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Neuritogenesis Induced by Thyroid Hormone-treated Astrocytes Is Mediated by Epidermal Growth Factor/Mitogen-activated Protein Kinase-Phosphatidylinositol 3-Kinase Pathways and Involves Modulation of Extracellular Matrix Proteins*

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Thyroid hormone (T3) plays a crucial role in several steps of cerebellar ontogenesis. By using a neuron-astrocyte coculture model, we have investigated the effects of T3-treated astrocytes on cerebellar neuronal differentiation *in vitro*. Neurons plated onto T3-astrocytes presented a 40–60% increase on the total neurite length and an increment in the number of neurites. Treatment of astrocytes with epidermal growth factor (EGF) yielded similar results, suggesting that this growth factor might mediate T3-induced neuritogenesis. EGF and T3 treatment increased fibronectin and laminin expression by astrocytes, suggesting that astrocyte neurite permissiveness induced by these treatments is mostly due to modulation of extracellular matrix (ECM) components. Such increase in ECM protein expression as well as astrocyte permissiveness to neurite outgrowth was reversed by the specific EGF receptor tyrosine kinase inhibitor, tyrphostin. Moreover, studies using selective inhibitors of several transduction-signaling cascades indicated that modulation of ECM proteins by EGF is mainly through a synergistic activation of mitogen-activated protein kinase and phosphatidylinositol 3-kinase pathways. In this work, we provide evidence of a novel role of EGF as an intermediary factor of T3 action on cerebellar ontogenesis. By modulating the content of ECM proteins, EGF increases neurite outgrowth. Our data reveal an important role of astrocytes as mediators of T3-induced cerebellar development and partially elucidate the role of EGF and mitogen-activated protein kinase/phosphatidylinositol 3-kinase pathways on this process.

Thyroid hormone (3,5,3′-triiodothyronine, T3)[1] is essential for normal development of the vertebrate nervous system (NS), influencing diverse processes of brain development such as neuronal migration, neurite outgrowth, synapse formation, myelination, and glial cell differentiation (1–5). Although the T3 role on central nervous system (CNS) morphogenesis is well documented, the precise mechanism of hormone action is not completely understood. To gain insights into T3 effects on CNS we have focused on the cerebellum ontogenesis, which is one of the most dramatically affected brain structures in hypothyroidism (6, 7).

Most of the granular cells of the cerebellum arise from the external granular cell layer (EGL). Postnatally, these cells migrate from the premigratory zone of the EGL to the internal granular layer, leaving their axons behind to produce the molecular layer. These events are accompanied by a progressive morphological differentiation of Purkinje cells characterized by perisomatic extensions and dendritic trees (8–10). Although cerebellar histogenesis is well studied, the molecular mechanisms that control proliferation and differentiation of granular cells are still unknown. These processes have been shown to undergo dramatic modulation by thyroid hormone (6, 10, 11). Besides a series of abnormalities found in the cerebellar cortex, hypothyroidism causes a decrease in EGL proliferation rate, increased neuronal death in the internal granular layer, impaired migration of granular cells, and a deficiency in the elaboration of Purkinje cell dendritic trees, spines, and synapses (6).

Although a few genes have been shown to be directly modulated by T3 in the cerebellum, the molecular mechanism of T3 action on this brain region is still controversial (12–14). It has been proposed that such endocrine regulation of cerebellar development might be the result of T3-dependent modulation of secretion of several growth factors such as neurotrophin 3, nerve growth factor, insulin growth factor, and brain-derived neurotrophic factor (15, 16).

Astrocytes have been pointed out as the major source of trophic factors in the CNS (17–19). The fact that thyroid hormone treatment of astrocytes is associated *in vitro* with the secretion of several growth factors makes the astrocyte a putative candidate for mediating T3 action on neural histogenesis (19, 20). Recently, we described a novel mechanism for T3 action over granular neurons mediated by astrocytes. We demonstrated that cerebellar astrocytes treated by T3 secrete a combination of growth factors such as epidermal growth factor (EGF) and tumor necrosis factor-β, which induces proliferation of cerebellar granular neurons *in vitro* (19).

In the present work, we used an *in vitro* system of neuron-astrocyte coculture to assess the effects of T3 mediated by

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[1] The abbreviations used are: T3, triiodothyronine; NS, nervous system; CNS, central nervous system; ECM, extracellular matrix protein; EGF, epidermal growth factor; EGFR, EGF receptor; EGL, external granular layer; MAPK, mitogen-activated protein (MAP) kinase; MEK, MAPK/extracellular signal regulated kinase kinase; PI3K, phosphatidylinositol 3-kinase; DMEM, Dulbecco's modified Eagle's medium; CM, conditioned medium.

astrocytes on another step of cerebellar morphogenesis such as granule cell differentiation. We provide evidence that EGF secreted by astrocytes in response to T3 presents a binary role in cerebellar ontogenesis; acting directly on neurons, EGF promotes proliferation of granular cell precursors, and indirectly, EGF increases neuronal morphological differentiation by modulating the content of two astrocytic extracellular matrix (ECM) proteins, laminin and fibronectin. Furthermore, we suggest that EGF modulation of ECM proteins is mainly mediated by activation of MAPK and PI3K pathways. Together, our work gives glial cells a novel attribute as mediators of the endocrine-regulated cerebellar development and describes an additional role for EGF on brain morphogenesis.

EXPERIMENTAL PROCEDURES

Astrocyte Primary Cultures—Primary astrocyte cultures were prepared from cerebella derived from newborn Wistar rats (Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brazil) after the procedure previously described (19, 21). After rats were decapitated, cerebella were removed and carefully stripped off the meninges. Tissues were washed in phosphate-buffered saline, 0.6% glucose (Sigma) and dissociated into single cells in a medium consisting of Dulbecco's modified Eagle's medium (DMEM) and nutrient mixture F-12 (Sigma) enriched with glucose (3.3×10^{-2} M), glutamine (2×10^{-3} M), and sodium bicarbonate (0.3×10^{-2} M). Cells were plated onto plastic culture flasks (Sigma) or glass cover slips (24 wells plates, Sigma), previously coated with polyornithine (1.5 μg/ml, M_r 41,000, Sigma) in DMEM/F-12 medium supplemented with 10% fetal calf serum (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, which usually occurred after 7–10 days.

T3 and EGF Treatment—After reaching confluence, glial monolayers were extensively washed with serum-free DMEM/F-12 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 (T3, Sigma) and/or 10 ng/ml of EGF (Invitrogen) in DMEM/F-12 for 3 days, which was renewed every day except after the third day. Control cultures were maintained in DMEM/F-12 without fetal calf serum with medium changes equivalent as those of T3/EGF-treated cultures.

Conditioned Medium (CM) Preparation—Conditioned medium was obtained as previously described (19). After the third day of T3 treatment, control and hormone-treated cultures were maintained for 2 days without medium change, and the CM was collected on the second day after the end of T3 treatment. CM derived from either T3-treated cells (T3CM) or control cultures (CCM) was clarified by centrifugation at $1500 \times g$ for 10 min and used immediately or stored in aliquots at −20 °C for further use. T3CM was confirmed to be free of residual T3 by radioimmunoassay as previously described (19).

Neuron Primary Cultures and Cocultures—Neurons were prepared from cerebella derived from 19-day Wistar rat embryos (E19) as previously described (19, 21). Briefly, cells were freshly dissociated from cerebellum, and 1×10^5 cells were plated onto glass cover slips previously coated with polyornithine (1.5 μg/ml, M_r 41,000, Sigma) in T3CM or CCM. For coculture assays, neurons were plated onto glial monolayer carpets nontreated or previously treated by T3. Cultures were kept for 24 h at 37 °C in a humidified 5% CO_2, 95% air atmosphere.

Inhibition Assays—Astrocyte monolayers were concomitantly treated by EGF (10 ng/ml) or T3 (50 nM) and specific signaling pathway inhibitors for 3 days accordingly to the previously described protocol. To prevent a direct action of the inhibitors on neurons on coculture assays, inhibitor-containing medium was replaced by drug-free medium immediately before neuronal plating. Cocultures were kept for 24 h. The following inhibitors were used: PD98059, MAPK-specific inhibitor (50 μM;); LY294002, PI3K-specific inhibitor (5 μM); genistein, tyrosine kinase inhibitor (2.5 μM); bis-tyrphostin, potent and specific inhibitor of the EGF receptor (EGFR) (500 nM); KT5720, specific inhibitor of protein kinase A (400 nM). All inhibitors were purchased from Calbiochem and diluted in methyl sulfoxide (C_2H_6OS, Sigma).

Immunocytochemistry—Immunostainning was performed as previously described (19). Briefly, cells were fixed with 4% paraformaldehyde for 3 min (for extracellular matrix protein labeling) or 20 min (for cytoskeleton protein labeling), extensively washed with phosphate-buffered saline, and in the case of cytoskeleton protein labeling, permeabilized with 0.2% Triton X-100. For peroxidase assays, endogenous peroxidase activity was abolished with 3% H_2O_2 for 15 min followed by extensive washing with phosphate-buffered saline. Cells were incubated with 5% bovine serum albumin (Invitrogen) in phosphate-buffered saline (blocking solution) for 30 min and subsequently with the specified primary antibodies, diluted in blocking solution, overnight at 4 °C. Primary antibodies were mouse anti-human β-tubulin III antibody (1:400 dilution; Sigma), rabbit anti-mouse laminin (1:30 dilution; Sigma), and rabbit anti-human fibronectin (1:200 dilution, Dako, Carpinteria, CA). Secondary antibodies were conjugated with Cy3 (sheep anti-rabbit, 1:3000 dilution, Sigma) or horseradish peroxidase (goat anti-mouse, 1:200; Invitrogen). Peroxidase activity was revealed with 3,3′-diaminobenzidine (Sigma). Negative controls were created by omitting primary antibodies during staining. In all cases, no reactivity was observed when the primary antibody was absent. Cell preparations were mounted directly on *N*-propyl gallate for fluorescence assays, or in the case of peroxidase reactions, they were dehydrated in a graded ethanol series, and coverslips were mounted in Entellan (Merck).

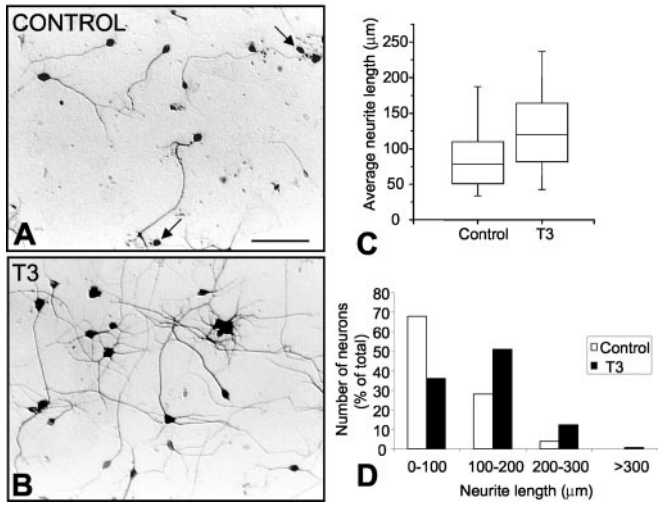

FIG. 1. **Astrocytes treated by T3 increase cerebellar neurite outgrowth.** Cerebellar neurons obtained from E19 rats were cultivated for 24 h onto control (*A*) and hormone-treated astrocyte monolayers (*B*). Subsequently, cells were fixed and immunostained using a monoclonal anti-β-tubulin III reagent as the primary antibody. Total neurite length (*C* and *D*) was obtained using the Sigma Scan Pro Software (Jandel Scientific). In all cases, at least 100 neurons randomly chosen were observed. Hormone treatment strongly enhanced astrocyte permissiveness to neurite outgrowth. *Arrows* in *A* shows aneuritic neurons frequently found on control carpets. A higher density of neurons as well as those with longer neurites can be observed on T3-carpets (*B*). Statistical significance was observed for all groups ($p < 0.05$). The *scale bar* corresponds to 50 μm.

Morphometry and Statistical Analysis—Neurons stained with anti-β-tubulin III antibody were photographed in a Nikon microscope (Nikon Eclipse TE300). Photos were scanned, and the numbers of neurites and total neurite length were analyzed using the Sigma Scan Pro Software (Jandel Scientific). In each experiment (at least three independent experiments were done), about 100 neurons per well, encompassing five fields randomly chosen, were analyzed. The data were stored, and graphical and statistical analyses were performed using the Microsoft Excel version 7.0.

RESULTS

Cerebellar Astrocytes Treated by T3 Enhance Number and Neurite Outgrowth—To investigate the role of astrocytes as mediators of T3 action in cerebellum ontogenesis, we analyzed outgrowth and number of neurites of cerebellar neurons cultivated with T3-treated astrocytes. Cerebellar neurons derived from 19-day embryonic rats (E19) were plated onto cerebellar astrocyte monolayers previously treated by T3. After 24 h, cells were immunostained for the neuronal marker, β-tubulin III, and number and total length of neurites were measured. Such analysis revealed a clear difference between neurons plated on the two carpets (Fig. 1). We observed a 40–60% increment on total neurite length of cells plated onto T3-treated astrocyte monolayers as well as an increased number of neurons as

expected due to the previous reported T3-astrocyte action on neuronal proliferation (19) (Fig. 1*C*). Neurite sprouting started as early as 2 h of culturing in both control and treated monolayers, although a significant difference in neurite length between these two conditions could already be noted at this time (1.6-fold increase) (data not shown). On T3-astrocyte carpets, most of the neurons developed neurites with average size between 100 and 200 μm, whereas most of those plated onto control astrocytes exhibited an average size between 0 and 100 μm (Fig. 1*D*). A major difference was observed for neurons with extensive neurites (200 μm or more). Whereas 13% of neurons presented this pattern of neuritogenesis when plated onto T3-treated astrocytes, only 4% of those plated onto control astrocytes developed neurites between 200 and 300 μm (Fig. 1*D*). Neurites longer than 300 μm were rarely observed in control condition (Fig. 1*D*).

Analysis of neuronal morphology revealed a dramatic improvement of neurite number of cells plated onto T3-treated astrocytes. As shown in Fig. 2*B*, there was a 50% decrease on the number of neurons without neurites on T3-treated astrocytes. Furthermore, a significant increase was also observed on the number of neurons with two or more neurites in this condition (Fig. 2*B*). A few neurons extended three or more neurites when plated onto T3-monolayers; on the other hand, they very seldom presented this pattern when plated onto control cultures (Fig. 2*A*). Taken together these data indicate that cerebellar astrocytes treated by thyroid hormone positively modulate neuritogenesis of cocultured neurons.

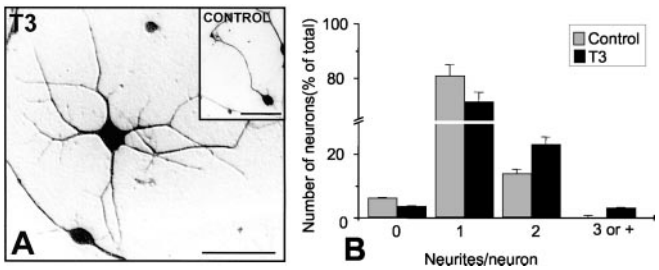

FIG. 2. **Astrocytes treated by T3 increase neuritogenesis.** Cerebellar neurons obtained from E19 rats were cultivated for 24 h onto hormone-treated (*A*) and control astrocyte monolayers (*inset*). After 24 h of coculture, neurons were morphologically characterized by β-tubulin III immunostaining, and number of neurites was obtained using the Sigma Scan Pro Software (Jandel Scientific) (*B*). In all cases, at least 100 neurons randomly chosen were observed. T3-astrocytes promoted neuronal arborization. A complex neuritic network was frequently observed on neurons plated onto T3-astrocytes. Statistical significance was observed for all groups ($p < 0.05$). *Scale bars* correspond to 25 μm.

T3-astrocyte-induced Neuritogenesis Is Indirectly Mediated by EGF—We previously described that astrocytes treated by thyroid hormone modulate neuronal proliferation by secreting growth factors, one of them identified as EGF (19). To evaluate the involvement of T3-astrocyte-derived EGF on neurite outgrowth, cerebellar astrocyte cultures were treated by EGF and T3 alone or in combination as described. After treatment, embryonic neurons were plated onto different astrocyte carpets, and the number and length of neurites were analyzed (Fig. 3).

Treatment of astrocytes by EGF induced a neurite outgrowth similar to that promoted by T3 treatment (Fig. 3*C*). Quantitative analyzes revealed that under this condition there was a significant increase in the average neurite length. Most of the neurons plated onto EGF-astrocytes extended neurites ranging from 100 to 200 μm in contrast to the great majority of those kept onto control astrocytes, which exhibit neurites shorter than 100 μm (Fig. 3*E*). It is noteworthy that a significant increase in the number of neurons presenting 200–300 μm neurites was observed in EGF-astrocyte carpets (150%) (Fig. 3*E*).

Neurite number was also affected by EGF treatment of astrocytes. The fraction of aneuritic neurons was significantly decreased by EGF treatment (50%, Fig. 3*F*), whereas neurons with two neurites were substantially increased (40%). Neurons with three or more processes, virtually absent from control cocultures, were often observed after EGF treatment (Fig. 3*F*). Surprisingly, the addition of EGF concomitantly to T3 did not enhance T3 effect on either number or length of neurites (Fig. 3, *E* and *F*).

To discriminate between a direct and indirect action of EGF on neuritogenesis, we cultivated embryonic neurons on conditioned medium derived from hormone-treated astrocytes (T3CM). After 24 h, outgrowth and number of neurites were evaluated as previously described. Culture of cerebellar neurons on T3CM did not promote significant increment in either total neurite length or number of neurites when compared with CCM (Fig. 4), indicating that astrocytic EGF secreted in response to T3 treatment is not directly implicated in modulation of cerebellar neuritogenesis.

To fully implicate EGF on T3 modulation of neurite outgrowth, we blocked its activity with genistein and tyrphostin,

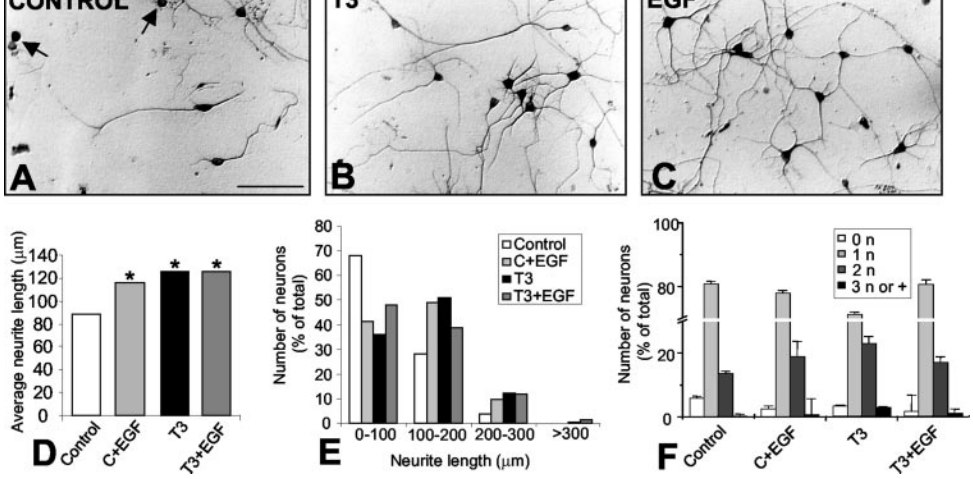

FIG. 3. **Effect of EGF on cerebellar neuritogenesis.** Cerebellar neurons obtained from E19 rats were plated onto astrocyte monolayers nontreated (*A*) or previously treated by T3 (*B*) or EGF (*C*) (10 ng/ml) alone or in combination. Cultures were kept for 24 h before quantification of length (*D* and *E*) and number of neurites (*F*) as previously described. EGF treatment of astrocytes significantly enhanced astrocyte permissiveness for neuritogenesis. *Arrows* in *A* show aneuritic neurons. The *asterisk* in *D* corresponds to $p < 0.001$; *n*, neurites; *C*, control. The *scale bar* corresponds to 50 μm.

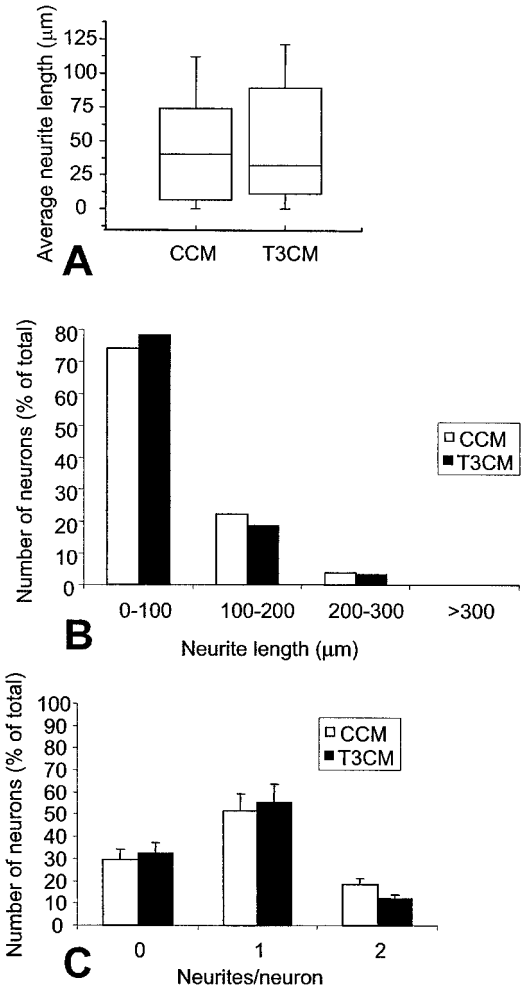

FIG. 4. **Conditioned medium derived from T3-treated astrocytes does not increase number and neurite outgrowth.** Cerebellar neurons obtained from E19 rats were maintained for 24 h on conditioned medium derived from nontreated (*CCM*) and T3-treated astrocytes (*T3CM*). Subsequently, β-tubulin III-positive cells were analyzed as described under "Experimental Procedures." Total length (*A* and *B*) and number of neurites (*C*) were obtained using the Sigma Scan Pro Software (Jandel Scientific). In all cases, at least 100 neurons randomly chosen were observed. T3CM did not affect the number and outgrowth of neurites. All groups analyzed did not present significant statistical relevance ($p > 0.05$).

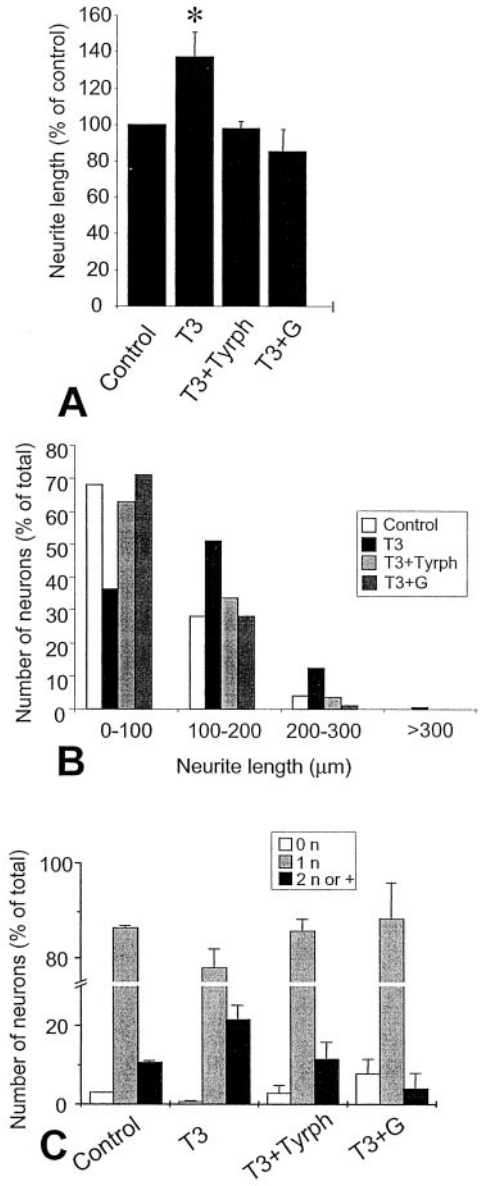

FIG. 5. **Effect of the EGFR tyrosine kinase inhibitors genistein and tyrphostin on neurite outgrowth induced by T3-astrocytes.** E19 cerebellar neurons were cultivated onto astrocyte monolayers previously treated by T3 alone or in combination with genistein (2.5 μM) (*G*) and tyrphostin (500 nM) (*Tyrph*). Inhibitor-containing medium was replaced by drug-free medium immediately before neuronal plating. After 24 h of culture the average of neurite length (*A* and *B*) and number of neurites (*C*) were analyzed as previously described. The *asterisk* corresponds to $p < 0.001$; *n*; neurites. Note that addition of genistein or tyrphostin completely inhibited T3-astrocyte effect on number and outgrowth of neurites. None of these inhibitors had a significant effect on control cultures (data not shown).

two EGFR inhibitors. Astrocytes were concomitantly treated by T3 and genistein or tyrphostin, as previously described (Fig. 5). Inhibitor-containing medium was replaced by drug-free medium immediately before to neuronal plating. After treatment, E19 cerebellar neurons were settled onto astrocyte carpets, and number and length of neurites were analyzed after 24 h of coculture. Trypan blue viability assays showed that cell viability was not altered by inhibitors (data not shown). Genistein and tyrphostin treatment of T3-astrocyte monolayers dramatically affected the average neurite length (Fig. 5*A*). Under these conditions, an increment was observed in the number of neurons extending short processes (0–100 μm) followed by a striking decrease in those with longer neurites (Fig. 5*B*). Similar results were obtained by concomitant treatment of astrocyte carpets with EGF and inhibitors (data not shown).

Number of aneuritic neurons was severely increased by concomitant treatment of astrocytes with T3 and genistein or tyrphostin, especially the former (7–10 times), whereas the fraction of more branched neurons (two or more neurites) clearly decreased after inhibitor treatment (Fig. 5*C*). Together, our data highly implicate EGF in mediation of neuritogenesis induced by T3-treated astrocytes. However, our data strongly suggest a diverse mechanism of action for EGF in neuritogenesis (indirect) from the one previously described by us to modulate neuronal proliferation (direct) (19).

EGF Effect on Cerebellar Neuritogenesis Is Mediated by Extracellular Matrix Proteins—Because we demonstrated that T3CM, which contains EGF, does not enhance neurite outgrowth, we assume that EGF action on neuritogenesis might be indirect, possibly modulating secretion of additional molecules by astrocytes. Neurite growth of CNS neurons is primarily dependent of ECM protein expression. Within ECM components, laminin and fibronectin play a major role in stimulating neurite outgrowth during NS development. To evaluate the

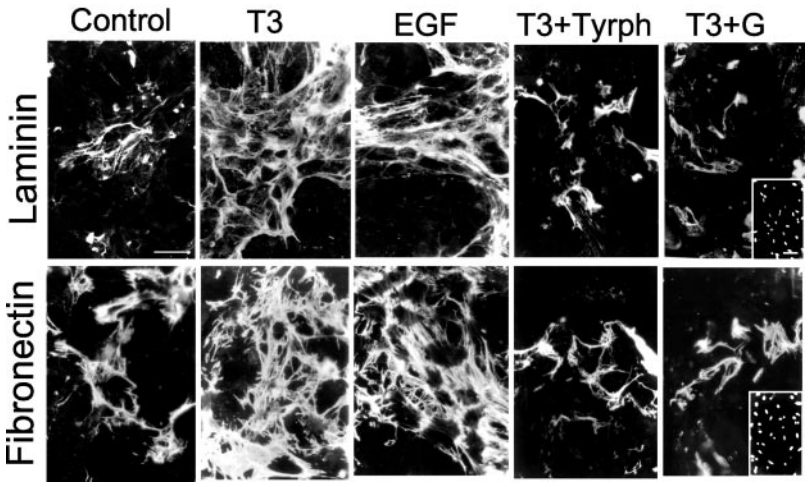

FIG. 6. **T3 and EGF modulate astrocyte production of extracellular matrix proteins.** Cerebellar astrocyte cultures treated by EGF (10 ng/ml) or T3 (50 nM) as described under "Experimental Procedures" were immunostained for the ECM proteins laminin (*upper panel*) and fibronectin (*lower panel*). Note that either T3 or EGF greatly potentiated ECM production by astrocytes. The addition of genistein or tyrphostin strongly reversed this phenomenon. *Control*, nontreated astrocytes; *T3*, astrocytes treated by T3; *EGF*, astrocytes treated by EGF; *T3+G*, astrocytes concomitantly treated by T3 and genistein; *T3+Tyrph*, astrocytes concomitantly treated by T3 and tyrphostin. *Insets* show a 4′,6-diamidino-2-phenylindole (DAPI) nuclear immunolabeling of a T3 and genistein astrocyte culture, which is equivalent for all conditions. *Scale bars* correspond to 100 μm.

involvement of these ECM proteins in EGF-induced neurite outgrowth, T3 and EGF-treated astrocytes were immunolabeled for laminin and fibronectin. As shown in Fig. 6, both proteins had their pattern of expression highly augmented after T3 and/or EGF treatments. Although in control cultures laminin and fibronectin were restricted to certain groups of cells, in treated cultures the staining was more uniform and widespread throughout cultures (Fig. 6). Staining was mostly extracellular with a network of thick and fibrous strands. Concomitant treatment of astrocytes with T3 and the EGFR inhibitors, genistein and tyrphostin, decreased fibronectin and laminin staining (Fig. 6), strongly implicating the EGF pathway in this process. Similar results were obtained by the addition of these inhibitors to EGF-treated astrocytes (data not shown). Together, these results strongly suggest that modulation of ECM protein expression might be the major mechanism by which EGF indirectly promotes neurite outgrowth of cerebellar neurons *in vitro*.

EGF Modulates Laminin and Fibronectin Expression through MAPK and PI3K Pathways—The biological response to EGF might be determined by activation of distinct signaling pathways. To define the signaling molecules involved in EGF-induced cerebellar neuritogenesis and laminin/fibronectin overexpression we used several kinase inhibitors (Figs. 7 and 8). Astrocyte monolayers concomitantly treated with EGF and the specific inhibitor for 3 days were used as carpets for cerebellar neurons. To prevent a direct action of the drug on neurons rather than a glia-mediated effect, inhibitor-containing medium was replaced by drug-free medium before neuronal plating. PD98059, a selective inhibitor of MEK (MAPK/extracellular signal regulated kinase kinase), completed blocked EGF-induced neuritogenesis (Fig. 7). Similar results were obtained by administration of LY294002, a specific inhibitor of the PI3K (Fig. 7). In contrast, KT5720, a specific inhibitor of protein kinase A, did not block EGF effects on neuritogenesis (Fig. 7). Neither inhibitor had effect on control astrocytes (data not shown). Trypan blue viability assays revealed that all inhibitors were used in nontoxic concentrations (data not shown).

To further correlate EGF-induced neuritogenesis with ECM expression, astrocytes carpets treated by EGF and kinase inhibitors were immunostained for laminin and fibronectin (Fig. 8). In agreement with their effects on neuritogenesis, PD98059 and LY294002 dramatically attenuated EGF-induced ECM overexpression (Fig. 8). As expected, KT5720 did not prevent laminin and fibronectin overexpression induced by EGF. These data highlight the straight correlation between EGF modulation of neuritogenesis and ECM overexpression and strongly implicate the MAPK and PI3K pathways in this process.

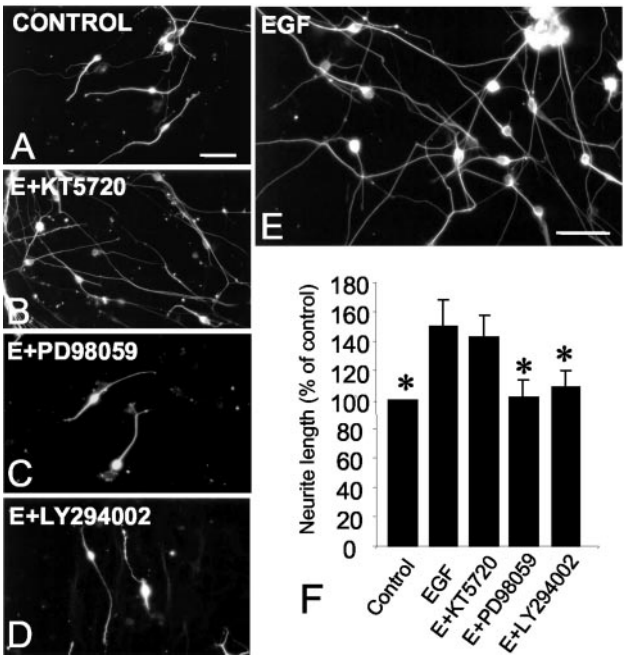

FIG. 7. **Effect of kinase inhibitors on EGF-induced neurite outgrowth.** E19 cerebellar neurons were cultivated onto astrocyte monolayers nontreated (*Control*; *A*) or previously treated by EGF (10 ng/ml) alone (*EGF*; *E*) or in combination with KT5720 (400 nM) (*E+ KT5720*; *B*), PD98059 (50 μM) (*E+ PD98059*; *C*), and LY294002 (5 μM) (*E+ LY294002*; *D*). Inhibitor-containing medium was replaced by drug-free medium immediately before neuronal plating. After 24 h of coculture, cells were immunolabeled for β-tubulin III (*A–E*), and the average of neurite length (*F*) was analyzed. EGF greatly potentiated neuritogenesis. The addition of PD98059 and LY294002 greatly prevented this phenomenon, whereas that of KT5720 had no effect on neuritogenesis. None of these inhibitors had a significant effect on control cultures (data not shown). Concomitant treatment of astrocytes with EGF and methyl sulfoxide did not impair EGF effect. The *asterisk* corresponds to $p < 0.001$ in comparison to EGF. The *scale bar* corresponds to 100 μm.

DISCUSSION

In the present work, we provide the first evidence that EGF secreted by T3-treated astrocytes induces EGL neurons to undergo differentiation initiated by outgrowth of neurites. Such an event is mediated by EGF modulation of laminin and fibronectin astrocytic expression through MAPK and PI3K pathways. The present findings together with those previously described by us (19) suggest a binary role for EGF on cerebellar ontogenesis, directly, on granular precursors proliferation and,

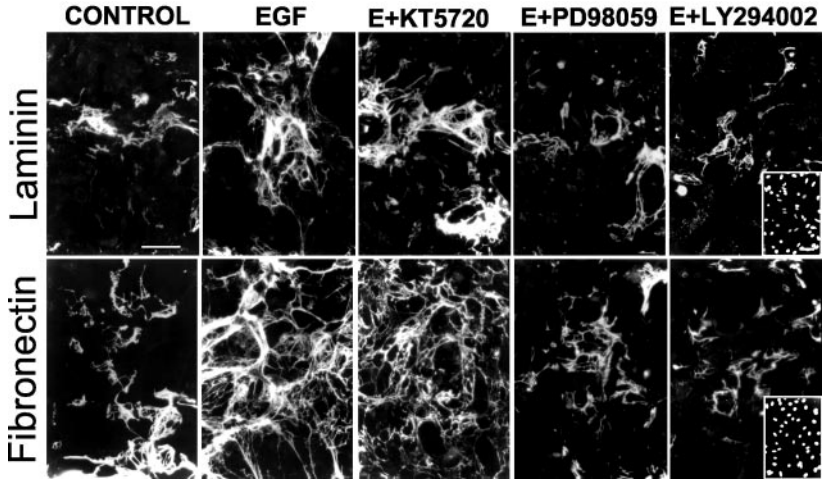

FIG. 8. **Effect of kinase inhibitors on EGF-induced astrocyte production of ECM proteins.** Astrocyte carpets were treated by EGF and specific inhibitors according to "Experimental Procedures." After treatments, cultures were immunolabeled for the ECM proteins laminin and fibronectin. EGF strongly potentiated ECM production by astrocytes. The addition of PD98059 and LY294002 greatly prevented this phenomenon, whereas that of KT5720 had no effect on ECM pattern. None of these inhibitors had a significant effect on control cultures (data not shown). *Control*, nontreated astrocytes; *EGF*, EGF-treated astrocytes; *E+KT5720*, astrocytes concomitantly treated by EGF and KT5720 (400 nM), *E+PD98059*, astrocytes concomitantly treated by EGF and PD98059 (50 μM), *E+LY294002*, astrocytes concomitantly treated by EGF and LY294002 (5 μM). *Insets* show a 4′,6-diamidino-2-phenylindole (DAPI) nuclear immunolabeling of an E+LY294002 astrocyte culture, which is equivalent for all conditions. *Scale bars* correspond to 100 μm.

indirectly, through ECM components in neurite outgrowth. Our data create a new scenario on the role of EGF and glial cells as mediators of T3 action on cerebellar development.

Astrocytes have been well recognized as the major source of ECM components including fibronectin and laminin both *in vivo* and *in vitro* (22–24). The pattern of these ECM proteins on the astrocyte surface, which is highly modulated by thyroid hormone, provides directional cues to neurite outgrowth (22, 25–28).

We now report that astrocytes treated by T3 or EGF greatly increased laminin and fibronectin fibrils in the extracellular space, thus providing a permissive substrate to neurite outgrowth. Our data contrast with those obtained from Farwell and Dubord-Tomasetti (29), who demonstrated that T4 but not T3 increases laminin expression. We believe, however, that this apparent discrepancy between these two works most likely reflect fundamental differences in the technical approaches such as hormone treatment schedule, hormone concentration, and differences in culture conditions. Furthermore, those authors have cultured astrocytes derived from whole brain, whereas we have used in our study astrocytes derived from cerebellum. It has been speculated that spatial differences in the expression of T3 receptors account for the variety of T3 response elicited in brain structures (3, 30).

The addition of the EGFR tyrosine kinase inhibitor, tyrphostin, to T3-treated astrocytes greatly inhibited the ECM increment elicited by the hormone as well as impaired astrocyte permissivity to neurite extension. These data strongly suggest that T3-induced ECM augmentation in astrocytes is mediated by EGF. Furthermore, because no additive effects on neurite outgrowth were observed in astrocytes treated by EGF and T3 in combination, it seems likely that the two growth factors act probably through the same pathway, *i.e.* induction of ECM components. Because T3-astrocytes already produce EGF (19), we assume that the addition of exogenous EGF raises the growth factor concentration beyond the saturation limit optimum for its effect.

T3 has been proposed to directly modulate some ECM genes (31, 32); however, a direct T3 regulation has not been undoubtedly reported for laminin and fibronectin. Our results do not completely rule out a T3 direct regulation of these proteins;

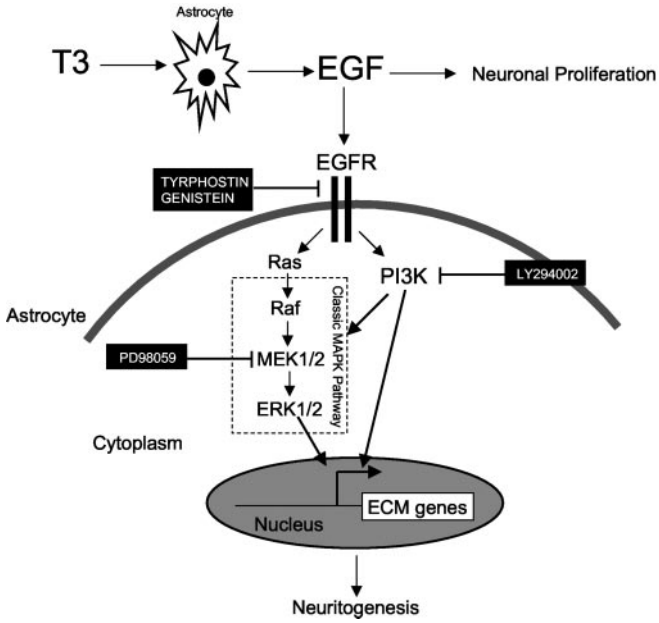

FIG. 9. **Schematic representation outlining the roles of MAPK and PI3K pathways in the EGF-induced cerebellar neuritogenesis.** Thyroid hormone induces astrocytes to secrete EGF. Directly, EGF induces neuronal proliferation; indirectly, by modulating the content of astrocytic ECM proteins, EGF increases neurite outgrowth. EGF effects are triggered by EGFR tyrosine kinase signaling mediated by cooperation between MAPK and PI3K pathways. Two possibilities are depicted; 1) PI3K and MAPK cascades are independently activated, or 2) The two pathways cross-talk somewhere. The influences of general tyrosine kinase inhibition (genistein), EGFR inhibition (tyrphostin), classic MAPK pathway inhibition (PD98059), and PI3K pathway inhibition (LY294002) are shown.

however, they reveal an additional new mechanism for ECM protein modulation mediated by EGF in the NS. Together with ours, the recent finding that fibronectin mRNA is increased by activation of the EGFR in cardiac fibroblasts (33) and EGFR gene amplification is associated with laminin overexpression in tumor cell lines (34) suggest that EGF modulation of laminin and fibronectin might be a more general process occurring in several tissues. We completely rule out a direct action of EGF

on cerebellar neurite outgrowth since addition of EGF (data not shown) or T3CM (which contains EGF) directly on neuronal cultures does not increase neuritogenesis. This is the first time a T3 action on ECM protein expression and neuronal outgrowth mediated by an intermediary growth factor in NS is clearly described.

EGF is implicated in widespread effects in CNS such as proliferation and differentiation of a variety of neuronal progenitors, postmitotic neurons, and glial cells (35, 36). EGF exerts most of its cellular actions through activation of the EGF receptor, which belongs to a family of structurally related tyrosine kinase receptors (37). Immunoreactivity for EGFR has been demonstrated in several regions of the embryonic and adult brains such as frontal cortex, hippocampus, cerebellum, and striatum (36, 38), which support a role for EGF during brain development. Signaling through EGFR is triggered by ligand binding, receptor dimerization, and tyrosine phosphorylation and is classically associated with activation of the Raf-MEK-MAP/extracellular signal-regulated kinase pathway (36, 39). In our work, the specific inhibitor of MEK1/2 kinase, PD98059, greatly inhibited laminin and fibronectin overexpression induced by EGF. Similar results were yielded by administration of the specific EGFR inhibitor, tyrphostin, which suggested that MAPK pathway is activated downstream of EGFR tyrosine kinase (data not shown). Although the molecular mechanism of ECM modulation by EGF in NS has not been described yet, EGFR transactivation was found to up-regulate fibronectin in a MEK-extracellular signal-regulated kinase-dependent manner in other systems (40–42). Activation of EGFR is followed by induction of the Ras signaling pathway characterized by a kinase cascade, including Raf, MAPK kinase, and MAPK. It has been suggested that activated MAPK can translocate into the nucleus where it phosphorylates and activates several transcriptional factors (36). Recently, it has been demonstrated that the Ras-MAPK cascade described above is just one of the transcytoplasmic nuclear-signaling pathways activated by EGF (43). This is the case of PI3K, the activity of which has been described to be stimulated by EGF. PI3Ks are a conserved family of lipid kinases that catalyze the phosphorylation of the 3′ position of the inositol ring of phosphoinositides (43). They produce lipids implicated in several cellular processes. Although the mechanism involved in EGFR activation of MAP does not display an obvious role for PI3K, pharmacological inhibitors of PI3K were found to strongly interfere with MAPK pathways in several systems (43–46). In agreement with these data, the addition of the PI3K pathway inhibitor, LY294002, completely abolished EGF-induced ECM overexpression. Recent evidence has been accumulated pointing a functional cross-talking between PI3K and MAP kinase pathways (43, 45–48).

Because we previously demonstrated that the effects of EGF on neuronal proliferation involved the protein kinase A-cAMP pathway, we sought to investigate the role of this pathway on EGF-induced neuritogenesis. The addition of the protein kinase A inhibitor KT5720 had no effect on EGF-induced ECM overexpression and neuritogenesis. Taken together, two models for the T3/EGF neuritogenesis induced by astrocytes might be proposed. Thyroid hormone induces cerebellar astrocytes to secrete EGF, which induces neuronal proliferation (Ref. 19 and Fig. 9). By autocrine mechanism, EGF activates astrocytic EGFR. Transactivation of EGFR leads to 1) induction of PI3K followed by MAPK pathway activation, or 2) alternatively, EGFR may activate two separate cascades, a PI3K-dependent pathway and the classical MAPK pathway (Fig. 9). The fact that the administration of LY294002 and PD98059 alone is sufficient to completely inhibit ECM overproduction, and concomitant addition does not yield additive inhibition (data not shown) call in favor of converging rather than independent pathways. Full elucidation of the molecular mechanisms implicating PI3K and MAPK pathways await further experiments.

We reported a new attribute of EGF as mediator of thyroid hormone action on cerebellar development. Our results suggest that EGF might play a crucial role in distinct aspects of granular cell development in culture. How these *in vitro* results could account for *in vivo* cerebellar ontogenesis? Expression of the EGFR and T3 receptor does appear to be temporally uncoordinated in cerebellum. The early germinative zone of the EGL (E15–19) was not undoubtedly reported to express T3 receptor, which will be expressed later in the development in the postmitotic premigratory zone of EGL and in the internal granular layer (49), whereas EGFR mRNA is highly expressed in the EGL (50). These data highlight the importance of a mediator for T3 activity (possibly glia cells via EGF secretion) at least on these early events of cerebellum ontogenesis.

Other factors also modulated by thyroid hormone such as the neurotrophins family have been also implicated in the regulation of several steps of cerebellar development (51). Our work points to EGF as an additional growth factor in the modulation of cerebellar granular cell ontogenesis, thus providing support for a multiple novel neurotrophic activity of growth factors in the development of cerebellar cortex. The fact that replacement of neurotrophin-3 or brain-derived neurotrophic factor results in some rescue of cerebellar development in hypothyroid animals (52) points to the possibility of using glia-derived growth factors as putative therapy to congenital hypothyroidism. Understanding the molecular relationship of thyroid hormones and neuron-astrocyte interactions could open in the future a new avenue to explore and rescue the abnormalities exhibited by the hypothyroid brain. Our work provides the first evidence that EGF secreted by astrocytes mediates thyroid hormone neuritogenesis in the cerebellum. The complexity of the processes underlying axonal growth suggests the existence of multiple sites of possible regulation. Therefore, it is likely that modulation of ECM proteins by EGF reported here in this paper might provide a potential mechanism by which this morphogenetic hormone exerts its effects on neurite outgrowth and establishment of neuronal connections.

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