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Cerebellar Astrocytes Treated by Thyroid Hormone Modulate Neuronal Proliferation

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ABSTRACT Thyroid hormones are important for neurogenesis and gliogenesis during brain development. We have previously demonstrated that triiodothyronine (T_3) treatment induced proliferation in primary culture astrocytes derived from the cerebellum of neonatal rats. Conditioned medium obtained from those T_3-treated astrocytes (T_3CM) mimicked the effect of hormonal treatment on these cells. Because neuron–glia interaction plays an important role in brain development, we tested the ability of such T_3-glial CM to influence neuronal physiology. With that aim, neurons from 19-day embryonic cerebella were cultivated for 24 h in the presence of CM obtained from T_3-treated cerebellar astrocytes. Interestingly, the cerebellar neuronal population increased by 60–80% in T_3CM. Addition of 5 μM forskolin enhanced the responsiveness of cerebellar neurons to astrocytes T_3CM, but it did not interfere with neuronal survival in control medium. Conversely, inhibition of adenylate cyclase by its specific inhibitor, SQ22536, reversed the T_3CM effect on neurons. These data strongly suggest that cAMP signal transduction pathways might be implicated in such an event. Analysis of bromodeoxyuridil incorporation revealed that the increase in neuron number in T_3CM was partially due to neuron proliferation, because the proliferation index was three times higher in T_3CM than in control medium. Neutralizing antibody assays demonstrated that T_3CM effects on neurons are due, at least in part, to the presence of tumor necrosis factor-β and epidermal growth factor. Thus, we report here a novel molecular mechanism of action of thyroid hormone on cerebellar neuronal cells: Thyroid hormone induces astrocytes to secrete growth factors that can interfere with neuronal proliferation via a paracrine pathway. *GLIA 25:247–255, 1999.*

INTRODUCTION

Thyroid hormones (mainly triiodothyronine [T_3]) exert profound effects on the growth, development, and homeostasis of vertebrate organisms (Skoloff and Kennedy, 1973; Legrand, 1982). Several steps of brain development (i.e., cell migration, outgrowth of neuronal processes, acquisition of neuronal polarity, synaptogenesis, myelination, glial cell proliferation, and neuronal cell death) seem to be affected by T_3 deficiency (Porterfield and Hendrich, 1993; Bernal and Nunez, 1995; Garcia-Segura et al., 1996). These events are seen in all the regions of the central nervous system (CNS) but are better observed in the developing cerebellum (Legrand, 1982; Bernal and Nunez, 1995). Thyroid hormone effects on the CNS are mediated by the binding of the

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hormone to its nuclear receptors, which have been recognized in neurons and astrocytes both in vivo and in vitro (Puymirat, 1992; Carlson et al., 1996).

In vivo, T_3 controls astrocyte number and maturation of Bergmann glia in the cerebellum (Clos et al., 1980). In vitro, T_3 treatment increases the proportion of Bergmann cell-like glial cells, with longer processes and rounded cell bodies, which replace the protoplasmic or velate astrocytes (Messer et al., 1985). In addition, T_3 treatment accelerates the morphological and biochemical differentiation of astrocytes from neonatal rat cortex (Aizenman and de Vellis, 1987; Gould et al., 1990; Gavaret et al., 1991). In fact, we previously demonstrated that T_3 treatment induces morphological changes in primary cultured astrocytes from newborn rat cerebral hemispheres, whereas the same treatment induces proliferation in cerebellar astrocytes (Trentin and Moura Neto, 1995; Trentin et al., 1995; Lima et al., 1997; Lima et al., 1998; Trentin et al., 1998). Such effects were mediated by growth factors secreted by astrocytes in response to T_3 treatment (Trentin et al., 1995; Lima et al., 1997; Trentin et al., 1998).

T_3 effects on neuronal and astrocytic cells are now well established. However, the precise mechanism by which thyroid hormone exerts its developmental effects on the brain remains unknown. Thyroid hormone is directly involved in the development of cultured granule neurons by affecting the survival and differentiation of these cells (Heisenberg et al., 1992). T_3 was reported to promote postmitotic survival by preventing the apoptosis of newly formed and early differentiated cerebellar granule neurons (Muller et al., 1995). T_3 effects on neurons might represent a direct action on these cells as well as an indirect one primarily on other cells, such as astrocytes.

In the present work, we propose a novel, indirect action of T_3 on cerebellar neurons that affects primarily the secretion of growth factor by astrocytes. As reported previously, T_3 treatment induces cerebellar astrocytes to secrete a combination of mitogenic growth factors that promote astrocyte proliferation (Trentin and Moura Neto, 1995; Lima et al., 1997; Lima et al., 1998). Here, we demonstrate that conditioned medium derived from T_3-treated cerebellar astrocytes induces cerebellar neuronal proliferation in vitro. In addition, we identify some of the factors involved in this phenomenon and some steps of their putative action pathway. These data highlight the importance of neuron-glia interactions in the establishment of brain architecture and development, and point to a distinct and indirect action of T_3 on neuronal cell physiology by way of glial cells.

MATERIALS AND METHODS

Primary Astrocyte Cultures

Primary astrocyte cultures were prepared from cerebella of newborn Wistar rats (UFRJ, Rio de Janeiro, RJ, Brazil), following the procedure previously described for astrocyte cultures (Garcia-Abreu et al., 1995a, 1995b; Trentin et al., 1995; Trentin and Moura Neto, 1995; Garcia-Abreu et al., 1996, Lima et al., 1997, 1998; Trentin et al., 1998). The rats were decapitated, and then cerebella were removed and carefully stripped of the meninges. Tissues were washed in phosphate-buffered saline (PBS)/0.6% glucose (Sigma, St. Louis, MO) and dissociated into single cells in a medium consisting of DMEM and nutrient mixture F12 (DMEM/F12; Sigma) enriched with glucose (3.3×10^{-2} M), glutamine (2×10^{-3} M), and sodium bicarbonate (0.3×10^{-2} M). Cells (3×10^5) were plated onto 25-cm^2 plastic culture flasks (Sigma) previously coated with polyornithine (1.5 μg/ml, 41,000 MW; Sigma) in DMEM/F12 medium supplemented with 10% fetal calf serum (FCS; Fazenda Pigue, Rio de Janeiro, RJ). The cultures were incubated at 37°C in a humidified 5% CO_2/95% air atmosphere. Cell culture medium was changed 24 h after plating and subsequently every third day until reaching confluence (usually after 7–10 days).

T_3 Treatment and Conditioned Medium Preparation

After reaching confluence, glial monolayers were washed three times with serum-free DMEM/F12 medium and incubated as previously described for an additional day in serum-free medium. After this period, cultures were treated with 50 nM 3,3′,5-triiodo-L-thyronine (T_3) in DMEM/F12 for three days, renewed every day except after the third day. Control cultures were maintained in DMEM/F12 without FCS. Control and hormone-treated cultures were then maintained for two days without medium change, and the conditioned medium (CM) was collected on the second day after the end of T_3 treatment. CM derived from either T_3-treated cells (T_3CM) or control cultures (CCM) was then recovered, centrifuged at 1,500 *g* for 10 min, and either used immediately or stored in aliquots at −20°C for later use. T_3CM was confirmed to be free of residual T_3 by radioimmunoassay (RIA), as previously described (Trentin et al., 1995; Lima et al., 1998).

CM Neutralization Assay

For CM neutralization assays, CM (obtained as previously described) was incubated for 2 h, at 37°C before use in neuronal cultures, in the presence of the following neutralizing antibodies: rabbit anti–tumor necrosis factor-β (anti-TNF-β; 1:1,750), rabbit anti-epidermal growth factor (anti-EGF; 1:500), or anti–nerve growth factor (anti-NGF; 1:4,800) (all from Sigma).

Neuron Primary Cultures

Neurons were prepared from cerebella or cerebral cortexes of day 19 Wistar rat embryos (E19) as previ-

ously described (Garcia-Abreu et al., 1995a, b; Gomes et al., 1996; Martins et al., 1997). Cells were freshly dissociated from cerebral structures and plated on 5.5-mm-diameter glass coverslips (24-well plates; Sigma) that had been previously coated with polyornithine (1.5 μg/ml, 41,000 MW; Sigma) in T_3CM or CCM. For CM neutralization assays, neurons were cultured in the presence of antibody-neutralized CM. In some experiments, 5 μM of forskolin (Sigma) in dimethyl sulfoxide (DMSO) was added 2 h after neuron plating to avoid adhesion impairment. Neuron cultures were kept for 24 h at 37°C in a humidified 5% CO_2/95% air atmosphere.

Adenylate Cyclase Inhibition Assay

Neuron cultures were prepared in T_3CM or CCM as previously described. After 2 h of culture, different concentrations (100, 200, and 500 μM) of the specific adenylate cyclase inhibitor 9-(tetrahydro-2-furanyl)9*H*-purin-6-amine (SQ22536; Research Biochemicals International [RBI], Natick, MA) were added to the cultures. All inhibitor dilutions were done in water. Cultures then were kept for 24 h at 37°C in a humidified 5% CO_2/95% air atmosphere.

Trypan Blue Viability Assay

After 24 h, cerebellar neuron cultures were incubated in the presence of a 0.4% trypan blue solution in PBS for 1 min, and the number of viable cells was quantified. At least five fields were counted per well. Experiments were done in triplicate, and each result represents the mean of three independent experiments. Statistical analysis was done by analysis of variance (ANOVA).

Quantitative Analysis of Neuron Number

To determine neuron number in different condition assays, cultures were immunostained for class III β-tubulin, a specific neuron marker, and positive cells were visualized and counted with the use of a Zeiss Axiovert 35 microscope. At least three fields were counted per well. The experiments were done in triplicate, and each result represents the mean of three independent experiments. Statistical analysis was done by ANOVA.

Bromodeoxyuridil Incorporation and Detection

Neuron cultures were incubated for 24 h in the presence of 1 μg/ml of 5-bromo-2′-deoxyuridine (BrdU; Sigma). To avoid adhesion impairment, neurons were allowed to settle 2 h before BrdU addition. After BrdU incubation, cells were fixed with 4% paraformaldehyde for 20 min. Cultures were washed twice with distilled water and then incubated in 2 N HCl at 50°C for 15 min, twice. Subsequently, neuron cultures were washed with 0.1 M borate buffer for 10 min at room temperature, twice. Then, after being washed with PBS, cells were immunoreacted with anti-BrdU antibody (as described below) and visualized using a Zeiss Axioplan microscope. The mitotic index was measured by counting the percentage of labeled cells in at least five different fields per coverslip.

Immunocytochemistry

For immunocytochemistry, cultured cells were fixed with 4% paraformaldehyde for 20 min, washed three times with PBS, and permeabilized with 0.2% Triton X-100 for 5 min at room temperature. After permeabilization, cells were washed twice with PBS. Immunocytochemistry was performed as previously described (Garcia-Abreu et al., 1995a; Martins et al., 1997). Cells were incubated with 10% normal goat serum (NGS; Vector Laboratories, Burlingame, CA) in PBS (blocking solution 10% NGS/PBS) for 1 h and subsequently with the specified primary antibodies, diluted in blocking solution, overnight at room temperature. Primary antibodies were rabbit anti-cow glial fibrillary acidic protein (GFAP) antiserum (1:50 dilution; Dako, Carpinteria, CA); mouse anti-human β-tubulin III antibody (1:200 dilution; Sigma); anti-CNPase (a gift from Dr. P. Braun, McGill University), and rat anti-BrdU (1:500 dilution; Accurate Chemical & Scientific Corp., Westbury, NY). After incubation of the primary antibodies, the cells were washed three times with blocking solution and then incubated with secondary antibodies for 2 h at room temperature. Secondary antibodies were conjugated with fluorescein isothiocyanate (FITC; goat anti-mouse, 1:50 dilution, or goat anti-rabbit, 1:50 dilution; Sigma) or with biotin (goat anti-rat, 1:400 dilution; Vector Laboratories). In cases of biotin-conjugated antibodies, the secondary antibody was revealed by incubating the cells with Texas red–streptavidin conjugate according to the manufacturer's instructions (Vector Laboratories). Negative controls were created by omitting primary antibodies during the staining. In all cases, no reactivity was observed when the primary antibody was absent. Cell preparations were mounted directly on *N*-propyl gallate. The coverslips were visualized by using a Zeiss Axioplan microscope.

RESULTS

Cerebellar Astrocyte CM Increases Neuron Number Regiospecifically in the Brain

Previous studies have shown that T_3CM induces cerebellar astrocytes to proliferate in vitro (Trentin and Moura Neto, 1995). To investigate whether T_3CM also has an influence on neuron population, cerebellar neurons derived from E19 rats were cultured in the presence of T_3CM. After 24 h of culture, T_3CM increased neuron population by 60–80% versus CCM (Fig. 1C).

Cultures were immunostained with anti–β-tubulin III, anti-GFAP, and anti-CNPase antibodies to reveal neuronal, astrocytic, and oligodendrocytic cells, respectively. Most T_3CM- and CCM-treated cells stained positive for β-tubulin III, attesting for their neuronal phenotype (see Fig. 2 for T_3CM). Under both conditions, after 24 h, only 1–2% of the cells were labeled by the GFAP antibody, demonstrating that only a minor population of astrocytic cells was present in the cultures (data not shown). In the presence of neither CCM nor T_3CM were neuronal cultures contaminated by oligodendrocytes, as revealed by the lack of anti-CNPase staining after 24 h of culture, under both conditions.

Because RIAs have previously proved T_3CM to be free of residual T_3, our data suggest that the T_3CM effect on neuronal population is due to factors secreted by cerebellar astrocytes instead of a direct action of the hormone.

Because the complexity and heterogeneity of neuron–glia interactions are hallmarks of the CNS, we decided to investigate whether T_3 cerebellar secreted factors had a similar effect in other brain region. For this purpose, cerebral cortex neurons obtained from E19 rats were cultured in the presence of T_3CM or CCM. As shown in Figure 1, T_3-cerebellar growth factors did not succeed in increasing cortical neuron number (Fig. 1C).

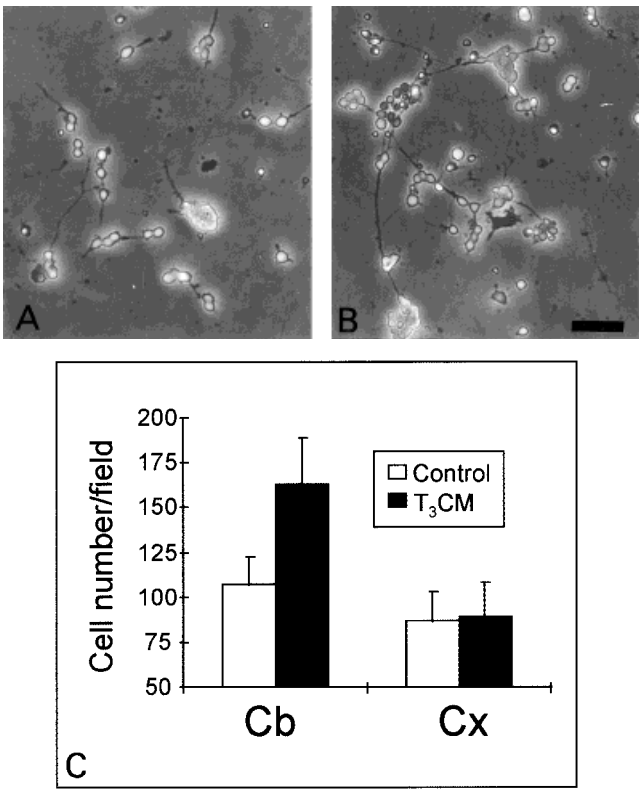

Fig. 1. Cerebellar glial CM increases neuron number. Cerebellar (Cb) or cerebral cortical (Cx) neurons obtained from E19 rats were maintained for 24 h on either CCM (**A**) or T_3CM (**B**), as described in Materials and Methods. **C:** Three fields were counted for each well of experiment. Each point represents the average of three independent experiments, done in triplicate. Values are means ± SD. Cb: $P < 0.05$; Cx: $P > 0.05$. Bar, 50 μm.

Cerebellar Astrocyte CM Did Not Alter Neuron Survival

To investigate whether neuron population augmentation was due to increased survival in T_3CM, we stained cells with trypan blue after 24 h of plating. Viable cells were quantified in five fields per well. As shown in Table 1, in T_3CM and CCM, cell viability was ~70%, although the absolute number of neurons was much higher in T_3CM. These data suggest that, at least after 24 h of culture, the main event involved in the neuron population increase is not modulation of neuronal survival.

T_3-Treated Cerebellar Astrocytes Secrete Factors That Increase Neuronal Proliferation

We previously demonstrated that T_3-treated cerebellar astrocytes secrete factors that can induce cerebellar astrocyte proliferation (Trentin and Moura Neto, 1995; Trentin et al., 1995). To investigate whether T_3CM also possesses such factors—or new ones that could increase neuronal progenitor proliferation—we incubated neuronal cells kept in either T_3CM or CCM in the presence of 1 μg/ml of BrdU for 24 h. After this period, cells were fixed and processed for β-tubulin III and BrdU immunostaining; positive cells were observed by using fluorescence microscopy and quantified.

As shown in Figure 3, at least three times more neurons kept in T_3CM (35%) incorporated BrdU than those maintained in CCM (11%), indicating that proliferation should be an important mechanism of neuron population augmentation.

Neuron Number Increase Induced by T_3-Glial Factors Involves a cAMP-Dependent Pathway

Differentiation and proliferation events usually involve a great diversity of second-messenger pathways. To study cAMP pathway participation in our model, 5 μM of forskolin, an adenylate cyclase activator, was added to neuron cultures, and the neuronal cells were counted as previously described. As shown in Figure 4A, forskolin addition had no effect on CCM, whereas it enhanced the T_3CM effect by 35%. These results point to a possible role for a cAMP pathway in mediating the effects of growth factors secreted by T_3-treated cerebellar astrocytes. To confirm this hypothesis, neuronal cultures kept in the presence of either T_3CM or CCM were treated with different concentrations of SQ22536, a specific adenylate cyclase inhibitor. As shown in Figure 4B, the T_3CM effect was reversed in a dose-dependent manner, and minor effects were observed with CCM. The IC_{50} for T_3CM inhibition was 74 μM.

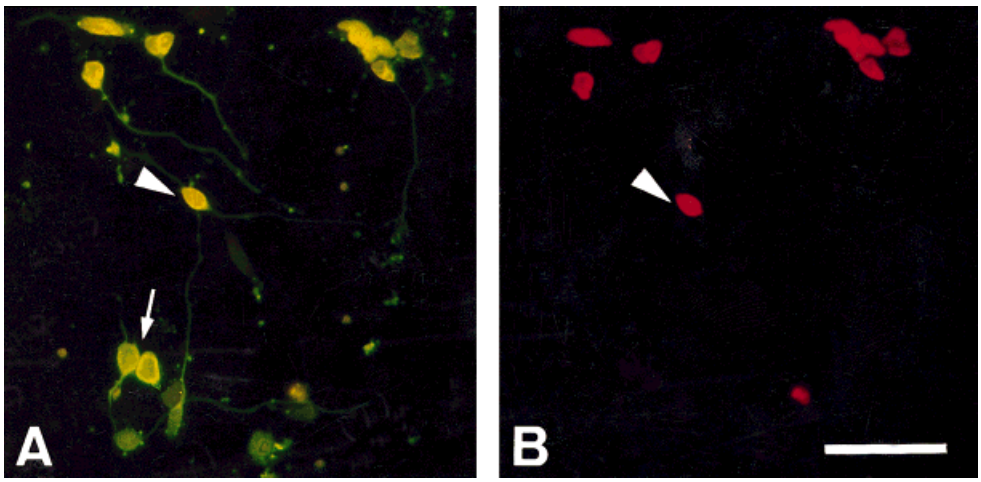

Fig. 2. Neuronal BrdU incorporation is induced by T_3 cerebellar glial CM. Cerebellar neurons obtained from E19 rats were maintained for 24 h on T_3CM in the presence of 1 μg/ml of BrdU. Subsequently, cells were fixed and double-stained for β-tubulin III (**A**) and BrdU (**B**). Arrowhead shows a neuronal body double-labeled for β-tubulin III and BrdU; arrow shows neuronal bodies labeled for β-tubulin III only. Bar, 50 μm.

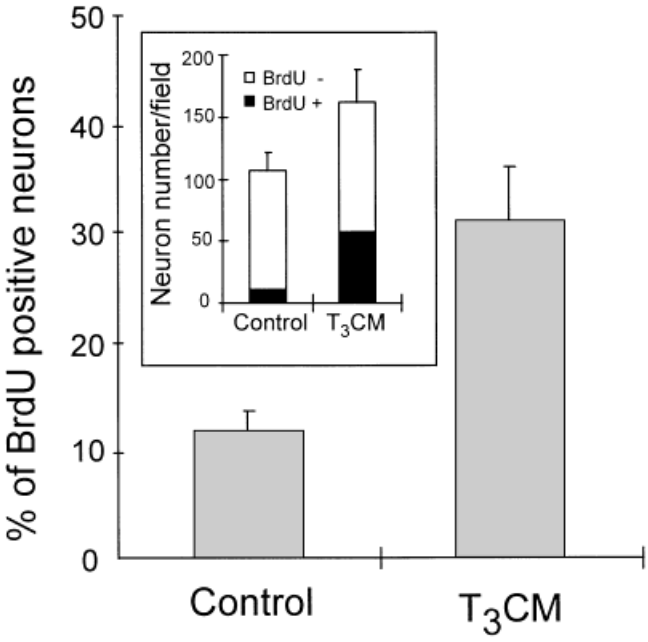

Fig. 3. Cerebellar glial CM induces neuron proliferation. Cerebellar neurons obtained from E19 rats were maintained for 24 h on T_3CM or CCM in the presence of 1 μg/ml of BrdU. Subsequently, cells were fixed and double stained for β-tubulin III and BrdU. Three fields were counted for each well of experiment. Each point represents the average of three independent experiments done in triplicate. Values are means ± SD. $P < 0.05$.

These data clearly point to a major role of a cAMP pathway in the T_3CM neuron effect.

Effect of Growth Factors Neutralizing Antibodies on Neuron Number

To analyze the growth factors involved in neuronal population increase, T_3CM and CCM were neutralized by antibodies against some known growth factors and tested for their ability to increase neuron number. As demonstrated in Figure 5A, addition of anti–TNF-β antibodies to T_3CM completely inhibited neuron increase in that medium. In addition, T_3CM treated with anti-EGF increased the neuron population by 37%, whereas T_3CM alone caused a 60% increase (Fig. 5B). We also have tested an antibody against NGF, the most studied neurotrophin. NGF seems not to play a key role in neuron proliferation induced by T_3 (Fig. 5C). Together, these data suggest that thyroid hormone treatment induces cerebellar astrocytes to secrete a combination of growth factors that act on neuronal cells.

DISCUSSION

The main finding of the present work is the demonstration that T_3 induces neuron proliferation through a glia-mediated process, instead of its exclusive and generally known direct action on neurons. This effect was based on the property of T_3 to induce synthesis and secretion of growth factors by glial cells, which was described previously (Trentin and Moura Neto, 1995; Trentin et al., 1995; Lima et al., 1997). Our data suggest a novel mechanism of interaction—trophic support dependent on hormonal action—between neurons and glial cells that might occur in vivo during development. This finding also is in agreement with the increasing amount of evidence indicating that neurotrophic support can be provided by glial cells (Banker, 1980; Denis-Donini et al., 1984; Engele and Bohn, 1991; Garcia-Abreu et al., 1996; Gomes et al., 1996; Maxwell et al., 1996; Choi-Lundberg et al., 1997; Kotzbauer et al., 1997).

It was previously demonstrated that T_3 induces cerebellar astrocyte proliferation in vitro (Trentin et al., 1995; Lima et al., 1997, 1998). Such proliferation seems to be solely mediated by soluble factors released by hormone-treated cells in the culture medium, because the proliferation effect was mimicked by the T_3CM obtained from those cultures (Trentin et al., 1995). These results suggested an indirect autocrine mechanism underlying the proliferative T_3 mode of action.

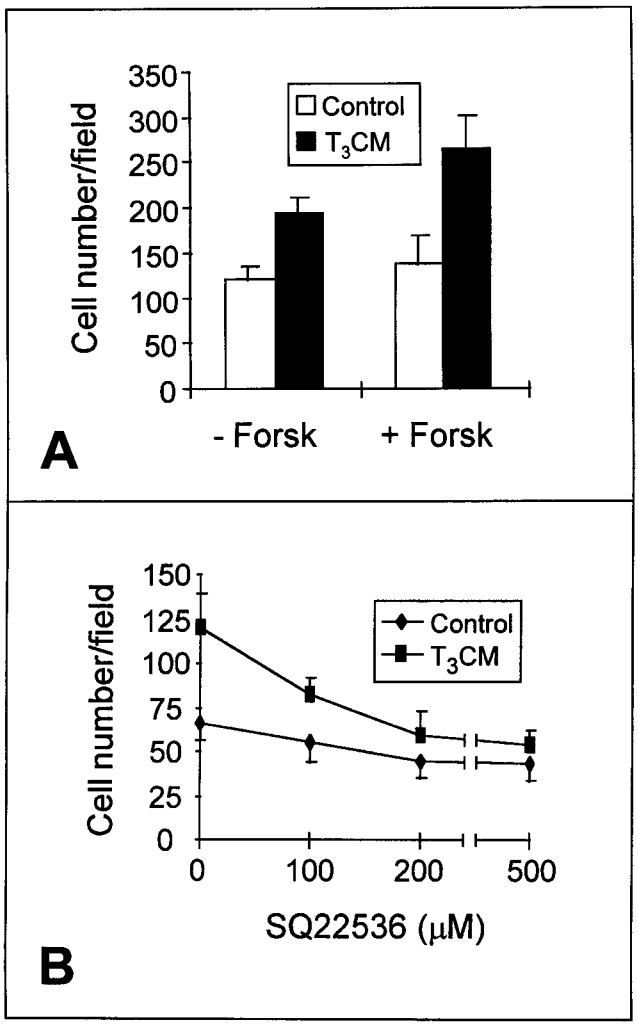

Fig. 4. T_3CM effects involve a cAMP-dependent pathway. **A:** Forskolin increases neuron number on glial T_3CM. Cerebellar neurons obtained from E19 rats were maintained for 24 h on either T_3CM or CCM; supplemented with 5 μM of forskolin (Forsk). Three fields were counted for each well of experiment. Each point represents the average of three independent experiments done in triplicate. Values are means ± SD. $P < 0.05$. **B:** Adenylate cyclase inhibition reverses the T_3CM effect in a dose-dependent manner. Cerebellar neurons were obtained as previously described and maintained for 24 h on CCM or T_3CM, supplemented with increasing concentrations of the adenylate cyclase inhibitor, SQ22536 (100, 200, and 500 μM). Five fields were counted for each well of experiment. Each point represents the average of two independent experiments done in duplicate. Values are means ± SD. $P < 0.05$ for 0, 100, and 200 μM; $P > 0.05$ for 500 μM.

To evaluate whether T_3CM could also affect neuronal physiology, we cultivated cerebellar neurons in T_3CM. This medium increased the cerebellar neuron population by 60–80%. There are three possibilities for such an increase in neuron number: first, an increase in proliferation, by which neuronal progenitors are specifically induced to proliferate by the T_3CM; second, an increase in differentiation, by which, for example, more cells differentiate into neurons from an undifferentiated precursor pool, with little change in proliferation, after T_3CM incubation; and third, an increase in survival, whereby more neurons are surviving after T_3CM incubation. To investigate which step of neuronal physiology was affected by factors released by T_3 astrocytes, we carried out trypan blue and BrdU incorporation assays.

We ruled out a survival effect of the T_3CM on these cells because the proportion of survival determined by trypan blue exclusion was exactly the same in neurons incubated with CCM or T_3CM (Table 1). We are currently undertaking a temporal study of the T_3CM influence on these cells to determine whether there is an early neurospecific effect on adherence and survival (before 24 h). Preliminary results did not indicate any major differences in cell survival (e.g., there was no increase in cells on the supernatant of the first wash).

BrdU incorporation by class III β-tubulin–positive cells increased three times during the incubation period with T_3CM. Because class III β-tubulin–positive cells are regarded as postmitotic neurons in the CNS—with only a few exceptions (Memberg and Hall, 1995; Menezes et al., 1995), all outside the cerebellum—this finding indicates that progenitor cells proliferate and generate more neurons with T_3CM than with CCM. It is also possible that immature neurons were differentiating in our cultures. This possibility remains to be investigated. Although we cannot rule out an enhancement in the differentiation of neuronal cells, a 35% increase of BrdU-labeled cells in the much larger population of T_3CM-treated cultures seems to account for most of the difference in absolute cell number between neuron cultures maintained in the presence of CCM or T_3CM (Fig. 3, inset).

Another argument that speaks against a major direct action on differentiation is the fact that T_3CM had no effect over cortical neurons at E19 (Fig. 1), when many neuronal cells are immature but mostly postmitotic because the cortical neurogenetic pool is dramatically reduced at this stage of development (Bayer and Altman, 1994). Data concerning cortical astrocytes may also suggest a developmentally regulated expression of either growth factors and their cognate receptors throughout the nervous system. In fact, changes in neurotrophin responsiveness during nervous system development has already been described for cerebellar granule neurons (Segal et al., 1992). Our findings raise the question of whether the fundamental mechanisms that control neurogenesis from different brain regions differ and highlight the great heterogeneity of neuronal development in the CNS.

Our data argue that the principal action of the T_3CM is to boost normal cerebellar neuron proliferation. This proliferative effect seems to be mediated by soluble factors secreted by the treated glial cells (Trentin et al., 1995). To identify some of the factors responsible for neuron proliferation in T_3CM composition, we performed assays using neutralizing antibodies to inhibit the T_3CM effect. Antibodies against specific neurotrophins were chosen based on their known effects on proliferative or immature neuronal cells. Two such antibodies, anti-EGF and anti–TNF-β, significantly inhibited neuron increase in T_3CM.

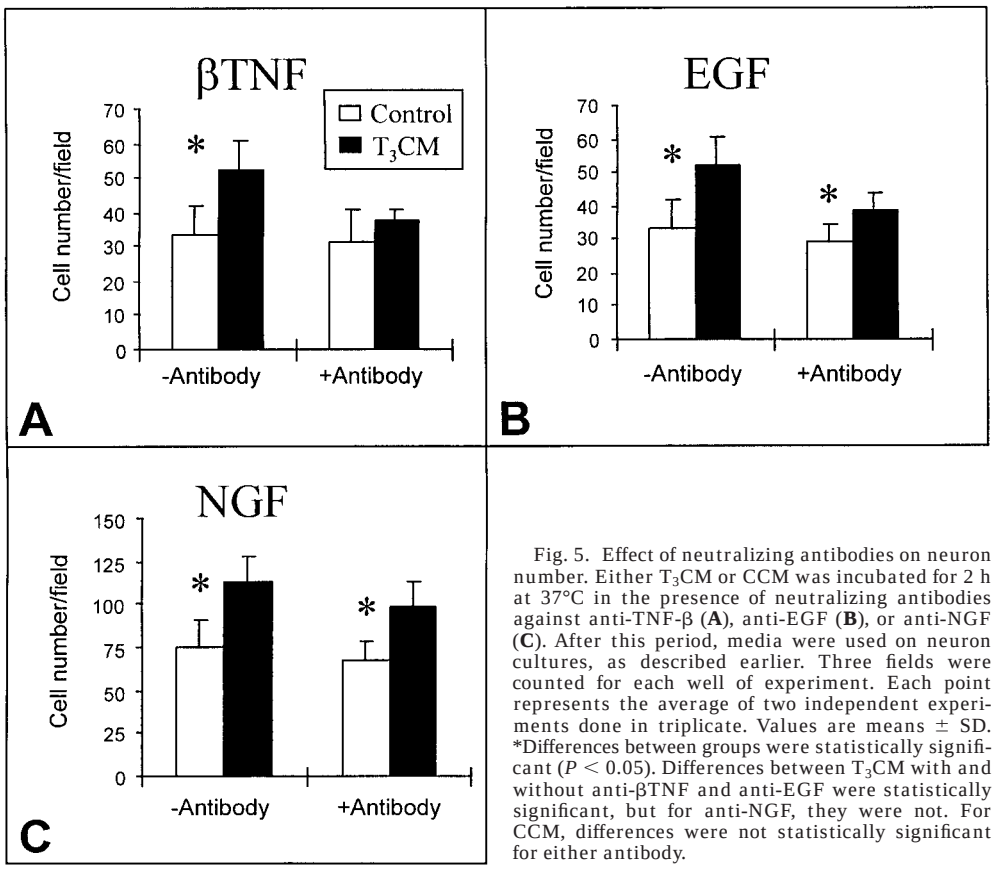

Fig. 5. Effect of neutralizing antibodies on neuron number. Either T_3CM or CCM was incubated for 2 h at 37°C in the presence of neutralizing antibodies against anti-TNF-β (**A**), anti-EGF (**B**), or anti-NGF (**C**). After this period, media were used on neuron cultures, as described earlier. Three fields were counted for each well of experiment. Each point represents the average of two independent experiments done in triplicate. Values are means ± SD. *Differences between groups were statistically significant ($P < 0.05$). Differences between T_3CM with and without anti-βTNF and anti-EGF were statistically significant, but for anti-NGF, they were not. For CCM, differences were not statistically significant for either antibody.

TABLE 1. Effect of cerebellar glial CM on neuronal survival[a]

	Live cells (%)	Dead cells (%)
CCM	68 ± 9	32 ± 9
T_3CM	69 ± 4.7	31 ± 4.7

[a]Cerebellar neurons obtained from E19 rats were kept for 24 h in either T_3CM or CCM. Cellular viability was accessed by trypan blue staining. Three fields were counted for each well of experiment. Each point represents the average of three independent experiments done in triplicate. Values are means ± SD. $P < 0.05$.

TNF has been reported to have a mitogenic effect on several neural cells (Selmaj et al., 1991). Recently, TNF-α was shown to prolong cerebellar granule neuron survival in culture (Luca et al., 1996).

Although EGF was originally identified as a potent epidermal proliferative agent (Cohen, 1965), increasing evidence supports its role in neuron differentiation (Pettmann et al., 1979; Rosenberg and Noble, 1989). It was demonstrated that intraventricular administration of EGF expanded proliferative progenitors in the subventricular zone of adult mice (Craig et al., 1996).

NGF was reported to be an intermediate of the thyroid hormone in controlling postmitotic survival of cerebellar granule cells by preventing apoptosis (Muller et al., 1995). However, in our cultures, anti-NGF-neutralizing antibodies had no apparent effect on neuron increase.

The signaling mechanisms by which growth factors promote neuronal survival and proliferation, particularly of CNS neurons, are not fully characterized. Our data demonstrated that forskolin, an agent that increases endogenous cAMP levels, enhances the responsiveness of cerebellar neurons to T_3CM (Fig. 4). Supporting the role of cAMP in mediating the T_3CM effect, SQ22536, a specific inhibitor of adenylate cyclase, reversed the T_3CM effect on neurons. In fact, increasing evidence demonstrates that intracellular cAMP pathways seem to be directly involved in distinct neuronal systems, such as dopaminergic cell survival (Michel and Agid, 1996). Although our data do not exclude the involvement of other signaling pathways, it demonstrates that a cAMP pathway must participate. It is possible that cAMP could enhance the release of factors that act collaboratively with glial T_3 growth factor. Another possibility is that cAMP improves the responsiveness of cerebellar neurons to those growth factors by increasing the number of their receptors or by potentiating their signal transduction pathways.

In the developing cerebellum, the first three postnatal weeks seem to be a period of particular sensitivity to thyroid hormones (Porterfield and Hendrich, 1993). During that time, granule cell precursors in the external granule layer proliferate copiously, producing new neurons. This layer gives rise to the cerebellar granule neurons, which divide and subsequently migrate inward to reach the internal granule layer. Granule neurons, then, establish synaptic contact with the developing Purkinje cells in the molecular layer (Altman, 1972). Genetic and epigenetic factors play an

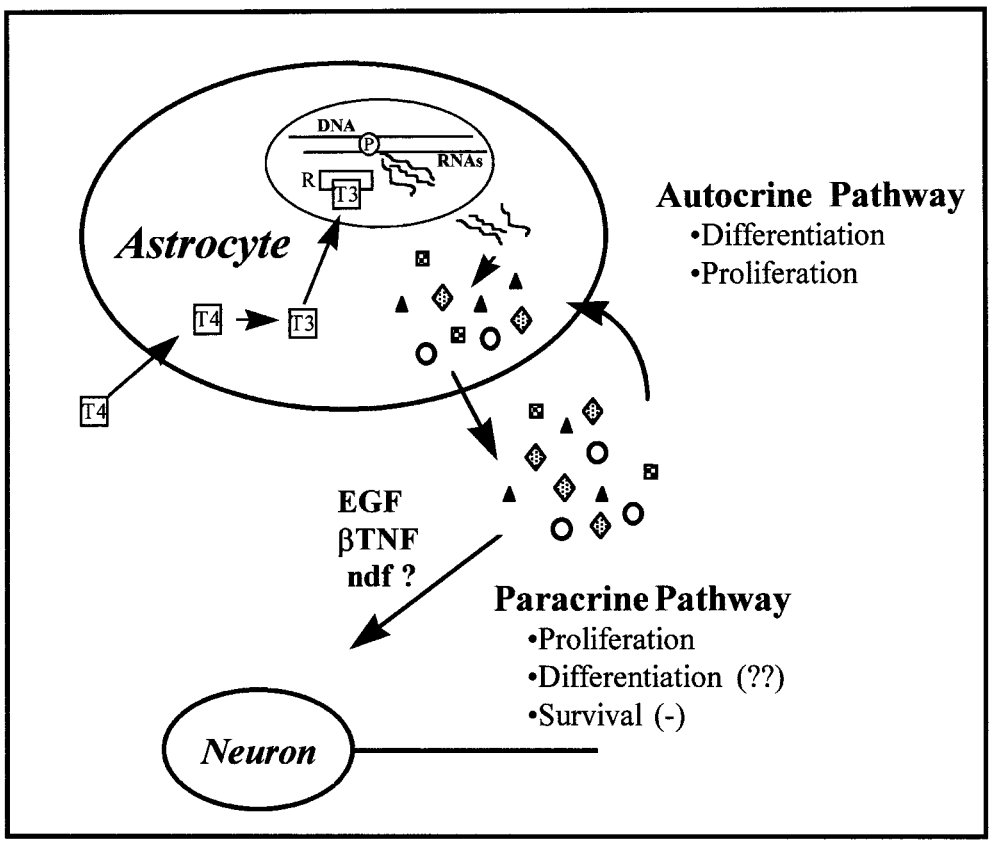

Fig. 6. Model for the T_3 effect on cerebellar neurogenesis mediated by glial cells. We propose that T_3 induces astrocytes to secrete a combination of factors that act by autocrine and paracrine pathways, modulating the proliferation of astrocytes and neurons, respectively. ndf = undetected known and unknown factors; T_4 and T_3-thyroid hormones; R = T_3 receptor; P = gene promoter.

important role during most of these processes (Baptista et al., 1994; Alder et al., 1996; Jankovski et al., 1996). Thyroid hormones have been reported to highly regulate some of the steps of cerebellar ontogenesis (Legrand, 1979). However, the mechanisms involved in this developmental hormonal regulation are largely unknown. Thus, it is possible that, in vivo as well as in vitro, T_3 induction of growth factor production and/or secretion by cerebellar astrocytes is critical for the regulation of cerebellum development.

In summary, our results suggest that, in addition to a direct action on neurons, T_3 can, through a glia-mediated process, influence neuronal physiology. Thus, we propose a novel molecular mechanism of T_3 action on neuronal cells: T_3 induces astrocytes to secrete a combination of factors that act by autocrine and paracrine (TNF-β, EGF, and possibly others not yet identified) pathways, modulating the proliferation of astrocytes and neurons, respectively. The demonstration that T_3 can indirectly affect neuronal development through the induction of growth factor release not only emphasizes the complexity of cell–cell interaction during CNS development but also demonstrates a primary role of astrocytes to the harmonious development of the brain.

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