

Neuro–glia interaction effects on GFAP gene: a novel role for transforming growth factor- β 1

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Abstract

Central nervous system (CNS) development is highly guided by microenvironment cues specially provided by neuron–glia interactions. By using a transgenic mouse bearing part of the gene promoter of the astrocytic maturation marker GFAP (glial fibrillary acidic protein) linked to the β -galactosidase (β -Gal) reporter gene, we previously demonstrated that cerebral cortical neurons increase transgenic β -Gal astrocyte number and activate GFAP gene promoter by secretion of soluble factors *in vitro*. Here, we identified TGF- β 1 as the major mediator of this event. Identification of TGF- β 1 in neuronal and astrocyte extracts revealed that both cell types might synthesize this factor, however, addition of neurons to astrocyte monolayers greatly increased TGF- β 1 synthesis and secretion by astrocytes. Further, by exploiting the advantages of cell culture system we investigated the influence of neuron and astrocyte developmental stage on such interaction. We demonstrated that younger neurons derived from 14 embryonic days wild-type mice were more efficient in promoting astrocyte differentiation than those derived from 18 embryonic days mice. Similarly, astrocytes also exhibited timed-schedule developed responsiveness to neuronal influence with embryonic astrocytes being more responsive to neurons than newborn and late postnatal astrocytes. RT-PCR assays identified TGF- β 1 transcripts in young but not in old neurons, suggesting that inability to induce astrocyte differentiation is related to TGF- β 1 synthesis and secretion. Our work reveals an important role for neuron–glia interactions in astrocyte development and strongly implicates the involvement of TGF- β 1 in this event.

Introduction

The vertebrate central nervous system (CNS) is composed of three major classes of neural cells: neurons, macroglial cells that include astrocytes and oligodendrocytes, and microglial cells. Interactions between those cellular components play an important role in several processes of brain development such as cell specification, neuronal proliferation and migration, axonal guidance, synapse formation and glial maturation (Garcia-Abreu *et al.*, 1995; Pfriederger & Barres, 1997; Fróes *et al.*, 1999; Gomes *et al.*, 1999a,b; Lim & Alvarez-Buylla, 1999; Noda *et al.*, 2000; Oppenheim *et al.*, 2000; Cui *et al.*, 2001; Gomes *et al.*, 2001a,b; Song *et al.*, 2002). It has been reported that neurons are able to modulate morphology, differentiation and gene expression of astrocytes (Hatten, 1985; Kentroti & Vernadakis, 1997; Swanson *et al.*, 1997; Gomes *et al.*, 1999a; Perego *et al.*, 2000; Rouach *et al.*, 2000). However, until now the mechanism involved in such interaction as well as a putative molecule implicated in this process has not yet been fully identified.

GFAP (glial fibrillary acidic protein) is the major intermediate filament of mature astrocytes and has been widely recognized as an astrocyte differentiation marker (Eng *et al.*, 1971; Bignami *et al.*, 1972; Gomes *et al.*, 1999a). The generation of GFAP-

deficient mice has recently provided new insight into the role of this protein, in addition to its structural function. It has been implicated in several processes of brain development including maintenance of CNS white matter architecture, blood brain barrier formation, myelination, CNS response to injury and neuron–glia interaction (Liedtke *et al.*, 1996; Wang *et al.*, 1997; Pekny *et al.*, 1998, 1999; Menet *et al.*, 2000, 2001). Understanding the factors that modulate GFAP gene expression might contribute to elucidate the molecular mechanisms involved in the physiology of normal and pathologic astrocytes.

By using a transgenic mouse bearing part of the GFAP gene promoter linked to the β -galactosidase (β -Gal) reporter gene, we demonstrated that cortical neurons are able to induce the GFAP gene, followed by transgenic astrocyte differentiation *in vitro* (Gomes *et al.*, 1999a). This event seems to be mediated by a secreted neuronal factor, because conditioned medium (CM) derived from virtually pure neuronal cultures mimicked astrocyte differentiation and GFAP gene promoter activation elicited by neurons (Gomes *et al.*, 1999a). In the present study, we demonstrated for the first time that cortical neurons induce GFAP gene promoter by secreting TGF- β 1 (transforming growth factor β 1). Furthermore, we exploited the advantages of cell culture system to understand neuron–astrocyte interaction mediated by TGF- β 1 and analysed the influence of neuron and astrocyte developmental stage on such interaction. Our data reveal an important role of neuronal cells on astrocyte differentiation and shed light into the role of TGF- β 1 in gliogenesis.

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Materials and methods

Astrocyte primary cultures

Astrocyte primary cultures were prepared from transgenic mice bearing part of the 5' flanking region of the murine GFAP gene linked to the *Escherichia coli* β -galactosidase (β -Gal) reporter gene (*lacZ*), as previously described (Galou *et al.*, 1994; Gomes *et al.*, 1999a). Cultures were prepared from cerebral hemispheres (CH) derived from embryonic, newborn and postnatal transgenic mice, following the procedure previously described (Gomes *et al.*, 1999a). All animals were kept under standard laboratory conditions according to NIH guidelines. After the mice were anaesthetized, they were decapitated, the cerebral hemispheres (CH) were removed and the meninges carefully stripped off. Tissues were washed in phosphate-buffered saline (PBS) with 0.6% glucose (Sigma Chemical Co., St Louis, MO, USA) and dissociated into single cells in a medium consisting of Dulbecco's minimum essential medium (DMEM) and nutrient mixture F12 (DMEM/F12, Gibco BRL, Rockville, MD, USA), enriched with glucose (3.3×10^{-2} M), glutamine (2×10^{-3} M) and sodium bicarbonate (0.3×10^{-2} M). A total of 3×10^5 cells were plated onto 15.5-mm diameter wells (24-wells plates, Sigma), previously coated with polyornithine (1.5 μ g/mL, M_r 41 000, Sigma), in DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS, Fazenda Pigue, Rio de Janeiro, Brazil). For immunocytochemistry assays, cells were plated on polyornithine-treated glass coverslips. The cultures were incubated at 37 °C in a humidified 5% CO₂, 95% air chamber for 10 days until reaching confluence.

Cocultures

After reaching confluence, glial monolayers were washed three times with serum-free DMEM/F12 medium and incubated as previously described for 2 additional days in serum-free medium. After this period, neurons freshly dissociated from the CH of Swiss mice at embryonic day 14 (E14) or E18 were obtained following a previously described procedure, and plated onto the transgenic glial monolayer carpets. Cocultures were kept for 24 h under the same conditions previously described (Gomes *et al.*, 1999a).

Detection of β -galactosidase (β -Gal) activity and quantitative analysis of β -Gal positive astrocytes

Glial cultures and cocultures were fixed in 4% paraformaldehyde in PBS and stained for β -Gal with 0.4 mg/mL of 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-Gal, US Biochemical Corp., Cleveland, OH, USA) as substrate in 4 mM potassium ferricyanide, 4 mM potassium ferrocyanide, 2 mM MgCl₂ and 0.001% Tween 20. Staining was allowed to occur for 16–20 h at 37 °C. Development of the β -Gal reaction was stopped after several washes with PBS. After β -Gal detection, transgenic cultures were analysed for β -Gal-positive astrocytes under a Zeiss Axiovert 35 microscope. At least three fields were counted per well. The experiments were carried out in duplicate or triplicate and each result represents the mean of three independent experiments. Statistical analyses were carried out using ANOVA.

Immunocytochemistry

Immunocytochemistry was performed as previously described (Gomes *et al.*, 1999a). Briefly, cultured cells were fixed with 4% paraformaldehyde for 30 min and permeabilized with 0.2% Triton X-100 for 5 min at room temperature. After permeabilization, cells were blocked with 10% normal goat serum (NGS, Vector) in PBS (blocking solution) for 1 h and incubated overnight at room temperature with the specified primary antibodies diluted in blocking

solution. For peroxidase staining, previous to the primary antibody incubation, endogenous peroxidase activity was abolished with 3% H₂O₂ for 10 min, followed by extensive washing with PBS. Primary antibodies were rabbit anti-(cow GFAP) serum (Dako; 1 : 50 dilution); mouse anti-(human β -tubulin III) antibody (Sigma; 1 : 200) and anti-(transforming growth factor- β receptor type II) (TGFRII) antibody (Santa Cruz Biotechnology, Inc.; 1 : 100 dilution). After primary antibody incubation, cells were extensively washed with PBS/10% normal goat serum (NGS) and incubated with secondary antibodies for 1 h, at room temperature. Secondary antibodies were conjugated with horseradish peroxidase (HRP) [goat anti-(mouse IgG), Gibco; 1 : 200] or with Cy3 [sheep anti-(rabbit IgG); Sigma; 1 : 3000]. Peroxidase activity was revealed with 3,3'-diaminobenzidine (DAB, Sigma). Negative controls were performed by omitting primary antibody during staining. In all cases no reactivity was observed when the primary antibody was absent.

Conditioned medium (CM) preparation

In order to prepare conditioned medium (CM) from neurons, neuronal cells derived from cerebral hemispheres (CH) of 14 days embryonic (E14) Swiss mice were plated on polyornithine-coated wells (or glass coverslips) (400 000 cells/well) in DMEM/F12 serum-free medium. The cultures were maintained for 24 h at 37 °C in a humidified 5% CO₂, 95% air chamber. The culture medium was then recovered, centrifuged at 1500 g for 10 min to remove the eventual cellular debris, and either used immediately or stored in aliquots at –20 °C for future use. Adherent cells on the coverslips were fixed with 4% paraformaldehyde and immunoreacted with antibody specific to the neuronal marker β -tubulin III. Approximately 95% of the cells stained with the antibody, confirming their neuronal phenotype. No GFAP-positive cells were found under these conditions. Conditioned medium (CM) from cocultures were prepared by cultivating 14 days embryonic (E14) neurons with newborn astrocytes for 24 h, as previously described (Gomes *et al.*, 1999a). After recovering of coculture conditioned medium (CM) the same procedure was followed as described for neuronal CM. Use of coculture or neuronal CM yielded similar results. We used coculture CM in most of experiments, except for identification of TGF- β 1 secreted by neurons (Fig. 3B). In this case, neuronal CM was used (Fig. 3B).

Treatment of transgenic astrocyte monolayers with CM and growth factors

Astrocyte monolayers derived from newborn transgenic mice CH were prepared as previously described. After 10 days *in vitro*, in presence of DMEM/F12 supplemented with 10% FBS, the cultures were incubated for 2 additional days with DMEM/F12 serum-free. The culture medium was then removed and replaced by the same volume of one of the CM described above. The following growth factors were added, alone or in combination, to serum-free medium (10 ng/mL): human transforming growth factor- β 1 (TGF- β 1) (R&D Systems, Buckinghamshire, UK); basic fibroblast growth factor (bFGF) (kindly provided by Mary Armelin, Institute Ludwig, São Paulo, Brazil); epidermal growth factor (EGF) (Gibco). Cultures were kept for an additional 24 h at 37 °C in a humidified 5% CO₂, 95% air chamber and then stained with X-Gal, as described previously.

Antibody blocking assays

For neutralization assays, CMs were incubated for 2 h, at 37 °C, prior use, in presence of 10 μ g/mL of a neutralizing antibody against transforming growth factor- β 1 (TGF- β 1) (chicken anti-human TGF- β 1 antibody; R&D Systems) or a control unrelated immunoglobulin.

In coculture experiments, the neutralizing antibodies were added onto astrocyte monolayers at the same time as neurons.

Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was isolated from cells using TRIZOL (Life Technologies), according to the protocol provided by the manufacturer. Single-stranded cDNA was synthesized using Moloney Murine Leukaemia Virus (M-MuLV) reverse transcriptase with the First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech). Briefly, 1–5 μ g of RNA was incubated with a reaction mixture containing 45 mM Tris, pH 8.3; 68 mM KCl; 15 mM dithiothreitol; 9 mM MgCl₂; 0.08 mg/mL BSA and 1.8 mM each dNTP; 5 μ g of oligo dT primer (5'-d[AACTGGAAGAATTCGCGGCCGAGGAAT18]-3'); 6.0 mM of dithiothreitol for 1 h at 37 °C. The reaction was stopped by heating the mixture to 95 °C for 5 min and then placing on ice. Amplification was performed on a thermal cycle (PCR Sprint Temperature Cycling System, Hybaid Limited) using *Taq* DNA Polymerase (Amersham Pharmacia Biotech) under the following conditions (33 cycles): denaturation at 92 °C for 2 min; annealing at 60 °C for 1 min 30 s and polymerization at 72 °C for 1 min 30 s. The first denaturation temperature was 94 °C and the final extension at 72 °C was allowed to continue for 7 min. Sense and antisense specific primers were used as follows, respectively: TGF- β 1: 5'-GCGGACTACTATGCTAAAGAAG-3' and 5'GTTGTGTTGG-TTGTAGAGGGCA-3' giving a product of predicted length of 745 bp; β -actin: 5'-GTGGGCGCGTCTAGGCACCAA-3' and 5'-CTCTTTGATGTCACGCACGATTTC-3' giving a product of predicted length of 550 bp; GFAP: 5'-CTTTGCTAGCTACATCGAGAAGGTCCGCTT-3' and 5'-CGATTCAACCTTCTCTCCAAATCCACACG-3' giving a product of predicted length of 281 bp. Amplified products were size-fractionated by electrophoresis using a 2% agarose gel. Negative controls for contamination from extraneous and genomic DNA were carried out. Under these conditions, no apparent band was detected.

Western blot analysis

To detect proteins of interest, cultures and cocultures were lysed in buffer A, containing 102 mM of MES, 0.51 mM of MgCl₂, 10 mM of EGTA, 0.08 mM of β -mercaptoethanol, 1 mM of phenylmethanesulfonyl fluoride and 1 mM of benzamidine. Cell lysates were mixed with LB (loading buffer) (450 mM of dithiothreitol, 345 mM of SDS, 400 mM of Tris pH 6.8 and 50% of Glycerol), and then boiled for 5 min before loading in the gel. Proteins from CM were obtained after precipitation with 10% trichloro-acetic acid (TCA) on ice for at least 30 min (or overnight at 4 °C). After precipitation, proteins were harvested at 10 000 g, at 4 °C for 10 min and washed twice with cold 100% ethanol. The pellet was lysed in buffer A and CM proteins were treated as previously described for the cellular extract samples. A total of 10 μ g protein per lane were electrophoresed in a 10% or 15% SDS/PAGE mini gel, according to the protein size. After separation, proteins were electrically transferred onto a membrane [Hybond-P poly(vinylidene fluoride) transfer membrane] for 1 h. Membranes were blocked in PBS/BSA 5% and primary antibodies added for 1 h at room temperature. After several washes, a peroxidase-conjugated secondary antibody was added to the membranes and incubated for 1 h at room temperature. Proteins were visualized using the enhancing chemiluminescence detection system (ECL-Plus, Amersham Pharmacia Biotech). The following primary antibodies were used: mouse anti-(β -actin) (Santa Cruz, 1 : 500 dilution); mouse anti-GFAP (Sigma, 1 : 2000 dilution) and mouse anti-(human TGF- β 1) (R&D Systems, 1 : 200 dilution). The follow-

ing secondary antibodies were used: goat anti-(rabbit IgG) (Gibco); goat anti-(mouse IgG) (Gibco), both 1 : 20000 dilution.

Results

TGF- β 1 is responsible for neuronal induced-astrocyte differentiation

We previously demonstrated that neuronal-induced astrocyte differentiation is followed by a decrease in cell proliferation and activation of GFAP gene promoter without changes in astrocyte morphology. Conditioned medium (CM) prepared from virtually pure neuronal cultures mimicked these effects, which suggested that these features were mainly mediated by a soluble factor secreted by neurons (Gomes *et al.*, 1999a and Fig. 1). As TGF- β 1 has been implicated in many of these effects, we sought to investigate the involvement of TGF- β 1 on neuronal-induced astrocyte differentiation. With this aim, CH astrocyte cultures derived from newborn transgenic mice were cultured in presence of TGF- β 1 for 24 h (Fig. 1A). The addition of 10 ng/mL of TGF- β 1 led to a 60% increase in β -Gal astrocyte number. This value resembles that obtained by the addition of neurons or CM (60–80%) (Fig. 1A).

The activity of members of TGF- β family is resistant to boiling and acid treatment and sometimes even potentiated by these processes (Flaumenhaft *et al.*, 1993; Dobbertin *et al.*, 1997; Mittaud *et al.*, 2002). In order to search for a TGF- β -like activity in the CM, this medium was boiled for 5 min prior to use. As it can be observed in Fig. 1A, boiling of CM did not impair its activity.

To fully identify TGF- β 1-like effect on neuronal-astrocyte cocultures and in CM, neutralizing antibodies directed against human TGF- β 1 were added to transgenic astrocytes cultured in presence of neurons, CM or TGF- β 1 (Fig. 1A). Addition of 10 μ g/mL of anti-(TGF- β 1) antibody fully prevented neuronal, CM and TGF- β 1 effects (Fig. 1A). Comparisons between anti-(TGF- β 1) antibody and an unrelated IgG directly confirmed the specificity of the blocking effects (Fig. 1A).

In order to address astrocyte differentiation mediated by TGF- β 1, we employed anti-(TGF- β 1) neutralizing antibodies in astrocyte monolayers cultured with neurons or in presence of CM and analysed GFAP levels in the cell extracts (Fig. 1B and C). Neurons and CM caused an approximately threefold increase in GFAP protein levels (Fig. 1B and C). Addition of anti-(TGF- β 1) antibody prevented GFAP level increase induced by both neurons and CM (Fig. 1B and C).

As TGF- β 1 action has been reported to be potentiated and sometimes even mediated by other growth factors such as basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) (Kriegelstein *et al.*, 1998a,b; Reilly *et al.*, 1998; Dhandapani *et al.*, 2002; Diez-Marques *et al.*, 2002), we sought to investigate the effect of these growth factors on TGF- β 1 action (Fig. 2). Astrocyte cultures were kept for 24 h in the presence of basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), and TGF- β 1 or in presence of a mixture of TGF- β 1/FGF or TGF- β 1/EGF (Fig. 2). bFGF or EGF did not significantly increase the number of β -Gal-positive astrocytes. Addition of either bFGF or EGF combined with TGF- β 1 did not enhance TGF- β 1 effect on astrocyte differentiation, rather both decreased TGF- β 1 effect (bFGF, 9% and EGF, 22% inhibition) (Fig. 2B and C). Concomitant addition of E14 neurons and TGF- β 1 did not enhance their effects, but instead slightly inhibited neuron and TGF- β 1 action on β -Gal astrocyte number (15% of inhibition) (Fig. 2A).

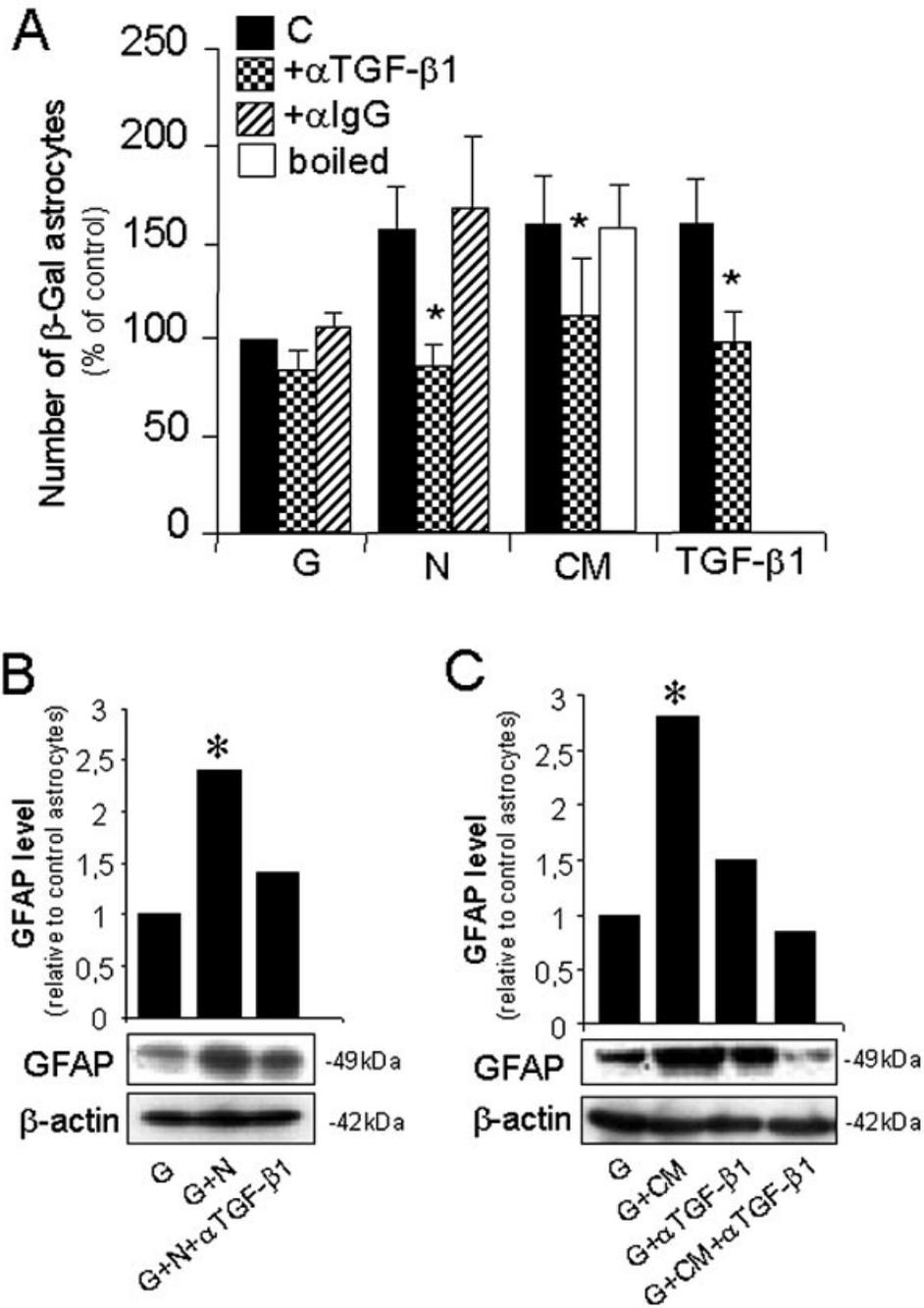


FIG. 1. Neutralization of neurons and neuronal CM effects on GFAP gene promoter by anti-(TGF-β1) antibodies. (A) Newborn astrocytes were cultured alone (G), with E14 neurons (N), conditioned medium (CM) or TGF-β1 (10 ng/mL) in presence or absence of 10 μg/mL of neutralizing antibodies. After 24 h of culture, β-Gal astrocyte number was analysed. Each point represents the average of three independent experiments carried out in triplicate. **P* < 0.001 in comparison to control, black bar (mean ± SD). C: control (without antibody). +αTGF-β1: addition of antibody against TGF-β1; +αIgG: addition of unrelated unspecific immunoglobulin; boiled: CM boiled for 5 min (without antibody). Neurons, CM and TGF-β1 dramatically increased β-Gal astrocyte number whereas addition of anti-TGF-β1 prevented this effect. Boiling of CM did not alter its effect on astrocyte monolayer. (B and C) Representative Western blots and graphic analysis of three independent experiments showing GFAP protein levels. After neutralizing antibody assays, equal amount of total protein (10 μg per lane) from astrocytes cultured with neurons (B) and CM (C) was resolved in a 10% polyacrylamide gel and analysed by immunoblotting for GFAP. Immune reaction for β-actin was used to monitor loading. Neurons and CM greatly increased GFAP levels, whereas addition of anti-(TGF-β1) prevented this overexpression. The levels of GFAP protein immunoreactivity are expressed relative to the levels observed in cultures of astrocytes alone (control astrocytes). **P* < 0.05 (mean ± SD). G: P2 astrocytes cultured alone; G + N: P2 astrocytes cultured with E14 neurons; G + CM: P2 astrocytes cultured with CM; αTGF-β1: depicts addition of 10 μg/mL of antibody against TGF-β1.

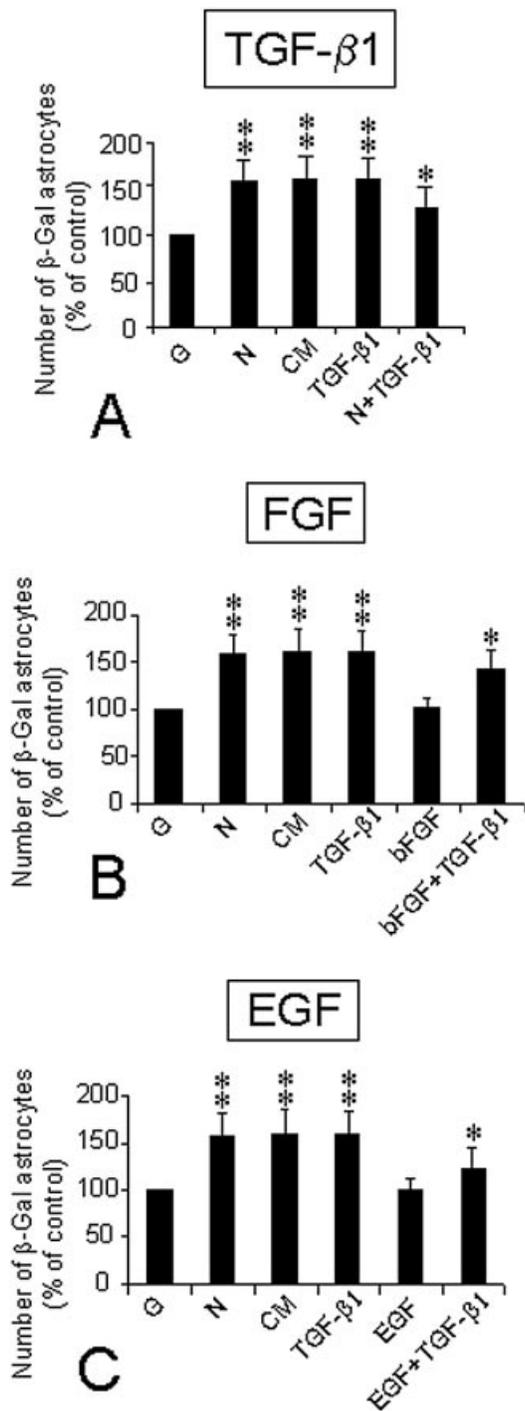


FIG. 2. Effect of TGF- β 1, bFGF and EGF on GFAP gene promoter-directed expression of lacZ. Cerebral hemisphere (CH) astrocytes derived from newborn transgenic mice were cultured alone (G), with E14 neurons (N), with conditioned medium (CM) or in presence of TGF- β 1, bFGF or EGF. Factors were added at final concentration of 10 ng/mL alone or in combination and kept for 24 h. After β -Gal activity detection, β -Gal positive astrocytes were quantified. Each point represents the average of three independent experiments carried out in triplicate. Statistical significance: * $P < 0.05$; ** $P < 0.00001$ (mean \pm SD). Addition of 10 ng/mL of TGF- β 1 greatly increased number of β -Gal astrocytes while addition of bFGF and EGF had no effect on this number. Administration of bFGF, EGF and neurons, together with TGF- β 1 decreased TGF- β 1 action.

These results indicate that neuronal-induced astrocyte differentiation and GFAP gene promoter induction could be mainly attributed to a TGF- β 1-like activity present in neuronal CM. Furthermore, under these conditions, TGF- β 1 effects are not synergistically enhanced by bFGF and EGF.

Characterization of TGF- β 1 synthesis and secretion by neurons and astrocytes

TGF- β 1 synthesis was analysed by Western blot assays of total protein extracts from E14 cultured cortical neurons and newborn astrocytes cultured alone or in presence of E14 neurons or CM (Fig. 3A). TGF- β 1 was identified in glial and neuronal extracts although the former showed to be a better source of TGF- β 1 (Fig. 3A). The addition of either neurons or CM significantly increased synthesis of TGF- β 1 by astrocytes (fivefold) (Fig. 3A).

We next investigated TGF- β 1 secretion by neurons and astrocytes (Fig. 3B). With this aim, proteins secreted by neurons, newborn astrocytes cultured alone or with neurons, were precipitated and analysed by Western blot (Fig. 3B). Secreted TGF- β 1 was observed in all conditions (Fig. 3B). Addition of E14 neurons drastically increased TGF- β 1 secretion by astrocytes (12-fold) (Fig. 3B).

Next, we directly assessed TGF- β 1 gene expression in neurons and astrocytes by RT-PCR (Fig. 3C). Amplification of β -actin mRNA was performed to assay RNA integrity and for loading control. Total mRNA from cultured neurons derived from E14 and E18 wild-type mice and from astrocytes cultured alone or with E14 neurons were reverse-transcribed and amplified by PCR. TGF- β 1 transcripts were detected in either astrocytes cultured alone or in presence of E14 neurons (Fig. 3C). We detected TGF- β 1 transcripts in E14 neurons, although the level of expression was lower than that of astrocytes (Fig. 3C). In order to ensure that the E14 neuron-derived TGF- β 1 product was from a pure neuronal source and not due to an astrocyte contamination, we performed RT-PCR for GFAP (Fig. 3C). We did not detect GFAP transcript in neuronal cultures, even in longer PCRs (40 cycles) (Fig. 3C and data not shown). We did not detect TGF- β 1 transcripts in older (E18) neurons (Fig. 3C). These results show that young neurons (E14) synthesize TGF- β 1. Furthermore, they strongly suggest that TGF- β 1 secretion by astrocytes is increased by neurons.

Neuron-astrocyte interaction mediated by TGF- β 1 is modulated by cellular age

Analysis of the time-course of neuron-glia interaction previously demonstrated that maximum effect of neuron on GFAP-lacZ construction was observed after 24 h of interaction (Gomes *et al.*, 1999a) and Fig. 4A. Neurons cocultured with astrocytes for longer periods, such as 48 and 72 h, were not able to sustain β -Gal astrocyte number increase (Fig. 4A). We wondered if neuron ability to induce astrocyte differentiation is lost during later stages of development. Therefore, E14 CH neurons were plated onto transgenic newborn astrocyte monolayers. After 48 h of coculture, E14 CH neurons were added again to the cocultures. The addition of new neurons resulted in an increase in β -Gal astrocytes (Fig. 4A).

In order to investigate a possible correlation between neuronal maturation *in vitro* and astrocyte differentiation (e.g. GFAP gene promoter induction), we performed neuronal morphological analyses. In fact, immunostaining of neurons by the antibody against the neuronal maturation marker β -tubulin III demonstrated that coculture progression was followed by an enhancement of neuronal complexity (Fig. 4B). After 3 days in coculture, an increase in neurite outgrowth was clearly observed. In order to go further in this question, we tested the ability of older CH neurons derived from E18 wild-type mice to increase β -Gal astrocyte number. Older neurons did not induce

astrocyte differentiation confirming the above results obtained from kinetic assays that as maturation proceeds; neurons lose their ability to induce astrocyte differentiation (Fig. 4A). These data, together with the observation that E18 neurons do not synthesize TGF- β 1 transcripts (Fig. 3C), suggest that neuronal ability to induce astrocyte differentiation and GFAP gene promoter might be directly associated to TGF- β 1 production and inversely correlated to neuronal age.

As responsiveness to TGF- β 1 is modulated during development, we next investigated the influence of astrocyte age on neuron/TGF- β 1-induced astrocyte differentiation *in vitro*. To do so, we prepared CH astrocytes cultures from E14, newborn and 10-day-old (P10) transgenic mice and tested the ability of such cultures to respond to E14 neurons (Table 1).

Newborn cultures, as previously demonstrated, increased β -Gal astrocyte number by 50–60% after being cocultured with 200 000 neurons (Gomes *et al.*, 1999a and Table 1). Interestingly, younger astrocytes derived from E14 mice increased the β -Gal astrocyte number by 250–300%, whereas older astrocytes failed to respond to neurons (Table 1). Western blot assays revealed that although E14 and P2 astrocytes increased GFAP synthesis in response to neurons, the level of this protein in P10 astrocytes was not affected by neurons (data not shown). The addition of increasing concentrations of E14 neurons demonstrated that younger astrocytes (E14) respond to neurons, referring to GFAP promoter-directed expression of *lacZ*, even when low concentrations of neurons (50 000 and 100 000) were plated onto the monolayer, in contrast to newborn astrocytes

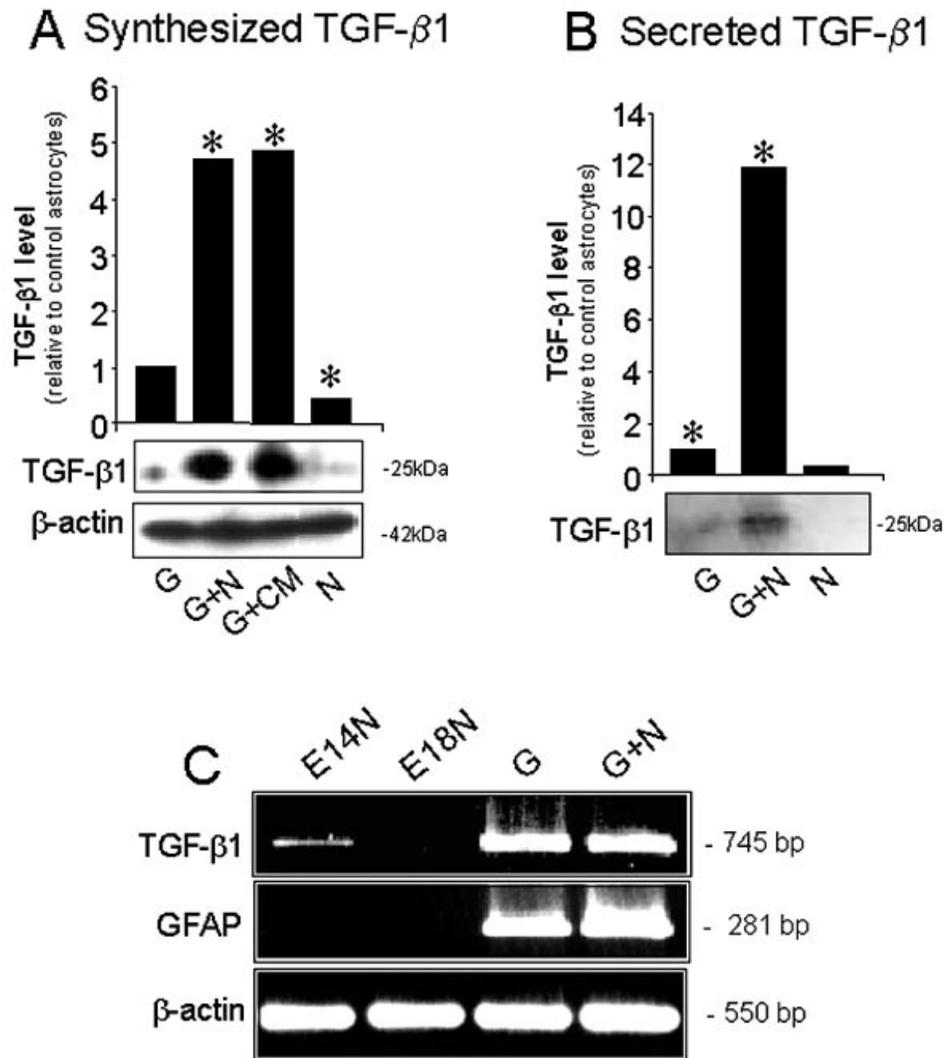


FIG. 3. Identification of TGF- β 1 content and mRNA expression in neurons and astrocytes. Representative Western blots and graphic analysis (A and B) of three independent experiments showing TGF- β 1 content. (A) Synthesized TGF- β 1: Equal amounts (10 μ g per lane) of total protein of cellular extracts of E14 neurons (N), P2 astrocytes cultured alone (G) or with E14 neurons (G + N) or with CM (G + CM) were resolved in 10% polyacrylamide gel and analysed by immunoblotting for TGF- β 1. Immune reaction for β -actin was used to monitor cellular protein loading. The levels of TGF- β 1 protein immunoreactivity are expressed relative to the levels observed in cultures of astrocytes alone. (B) Secreted TGF- β 1: Media containing total proteins secreted by E14 neurons (N), P2 astrocytes cultured alone (G) or with E14 neurons (G + N) were precipitated with 10% TCA and analysed by immunoblotting. Addition of neurons and CM substantially increased TGF- β 1 synthesis and secretion by astrocytes. * $P < 0.05$ (mean \pm SD). (C) RT-PCR of TGF- β 1 mRNA. Total RNA was extracted from E14 and E18 neurons (E14N and E18N) and from astrocytes cultured alone (G) or in presence of E14 neurons (G + N). Reverse-transcribed RNA was amplified by PCR using specific primers for TGF- β 1, GFAP and β -actin. Amplified products were size-fractionated by electrophoresis through a 2% agarose gel and visualized by ethidium bromide staining. Message for TGF- β 1 was not detected in E18 neurons whereas young neurons (E14) and astrocytes showed a clearly amplification product.

(Fig. 5A). These data demonstrate that astrocyte differentiation in response to neurons is down-regulated by astrocyte age, at least concerning GFAP gene expression.

In order to correlate neuronal astrocyte-responsiveness to synthesis of TGF- β 1, we investigated TGF- β 1 levels in astrocytes from different ages cultured alone or in presence of neurons (Fig. 5B). TGF- β 1 expression by astrocytes decreased with age. TGF- β 1 level was 40% higher in E14 astrocytes than P2 astrocytes (Fig. 5B). TGF- β 1 expression was barely detected in astrocytes derived from P10 mice (Fig. 5B). Addition of neurons onto E14, P2 and P10 astrocytes increased the level of TGF- β 1, although this increment tended to decrease as astrocyte age advances (Fig. 5B). Similar results were obtained by employing CM, instead of neurons (data not shown). These results suggest that astrocyte responsiveness to neurons is

closely related to TGF- β 1 synthesis. However, older astrocytes, which do not respond to neuronal influence, in respect of GFAP-gene promoter induction, synthesized TGF- β 1 in response to neurons. Thus, we sought to investigate if such differences in astroglia responsiveness might reflect differences in their expression of TGF- β receptors (Fig. 6). Immunocytochemical detection of transforming growth factor- β receptor type II (TGFRII) in P2 and P10 astrocytes revealed significant differences in both intensity and pattern of expression of this protein (Fig. 6). The intensity of TGFRII expression was greatly enhanced in P2 astrocytes (Fig. 6). Furthermore, the pattern of expression in P2 astrocytes was punctual and spread all over the cytoplasm (Fig. 6A, B, I and J), while in P10 astrocytes TGFRII was diffuse and less localized (Fig. 6E and F). The addition of neuron did not alter either the pattern of expression or intensity of TGFRII in both cocultures (P2 and P10; data not shown).

TABLE 1. Astrocyte responsiveness to neurons depends on astrocyte age

Astrocyte age	Percentage increase of β -Gal astrocyte number by neurons
E14	250–300%
P2	50–60%
P10	No increase

Astrocyte monolayers derived from E14, newborn (P2) and 10-day-old (P10) CH were cultured in presence of 200 000 E14 neurons. Cocultures were maintained for 24 h. After X-Gal development, β -Gal astrocyte number was analysed.

Discussion

Previous studies have suggested that neurons modulate astrocyte morphogenesis, but a putative molecule as well as the mechanism underlying this effect remains poorly understood. By using a GFAP-*lacZ* transgenic mouse lineage, we demonstrated that neurons activate the GFAP gene promoter inducing astrocyte differentiation *in vitro* (Gomes *et al.*, 1999a). In the present study, we provide evidence that such an event is mediated by TGF- β 1 and modulated by the developmental stages of neurons and astrocytes. As far as we know, this is the first time that the induction of astrocyte differentiation and

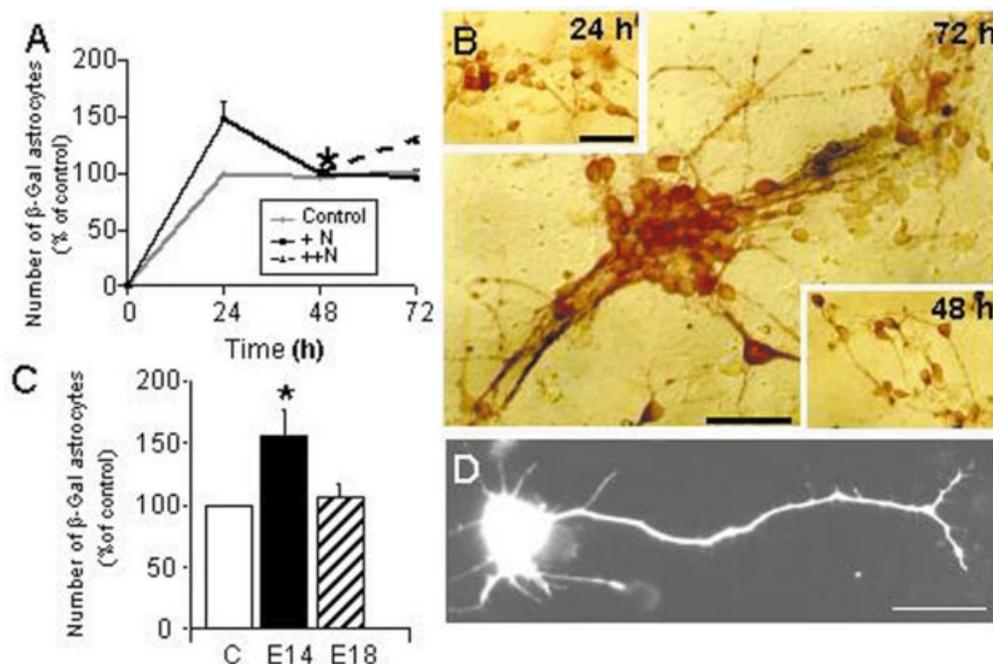


FIG. 4. Neuronal ability to induce GFAP gene promoter is modulated by neuron age. (A) Temporal course of neuron–glia interaction: cocultures of 200 000 E14 neurons and newborn astrocytes derived from CH were prepared as previously described in Materials and methods. Cell fixation and X-Gal revelation were performed at different times after neuron plating. Results express the mean of three independent experiments carried out in triplicate. Maximum effect of neurons on *lacZ* expression was observed at 24 h of coculture. After 48 and 72 h of coculture, β -Gal astrocyte number decreased to control levels. $p < 0.05$ for 24 and 72 h. The asterisk (*) represents addition of E14 neurons after 48 h of coculture. Such addition increased β -Gal astrocyte number again. (B) Neurons were immunostained by peroxidase immunoreactivity to the neuronal marker β -tubulin III after different times of coculture. Progression of coculture was followed by an increase in the complexity of neuronal arborization. (C) Newborn transgenic mice astrocytes were cultured in presence of E14 or E18 neurons for 24 h. After X-Gal revelation, β -Gal positive astrocyte number was analysed. Each point represents the average of three independent experiments carried out in triplicate (mean \pm SD). * $P < 0.00001$ (mean \pm SD). E18 neurons did not increase GFAP promoter-directed expression of *lacZ* from newborn astrocyte. (D) An E14 neuron maintained for 48 h in serum-free medium, immunostained for β -tubulin III. In this condition, more than 95% of the cells displayed this typical morphology and express the specific neuronal marker attesting the purity of neuronal cultures. Scale bars, 50 μ m.

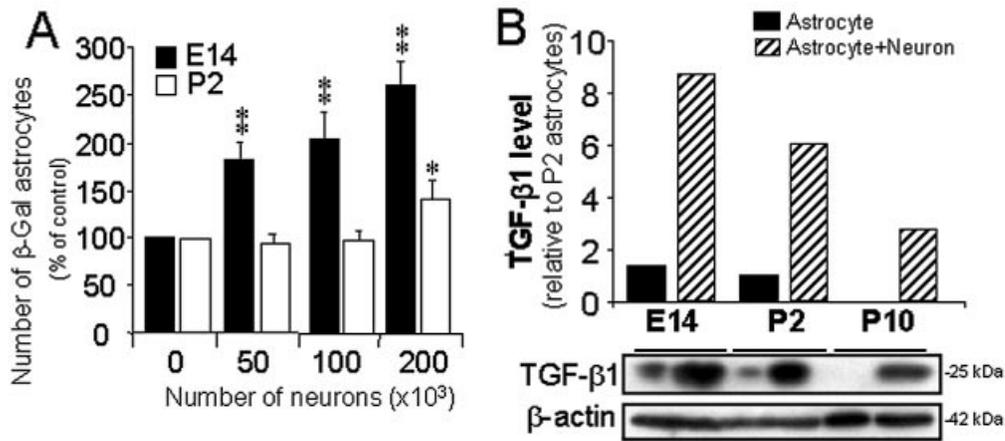


FIG. 5. Astrocyte responsiveness to neurons is modulated by astrocyte age and associated with TGF-β1 synthesis. (A) Astrocytes derived from P2 or E14 transgenic mice were cultured alone or in presence of increasing concentrations of E14 neurons for 24 h. After development of X-Gal reaction, β-Gal astrocytes were quantified. Each point represents the average of three independent experiments carried out in triplicate. Statistical significance: * $P < 0.00001$; ** $P < 0.000001$ (mean \pm SD). Younger astrocytes exhibited increased responsiveness to neurons in comparison to newborn ones. (B) Representative Western blot and graphic analysis of three independent experiments showing TGF-β1 content: Equal amounts (10 μg per lane) of total protein of cellular extracts of astrocytes derived from E14, P2 or P10 transgenic mice cultured alone or with E14 neurons were resolved in 10% polyacrylamide gel and analysed by immunoblotting for TGF-β1. Immune reaction for β-actin was used to monitor protein loading. The levels of TGF-β1 protein immunoreactivity are expressed relative to the levels observed in cultures of P2 astrocytes alone. Expression of TGF-β1 decreased with astrocyte age. Neurons greatly increased astrocytic TGF-β1 expression in despite of animal age, although such phenomenon decreased with astrocyte age.

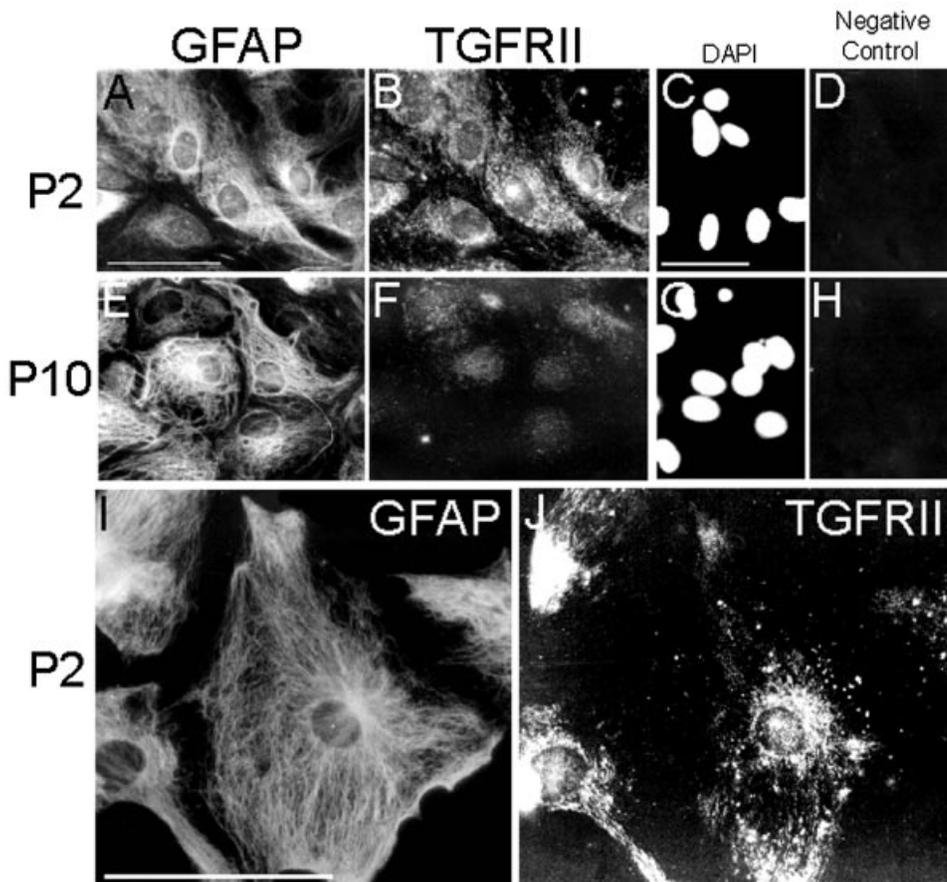


FIG. 6. Immunolocalization of TGFRII in primary cultured astrocytes. Astrocytes derived from newborn (P2) and 10-day-old (P10) transgenic mice were cultured in absence of neurons for 10 days as described in the Materials and methods. P2 (A–D, I and J) and P10 (E–H) astrocytes were immunostained for GFAP (A, E and I) and TGFRII (B, F and J). (C and G) DAPI nuclear labelling of the culture (same field as the negative controls). (D and H) Negative controls for TGFRII and GFAP immunostaining. Scale bars: 50 μm. Immunostaining of TGFRII was hardly diminished in older astrocytes.

GFAP gene expression by neuron-secreted TGF- β 1 has been reported.

TGF- β 1 mediates neuronal-induced astrocyte differentiation

The TGF- β superfamily is constituted by multifunctional polypeptide members, which perform critical functions in regulating CNS developmental processes such as cell adhesion, migration and proliferation (Böttner *et al.*, 2000; Unsicker & Strelau, 2000; Abreu *et al.*, 2002). TGF- β 1 inhibits astrocyte proliferation, increase GFAP expression *in vivo* and *in vitro* and modulate several extracellular matrix proteins and ionic channels (Laping *et al.*, 1994; Rich *et al.*, 1999; Perillan *et al.*, 2002). Despite the widespread effects reported for TGF- β 1 in CNS pathology, where it has been implicated in organization of the glial scar in response to injury (Moon & Fawcett, 2001; Zhu *et al.*, 2002), relatively little has been reported on its role on physiological situations.

We have demonstrated that embryonic cortical neurons activate GFAP gene by secreting TGF- β 1. As we used a neutralizing antibody specific for the isoform 1 of TGF- β , we undoubtedly exclude TGF- β 2/3 participation in this event. In addition, analysis of TGF- β 1 synthesis by Western blot and PCR assays showed that neurons secreted TGF- β 1 and enhanced its secretion by astrocytes. Accuracy of these assays was supported by using antibodies and primers specific for the isoform 1 of TGF- β . A positive feedback of TGF- β 1 expression has recently been described for other systems where TGF- β 1 modulates its own synthesis (Morgan *et al.*, 2000; Diez-Marques *et al.*, 2002).

Astrocytes synthesized and secreted TGF- β 1 even in absence of neurons. However, the addition of glial derived CM did not increase β -Gal astrocyte number (data not shown). TGF- β 1 is generally secreted as a latent TGF- β 1 complex unable to bind to TGF β R and requiring extracellular activation for biological activity (Flaumenhaft *et al.*, 1993). One possibility is that TGF- β 1 requires an additional neuronal growth factor to become biological active. A second possibility is that neuronal effect on astrocyte might represent a synergistic action between TGF- β 1 and other nonyet identified growth factor. Our data, however, do not support a role for bFGF or EGF, known to act in concert with TGF- β 1 in several systems (Hunter *et al.*, 1993; Kriegstein *et al.*, 1998a,b; Reilly *et al.*, 1998; Dhandapani *et al.*, 2002), on neuronal-induced astrocyte differentiation. Treatment of transgenic astrocytes with TGF- β 1 and bFGF or EGF in combination resulted in decrease of β -Gal astrocyte number compared to treatment with TGF- β 1 alone. bFGF and EGF are known as potent stimulator of cell proliferation (Laping *et al.*, 1994; Gomes *et al.*, 1999b; Levison *et al.*, 2000; Nakatsuji & Miller, 2001; Goddard *et al.*, 2002). Our previous work has demonstrated that GFAP-*lacZ* induction by neurons is followed by a decrease in BrdU incorporation and hence astrocyte proliferation (Gomes *et al.*, 1999a). Together, these data suggest a differential (probably antagonist) effect of these factors on proliferation and differentiation of astrocytes.

Although we cannot completely rule out the involvement of other growth factors in this study, if it is the case, their activity might depend on TGF- β 1 because neutralizing antibodies against TGF- β 1 completely prevented neuronal effect on GFAP gene promoter. Moreover, two additional arguments favour TGF- β 1 involvement: (1) boiling of CM did not impair its activity, a characteristic that has been related to the presence of disulphide bonds in TGF- β 1 (Flaumenhaft *et al.*, 1993; Dobbertin *et al.*, 1997; Mittaud *et al.*, 2002); (2) no additive effect was observed after concomitant administration of neurons and TGF- β 1 to astrocytes cultures which suggest that the same pathway might be activated. Further, it showed to be slightly

inhibitory, which is consistent with a previously reported negative feedback in the TGF- β 1 pathway following increased stimulus (Vivien *et al.*, 1998).

Neuron–glia interaction is developmentally regulated by TGF- β 1

We detected TGF- β 1 mRNA transcripts and protein in young (E14) neurons. These data argue against the current idea of TGF- β 1 as a solely injury-related astrocytic-cytokine (Böttner *et al.*, 2000). Although immunoreactivity for TGF- β 1 in healthy brain is barely detected, mRNA and bioactive TGF- β 1 can be found in embryonic brain (E15) (Flanders *et al.*, 1991), which supports a role for TGF- β 1 in nervous system development. Additional evidence of TGF- β 1 synthesis by young neurons was recently provided by Mittaud *et al.* (2002) who reported that TGF- β 1 secreted by hypothalamic neurons modulate the oxytocin receptor in rat-cultured astrocytes. We failed to detect TGF- β 1 expression in older neurons derived from E18 mice. Our data are in agreement with others who observed that cortical neurons derived from E17 or newborn rodents do not express TGF- β 1 transcripts (Dobbertin *et al.*, 1997; Vivien *et al.*, 1998). Thus, neuronal differentiation is followed by a decrease in TGF- β 1 synthesis and secretion, which is highly correlated to a failure to activate GFAP gene promoter. This hypothesis is also supported by our observation that either E14 neurons maintained for 3 days in coculture or E18 neurons failed to induce GFAP gene promoter. This time course of TGF- β 1 expression and neuronal morphological differentiation observed in our *in vitro* assay system correlates with the schedule of neuronal maturation *in vivo*.

By E14–16, the peak of neurogenesis in mice, most of the neurons are highly proliferative whereas on E18 most of them are already committed to a differentiated pathway (Burrows *et al.*, 1997). We suggest that neuronal maturation is followed by a downregulation of TGF- β 1 synthesis and secretion. This hypothesis complements previous observation that in CNS TGF- β 2/3 expression is observed in later stages (Flanders *et al.*, 1991; Dobbertin *et al.*, 1997; Vivien *et al.*, 1998). This might suggest a differential and complementary role for TGF- β isoforms during neurogenesis. Furthermore, we propose that neuronal ability to induce astrocyte differentiation is strictly correlated to TGF- β 1 secretion and inversely correlated to neuronal maturation.

There is a decreasing gradient of astrocyte response to neuronal differentiation factor. Whereas embryonic and newborn astrocytes are still competent to respond to local signals that regulate their differentiation, older astrocytes progressively loose such property. Such astrocyte responsiveness to neuronal influence is strongly correlated to TGF- β 1 synthesis. Addition of neurons induced TGF- β 1 synthesis in all astrocyte cultures independently of the astrocyte age. However, the intensity of such event decreased with astrocyte age. Although astrocytes are well recognized as potent source of TGF- β 1, as far as we know, it is the first time to be described a modulation of astrocytic TGF- β 1 by neurons.

We failed to detect TGF- β 1 in P10 astrocytes unless they were cocultured with neurons. We confirmed that TGF- β 1 detected in P10 astrocyte-neuronal cocultures was due to astrocytic cells rather neuronal secretion as incubation of P10 astrocytes with CM also induced TGF- β 1 synthesis by those cells (data not shown). It was intriguing however, that although P10 astrocytes synthesized TGF- β 1 they did not respond to this cytokine.

TGF- β 1 exert their effects through binding to specific cell-surface receptors identified as serine-threonine kinase transmembrane proteins of 53- and 75-kDa, known as type I and II receptors (TGFRI and TGFRII) (Massagué, 1992, 2000). Upon ligand binding,

TGFRI and TGFRII form a heteromeric complex and transduce intracellular signals. TGFRII mRNA seems to be widely distributed on the CNS but their cellular localization and expression by neurons or glial cells have not been carefully documented. Some *in vivo* and *in vitro* studies detected both receptor proteins and mRNA in some subpopulations of astrocytes and neurons (Böttner *et al.*, 1996; Vivien *et al.*, 1998). We demonstrated that older astrocytes present a scarce and diffuse pattern of expression of TGFRII when compared to newborn astrocytes. Whereas TGFRI mRNA expression has been described in the adult and developing rat nervous system, available data on TGFRII localization in the adult rat is conflictive (Lorentzon *et al.*, 1996; Tomoda *et al.*, 1996; Vivien *et al.*, 1998). Our data are in agreement with those from Böttner *et al.* (1996) who detected by RT-PCR, TGFRII transcripts in rat cortex, midbrain, cerebellum and hippocampus. TGFRII expression clearly decreased from embryonic to adult stage (Böttner *et al.*, 1996). Although full elucidation of the mechanism underlying unresponsiveness of P10 astrocytes to neurons should await additional data, differences in TGFRII expression might account for such phenomenon. Further studies on the signalling pathways triggered by TGFRI should provide elucidative data on this mechanism.

Physiological significance of neuronal-derived TGF- β 1 on NS development

Radial glial cells transformation into astrocytes is followed by an increase in GFAP expression and down regulation of vimentin (Pixley & De Vellis, 1984; Campbell & Gotz, 2002). In our work, GFAP increase induced by neuronal CM was also followed by a decrease in vimentin expression (data not shown). The fact that astrocyte responsiveness to neurons as well as neuronal action is increased in the embryonic period suggests a possible role for neuron–glia interaction in the vimentin–GFAP switch. The time-course observed in our *in vitro* assays system correlates with the schedule of disappearance of radial glia from the developing murine neocortex; *in vivo*, radial glia are most fully differentiated during the period E14–18 (Cameron & Rakic, 1991). Several studies have suggested that neurons could modulate astrocyte and radial glia development, however, none of them succeeded in identifying the effective molecule (Hatten, 1985; Sotelo *et al.*, 1994; Hunter & Hatten, 1995; Soriano *et al.*, 1997; Swanson *et al.*, 1997). The developmentally regulated expression of TGF- β 1 demonstrated in the present work makes this growth factor a putative candidate for this event.

Taken together, the present data argue that astrocytes display a considerable degree of plasticity, which follows a progressive restricted program during development. This implies that as neuronal environment changes, immature astrocytes may redefine their ability of interactions to accommodate the next set of neurons. This idea suggests that during development the function of immature astrocytes is dynamically regulated by developing neurons. Our data support the concept that within the context of brain development, neuronal signals might provide a source responsible for astrocyte development and strongly implicates TGF- β 1 on this process. We introduce a novel role for TGF- β 1 as a mediator of neuron–astrocyte interactions during physiological situations apart from its known role in pathological conditions of the CNS.

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Abbreviations

bFGF, basic fibroblast growth factor; β -Gal, β -Galactosidase; CH, cerebral hemispheres; CM, conditioned medium; E14 and E18, 14 and 18 embryonic days mice, respectively; EGF, epidermal growth factor; GFAP, glial fibrillary acidic protein; P2 and P10, 2- and 10-day-old mice, respectively; TGF- β 1, transforming growth factor-beta 1; TGFRI, transforming growth factor-beta receptor.

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