of hypothyroid than in normal cerebella (black arrows). While EGFR-IR was present in the cell bodies (white arrowheads), apices and apical dendrites (dotted arrow) of Purkinje cells in normal animals, only the cell bodies of Purkinje cells were immunopositive in hypothyroid tissue. Moreover, many EGFR-IR cells were found in the molecular layer in proximity to Purkinje cells in normal cerebella (white arrows), but not in hypothyroid tissue. These cells may represent migrating granule cells nearing their destination (the IGL) and/or basket cells. However, in the hypothyroid tissue, there was no immunostaining within the molecular layer at P14. In normal animals, the distribution of EGFR-IR in the IGL was patchy. In contrast, little to no EGFR-IR was observed in hypothyroid cerebella.

At P22 (panels G and H), strong immunoreactivity was present in cell bodies and apical dendrites (white arrowheads and black dotted arrow) of Purkinje cells of both normal and hypothyroid cerebella. There was a decrease in the number EGFR-IR cells in the molecular layer of normal tissue and an increase in the apparent density of EGFR-IR cells in the molecular layer of hypothyroid tissue (white arrows).

The EGL consists of proliferating and postmitotic cells preparing to migrate to the IGL. The localization of EGFR-IR to the internal segment of this layer indicates that this receptor may play a role in post-mitotic pre-migratory events. From the previous experiment, it appeared that in the absence of a thyroid hormone-dependent signal, the pattern of EGFR-IR staining in the EGL was altered. In order to quantify the effect of thyroid hormone on the expression of EGFR in granule cells of the EGL, I calculated the thickness of EGFR-IR labeling at postnatal ages P6, P9, P12, P14, and P22. EGFR-IR was restricted to the inner segment of the EGL, as described above. Percentages of EGFR-IR cells in the EGL

was calculated by counting EGFR-IR profiles in a strip extending from the end of the molecular layer to the pial surface, and dividing by the total number of cell profiles, identified by toluidine blue, in the same strip. Counts from adjacent sections (n= 2-3) and fields (n= 4-5) and two separate experiments contributed to the mean (Figure 7). Results demonstrate that at P6, the percentage of immunoreactive profiles in the EGL was significantly lower in hypothyroid tissue than in normal tissue ($p < .02$). No significant differences in the percentage of EGFR-IR profiles in the EGL between normal and hypothyroid animals were observed at P9 and P12, but at P14, the situation reversed, and the percentage of EGFR-IR in the EGL of hypothyroid cerebella was significantly greater than that of normal animals ($p < .05$).

EGFR immunoreactivity does not co-localize with GFAP expression in normal P7 mouse cerebellum

EGF receptors are expressed on various neuronal subtypes (Gomez-Pinilla et al., 1988; Werner et al., 1988; Birecree et al., 1991), but EGF-family ligands are also well known to be mitogenic for astrocytes (Leutz and Schachner, 1981; Honegger and Guentert-Lauber, 1983; Huff et al., 1990; Casper et al., 1991). In order to determine whether the pattern of EGFR-IR represented neuronal or glial expression, I performed immunohistochemistry on tissue sections for glial fibrillary protein, an astrocyte-specific marker (Bignami and Dahl, 1974), and EGFR immunohistochemistry on adjacent sections. Figure 8 illustrates the distribution of both antigens within cerebella of P7 mice. GFAP-IR was localized to Bergmann glia, extending from the pial surface through the EGL in evenly spaced, winding fibers (white arrowheads; panel A), becoming more uniformly and diffusely distributed in the molecular layer (black arrowheads). In contrast, EGFR-IR was more cellular (black arrows; panel B), staining rounded cell bodies

within the internal segment, as described and illustrated earlier (Figure 6). This non-overlapping distribution suggests that EGFR-IR is localized to developing granule cells.

Discussion

The study presented here was initiated to investigate the role of thyroid hormone, and the lack thereof, on the expression of EGFR and its ligand, TGF-α, in the developing cerebellum of the mouse. Thyroid hormone is known have significant effects on the developing cerebellum of rats. Studies have demonstrated that hypothyroidism delays the disappearance of the EGL by extending the proliferation phase of presumptive granule cells and slowing the migration of granule cells towards the IGL (Nicholson and Altman, 1972; Lauder, 1979). Furthermore, parallel fiber outgrowth is retarded, resulting in a permanent deficit in their length (Lauder, 1978). The resulting granule cell death seen in hypothyroidism is believed to result from the failure of parallel fibers to make connections with Purkinje cell dendritic spines that may offer trophic support (Lauder, 1978). This failure, in turn, delays the differentiation and maturation of Purkinje cells (Rabie et al., 1979).

Thyroid hormone's effects are largely believed to be mediated by thyroid hormone receptors which are nuclear binding proteins acting as positive and negative regulators of gene transcription. Peak expression of thyroid hormone receptor (TR) -α1 is reported during the first three postnatal weeks in the cerebellum, coinciding with the postnatal increase in serum thyroxine levels (Mellstrom et al., 1991; Bradley et al., 1993); additionally, TR-β2 has been localized in Purkinje cells (Strait et al., 1991).

TGF–α and EGFR are reported to be present and highly regulated in germinal and differentiating fields throughout embryonic and postnatal rodent

CNS development, suggesting that they play important roles, and may do so in a time- and region-specific manner (Gomez-Pinilla et al., 1988; Seroogy et al., 1993; Seroogy et al., 1995; Kornblum et al., 1997). It has been reported that *weaver* mice were hypothyroid and had low levels of striatal TGF-α mRNA and thyroid hormone administration to P26 mice up-regulated TGF-α expression (Blum et al., 1999). My current study suggests that thyroid hormone regulates the expression of TGF-α and the expression and distribution of EGFR in the cerebellum.

Potential function of thyroid hormone in the regulation of TGF-α and EGFR gene expression

Granule cells in the EGL reach their peak of proliferation and migration between P5 and P10 in mice (Miale and Sidman, 1961; Fujita, 1967). This time frame coincides with; 1) the period when serum thyroxine levels begin rise (Figure 4) and 2) the period when TGF-α and EGFR mRNA levels are highest (Figure 5). These profiles suggest that thyroid hormone may initially stimulate EGFR and TGF-α expression, but does not maintain these levels. This is supported by the observation that in hypothyroid animals, these mRNAs do not achieve the high levels seen at P6-P10, but are seen to increase at P14. Thus, it is possible that at low concentrations, thyroid hormone induces TGF-α and EGFR, but at high concentrations, it decreases their expression. Alternatively, the presence of other factors may contribute to the down-regulation. Although in my experiments, serum thyroid hormone levels dropped below levels of detection in PTU-treated animals at all time points measured, the delayed but increased expression of TGF-α and EGFR mRNAs suggests that other factors are also responsible for their regulation. Since the nuclease protection assay measures only steady state mRNA levels, it is possible that these alterations are achieved at the transcriptional or post-transcriptional levels. The mechanism of

action of thyroid hormone on TGF-α, and EGFR gene expression remains to be determined. It is safe to speculate that thyroid hormone's mechanism of action is at the transcriptional level given that a segment of the TGF-a gene promoter has been found to be responsive, in a dose dependent manner, to thyroid hormone (Raja et al., 1991). Additionally, binding sites for thyroid hormone receptors have been found in the EGFR gene promoter (Thompson et al., 1992; Xu et al., 1993).

Potential role of EGF-family ligands in the EGL

The induction and subsequent regulation of EGF-family members during the cerebellum's active phase of development suggested potential roles in the proliferation, differentiation, and/or migration of granule cells. In order to determine the target of EGF-family-mediated effects, immunohistochemical studies were performed on cerebellar tissue sections from normal and hypothyroid mice during postnatal development. I found that EGFR-IR (Figure 6) largely paralleled EGFR and TGF-α mRNA levels as determined by nuclease protection assays (Figure 5 B). Moreover, EGFR protein expression was coincident with the neuroanatomical patterns of post-mitotic granule cells in both the normal and hypothyroid states (Figure 6). EGFR immunoreactivity was co-localized to the EGL's inner zone at P6. At later time points, P14 (Figure 6 C and D) and P22 (6 Figure G and H) staining diminished in the EGL, but persisted in granule cells migrating towards the IGL. The localization of EGFR protein within these cells implicates that granule cells are targets for EGF-family ligands in vivo. Moreover, their localization to granule cells in the pre-migratory, migratory, and post-migratory zones (inner EGL, molecular layer, and IGL, respectively) suggests that granule cells respond to their ligands after they have ceased to proliferate. If EGFR's were involved in the proliferation of granule cells, then EGFR-IR would have been located in the proliferative zone of the EGL.

Given that hypothyroidism has been reported to extend the proliferation phase in the EGL (Nicholson and Altman, 1972), I might have expected that EGFR would be expressed on a higher percentage of cells in the EGL. In contrast, my results demonstrate that the percentage of immunoreactive cells in the EGL of hypothyroid mice was even lower than that in normal mice at P6-P12 (Figure 7), when the peak of granule cell proliferation has been reported to occur (Fujita, 1967; Fujita et al., 1967). Peak expression of EGFR-IR (expressed as the percentage of EGFR-IR cells) in the EGL of hypothyroid mice occurred at P14 (when the EGL is significantly thinner), in contrast to P6- P12 in normal cerebella, suggesting a shift EGFR expression in this geminal zone.

Interestingly, although I observed significant shifts in the proportion of cells exhibiting EGFR-IR, I did not observe significant differences in the thickness of the EGL's postnatal profile (defined as the mean number of cells counted across the EGL along 2-3 cerebellar folia in 2-3 adjacent sagital sections) between treatment groups at all time points measured and in two separate experiments (data not shown). Therefore, it is unlikely that there was an increase in proliferation of granule cells in hypothyroid mice, but rather a delay in postmitotic events related to migration of granule cells out of the EGL. Although one would expect a build up of of total cells due to a delay in migration, I speculate that a percentage of post-mitotic cells die, perhaps to this delay in EGFR expression. One possibility is that EGFR is necessary for the extension of processes necessary for migration and /or trophic support from other cells, e.g., Bergmann glia, given that EGFR signaling has been shown to be involved in the survival of post-mitotic neurons in EGFR null mice (Sibilia et al., 1998).

Thus, EGFR appears to play a role in the migration of granule cells, as previously suggested (Seroogy et al., 1995; Kornblum et al., 1997). EGFR and its ligands have been shown to aid migration of many cell types, including corneal,

liver, epithelial, and neural precursors (Bade and Feindler, 1988; Chen et al., 1994; Chen et al., 1994; Berens et al., 1996; Craig et al., 1996; Thalmann-Goethsch et al., 1997; Xie et al., 1998). Threadgill et al., (Threadgill et al., 1995) reported that EGFR null mice have retarded migration of the EGL. Futhermore, EGF-like motifs are common in certain molecules with migratory function, such as astrotactin and reelin (Hirotsune et al., 1995; Zheng et al., 1996)

During normal development of the cerebellum, pre-migratory cells in the EGL are detected by P3 in mice (Fujita, 1967; Fujita et al., 1967). Two axonal processes are extended parallel to the long axis of the folia (parallel fibers), followed by the extension of a third process that is oriented perpendicular to the EGL, which descends into the underlying molecular layer, and grows toward the IGL (Miale and Sidman, 1961; Fujita, 1967; Fujieda, 1993). These fibers associate with radial fibers of Bergmann glia, believed to aid the migration of granule cells towards the IGL (Rakic, 1971). In hypothyroidism, the migration of granule cells out of the EGL is delayed and is believed to be related, in part, to retarded growth of both the parallel fibers and Bergmann glia (Lauder, 1979; Clos et al., 1980). Recent evidence shows that before post-mitotic granule cells migrate out of the pre-migratory zone of the EGL, they migrate medially and laterally (Ryder and Cepko, 1994). It is significant that at P6 when the densest EGFR-IR (Figure 6 A and B) in the pre-migratory zone was observed, coincides with medial-lateral migration and extension of parallel fibers. Also, I found that EGFR-IR was expressed in "spindle-shaped" cells and leading processes (Figure 6 A, inset), suggestive of migrating granule cells (Altman, 1972; Altman and Bayer, 1997). It is likely that EGFR is located in these processes, rather than Bergmann glia given that the pattern of GFAP and EGFR immunoreactivity observed in the EGL of P7 cerebella were observably different (Figure 8 A and B).

The presence of strong EGFR-IR in cells of the IGL, next to the Purkinje cell layer, in normal cerebella but not in hypothyroid cerebella, at P14, may represent granule cells that have recently arrived (Figure 6 E and F). EGFR-immunopositive cells were observed in the molecular layer, near Purkinje cells, of normal tissue but not hypothyroid tissue at P14 (Figure 6 E and F). These immunopositive cells in the normal cerebella may represent migrating granule cells. Moreover, the number of immunopositive cells and EGFR decreased by P22; conversely, an increase in EGFR-IR cells was observed in hypothyroid cerebella, comparable to that observed in normal tissue at P14. Also, I observed dense EGFR mRNA labeling in the EGL, molecular layer and IGL of hypothyroid cerebella than in normal cerebella, in which EGFR mRNA appeared to be down-regulated. This is consistent with Lauder's (1979) study in which she found that, although the rate of migration of granule cells out of the EGL was not delayed in hypothyroid animals, the rate of migration in the molecular layer was slower than in normal animals, consequently producing a "pile up" of migrating granule cells near the Purkinje cell layer.

At P22 I did not observe any EGFR-IR in the EGL between treatment groups (data not shown). Moreover, I found that the EGL was reduced to 0-2 cells thick, in both treatment groups, in numerous sections analyzed. In the IGL, at this time point, EGFR-IR was diffuse around cell nuclei, suggesting a decrease in the expression of EGFR in this zone. Moreover, the decrease in EGFR in the IGL suggests that this receptor may not be involved in the maintenance and/or survival of mature granule cells. The presence of EGFR-IR in the cell bodies and apical dendrites of normal Purkinje cells at P14, and delayed expression in Purkinje cell processes from hypothyroid animals until P22 also suggests that EGFR-family ligands play a role in the maturation of Purkinje cells.

EGF-receptor ligands are known to be mitogens for a variety of cell types (Anchan et al., 1991; Casper et al., 1991; Reynolds et al., 1992; Santa-Olalla and Covarrubias, 1995), however evidence exists that these factors also have post-mitotic activities (Abe et al., 1991; Casper et al., 1991; Abe and Saito, 1992; Alexi and Hefti, 1993; Casper et al., 1994). Misumi and Kawana (1998) EGFR mRNA expression overlapped with the neuronal marker protein gene product 9.5 (PGP 9.5), in differentiating fields of embryonic rats. In contrast, the proliferating neuroepithelium of the germinal trigone was devoid of both EGFR mRNA and PGP 9.5. Other studies suggest that EGFR might control post-mitotic events, since this receptor has been shown to be expressed in cerebellar Purkinje cells and cerebral cortical neurons throughout adulthood (Gomez-Pinilla et al., 1988; Werner et al., 1988; Tucker et al., 1993). Additionally, EGFR null mice exhibit degeneration of the forebrain during the second postnatal week, suggesting that this receptor and its ligands are critical for the survival of postmitotic neurons. In conclusion, this study shows that thyroid hormone is implicated in the induction of EGFR and its ligand, TGF-α. EGFR-IR and mRNA were largely expressed in post-mitotic cells and not in proliferating progenitor cells of the EGL, supporting the hypothesis that cells proliferate independently of EGFR (Kornblum et al., 1997; Misumi and Kawano, 1998). Moreover, I show that EGFR may be implicated in the differentiation, process outgrowth, and/or migration of granule cells. Thus, the delay in granule cell migration observed in the cerebella of hypothyroid animals may occur, partly, to a delay in EGFR expression and its ligands, suggesting that thyroid hormone directly or indirectly regulates their expression.

Figure 4: Developmental profiles of serum T_4 in C57Bl6/ J X CBA F1 mice: control vs. PTU treatment. Serum T_4 levels were measured by radioimmunoassay, as described in the Materials and Methods section, and are expressed as concentrations in $\mu g/dL$. Each data point represents the mean for n=5-15 samples (± SEM) for control (closed squares) and PTU-treated (open circles) animals. Note that serum T_4 levels of hypothyroid mice were below the minimum detectable dose of the assay ($1\mu g/dL$).

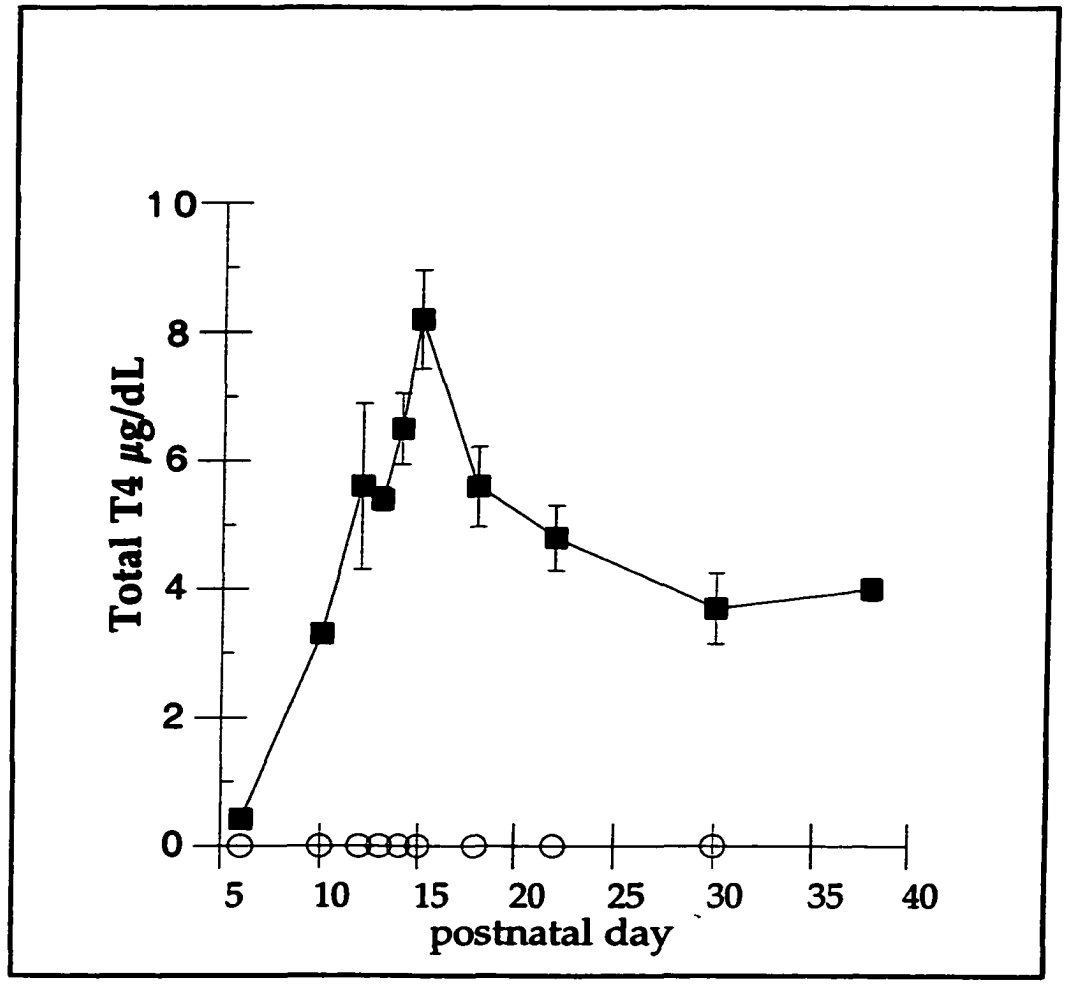


Figure 5. Developmental expression of TGF–α and EGFR mRNAs in the cerebellum of normal and hypothyroid C57Bl6/J X CBA F1 mice Messenger RNA abundance was determined by nuclease protection assays, as described in the Materials and Methods section. Panel A: data points represent mean values of TGF-α mRNA abundance, expressed in pg/μg total RNA, for normal (closed squares; n=4-14 per time point) and hypothyroid (open circles; n=2-7 per time point) mice ± SEM. Asterisks denote statistical significance between control and hypothyroid values at P6, p **< 0.003. Panel B: data points represent mean values for EGFR mRNA abundance in the cerebellum of normal mice (closed squares; n=3), and hypothyroid mice (open circles; n=3). Asterisks denote statistical significance between control and hypothyroid values for particular time points, p * < 0.03, p ***< 0.0003.

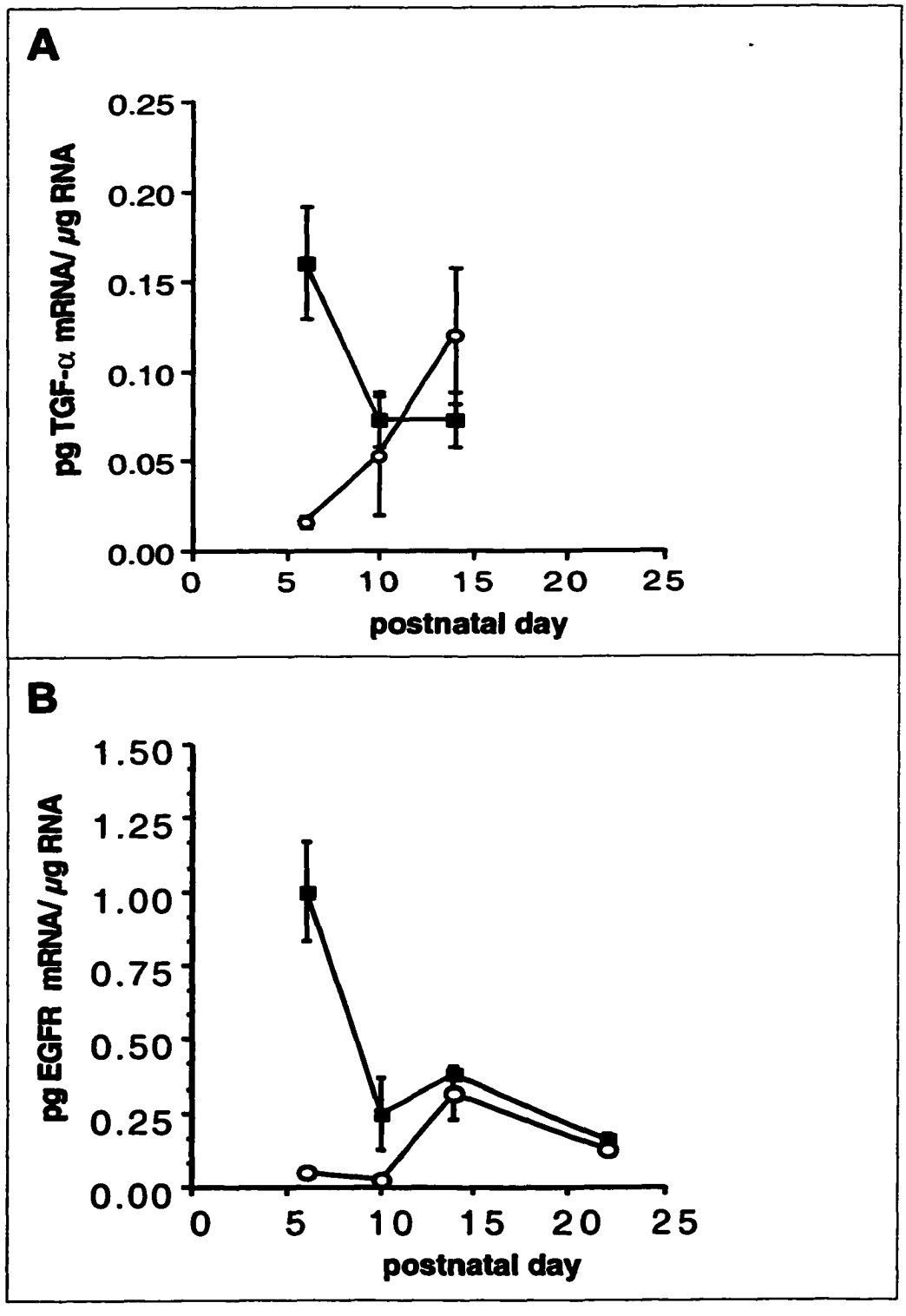


Figure 6: Immunohistotochemical localization of EGFR in postnatal cerebella of normal and hypothyroid mice. Tissue sections from control and PTU-treated mice were processed for immunohistochemistry with a polyclonal antibody to EGFR. All panels are bright-field photomicrographs of sagital sections in which immunoreactivity is brown, and counterstained nuclei are blue. Panels A, C, E, and G depict staining in normal animals; panels B, D, F, and H depict staining in hypothyroid animals, at postnatal days 6, 9, 14, and 22 for both. Black arrows point to immunoreactivity in the inner segment or pre-migratory zone of the EGL. White arrows point to EGF-IR "spindle- shaped" cells with leading processes (panel A, inset), and EGF-IR cells in the ML (panels A, C, E, G, and H). White arrowheads points to immunoreactivity in the Purkinje cell soma (panels C-H); black arrowheads points to EGF-IR in the IGL (panels C, D, E, and H). Broken black arrows identify EGF-IR apical dendrites (panels E and G). Scale bar = 100 μm for all micrographs. Panel I: EGFR western blot of A431 cell lysate (positive control; 2 μg), and mouse cerebellar homogenate (Cb; 9 μg), demonstrating a major band at MW 170 kDa.

Figure 7: Hypothyroidism produces a delayed increase in the percentage EGFR-IR cells in the EGL of postnatal mice. Percentages of EGF-IR cells the EGL were calculated to be the ratio of immunolabeled cell profiles over the total number of cell profiles (x 100) that spanned the width of the EGL at specified times in postnatal development. Data points represent mean values for normal (n=3-4; closed circles) and hypothyroid (n=3-4; open circles) (±SEM). Note that at P6, this percentage was significantly lower in hypothyroid cerebella (p **< .02) compared to normal cerebella; whereas, at P14 the percentage of EGFR-IR was significantly greater in hypothyroid cerebella than in normal normal cerebella by P14 (p *< .05).

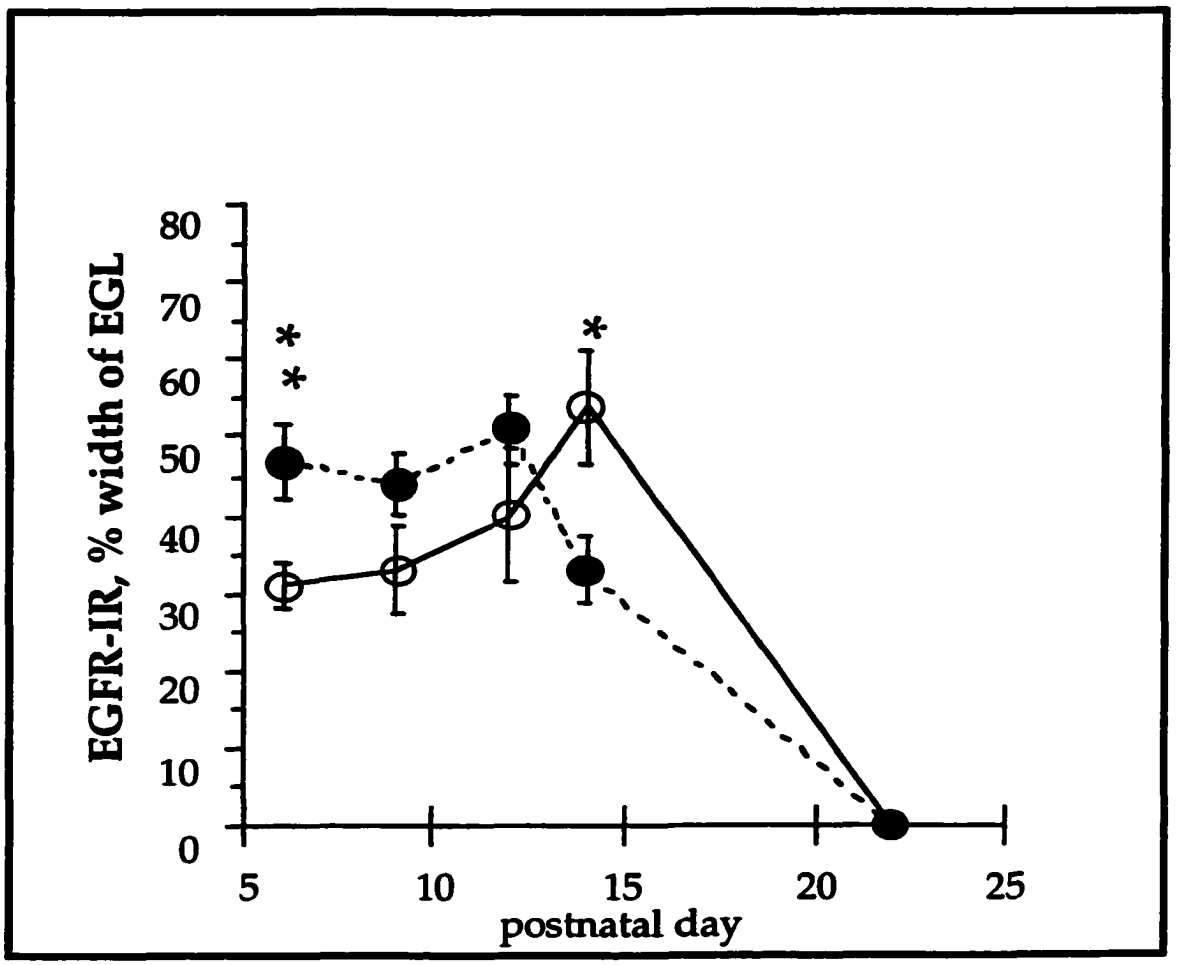
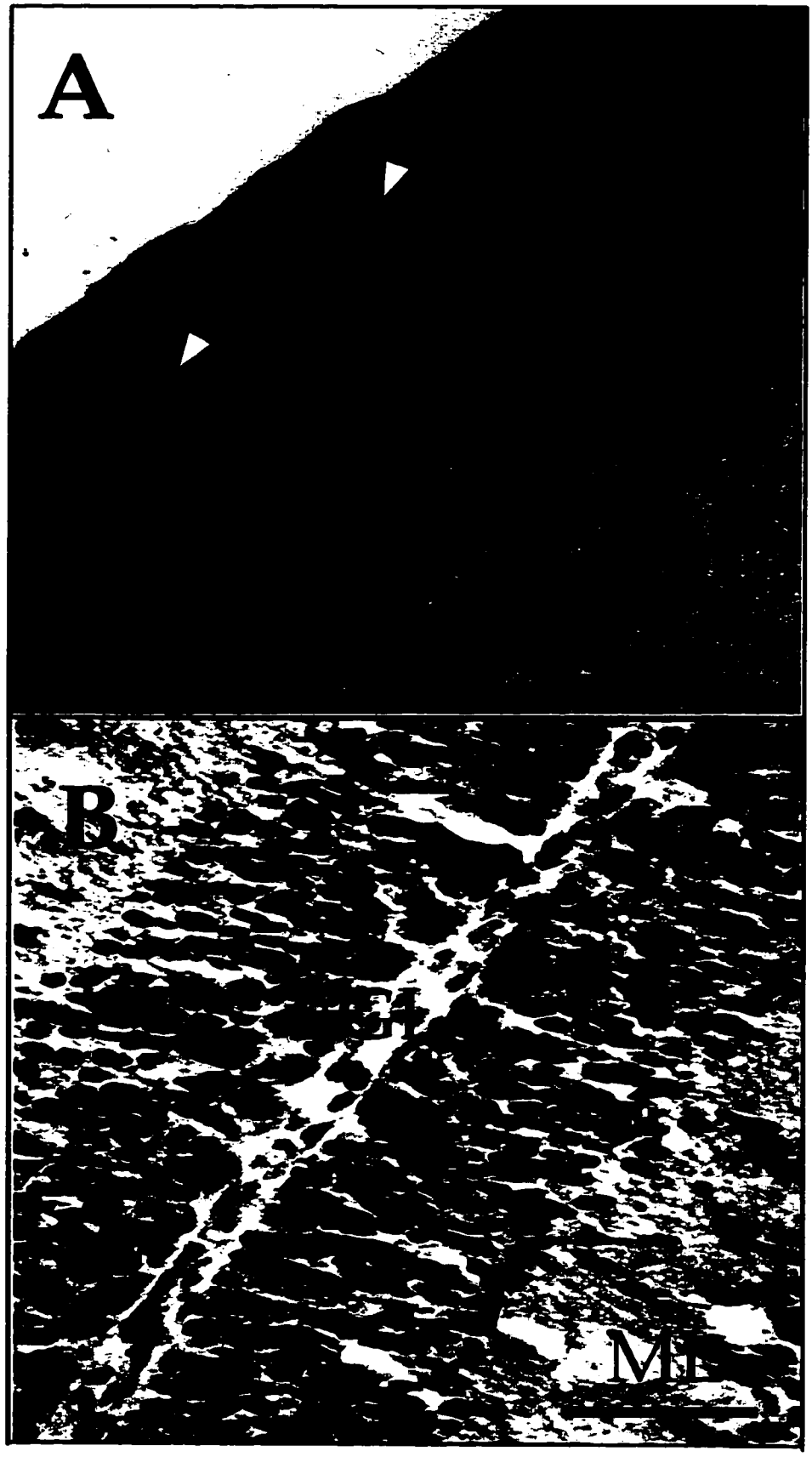


Figure 8: GFAP-IR and EGFR-IR in the EGL and molecular layer of postanatal cerebella. All panels are bright field sagittal sections. Panel A shows the immunocytochemical localization of GFAP-IR in P7 hypothyroid cerebella; white arrowhead shows immunoreactivity in the EGL; black arrowhead shows immunoreactivity in the molecular layer (ML). Panel B shows EGFR-IR in the EGL of P6 hypothyroid cerebella; black arrows shows immunoreactivity in the EGL; Scale bar = 100 μm.



Chapter 3

Thyroid Hormone Regulates Cerebellar Development: The Roles of Epidermal Growth Factor-Ligands and Basic Fibroblast Growth Factor In Vitro

Abstract

Hormones and growth factors are known to have significant effects on cerebellar cell development, but the relationship between these two types of molecules is not well understood. While previous studies utilized culture systems with purified or enriched populations of neurons or glia, I examined the effects of thyroid hormone on mixed cultures of cerebellar granule neurons and several types of glia, thus permitting biologically relevant neuronal-glial interactions. I found that L-triiodothyronine (T_3) had differential effects on neurons and glia, depending on cell density. At low plating densities, T_3 accelerated neuronal and glial differentiation and a switch in astrocyte morphology from radial to vellate. At high densities, T_3 decreased proliferation of non-neuronal cells. Western blotting of cell lysates showed that T_3 treatment regulated levels of epidermal growth factor receptor (EGFR), and basic fibroblast growth factor (bFGF) proteins in opposite directions; attenuating bFGF, but inducing EGFR. Taken togehter these results suggest that the actions of T_3 are mediated by down-regulating bFGF, which may be involved in earlier developmental events such as proliferation, and by up-regulating EGFR, which may mediate later, post-mitotic events such as differentiation.

Introduction

Thyroid hormone has a significant influence on cerebellar development, and in particular, events associated with granule cell proliferation and differentiation. While experimental hyperthyroidism causes premature termination of granule cell proliferation the external granule cell layer (EGL) and accelerated granule cell migration toward the internal granule cell layer (IGL) (Nicholson and Altman, 1972; Lauder, 1979), hypothyroidism delays the disappearance of the EGL, resulting in the delayed migration of granule cells toward to IGL (Nicholson and Altman, 1972; Lauder, 1977; Lauder, 1979; Carrasco et al., 2001). A number of studies have also investigated the role of thyroid hormone on granule cell development, but their results have disagreed regarding whether granule cells are a direct or indirect target of thyroid hormone (Balazs et al., 1985; Messer et al., 1985; Heisenberg et al., 1992). These studies used purified neuronal cultures plated at similar densities in serum-free medium and treated with similar concentrations of T_3, the biologically active form of thyroid hormone. The factor that varied among them was schedule of T_3 treatment; a siginicant increase in granule cell survival and differntiation was observed only in cultures treated with a single dose of T_3 (Heisenberg et al., 1992). Additional studies have reported that conditioned medium obtained from astrocytes treated with T_3 increased proliferation of embryonic cerebellar neuroblasts (Gomes et al., 1999), induced morphological changes of early postnatal cortical astrocytes and proliferation in cerebellar astrocytes (Trentin et al., 1995). These results suggest that T_3 may stimulate the secretion of other factors, essential for neural and glial development. In vivo and in vitro studies have shown that thyroid hormone influences mRNA and protein expression of other neurotrophic factors such as neurotrophins (Lindholm et al., 1993; Neveu and Arenas, 1996).

Members of the epidermal growth factor (EGF) and fibroblast growth factor (FGF) families are potent mitogens for cells of the CNS (Leutz and Schachner, 1981; Simpson et al., 1982; Kniss and Burry, 1988; Huff et al., 1990; Kitchens et al., 1994) though they have also been shown to promote a range of developmental functions, including survival, migration and differentiation (Morrison et al., 1986; Hatten et al., 1988; Morrison et al., 1988; Luo et al., 1997; El Idrissi et al., 1998). Their influence appears to depend on the biological, spatial, and temporal context in which they are placed (Abe et al., 1990; Abe et al., 1991). .

Basic FGF (bFGF) is reported to play a role in the development of granule cells, and in vitro, affecting a wide range of developmental events, depending on culture conditions. For example, in re-aggregates of granule cell neuroblasts, bFGF stimulated proliferation, whereas in monolayers, it stimulated differentiation (Gao et al., 1991). In vivo, bFGF has been implicated in cerebellar neurogenesis (Hatten et al., 1988). Peripheral injections of bFGF increased the proportion of mitotic granule cells (Tao et al., 1996; Cheng et al., 2001), and conversely, intraventricular treatment with anti-bFGF antibodies decreased DNA synthesis in the cerebellum (Tao et al., 1997). From what is known about the actions of thyroid hormone, the actions of bFGF clearly overlap, but little is known about the interaction between thyroid hormone and bFGF in the CNS. One study showed that bFGF increases the mRNA expression of type 3 iodothyronine deiodinase (an enzyme that metabolizes thyroid hormone to inactive metabolites) in cultured rat astrocytes (Pallud et al., 1999). A second study found that high concentrations of bFGF, in cardiac ventricles, were associated with physiological and experimentally induced hypothyroidism in rats (Liu et al., 1993).

Thyroid hormone upregulates the number of EGF binding sites or receptors (EGFR) in brain, but not in lung (Sadiq et al., 1985). In chapter 2 , I

provide evidence implicating a role for EGF-family ligands in the post-mitotic development of granule cells, and the regulation of this pathway by thyroid hormone (Carrasco et al., 2001). I found that hypothyroidism transiently attenuated the expression of EGFR mRNA in mouse cerebellum and that EGFR mRNA and protein was localized to the pre-migratory zone of the EGL, where post-mitotic granule cells are found.

Previous studies that have investigated the effect of thyroid hormone on cerebellar cells have used either pure neuronal or pure glial cultures. In contrast, the culture system I used for this investigation is characterized by the presence of granule neurons, glial cells consisting of mainly astrocytes, and fibroblasts (Trenkner, 1991). I chose this system because it best simulates the *in vivo* environment, in that it allows for neuronal-glial interactions that may dictate, or modulate developmental events. In this study Iexamined the effects of T_3 on proliferation and differentiation of neurons and glia, and analyzed the effects of T_3 on the expression of EGFR and bFGF.

Materials and Methods

Primary cultures of dissociated postnatal mouse cerebellum

Animals were cared for and sacrificed according to institutional guidelines. Cerebellar mixed cell cultures were prepared from postnatal days 6 and 7 C57Bl6/J mice, as previously described (Trenkner, 1991). After enzymatic and mechanical dissociation, single-cell suspensions were plated on poly-D-lysine-coated 24-well plates. Cells were plated at 1.5×10^5 cells/1.8 cm^2 and 2.0×10^5 cells/1.8 cm^2 in Eagle's Basal Minimal Essential Medium (DMEM; Sigma, St. Louis, MO) supplemented with 0.25% glucose, 2 mM glutamine, 10% fetal calf serum, 10 U/ml penicillin and 10 μg/ml streptomycin. The day of plating was defined as day <u>in vitro</u> zero (DIV 0). On DIV 1, the medium was changed to chemically

defined medium, containing equal volumes of Minimal Essential Medium with Earle's salts and Glutamax I, and Ham's F-12 nutrient mixture, and supplemented with 25 mM glucose, 25 mM sodium bicarbonate, 15 mM HEPES, 25 μg/ml of insulin, 100 μg/ml of transferrin, 60 μM putrescine, 20 nM progesterone, 30 nM sodium selenite, 50 U/ml penicillin and 50 μg/ml streptomycin, as described previously (Casper et al., 1991).

Thyroid hormone treatments were given by the addition of a concentrated T_3 (Sigma) ethanol stock solution to the medium, yielding a final concentration of 2 ng/ml, a physiological dose (Messer et al., 1984; Lindholm et al., 1993; Muller et al., 1995), with a final ethanol concentration of 0.14%. T_3-treated cultures were compared to control cultures with ethanol. I found no significant differences between control cultures and control cultures containing ethanol in all parameters measured ($p > 0.05$). Cultures were maintained in a humidified incubator in an atmosphere of 5% CO_2, 95% air. Cells were fixed and processed for the analyses described below on DIV 3, DIV 5 and DIV 7.

Immunocytochemistry

After T_3 treatments, cultures were fixed for 30 minutes at room temperature in 4% paraformaldehyde in phosphate buffered saline (PBS; 10 mM sodium phosphate, 140 mM sodium chloride, pH 7.4). After fixing, cultures were washed twice for 5 minutes with PBS and permeabilized with 95% ethanol/5% acetic acid for 5 minutes at -20°C. This was followed by blocking non-specific binding sites with 3% horse serum and 0.2% Triton-X in PBS for 30 minutes. Cultures were then incubated overnight at 4°C with primary antibodies. PHF-1, an antibody raised against paired helical filaments that stains rat neurons, was used to identify neurons (Greenberg et al., 1992); diluted 1 to 10); anti-glial fibrillary acidic protein (GFAP) was used to identify astrocytes (Boehringer-

Mannheim; diluted 1 to 50); O1 (Boehringer-Mannheim) and galactocerebroside (GC, Chemicon, Temecula, CA) antibodies were used to identify oligodendrocytes. Double label immunocytochemistry for GFAP and A2B5 (Boehringer Mannheim) was used to identify type 2 astrocytes. After incubation with primary antibodies, cultures were washed with PBS and incubated with biotin-conjugated secondary antibodies. For visualization of single-label immunocytochemistry, the peroxidase Vectastain anti-mouse ABC kit with diaminobenzidine (DAB) was used (Vector Laboratories, Burlingame, CA). For double-label immunocytochemistry, alkaline phosphatase-conjugated streptavidin (Vector) and fast red (Sigma) were used to visualize a second epitope. Cells that displayed immunoreactivity for these antibodies are designated with a "$^{+}$" superscript. Total cell number and neuronal, astrocytic, and oligodendrocytic elements were quantified by counting cells of each type in 4-6 fields of 3-4 replicate dishes under 200 X magnification under phase optics for total cell counts, or bright-field optics to quantify immunostained cells.

BrdU incorporation

Proliferating cells were identified by 5-bromo-2'-deoxyuridine (BrdU) incorporation into cellular DNA. BrdU (Amersham, Arlington, IL; diluted 1:1000) was diluted in the culture medium for 21 hours, after which the cells were fixed and processed for immunocytochemistry as described above, with an anti-BrdU mouse monoclonal antibody (Amersham; diluted 1:5). BrdU-labeled cells and total cells were counted in 4-6 fields of 3-4 replicate dishes under 200 X magnification using bright-field and phase optics, respectively. A proliferation index was calculated by dividing the number of $BrdU^{+}$ cells by the number of total cells x 100.

Western blotting

EGFR and bFGF protein levels were quantified in lysates of mixed cerebellar cultures by Western blot analysis. Cells were harvested at 4°C in lysis buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% deoxycholate, 150 mM NaCl, 1 mM EGTA, 2 mM PMSF, 4.4 μM leupeptin, and 0.83 μM aprotinin). The lysates were then sonicated for 5 seconds. Aliquots were stored at -70°C until protein determination and SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis). Protein content was determined by the Biorad protein assay, a Bradford based assay (Bio-Rad, Herculis, CA). Twenty microgram protein aliquots were loaded onto 7.5% acrylamide gels for EGFR blots (MW 170 kD) or 50 μg protein aliquots were loaded onto 12% acrylamide gels for bFGF blots (17-21 kD). Proteins were electro-transferred to 0.45 μm PVDF membranes (Millipore, Bedford, MA). When transfers were complete, membranes were incubated with 2 μg/ml of sheep anti-human EGF Receptor (Upstate Biotechnology Inc. (UBI), Lake Placid, NY), 5 μg/ml of mouse anti-human bFGF (UBI) and 2.5 μg/ml of mouse anti-actin (Calbiochem, San Diego, CA). Both of these antibodies cross-react with rodent homologues. After incubation with primary antibodies, membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies (Amersham). Enhanced chemiluminescence (ECL or ECL Plus, Amersham), followed by exposure to X-ray film, was used to detect protein signals. Signal intensities were quantified using densitometry on a UNIX workstation (BioImage, Millipore). Protein bands at molecular weights corresponding to EGFR and bFGF were normalized against actin signals and compared with respect to T_3 treatment.

Statistical Analysis

Statistical analysis was done using Statview (Abacus Concepts, Berkeley, CA.). A factorial (two- way) ANOVA with a post hoc Fisher *F* test was performed for all analyses. Cells were counted in 4-6 fields in replicate cultures (3-4 per treatment) at 200 X magnification. Cell counts for each culture were represented by the mean cell numbers per field. A factorial ANOVA was performed to assess the effect of T_3 treatment, cell density and DIV on PHF-1^+ and $GFAP^+$ cells. Data are presented as the mean ± standard error (S.E.M ±) of measurement. Significance was set at $p < 0.05$.

Results

Effects of T_3 on neuronal and astrocyte differentiation are density-dependent

Published studies have shown that the response of cerebellar granule cells to developmental stimuli depends greatly on culture conditions, including the densities at which cells are initially plated (Gao et al., 1991; Matsuda et al., 1994; Wells, 1999; Young et al., 2000). In order to establish a density that promoted cell survival, but also enabled us to see the effects of T_3 on differentiation, dissociated mixed cerebellar cells were plated at two densities: 1.5×10^5 cells/1.8 cm^2 ("lower" density) and 2.0×10^5 cells/1.8 cm^2 ("higher density"), and half of each group was treated with T_3. After 3, 5, and 7 DIV, cultures were processed for immunocytochemistry with PHF-1, an antibody that stains neuronal cell bodies and processes (Greenberg et al., 1992), and anti- GFAP, to identify differentiating astrocytes (Bignami and Dahl, 1973). Results from this experiment are presented in Fig. 1. PHF-1^+ and $GFAP^+$ cells are expressed as a percentage of the total cells per field. In panels 1A and 1C, the percentages of PHF-1^+ cells and $GFAP^+$ cells with respect to total cell numbers are reported in control and T_3-treated lower

density cultures. In panels 1B and 1D, the percentages of these cells are reported for higher density cultures. Panels 1E and 1F illustrate total cell numbers for each time point in control and treated cultures at each cell density. Interestingly, significant effects of T_3 on the proportions of neurons and glia, as represented by the percentages of PHF-1^+ and $GFAP^+$ cells, were only observed in cultures plated at the lower density.

T_3 treatment significantly increased the proportion of PHF-1^+ cells in cultures plated at the lower density at DIV 3 and DIV 5 (Fig. 1A). On DIV 3, 82 ± 1.5% of the total cells in T_3-treated cultures were PHF-1^+, compared to 66 ± 4.3% in control cultures ($p < 0.001$). On DIV 5, 74 ± 3.8% of cells were PHF-1^+ in T_3 treated cultures, compared to 58±3.9% in untreated cultures ($p < 0.01$). On DIV 7, the percentage of PHF-1 cells decreased in T_3-treated cultures to 50±4.1%, but the percentage of PHF-1^+ cells did not change in control cultures (61± 2.6%). In addition, the relative number of PHF-1^+ cells was significantly lower DIV 7 compared to DIV 3 in T_3-treated cultures ($p < 0.0002$). A the higher density, there were no significant differences in the percentages of PHF-1^+ cells in T_3 treated cultures compared with controls (Fig. 1B: 87 ± 2.0% vs. 83 ± 1.5% at DIV 3; 89±5.9% vs. 88 ± 3.5% at DIV 5, and 85 ± 3.5% vs. 84±1.8% at DIV 7 for T_3 and control cultures, respectively). This may be accounted for by the increased survival at high density, eliminating the decline in neuronal numbers with time (Figs. 1A and 1B).

Astrocytes undergo a switch in the composition of their cytoskeletons during early postnatal development from vimentin-based intermediate filaments (Dahl, 1981; Dahl et al., 1981) to GFAP-based intermediate filaments (Eng, 1985). In hypothyroid rats, this transition is delayed, and GFAP concentrations are 43% lower at P35 (Faivre-Sarrailh et al., 1991), accompanied by marked changes in astroglial morphology (Clos et al., 1973; Pesetsky, 1973). Likewise, T_3 treatment

significantly increased the differentiation of astrocytes in cultures plated at low density in a similar fashion to neuronal differentiation, but with a more protracted time course (Fig. 1C). On DIV 5 and 7, $GFAP^+$ cells constituted 14 ± 1% of the cell population in T_3-treated cultures, compared to 9±1.1% and 10 ± 1.5% at DIV 5 and DIV 7, respectively, in control cultures ($p < 0.02$). In contrast, there were no differences in the percentages of $GFAP^+$ cells in response to T_3 treatment at the higher plating density for all time points analyzed. However, I did observe a 1.5 ~ 2 fold increase in the percentage of $GFAP^+$ cells in both treatment groups on DIV 7 compared with DIV 3 and DIV 5 ($p< 0.0002$). This increase was comparable to that observed on DIV 5 in response to T_3 treatment at lower density (Fig. 1A), suggesting that T_3 accelerated astrocyte differentiation, or that differentiation was delayed by unidentified factors present in higher density cultures.

Since thyroid hormone influences cell proliferation and survival as well as differentiation, I examined the effects of T_3 on total cell numbers. In cultures plated at low density, there were no significant differences in total cell numbers with T_3 treatment at DIV 3 or DIV 5. On DIV 7, however, I observed a significant ($p <0.02$) decrease (24%) in total cell number in T_3 treated cultures (106 ± 12.4) relative to control cultures (81 ± 3.0). At high density, there were several fold-higher cell numbers at DIV 3 than at the later time points, where cell numbers were only slightly higher than at the low density. Interestingly, T_3 treatment had no effect on cell numbers at the higher plating density (Fig. 1F: DIV 3: 347 ±8.1 vs. 322 ±20.4; DIV5: 131± 3.3 vs. 133 ±1.5; DIV 7: 109 ± 6.3 vs. 111 ± 8.1). While thyroid hormone has been shown to increase (indirectly) granule cell proliferation of embryonic granule cells (Gomes et al., 1999) and survival of postnatal granule cells in vitro (Heisenberg et al., 1992), my results are not surprising given that the cells for my cultures were obtained from postnatal day

6 and 7 mice and the cellular composition of my culture system differs from those of other groups (see Discussion). In order to distinguish between effects on cell survival and proliferation, I performed trypan blue exclusion assays in cultures plated at the lower density. Trypan blue, a vital dye, is taken up only by dying or dead cells in culture. Significant differences in trypan blue staining was observed on DIV 5 between T_3-treated cultures (47% dead versus 53 ± 3.1%, live cells) and untreated cultures (28 % dead versus 72 ± 3.8%-live cells) ($p < 0.03$), indicating that T_3 decreased cell survival. Taken together, these data suggest that T_3 accelerates neuronal and glial maturation, but it may also accelerate cell death. At this time it is not known whether or not the maturation process is a prerequisite for neuronal death, although these two events occur in sequence in the natural course of brain development.

T_3 reduces cell proliferation

It has been suggested that thyroid hormone is, in part, responsible for signaling granule cells to exit the proliferation cycle and to begin the differentiation process (Nicholson and Altman, 1972). Similarly, non-neuronal cells cease cell division in order to differentiate. To determine if T_3 treatment influenced cell proliferation in my system, I plated cerebellar cultures at the higher of the two densities (2.0×10^5 cells/1.8 cm^2), described for the previous experiment. Cell-cell contact has been demonstrated to be essential for proliferation of cerebellar cells (Gao et al., 1991). Cultures were incubated, in the presence or absence of T_3, with BrdU for 21 hours, and subsequently fixed and processed for BrdU immunocytochemistry. Total cells and cells with $BrdU^+$ nuclei were quantified (Fig. 2). The numbers of labeled nuclei were expressed as a percentage of total cells per field. Under control conditions, BrdU labeled 5±0.5% of the cells in this time frame. In contrast, only 1±0.5% of cells were labeled in T_3

treated cultures (Fig. 2A), a statistically significant decrease ($p < 0.003$). From the large sizes and oval shapes of labeled nuclei, it appeared that the majority (~ 90-95%) of BrdU-labeled cells were of non-neuronal origin. Furthermore, from their branched morphology, and double-labeling with anti-GFAP (Fig. 2B), it was confirmed that these cells were type 1 astrocytes.

The effects of T_3 on glial cell differentiation

In the rat cerebellum, thyroid hormone regulates the number of astrocytes and the maturation of Bergmann glia (Clos et al., 1980). To examine the effect of T_3 on glial differentiation in my culture system, I plated cultures at the relatively lower density ($1.5 X10^5$ cells/1.8 cm^2). The choice of the lower plating density was supported by the results of my previous experiment (Fig. 1) and several published studies demonstrating that various factors induce differentiation in vitro at low density (Hatten et al., 1988; Gao et al., 1991; Lowenstein and Arsenault, 1996; Young et al., 2000). Cultures were incubated in the presence or absence of T_3, and analyzed on DIV 5 to determine the relative numbers of glial subtypes using immunocytochemistry. An antibody to GFAP was used to identify astrocytes (Bignami et al., 1972), and double-labeling of GFAP with A2B5, an antibody to a membrane glycosphingolipid (Raff et al., 1983), was used to identify type -2 astrocytes. Both GC and O1 antibodies were used to identify oligodendrocytes, recognizing membrane galactocerebroside and the surface lipid O1, respectively (Raff et al., 1979; Sommer and Schachner, 1981). Immunostained cells were counted in replicate culture wells, and mean values are expressed as a percentage of the total cells per field (Fig. 3). Results illustrate that the percentages of all glial subtypes were significantly higher in T_3-treated, compared to untreated cultures ($p < 0.05$). The percentage of total (type 1 and type 2 astrocytes) $GFAP^+$ cells was 14±3.6% in T_3-treated cultures,

compared to 7±1.1% in control cultures (Fig. 3A), consistent with my previous experiment (Fig. 1B), and indicating a doubling of astrocyte numbers with T_3 treatment. A2B5 and GFAP double-label immunocytochemistry, revealed that type-2 astrocytes constituted 0.7±0.3% of the total cell population in control cultures, and 2.0± 0.5% of total cells in T_3-treated cultures (Fig. 3B). GC^+ cells made up 8 ± 0.7% of T_3 treated cultures, compared to 5 ± 0.6 % of untreated cultures (Fig. 3C), and $O1^+$ cells made up 11 ± 1.1% of T_3 treated cultures, compared to 8 ± 0.7% of control cultures (Fig. 3D). There was no significant difference ($p > 0.05$) in total cell numbers between treatments (107±13 versus 105±12). This is consistent for all experiments at the lower cell density, where T_3 has no effect on total cell numbers at 5 DIV.

Effects of T_3 on astroglial morphology

Previous studies of the effects of T_3 on astroglial morphology were carried out using either purified or enriched astroglial cultures, and confluent or sub-confluent conditions (Trentin and Neto, 1995; Trentin et al., 1995; Lima et al., 1997). However, several studies have demonstrated that glial morphology is greatly influenced by the presence of neurons in culture (Hatten, 1985), including cultures of cerebellar astrocytes with granule cells (Hatten et al., 1984). Therefore, I investigated the effect of T_3 on astroglial morphology in a mixed cerebellar cell population. I plated cells at 1.5 x10^5 cells/1.8 cm^2 as described above, treated half of the cultures with T_3, and fixed them on DIV 5 for GFAP immunocytochemistry. $GFAP^+$ cells were examined under 200 X magnification. Three subtypes of astrocytes were identified based on morphology: 1) "radial-like," with large cell bodies and long and thin branched processes (Fig. 4B), 2) stellate, with condensed cables of $GFAP^+$ filaments in a "star-like" pattern (Fig. 4D), and 3) velate, with large cell bodies surrounded by cytoplasm resembling a

"veil" (Fig. 4F). Numbers of the various morphological subtypes are expressed as a percentage of the total number of $GFAP^+$ cells per field (Fig. 4). On DIV 3, radial-like astrocytes constituted the majority of $GFAP^+$ cells in both T_3-treated cultures (96±2.9%) and control cultures (96±2.0%). By DIV 5, there was a sharp decrease in this population in both treatment groups, but this decrease was greater in T_3-treated cultures. In T_3 treated cultures, radial-like astroglia comprised 18±5.0% of total astrocytes, compared to 37±6.8% in untreated cultures. By DIV 7, the percentage of "radial-like" astroglia in T_3 treated cultures had reached a plateau at 23± 5.6%, whereas in control cultures the percentage continued to decrease to 7±3.0%.

Fig. 4C shows that on DIV 3 the percentage of stellate astrocytes was very small in both T_3 treated (1.1±1.0%) and untreated (4.0±2.9%) cultures. In T_3-treated cultures the percentage of stellate astroglia increased on DIV 5 (26±5.5%) and had reached a plateau by DIV 7 (28±6.0%). In control cultures, in comparison, the percentage of stellate astroglia increased dramatically on DIV 5 (42±6.0%) and decreased by DIV 7 (28±6.0%).

Figure 4E demonstrates that vellate astrocytes comprised only 1-2% of $GFAP^+$ cells on DIV 3 in both T_3-treated and untreated cultures. In T_3-treated cultures the percentage of vellate astrocytes increased to 56±7.1% on DIV 5 and reached a plateau by DIV 7 (50±6.7%). In control cultures, the percentage of vellate astrocytes increased on DIV 5 to only 21±4.6% and continued to significantly increase on DIV 7 (68±4.3%).

T_3 and EGFR and bFGF expression

It has been proposed that thyroid hormone act in the development of the CNS in part through regulating the expression of growth factors (Fisher et al., 1982; Charrasse et al., 1992; Giordano et al., 1992; Figueiredo et al., 1993; Figueiredo et al., 1993; Lindholm et al., 1993; Alvarez-Dolado et al., 1994; Neveu and Arenas, 1996; Calza et al., 1997). Both EGF and bFGF, depending on in vitro conditions, promote mitosis, survival, or differentiation in granule cells (Hatten et al., 1988; Abe et al., 1991; Gao et al., 1991). Both EGF and TGF-α mRNAs are expressed in the cerebellum of mice, and their developmental profiles are parallel except that TGF-α mRNA levels are 15 -170 times higher then those of EGF (Lazar, 1991; Lazar and Blum, 1992). Since both factors bind to EGFR (also called ErbB-1, a member of the ErbB/EGFR family of receptors) with equal affinities (Massague, 1983; Ebner and Derynck, 1991; French et al., 1995) I chose to investigate the expression of EGFR.

The FGF receptors, transmembrane proteins, are expressed widely the developing CNS, including cerebellum (Miyake et al., 1995; Ozawa et al., 1996). bFGF is reported to function as a survival, mitogenic and differentiation factor for granule cells of the cerebellum depending on the culture conditions (Hatten et al., 1988; Morrison et al., 1988; Abe et al., 1991; Gao et al., 1991; Trenkner et al., 1996; Luo et al., 1997; El Idrissi et al., 1998). For example, Hatten (Hatten et al., 1988) showed that neurite extension by granule cells was dependent on astroglial derived bFGF, additionally, the influence of bFGF on neurite extension and survival was dependent on plating cell density.

To investigate whether thyroid hormone regulated the expression of EGFR and bFGF in my system, mixed cerebellar cells were cultured at 1.5×10^5 cells/1.8 cm^2 with and without T_3 for 5 days. Cells were harvested and processed

for protein SDS-PAGE, followed by EGFR, bFGF and actin immunoblotting. Due to the variability introduced into each experiment by unavoidable variations in culture conditions, SDS-PAGE sample loading, transfer efficiencies, and immunoblotting signal intensity, I report the results of two individual experiments (Fig. 5). Optical densities (OD's) of EGFR- and bFGF-immunoreactive bands were normalized to the OD of actin in each lane. Values were then expressed as percents of control levels. Results demonstrate the presence of one EGFR-immunoreactive band at 170 kDa in control cultures (Fig. 5A), identical to that exhibited by A431 cells, which have amplification of EGFR DNA (Merlino et al., 1984). The intensity of this band increased with T_3 treatment by 45% and 60%, respectively, when normalized against the actin signal at 41 kDa and the normalized control signal (Fig. 5B). Blots processed for bFGF immunoreactivity exhibited bands at 17.5 kDa. T_3-treated cultures displayed a reduced intensity of this band to 55% of the intensity of the control band in one experiment, and to undetectable levels in the other, when normalized to the actin signal (Figs. 5C and 5D). In the same culture system in which I demonstrated that T_3 treatment results in the attenuation of glial proliferation and the induction of neuronal and glial phenotypes, the growth factors EGF and bFGF are concomitantly up- and down-regulated, respectively.

Discussion

Thyroid hormone is known to be essential for normal brain development, and the macroscopic effects of altered thyroid hormone states in the brain have been extensively studied in the intact cerebellum (see (Grave, 1977; Porterfield and Hendrich, 1993) for references). Because of the complexity of the nervous system, studies that address the mechanism of action of thyroid hormone at the molecular level have been performed in vitro. In this manner, cerebellar cells are

removed from their complex environment and placed into a simpler context where they can be more easily manipulated. While previous studies have reported the effects of thyroid hormone on purified neuronal or astroglial populations (Nunez et al., 1991; Heisenberg et al., 1992; Trentin and Neto, 1995; Trentin et al., 1995; Lima et al., 1997; Lima et al., 1998; Gomes et al., 1999), these culture systems did not take into account the important interactions between neurons and glia that significantly influence cerebellar development (Hatten and Liem, 1981; Hatten et al., 1984; Hatten, 1985; Nagata et al., 1986; Hatten, 1987; Hatten et al., 1988; Gao et al., 1991). In the study presented here, I examined the effects of thyroid hormone in mixed cerebellar cell cultures, allowing for neuronal-glial interactions.

Thyroid hormone receptors (TRs) have been shown to be expressed in both type and type 2 astrocytes in vitro (Carlson et al., 1996; Lou and Miller, 1996). However, the distribution of TRs is heterogeneous. For example, while only TRβ2 mRNA has been detected in type 1 astrocytes, mRNA for all of the isoforms (α1, α 2, β1, β 2) have been detected in type 2 astrocytes and oligodendrocytes (Carlson et al., 1996; Baas et al., 1998; Carre et al., 1998). Thus, the expression profiles of various TR subtypes in different precursor populations may transduce specific differentiation signals, resulting in the generation of heterogeneous subtypes.

The effects of T_3 on cerebellar development are cell density-dependent

To characterize the effects of T_3 on cerebellar development, I plated mixed cerebellar cells at two different densities, thus maximizing the effects of proliferation and differentiation. It is known that plating cells at a high density promotes rapid conditioning of the medium that can override the effects of exogenous factors, such as T_3 (Balazs et al., 1985. Plating at a very low density

could magnify the effects of T_3, but lead to rapid cell death of control cultures or cause cells to differentiate prematurely, even without T_3 (Barres et al., 1994). A study by Young and colleagues reported a critical plating density for neuronal survival, below which survival was poor (Young et al., 2000). In their experiments, conditioned medium from high-density cultures increased survival in the low density-cultures, suggesting that soluble factors mediated cell survival. In general, protein synthesis and accumulation in culture have been shown to depend on cell density (Yamada et al., 2000). In a similar fashion, the biological functions of EGFR and bFGF vary depending on their environmental context, including cell density (Hatten et al., 1988; Matsuda et al., 1990; Gao et al., 1991; Engele and Bohn, 1992; Wells, 1999). I found that plating density was a crucial parameter for characterizing the effect of T_3 on development of neurons and glia: cultures plated at 1.5 $X10^5$ cells/1.8 cm^2 were suitable for characterizing T_3's effect on cell differentiation, and cultures plated at 2.0 X 10^5 cells/1.8 cm^2 were more sensitive to the effect of T_3 on cell proliferation. Since the relative densities differ by only 33%, this supports the notion of a threshold for cell-cell interactions in my culture system. It appears that the temporal course of development was slower at low density than at high density, and accelerated by thyroid hormone. It has been suggested that thyroid hormone provides a signal for granule cells to exit the proliferative cycle and to begin the differentiation process (Nicholson and Altman, 1972). In the hyperthyroid state, this leads to premature granule cell differentiation ultimately resulting in fewer granule neurons (Nicholson and Altman, 1972; Lauder, 1977). On the subcellular level, thyroid hormone influences the development of the neuronal cytoskeleton, including microtubule assembly (Nunez et al., 1991), and the expression of microtubule-associated proteins, including tau (Francon et al., 1977; Benjamin et al., 1988). The PHF-1 antibody recognizes phosphorylated tau in developing brain, a subpopulation of

vulnerable neurons in Alzheimer's disease (Greenberg et al., 1992), and stains all rodent neurons in culture to varying degrees. All granule neurons in my culture system, as defined by their morphological characteristics, displayed PHF-1-immunoreactivity. With this information, I interpreted the appearance of the PHF-1 epitope to represent neuronal differentiation, as it does in human development. Interestingly, PHF-1 expression was followed, to a certain degree, by cell death in T_3-treated cultures. However, it is not known whether the expression of this phospho-epitope is causally linked to neuronal vulnerability in rodent cultures.

Developing astrocytes contain vimentin, a ubiquitous cytoskeletal protein (Dahl, 1981). As they mature, they replace vimentin with GFAP (Eng, 1985). This is accompanied by morphological transformations (Dahl, 1981; Dahl et al., 1981). Numerous studies have demonstrated that astrocyte development, as well as the development of other types of glia is regulated by thyroid hormone (Clos et al., 1980; Aizenman and de Vellis, 1987; Gould et al., 1990; Gavaret et al., 1991; Trentin et al., 1995; Moura Neto et al., 1996; Paul et al., 1996; Trentin, 1997; Lima et al., 1998). Since my culture system contained numerous types of glia, I used immunocytochemical markers to identify and monitor and quantify the development of each type, and the effects of thyroid hormone on this development.

My results demonstrate that T_3 had significant effects on neuronal and glial differentiation in cultures plated at the lower density (see above) at all time points analyzed (Fig. 1, 3, and 4). Specifically, I observed increases in the percentages of PHF-1^+ neurons in T_3-treated cultures after 3 and 5 days of treatment, and a decrease relative to control cultures after 7 days of treatment.

T_3 has been postulated to signal granule cells to exit the proliferative cycle and begin the differentiation process (Nicholson and Altman, 1972; Lauder, 1977)

(Hadj-Sahraoui et al., 2000). I found that T_3 significantly decreased cell proliferation in cultures plated at 2.0×10^5 cells/1.8 cm^2, as demonstrated by BrdU labeling (Fig. 2). From their large, oval nuclei it appeared that the majority of BrdU-labeled cells (90-95%) were of non-neuronal origin, and that some of these cells were astrocytes, demonstrated by double-labeling with BrdU and GFAP (Fig. 2B). Granule neurons did not proliferate in the presence or absence of T_3 in my culture system, as others have demonstrated in purified granule cell cultures using conditioned medium from T_3-treated astrocytes (Gomes et al., 1999). Interestingly, there were no differences in BrdU incorporation between control and T_3-treated cultures at the lower density (data not shown). These cultures displayed a minimal amount of BrdU labeling (<1%) in the presence or absence of thyroid hormone. This may be due to a lack of cell-cell contact, or reduced concentrations of growth factors that enable the cell cycle.

In contrast to my observations, Trentin and colleagues have reported that T_3 treatment increased astrocyte proliferation in vitro (Trentin and Neto, 1995; Trentin et al., 1995). However, their cultures were enriched for astrocytes and established from prenatal rat cerebella, so they did not contain granule neurons, important determinants of glial cell morphology and activity. A study by Gao and colleagues demonstrate that glia cease to proliferate when they are cultured with neurons (Gao et al., 1991). Therefore, these seemingly opposite actions of thyroid hormone may depend on the substrate on which cells were plated, the cell density, allowing or inhibiting cell-cell contact, or the relative proportions of neurons and glia.

In my culture system T_3 significantly increased the proportion of GFAP+ cells at DIV 5, (Figs. 1C and 4A), consistent with studies where T_3 treatment increased the appearance GFAP+ cells in cultures of forebrain and mesencephalon, (Gould et al., 1990; Lima et al., 1997) and in intact rat cerebellum

(Faivre-Sarrailh et al., 1991). The mechanism for this increase can be due to differentiation of existing precursors, or by expansion of the cell type by proliferation. Several studies demonstrated that the numbers of astrocytes increased due to glial proliferation (Trentin and Neto, 1995; Trentin et al., 1995). I see a decrease in proliferation (Fig. 2).

T_3 increases differentiation of glial subtypes

O2-A precursor cells divide a limited number of times before they differentiate. The time of differentiation is dependent on intrinsic and extrinsic signals. Thyroid hormone and retinoic acid have been shown to be two of the extrinsic signals that trigger O-2A cells to stop dividing and begin differentiating in purified O2-A cell cultures (Barres et al., 1994). Type 2 astrocytes, identified by immunocytochemistry for A2B5, a cell surface ganglioside, in conjunction with GFAP, develop from O-2A precursor cells in culture medium containing 10% serum, whereas in medium without serum the precursors differentiate into oligodendrocytes (Raff et al., 1983). This cell type constituted a very small percentage (0.5- 2.0%) of the total cell population in my culture system. T_3 significantly increased the numbers of $A2B5^+/GFAP^+$ cells compared to untreated cultures after 5 days in serum free medium (Fig. 3B). All four TR isoforms have been detected in type 2-astrocytes (Carlson et al., 1996), suggesting that they are direct targets for the actions of T_3.

It is also known that neurons and astroglia secrete factors that influence oligodendroglial differentiation (Hardy and Reynolds, 1993). Therefore, I decided to assess whether the presence of neurons and astroglia in my culture system would modify these effects. Results demonstrate that T_3 significantly increased the number of $O1^+$ and GC^+ cells (Figs. 3C and 3D), suggesting that T_3

is necessary for the differentiation of these cells even in the presence of neurons and astrocytes.

T_3 accelerates the switch of astrocyte morphology from "radial-like" to velate

Several studies on the effect of T_3 on astroglial morphology have been carried out using purified or enriched astroglial cultures in confluent or sub-confluent conditions (Trentin and Neto, 1995; Trentin et al., 1995; Lima et al., 1997). T_3 treatment induced process formation in astroglia isolated from P1-P2 cerebellar tissue, as well as induce proliferation (Trentin and Neto, 1995; Trentin et al., 1995; Lima et al., 1997). My results differ from these in that most of the $GFAP^+$ cells in my culture system, derived from P6-P10 cerebellar tissue, started out with morphology resembling radial glia, also known as Bergmann glia. Hatten's group describes 2 types of astrocytes, which they name "Bergmann-like and astrocyte" (Hatten et al., 1984). While the proportion of radial-like glia decreased, the proportion of stellate and velate astrocytes increased, suggesting that the radial-like glia underwent a morphological transformation from one phenotype to the other. T_3 treatment accelerates this shift, maximizing the proportion of velate astrocytes at DIV 5, whereas in control cultures this occurs at DIV 7. Also, it appeared that the stellate morphology (Fig. 4B) was a transitory stage between radial and velate astroglia. These morphological phenotypes are reminiscent of those found in the immature cerebellum. <u>In vivo,</u> Bergmann glia, extend radial processes that are believed to aid migration of postmitotic granule cells from the pial surface to the internal granule layer. Within the internal granule cell layer velate "protosplasmic" astrocytes extend branched fibers that organize the mature granule cells into compartments (Rakic, 1971). The influence of T_3 on the kinetics of these transitions suggests that thyroid hormone plays a role in these morphological transformations,

orchestrating granule cell migration, astrocyte differentiation, etc. It follows that if hyperthyroidism prematurely inhibited proliferation of glial precursors, and accelerated astroglial maturation during this critical stage of development, it could result in reduced numbers of mature astrocytes in the IGL. This in turn may affect cerebellar cytoarchitecture, the survival of granule neurons, and their synaptic connections with Purkinje cells.

The effects of T_3 on neuronal and glial development may be mediated by bFGF and EGFR

Over the last decade much attention has been paid to the multiple roles played by neurotrophic factors in brain development. Previous studies have suggested that the neurotrophins and insulin-like growth factors have demonstrated effects on granule cell development, and are regulated by thyroid hormone (Walker et al., 1979; Timiras and Nzekwe, 1989; Hashimoto et al., 1994; Neveu and Arenas, 1996; Elder et al., 2000). There is also ample evidence that members of the EGF and FGF growth factor families play a role in cerebellar development. Moreover, thyroid hormone regulates EGFR expression in brain (Stein et al., 1989; Stein et al., 1991) and in other tissues (Gresik, 1981; Kesavan et al., 1991; Hume et al., 1992; North et al., 1992; Rogers et al., 1995; Rogers et al., 1996). Granule cells express high levels of FGF receptors in the developing and adult cerebellum (Miyake et al., 1995; Ozawa et al., 1996), supporting the notion that astroglial bFGF could directly influence the development of cerebellar granule cells. Basic FGF has been shown to promote both the migration of granule cells along "Bergmann-like" astrocytes, and neurite outgrowth, in cultures where granule cells were dispersed as a monolayer. In contrast, bFGF promoted granule cell proliferation when injected into mice (Tao et al., 1996; Cheng et al., 2001), or cultured in macrocellular aggregates (Hatten et al., 1988;

Gao et al., 1991). In dispersed cultures, cerebellar astrocytes expressed an 18 kDa protein that was immunoprecipitated by antibodies to bFGF. These antibodies were able to neutralize the effects of astrocytes on neuronal migration, suggesting that bFGF mediated cell-cell interactions. Other studies have shown that exogenous bFGF increased neurite outgrowth and survival of granule cells (Morrison et al., 1988; Abe et al., 1991). This suggests that bFGF can function as a mitogen, a differentiation factor, a survival factor, and a migration factor for granule cells, but it is unclear how the signal transduced by bFGF is interpreted to result in one type of response in this wide range of possibilities. There are many possible explanations. At the cellular level, it has been demonstrated that both plating density and neuron/glia interactions have significant effects on the activity of bFGF (Hatten et al., 1988; Gao et al., 1991) . At the molecular level, the abundance of bFGF may determine the cellular response. For example, it has been demonstrated that low concentrations of bFGF are neurotrophic, while higher concentrations are needed to stimulate cell proliferation under identical culture conditions (Casper et al., 1994). It is also known that bFGF can serve as either an intracellular or intranuclear signal (Walicke and Baird, 1991; Giordano et al., 1992; Woodward et al., 1992). Perhaps variations in intracellular compartmentalization may account for its pleiotrophic actions. Another possibility is based on evidence that bFGF exists in several isoforms generated from RNA processing (Giordano et al., 1992), and can be metabolized to smaller fragments as the result of proteolysis (Walicke and Baird, 1991). I observed a decrease in the 17.5 kDa isoform of bFGF at DIV 5 in T_3 treated cultures, coinciding with the increase in neuronal and glial differentiation. Therefore, the decrease in the 17.5 kDa isoform may reflect down-regulation at several levels, including transcription, translation, and post-translational modifications. Although I did not observe any other bFGF-immunoreactive bands on the

western blot, this may be due to the optimization of electrophoresis and blotting parameters for detection of the 17.5 kDa species. It is also possible that the antibody I used did not recognize other isoforms of bFGF that appeared as the signal at 17.5 kDa disappeared. Finally, since bFGF binds to a family of membrane receptors (Unsicker et al., 1992) it is possible that this ligand stimulates more than one signal transduction pathway, resulting in dramatically different responses on the cellular level. The larger question of how processing and receptor expression may be regulated is unknown.

Basic FGF is also known to be mitogenic for astrocytes and O-2A cells in vitro (Engele and Bohn, 1992). I showed a significant increase in the percentage of astrocytes and oligodendrocytes in T_3-treated cultures at DIV 5, coincident with a decrease in bFGF expression. This suggests that T_3 may trigger O-2A precursors to cease proliferation and differentiate by down-regulating the expression of bFGF. This is not reflected in a change in total cell numbers, because these cells comprise only a small fraction of the total cells.

The EGF family of growth factors also have been shown to have pleiotrophic actions on many cell types, including neurons and glia (see (Morrison, 1993) for review and references). Results from previous studies in vivo have suggested that EGF ligands play a role in cerebellar development (Lazar and Blum, 1992; Seroogy et al., 1993; Seroogy et al., 1995; Kornblum et al., 1997). Moreover, Seroogy (Seroogy et al., 1995) and Kornblum (Kornblum, 1997 They found EGFR mRNA expressed throughout the EGL, but with an increasing hybridization gradient from the pial aspect to the premigratory (inner zone of the EGL) zone, consistent with my findings in vivo.

In the accompanying study I demonstrated that experimental hypothyroidism transiently attenuated the expression of EGFR mRNA, and that of its ligand, TGF-α, in the postnatal cerebellum. In addition, the percentage of

EGFR-IR cells in the pre-migratory zone of the EGL was lower in hypothyroid animals. This suggests that EGF ligands may play a role in the post-mitotic development of granule neurons. Thyroid hormone has been postulated to signal granule cells to exit the proliferation cycle and begin the differentiation process (Nicholson and Altman, 1972; Lauder, 1977) (Hadj-Sahraoui et al., 2000). Therefore, is possible that one mechanism of thyroid hormone's actions is, either directly or indirectly, through up-regulation of EGFR.

In vitro, EGF has been shown to increase neuronal survival (Morrison et al., 1988; Abe et al., 1990; Abe et al., 1991), but under certain conditions, EGF treatment induces neurogenesis and gliogenesis (Leutz and Schachner, 1981) (Morrison et al., 1988). In the present study I found that EGFR protein levels increased in T_3-treated cerebellar cells at DIV 5, coincident with neuronal and glial differentiation. As described for the actions of bFGF and thyroid hormone, the different responses to EGF family ligands may be due to cellular and molecular factors (Avola et al., 1991). For example, co-culture of neurons and astrocytes demonstrate very different characteristics, including the cessation of glial proliferation, changes in morphology, and increased neuron-glial associations, than the culture of neurons or glia alone (Hatten, 1985). Also, younger (progenitor) cells may proliferate in response to EGF, while older (post-mitotic) cells may differentiate. Based on the distribution and time course of expression of EGFR, and its up-regulation after thyroid treatment, the effects of EGF-family ligands are most likely related to post-mitotic events in cerebellar development, such differentiation, migration and survival. On the molecular level, it is possible that the relative abundance of EGF receptors may determine whether cells proliferate or differentiate. Studies have shown that low levels of EGFR expression enhance proliferation whereas high levels result in premature differentiation of retinal and cortical progenitor cells (Burrows et al., 1997; Lillien

and Wancio, 1998). Moreover, EGF induced PC 12 cells to differentiate into a sympathetic-neuron like phenotype in cells that over-expressed EGFR (Traverse et al., 1994), but wild-type PC12 cells proliferate in response to EGF. In immortal cell lines, different signaling pathways control the various actions of EGFR, ranging from mitogenesis, differentiation, dedifferentiation and migration, and that these pathways activated are subject to spatial and temporal control (Wells, 1999). Future studies are warranted to examine the response of the various EGF-induced intracellular signaling pathways of thyroid hormone.

In summary, my results demonstrate that T_3 sets in motion a program of coordinated differentiation of neural cells, such as granule neurons, astrocytes, and oligodendrocytes. These effects are density-dependent. Based on evidence that T_3 treatment results in different effects in purified cell cultures, the effects I observed probably depend on neuronal-glial interactions. I also demonstrate that T_3 treatment induced EGFR and down-regulated bFGF proteins at a time point (DIV 5) when a significant increase in neuronal and glial differentiation was observed. Since many of the actions of EGF and bFGF overlap with the effects seen with changes in levels of thyroid hormone in vivo and in vitro, it is possible that these factors may mediate the effects of thyroid hormone.

Figure 9. The effects of T_3 on neuron and astrocyte differentiation are cell-density dependent. Primary cultures of dissociated postnatal mouse cerebellum were established as described in the MATERIALS and METHODS section. Cells were plated at 1.5 X10^5/ 1.8 cm^2 and 2.0 X 10^5/ 1.8 cm^2. Cultures were treated with 2 ng/ml (final concentration) on DIV 1 and subsequently fixed on DIV3, 5, and 7 followed by routine immunocytochemistry. Neurons and astrocytes were identified by staining for PHF-1 and GFAP, respectively. Total cell number, neuronal and astrocytic elements were quantified by counting cells of each type in 4-6 fields of 3-4 replicate dishes under 200X magnification under phase optics for total cell counts, or bright field optics for immunostained cells. PHF-1^+ and GAFP $^+$ are expressed as a percentage of the total cells per field. A) The percentage of PHF-1^+ cells plated at 1.5 X10^5 cells/1.8 cm^2 on DIV 3 and DIV 5 and DIV 7. (B) The percentage of PHF-1^+ cells plated at 2 X 10^5 cells/1.8 cm^2 on DIV 3, 5, and 7 (C) The percentage of $GFAP^+$ cells in cultures plated at 1.5 X10^5 cells/1.8 cm^2 on DIV3, 5 and 7. (D) The percentage of GFAP $^+$ in cultures plated at 2.0 X 10^5 cells/1.8 cm^2 on DIV 3, 5 and 7. (E) Total cell number in cultures plated at 1.5 X105 on DIV3, 5 and 7. (F) Total cell number in cultures plated at 2.0 X10^5. Data are ± SEM (error bars) values of 3-4 cultures per treatment. Statistical analysis was done by a factorial ANOVA with a post hoc Fisher PLSD test. Asterisks denote values that are significantly different between treatments (*$p < .03$); within treatment (** $p < .002$); between and within treatment (***$p < .0003$).

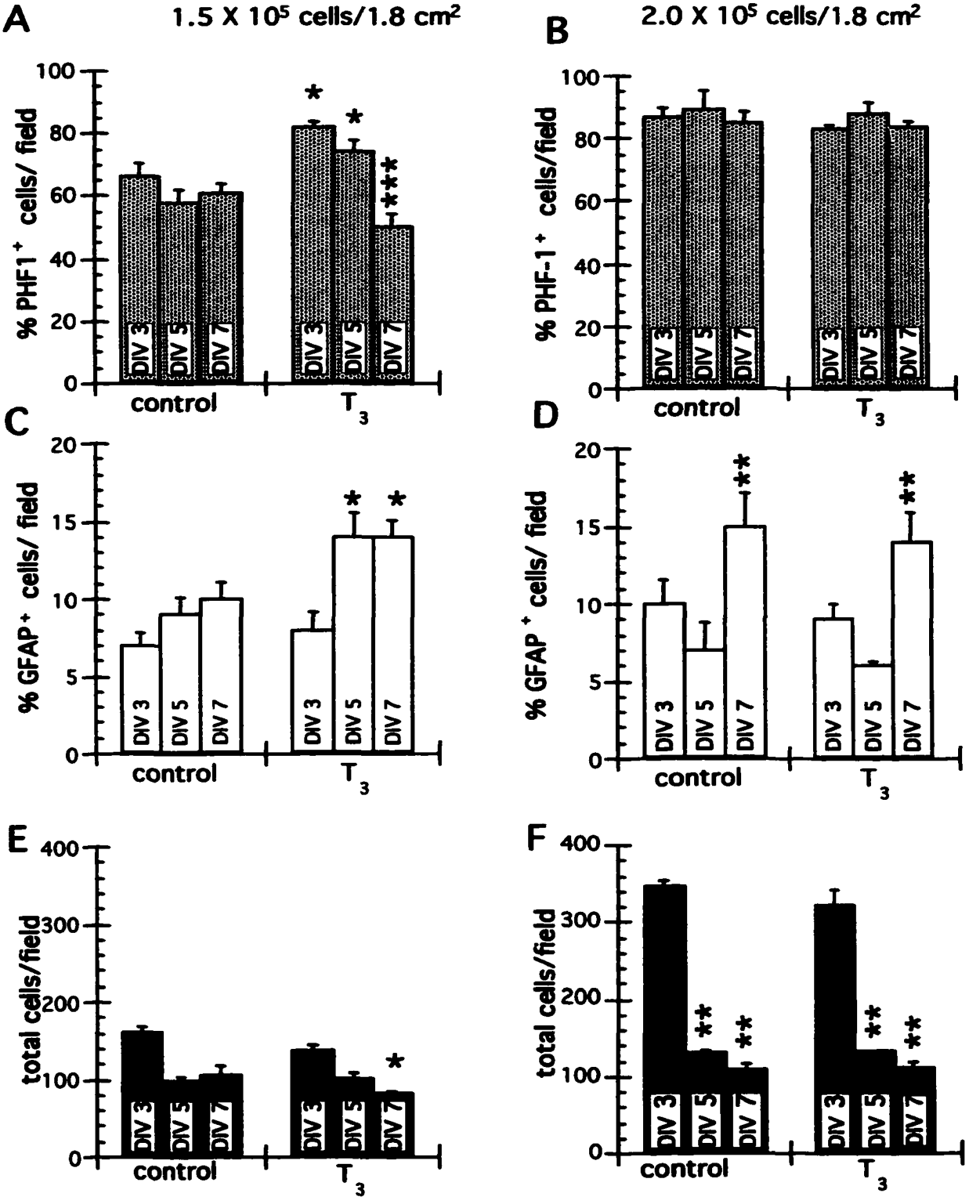


Figure 10. T_3 decreases cell proliferation of non-neuronal cells. Cerebellar mixed cell cultures were prepared from early P6 or P7 mice and treated with T_3 at DIV 1 and incubated with BrdU at DIV 4 for 21 hours. Cultures were then fixed and processed for immunocytochemistry on DIV 5. **(A)** Percentage of BrdU labeled cells in mixed cerebellar cell cultures at DIV 5 plated at $2.0 \times 10^5/1.8$ cm^2. **(B)** BrdU (black arrowhead) and GFAP (white arrowhead) double labeled immunocytochemistry. Data are ± SEM (error bars) values of 3-4 cultures per treatment. A one-way ANOVA with a Fisher PLSD post hoc analysis was done. Asterisks denote values that are significantly different ($p < .02$) from control. The figure contains data from one representative experiment. Scale bar = 50 μm.

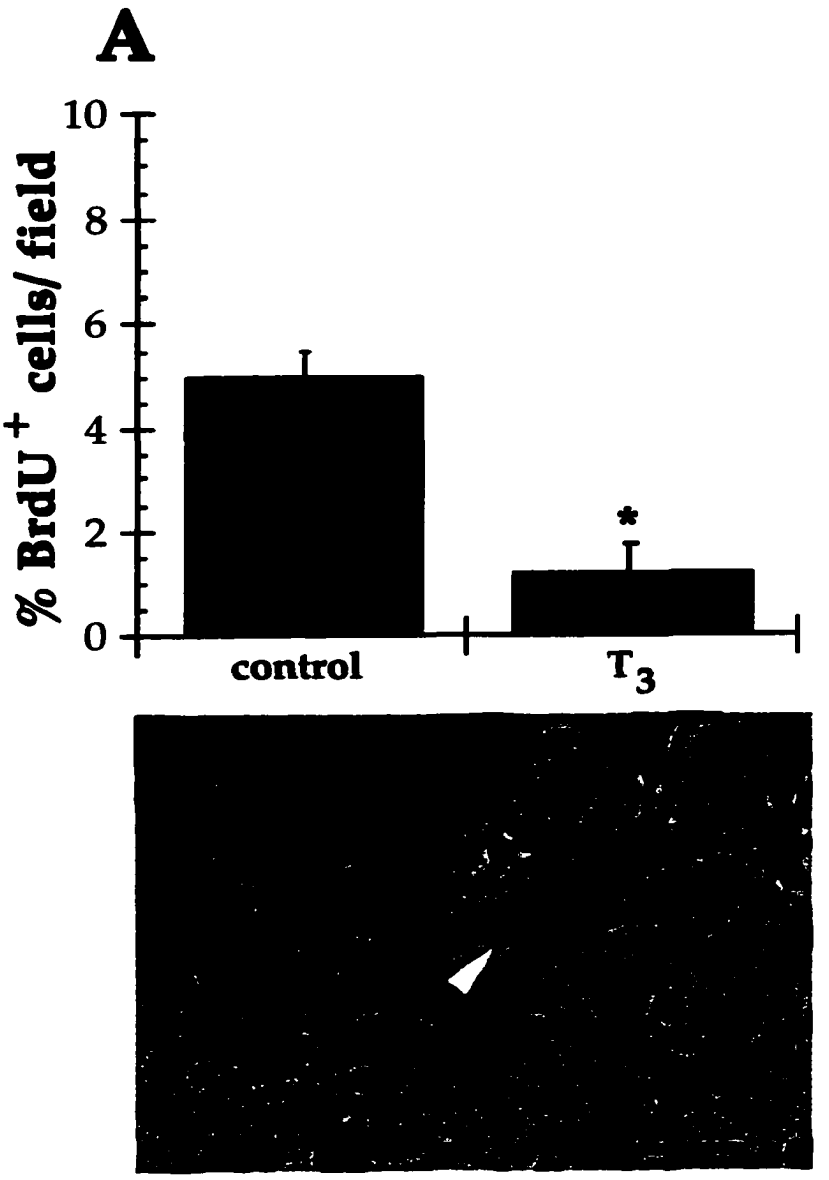


Figure 11. T_3 increases differentiation of glial subtypes. Replicate cultures of mixed cerebella cells were treated with T_3 for 5 days, then fixed and immunocytochemistry for **(A)** GFAP **(B)**A2B5 **(C)** GC and **(D)** O1 was performed. Double label immocytochemisty for A2B5 and GFAP was also performend. Data are ± SEM (error bars) values of 3-4 cultures. A one-way ANOVA followed by a Fishers PLSD post hoc was performed comparing the cells of each subtype. Asterisks denote values that are significantly different ($p < .05$) from control.

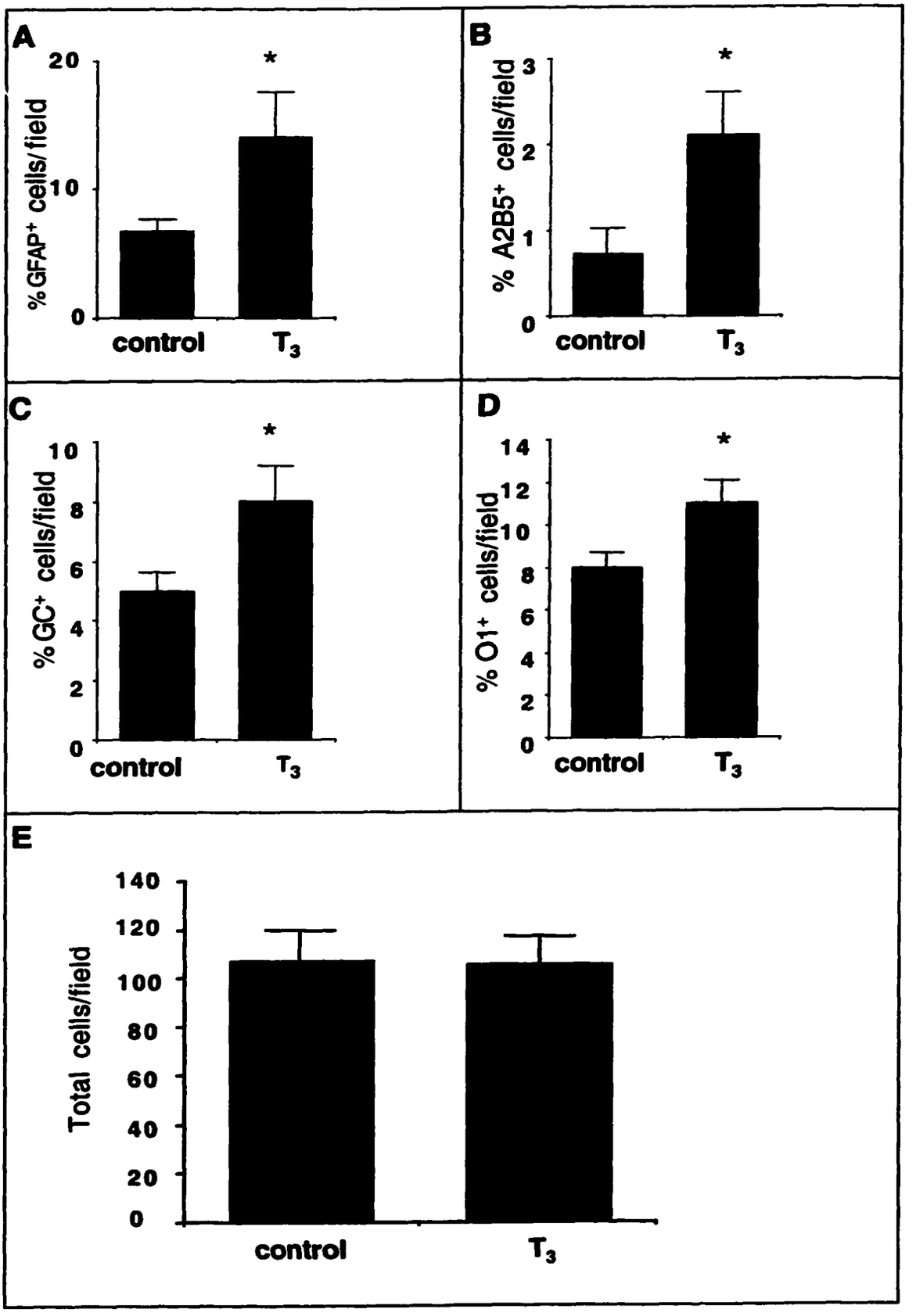


Figure 12. T_3 treatment accelerates morphology of astrocytes from "radial-like" and stellate to velate. Cultures were established as described and treated for 3, 5 and 7 days with T_3. Cultures were fixed and immunocytochemistry was performed for GFAP. Open circles represent T_3-treated cultures; black circles represent control cultures **(A)** "radial -like" astrocytes time course **(B)** "radial-like astrocyte morphology **(C)** stellate astrocytes time course **(D)** stellate astrocyte morphology **(E)** velate astrocytes time course **(F)** velate astrocyte morphology. Data are ± SEM (error bars) values of 3-4 cultures. A factorial ANOVA followed by a Fishers PLSD post hoc was performed comparing the $GFAP^+$ cells of each morphology. Astericks denote values that are significantly different ($p < .01$) Scale bar = 50 μm

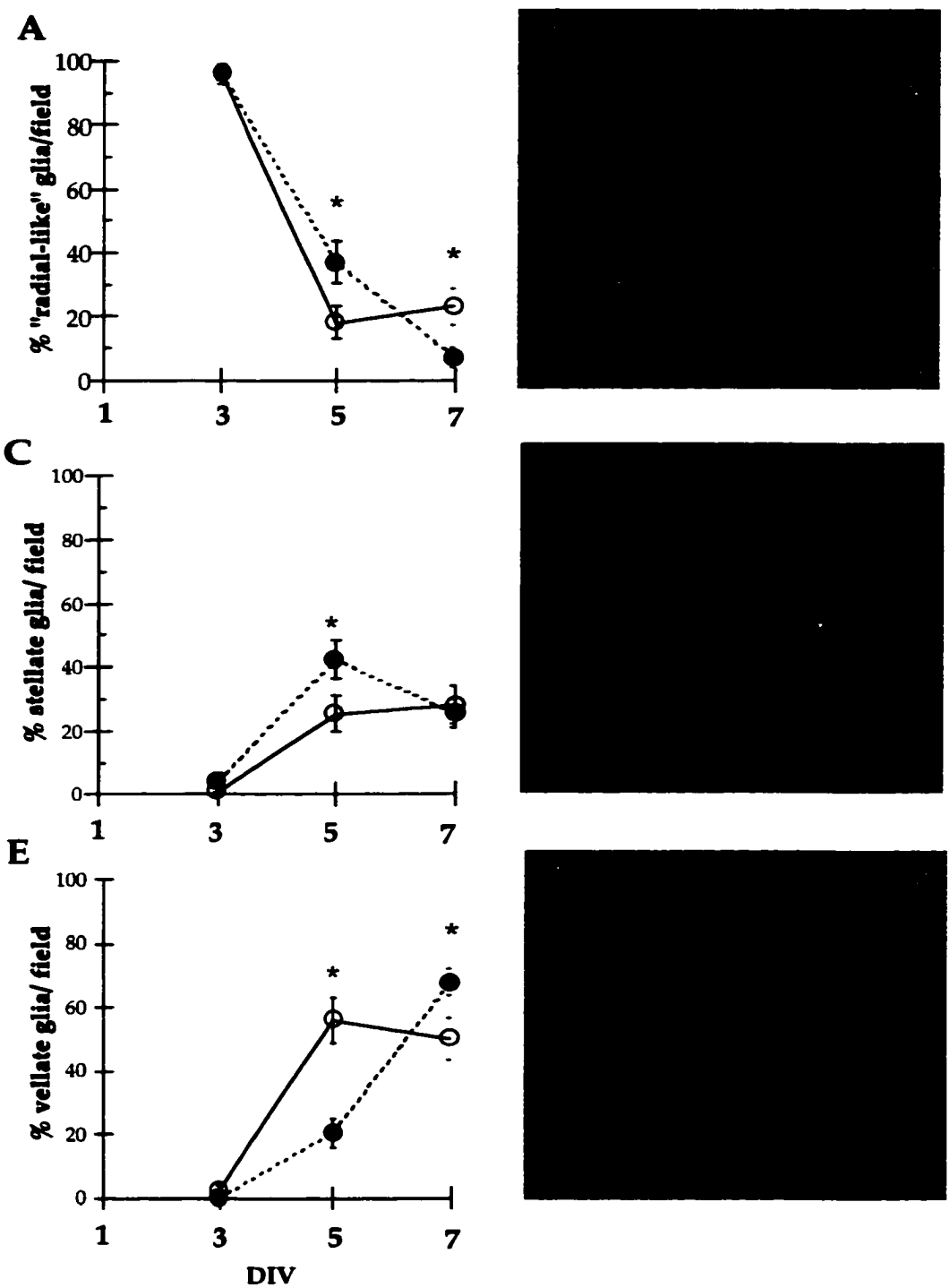
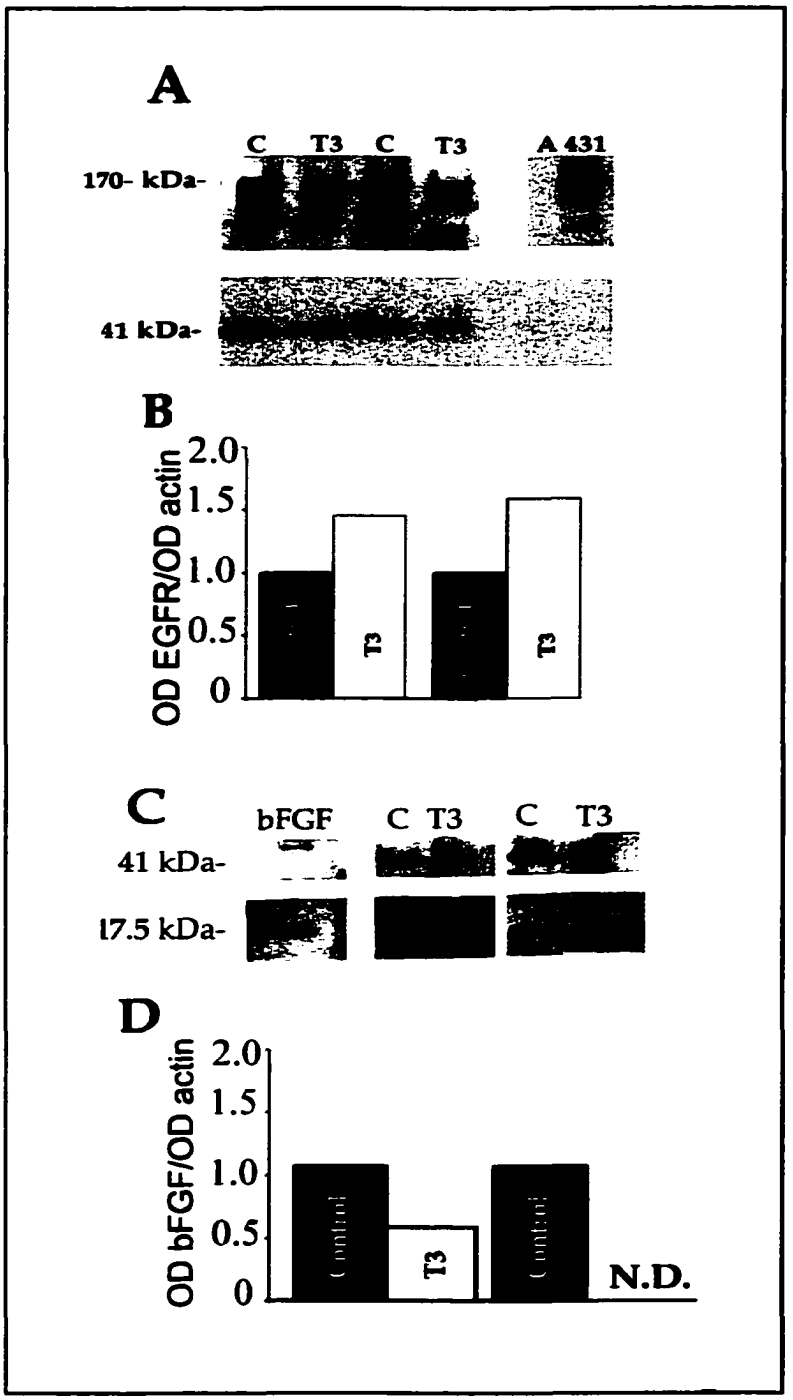


Figure 13. T_3 regulates EGFR and bFGF proteins. Cerebellar cell cultures were established and treated with T_3, as described. Cell lysates were collected on DIV 5 and processed for immunoblotting. (A) EGFR expression in control and T_3-treated cultures. A431 cell lysate (2 μg) was used as a positive control. Bands at 170 kDa identify EGFR. Bands at 41 kDa represent actin. (B) Relative abundance of EGFR normalized for actin expression. (C) bFGF expression in control and T_3-treated cultures. Bands at 17.5 kDa identify bFGF, as labeled in the positive control blot of recombinant bFGF protein. Bands at 41 kDa represent actin. (D) Relative abundance of bFGF normalized for actin expression. N.D. = not detectable.



Chapter 4

General Discussion

It is well established that thyroid hormone is essential for normal CNS development. The main mechanism of action of thyroid hormone is transcription of target genes. Growth factors are also suggested to be essential for normal development of neurons and glia. Thus, the effects of thyroid hormone are suggested to be mediated through growth factors. An interaction between thyroid hormone and the neurotrophins, such as NGF, has been shown in the CNS. Reports on the effect on the epidermal growth factor family are limited, however, even though its members are widely distributed in the developing and adult CNS. Members of the epidermal growth factor family, such as epidermal growth factor (EGF), transforming growth factor-alpha (TGF-α) and their common receptor, the epidermal growth factor receptor (EGFR) are reported to be "potent mitogens" for somatic and CNS cells. I chose to investigate the effects of thyroid hormone on EGFR and TGF-α expression in the developing cerebellum of the mouse based on the correlational observation described below.

A brief review on the postnatal development of the cerebellum, postnatal thyroid hormone function, TGF-α and EGF expression is provided here: the fourth ventricle gives rise to the deep cerebellar neurons, besides Purkinje and Golgi II cells. Also, some of the progenitors located in the progenitor zone migrate to the external surface of the cerebellum. The presumptive granule neurons, continue to proliferate until the entire surface of the cerebellum is covered, forming the EGL. The EGL reaches maximal thickness between P5 and P10. It has been hypothesized that thyroid hormone provides the signal that terminates granule cell proliferation and stimulates their differentiation, since in hypothyroid animals the disappearance of the EGL is retarded and in hyperthyroid animals it is accelerated. Thyroid hormone levels

levels in the rodent are baseline until postnatal day 6 when they begin to rise to reach peak levels by postnatal day 14-15. This rise in (P6 -P10) thyroid hormone is positively correlated with the peak of presumptive granule cell proliferation, granule cell migration and EGF and TGF-α mRNA gene expression. Second, the peak of thyroid hormone concentration is negatively correlated with the decrease in granule cell proliferation and migration, and TGF-α and EGF gene expression.

Since EGF and TGF-α and their receptor are usually associated with proliferation, I hypothesized that one way thyroid hormone might terminate proliferation of presumptive granule cells would be by down regulating TGF-α and/or EGFR gene expression. Thyroid hormone has been shown to down-regulate the expression of these factors in tissue outside the CNS. Thus, inhibiting thyroid hormone production should maintain the mRNA levels of these polypeptides elevated past P10. Moreover, EGFR expression should be densest in the outer (proliferative zone) segment of the EGL.

In chapter 2, I showed that the results did not support my hypothesis. Hypothyroidism significantly but transiently attentuated TGF-α and EGFR mRNA expression during the early postnatal period in the cerebellum. This suggest that it could be the rise in thyroid hormone concentration that is necessary for the timely expression of these genes. Additionally, the spatial and temporal distribution of EGFR suggests that this polypeptide is correlated with postmitotic not mitotic events in the developing cerebellum. This is of interest given that the EGF family of growth factors is generally thought of as mitogens.

In chapter 3, I showed that thyroid hormone accelerated neuronal differentiation when cells were plated at an optimal density, decreased proliferation of cells of non- neuronal origin, increased glial differentiation,

and accelerated a switch in astrocyte morphology from "radial-like" to velate. Analyses of mixed cerebellar cell lysates by Western blotting showed that thyroid hormone increased EGFR but reduced basic fibroblast growth factor (bFGF) expression during cell differentiation. Taken together these results suggest that thyroid hormone effects on neuron differentiation are indirect, and may be mediated by regulation of growth factor expression. Additionally, it confirms the in vivo observation that EGFR is implicated in postmitotic developmental events. Moreover, bFGF may be implicated in the early phase of cerebellar cell development, for its expression decreases with differentiation. Since I discussed in detail the significane of the results for each study in the discussion section of chapters 2 and 3, remainder of this chapter focuses on what I suggest to be the most significant finding of this study

EGFR expression is regulated by thyroid hormone in vivo and in vitro

Using a nuclease protection assay I showed that postnatal hypothyroidism attenuated significantly but transiently EGFR and TGF-α in the cerebella of hypothyroid mice (Fig. 2B). The distribution of EGFR immunoreactivity was confined to the inner segment (premigratory zone) of the EGL at P6 and P9, the peak of granule cell proliferation and migration in both normal and hypothyroid cerebella. This confirmed that cells of the outer segment (proliferative zone) of the EGL proliferated independently of EGFR and its ligands. Nonetheless, the percentage of EGFR immunopositive cells in the premigratory zone of the hypothyroid cerebella were significantly lower than that of the normal cerebella. Furthermore, the peak of EGFR immunoreactivity in the EGL was shifted to P14 in the hypothyroid cerebella, compared to P12 in the normal cerebella. This shift partly reflects the delay in

mRNA expression discussed above. In line with these results, EGFR protein content increased in mixed cerebellar cell cultures treated with T_3 for 5 days.

The exact mechanism of thyroid hormone action whether transcriptional or post-transcriptional remains to be determined. Thyroid hormone may regulate the expression of TGF-α and EGFR at the transcriptional level during the early postnatal period, resulting in a delay in the accumulation of the transcript perhaps, because of the presence of minimal circulating concentrations of thyroid hormone and/or a delay in the upregulation of thyroid hormone receptors. Additionally, there may be other transcription factors also involved in regulating gene expression of these polypeptides, especially during later postnatal development. Another possibility is that mRNA stability decreases in the hypothyroid state causing a delay in mRNA accumulation. Thyroid hormone may contribute to gene expression of EGFR and TGF-α by stabilizing their newly transcribed mRNAs, perhaps through the regulation of proteins that aid mRNA stability. In support of this hypothesis, parallel results to those shown here have been reported for myelin associated glycoprotein (MAG) and myelin associated proteolipid (MAL) mRNAs in the cerebellum of hypothyroid rats (Rodriguez-Pena et al., 1993; Ibarrola and Rodriguez-Pena, 1997). Thyroid hormone regulation of mRNA stability is considered to be an important mechanism controlling the expression of thyroid hormone-responsive genes (Glass and Holloway, 1990). Numerous studies have shown that mRNA half life either increases or decreases in response to T_3 (Diamond and Goodman, 1985; Simonet and Ness, 1989; Kesavan et al., 1991; Krane et al., 1991).

A potential role for EGFR in granule cell migration.

A significant finding in the present study is that EGFR immunoreactivity was located in the premigratory zone of the EGL at P6 and P9 (peak of granule cell migration) in both normal and hypothyroid cerebella. Of greater significance is that EGFR immunoreactivity was detected in the leading process of "spindle-shaped" cells, suggestive of migrating granule cells.

In normal development, granule cells make their final division in the outer segment of the EGL (proliferative zone) and then pass to the inner segment (premigratory zone) where neurite outgrowth is initiated. Two axonal processes are extended to the long axis of the folia (parallel fibers), followed by the emergence of a third vertically oriented (leading) process that descends into the underlying molecular layer. These fibers associate with radial fibers of Bergmann glia that are presumed to aid their migration towards the IGL (Rakic, 1971).

In hypothyroidism the migration of granule cells is delayed and is believed to be related, partly, to retarded growth of the parallel fibers and Bergmann glia (Clos, et al.,1980; Lauder, 1979). The strong EGFR immunoreactivity in the premigratory zone and in the leading process of spindle shaped cells found in the present study suggests that EGFR may be involved in this process outgrowth. Since Bergamann glia are distributed along the EGL and are closely associated with granule cells at this time, they may release the ligand (eg, EGF or TGF-α) that activates this receptor and or the signal that stimulates EGFR synthesis. In support of this hypothesis EGF has been shown to stimulate process outgowth of mesencephlaic, cerebellar, and cortical neurons in vitro (Abe et al., 1991; Casper et al., 1991; Abe and Saito, 1992; Casper et al., 1994). The delay in the peak of EGFR expression

observed in the hypthyroid cerebella may be secondary to the retarded growth and maturation reported for Bergmann glia.

Lauder (1979), also showed that hypothyroidism significantly reduced the rate of migration of granule cells through the molecular layer, but the rate of exit from the EGL was not affected; producing a "pile up" of migrating cells in the molecular layer next to the Purkinje cells. In line with this report I found a greater number of EGFR immunopositive cells in the same region at P22 in the hypothyroid cerebella, reflecting a "pile up" of migrating granule cells. These results suggest that EGFR is localized in premigratory and migrating granule cells, implicating this receptor and one or more of its ligands in migration. In support for a role of EGFR in granule cell migration this receptor and its ligand have been shown to aid migration of other cells, including corneal, liver, epithelial, and neural precursors (Watanabe et al., 1987; Bade and Feindler, 1988; Chen et al., 1994; Chen et al., 1994; Craig et al., 1996; Thalmann-Goethsch et al., 1997; Xie et al., 1998). Moreover, EGF-like motifs are common in certain molecules with migratory function, such as astrotactin and reelin (Hirotsune et al., 1995; Zheng et al., 1996). Also, the neuregulin, a member of the EGF family of ligands, and its receptor erbB4 have been shown to be essential for granule cell migration along Bergmann fiber (Rio et al., 1997).

Neruronal migration is an essential step in the developement of the nervous system , especially laminated regions, such as the cerbral cortex and the cerbellum (Hatten, 1999). Abnormal migration of neurons is linked to mental retardation, cognitive deficits, and motor disorders (Eksloglu et al., 1996; Howell et al., 1997; des Portes et al., 1998). A number of neuronal genes have been identified as regulated by thyroid hormone (see Chapter 1). However, only reelin is directly involved in neuronal migration. The data

presented here suggest that the migration deficits obderved in the hypthyroid brain may partly be caused by alterations in EGFR expression. Therefore, the role of EGFR and/or its ligands in migration of granule cells in vivo and/or in vitro remains to be determined. Such studies should include, characterizing the dimerization pattern with other erbBs and signal pathways involved. Also, the availability of EGFR knockout mice should facilitate determining whether EGFR is involved in the migration of granule neurons.

Transformation of astrocyte morphology in vivo and in vitro

The first immature granule neurons arrive on the surface of the embryonic cerebellum at embryonic day 13 (E13) in the mouse (Miale and Sidman, 1961). Between E13 and birth they spread across the pial surface as layer of proliferating cells, the EGL. During the last few days of gestation, the Bergmann glia differentiate just beneath the EGL (del Cerro and Swarz, 1976). Bergmann glia are a specialized type of astrocytes that extend thick radial processes in the early postnatal cerebella and guide the migration of granule cells towards the IGL (Rakic, 1971). Within the IGL velate protoplasmic astrocytes extend numerous branched fibers that organize the mature granule cells and their dendrites into compartments (Rakic, 1971). In vivo, thyroid hormone appears to be necessary for the timely morphological maturation of astrocytes. In the adult hypothyroid cerebella the percentage of radial glia is higher compared to normal cerebella, suggesting a delay in morphological transformation (Clos et al., 1980).

In vitro studies on T_3's effect on astroglia morphology have been carried out using purified or enriched astroglial cultures in confluent or subconfluent conditions (Lima et al., 1997; Trentin et al., 1995; Trentin and Neto, 1995). In the mixed cerebellar cell culture system used in this study, morphological

the mixed cerebellar cell culture system used in this study, morphological transformation of astroglia most closely resembled the in vivo sequence. That is, radial astroglia are displaced by flat velate astroglia in the IGL. Also, T_3 appeared to be partly responsible for this morphological transformation. Thus, T_3 during critical morphological development of astroglia could prematurely accelerate astroglial maturation and ultimately result in a reduced number of mature astrocytes in the IGL, subsequently affecting the cytoarchitecture of the IGL, survival of granule neurons and their synaptogenesis with Purkinje cells.

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