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Glial fibrillary acidic protein gene promoter is differently modulated by transforming growth factor-beta 1 in astrocytes from distinct brain regions

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Abstract

The expression of glial fibrillary acidic protein (GFAP), the major intermediate filament protein of mature astrocytes, is regulated under developmental and pathological conditions. Recently, we have investigated GFAP gene modulation by using a transgenic mouse bearing part of the GFAP gene promoter linked to the β-galactosidase reporter gene. We demonstrated that cerebral cortex neurons activate the GFAP gene promoter, inducing transforming growth factor-beta 1 (TGF-β1) secretion by astrocytes. Here, we report that cortical neurons or conditioned medium derived from them do not activate the GFAP gene promoter of transgenic astrocytes derived from midbrain and cerebellum suggesting a neuroanatomical regional specificity of this phenomenon. Surprisingly, they do induce synthesis of TGF-β1 by these cells. Western blot and immunocytochemistry assays revealed wild distribution of TGF receptor in all subpopulations of astrocytes and expression of TGF-β1 in neurons derived from all regions, thus indicating that the unresponsiveness of the cerebellar and midbrain GFAP gene to TGF-β1 is not due to a defect in TGF-β1 signalling. Together, our data highlight the great complexity of neuron–glia interactions and might suggest a distinct mechanism underlying modulation of the GFAP gene in the heterogeneous population of astrocytes throughout the central nervous system.

Introduction

Glial fibrillary acidic protein (GFAP) is the major intermediate filament of mature astrocytes. In astrocyte precursors of the embryonic central nervous system (CNS), intermediate filaments usually consist of vimentin and nestin which are gradually replaced by GFAP during astrocyte maturation (Pixley & De Vellis, 1984; Pekny, 2001). Although GFAP has been widely recognized as an astrocyte differentiation marker (Eng *et al.*, 1971; Bignami *et al.*, 1972; Gomes *et al.*, 1999b), its function is not yet fully understood. Generation of GFAP-deficient mice has recently provided new insights into the role of this protein apart from 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; Menet *et al.*, 2001). The fact that GFAP-enriched glial scars constitute a histological marker of several diseases of the CNS strongly implicates this protein not only in the physiology of the CNS but also in the development of neuronal diseases (Titeux *et al.*, 2002).

Although GFAP has been viewed historically as a marker for differentiated astrocytes, the presence of GFAP in neuronal progenitor cells has put this concept under revision (Imura *et al.*, 2003). Moreover, identification of GFAP expression in various cell types inside and outside the CNS, including liver, gut, kidney, lung and others (Bush *et al.*, 1998; Eng *et al.*, 2000), clearly demonstrates that understanding the factors that modulate GFAP gene expression might contribute to elucidating the molecular mechanisms involved in cell specification in the nervous system.

The close association between neurons and astrocytes suggests that gene expression in these cells is likely to be influenced by mutual interactions. Increasing evidence has pointed to the modulation of the GFAP gene as a key step in such interactions both in physiological as well as pathological situations of the CNS (Steward *et al.*, 1990; Chen & Liem, 1994; Lefrançois *et al.*, 1997; Menet *et al.*, 2001; Kommers *et al.*, 2002). By inhibiting GFAP translation with an antisense mRNA, Lefrançois *et al.* (1997) demonstrated that GFAP expression is involved in the functional shift from neurite-promoting to neurite-inhibiting properties of reactive glia normally observed after a lesion. More recently, inactivation of the GFAP gene in GFAP knock-out mice greatly improved neuronal survival and neurite outgrowth onto astrocyte carpets *in vitro* (Menet *et al.*, 2001).

Additional works have suggested that the GFAP gene might be a potential target for neuronal modulation. This idea is supported by the fact that neuronal impairments, such as those derived from nervous system lesions, greatly increase GFAP expression (Laping *et al.*, 1994b). Further, neurotransmitters have been reported to induce phosphorylation of GFAP (Kommers *et al.*, 2002). Although

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there is some evidence that the GFAP gene is under neuronal modulation, a mechanism underlying this event has only recently been suggested. By using a transgenic mouse bearing part of the GFAP gene promoter linked to the β-galactosidase (β-Gal) reporter gene, we recently demonstrated that cortical neurons induce the GFAP gene promoter of cerebral cortex (Cc) transgenic astrocytes by inducing transforming growth factor-beta 1 (TGF-β1) secretion (de Sampaio e Spohr *et al.*, 2002). Here, we extend our investigation of GFAP regulation by analysing the neuroanatomical regional specificity of this phenomenon. We report that cortical neurons do not activate the GFAP gene promoter of transgenic astrocytes derived from midbrain (M) and cerebellum (Cb). Surprisingly, they induce synthesis of TGF-β1 by these cells. Our data highlight the great complexity of neuron–glia interactions and suggest a distinct modulation of the GFAP gene in the heterogeneous population of astrocytes throughout the CNS.

Materials and methods

Astrocyte primary cultures and cocultures

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-Gal reporter gene (*lacZ*) as previously described (de Sampaio e Spohr *et al.*, 2002). Briefly, cultures were prepared from Cc, Cb and M derived from newborn transgenic mice. All animals were kept under standard laboratory conditions according to NIH guidelines. After mice were anaesthetized by hypothermia, they were decapitated, brain structures were removed and the meninges were carefully stripped off. Dissociated cells were plated onto 15.5-mm diameter wells (24-well plates; Corning Inc., NY, USA), previously coated with polyornithine (1.5 μg/mL, molecular weight 41 000; Sigma Chemical Co., St Louis, MO, USA), in Dulbecco's modified Eagle's medium/F12 medium supplemented with 10% fetal calf serum (Invitrogen, Carlsbad, CA, USA). For immunocytochemistry assays cells were plated on polyornithine-treated glass coverslips. The cultures were incubated at 37 °C in a humidified 5% CO_2, 95% air chamber for 10 days until reaching confluence. Glial monolayers were then incubated for an additional day in serum-free medium and used as substrate in coculture assays. Neurons freshly dissociated from 14-day (E14) embryonic Cc and M or newborn (P0) Cb from Swiss mice were obtained following the same procedure as previously described and plated onto the transgenic glial monolayer carpets. Homotypic cocultures consisted of neurons and astrocytes derived from the same regions. Heterotypic cocultures consisted of neurons and astrocytes from different regions. Cocultures were kept for 24 h under the same conditions as previously described (Gomes *et al.*, 1999a).

Detection of β-galactosidase activity and quantitative analysis of β-galactosidase-positive astrocytes

Glial cultures and cocultures were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) and stained for β-Gal with 0.4 mg/mL of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (U.S. Biochemical Corp., Cleveland, OH, USA) as substrate in 4 mM potassium ferricyanide, 4 mM potassium ferrocyanide, 2 mM $MgCl_2$ 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 done in triplicate and each result represents the mean of three independent experiments. Statistical analyses were done by ANOVA.

Conditioned medium preparation

Neuronal conditioned medium (CM) was prepared as previously described (Gomes *et al.*, 1999a; de Sampaio e Spohr *et al.*, 2002). Briefly, neurons derived from E14 Cc, E14 M or P0 Cb from Swiss mice were kept on polyornithine-coated wells (400 000 cells/well) in Dulbecco's modified Eagle's medium/F12 serum-free medium for 24 h at 37 °C in a humidified 5% CO_2, 95% air chamber. The culture medium was then recovered, centrifuged at 1500 ***g*** for 10 min to get rid of eventual cellular debris and used immediately or stored in aliquots at −20 °C for further use. Adherent cells on the coverslips were fixed with 4% paraformaldehyde and immunoreacted with antibody to the neuronal marker β-tubulin III. Approximately 95% of the cells stained with the antibody, attesting their neuronal phenotype. No GFAP-positive cells were found under these conditions. The CM from cocultures was prepared by cultivating embryonic neurons with newborn astrocytes for 24 h as previously described (Gomes *et al.*, 1999a). After recovery of coculture CM it followed the same procedure as described for neuronal CM. Use of coculture or neuronal CM yielded similar results. We used coculture CM in most experiments.

Treatment of transgenic astrocyte monolayers with conditioned medium and growth factors

Astrocyte monolayers derived from newborn transgenic mice Cc were prepared as previously described. After 10 days *in vitro*, in the presence of Dulbecco's modified Eagle's medium/F12 supplemented with 10% fetal calf serum, the cultures were incubated for an additional day with serum-free Dulbecco's modified Eagle's medium/F12. 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 to serum-free medium (10 ng/mL): human TGF-β1 (R & D Systems, Buckinghamshire, UK); basic fibroblast growth factor (bFGF; kindly provided by Dr P.L. Ho, Butantan Institute, São Paulo, Brazil) and epidermal growth factor (EGF; Invitrogen). Cultures were kept for an additional 24 h at 37 °C in a humidified 5% CO_2, 95% air chamber and then stained with 5-bromo-4-chloro-3-indolyl-β-D-galactoside as described. In order to rule out the possibility that GFAP genes from different brain regions are simply insensitive to GFAP inducers, we have employed other growth factors and molecules which are known to modulate GFAP expression. At least one of them was able to induce the GFAP gene from M, Cb or Cc (data not shown).

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 (Vector Laboratories, Inc., Burlingame, CA, USA) in PBS (blocking solution) for 1 h and incubated overnight at room temperature with the specified primary antibodies diluted in blocking solution. In case of peroxidase staining, previous to the primary antibody incubation, endogenous peroxidase activity was abolished with 3% H_20_2 for 10 min, followed by extensive washing with PBS. Primary antibodies were rabbit anticow GFAP antiserum (Dako; 1 : 50); mouse antihuman β-tubulin III antibody (Promega Corporation; 1 : 500); rabbit antiTGF-β1 receptor type II (TGFRII; Santa Cruz Biotechnology, Inc.; 1 : 100 dilution) and rabbit antiTGF-β1 (Sigma Chemical Co.; 1 : 50). After primary antibody incubation, cells were extensively washed with PBS/10% normal goat serum and incubated with secondary antibodies for 1 h at room temperature. Secondary antibodies were conjugated with horseradish peroxidase (goat antirabbit; Sigma Chemical Co.; 1 : 200) with Cy3 (sheep

antirabbit; Sigma Chemical Co.; 1 : 5000) or with fluorescein isothyocyanate (sheep antimouse; Sigma Chemical Co.; 1 : 400). Peroxidase activity was revealed with 3,3′-diaminobenzidine (DAB peroxidase substrate kit; Vector Laboratories, Inc.). Negative controls were performed by omitting the primary antibody during staining. In all cases no reactivity was observed when the primary antibody was absent.

Western blot analysis

Biochemical characterization of proteins was done according to Abreu *et al.* (2002). Cultures were lysed in 2× loading buffer (100 mM Tris-Cl, pH 6.8, 4% of sodium dodecyl sulfate, 0.2% of bromophenol blue, 20% of glycerol, 200 mM of dithiotreitol) and then boiled for 5 min before loading in the gel. Approximately 5–10 μg of protein per lane were submitted to electrophoresis in a 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis mini gel. After separation, proteins were electrically transferred onto a Hybond-P polyvinylidene difluoride transfer membrane (Amersham Biosciences) for 1 h. Membranes were blocked in PBS–milk 5% and primary antibodies added for 1 h at room temperature. After several washes, peroxidase-conjugated secondary antibody was added to the membrane and incubated for 1 h at room temperature. Proteins were visualized using the enhancing chemiluminescence detection system (ECL-Plus; Amersham Pharmacia Biotech, Miami, FL, USA). The following primary antibodies were used: mouse antiβ-actin (Santa Cruz Biotechnology; 1 : 500), mouse antihuman TGF-β1 (R & D Systems; 1 : 200), rabbit anticow GFAP (Dako; 1 : 4000) and rabbit antiTGFRII (Santa Cruz Biotechnology, Inc.; 1 : 500). The following secondary peroxidase-conjugated antibodies were used: goat antirabbit IgG and goat antimouse IgG (Amersham Biosciences; 1 : 1000). In some cases, Coomassie blue staining of the gel was used to monitor protein loading.

Results

Glial fibrillary acidic protein promoter-lacZ activity on astrocyte cultures

In order to verify astrocytic-specific expression of the GFAP-*lacZ* gene, confluent astrocyte monolayers derived from Cc, M and Cb of newborn transgenic mice were incubated for 2 days in serum-free medium and subsequently reacted for β-Gal activity and GFAP immunostaining. In all astrocyte monolayers, approximately 95% of the cells were recognized by antiGFAP antibody attesting, in the majority, their astrocyte phenotype (Fig. 1). Most of the cortical astrocytes showed a protoplasmic morphology with a large spread cell body presenting a cytoplasmic filamentous pattern of GFAP staining (Fig. 1A–C). In cerebellar and midbrain cultures, several astrocytes presented a process-bearing appearance with strong staining of GFAP along the process (Fig. 1D–I). β-Galactosidase activity could be detected as a blue nuclear staining in several, but not all,

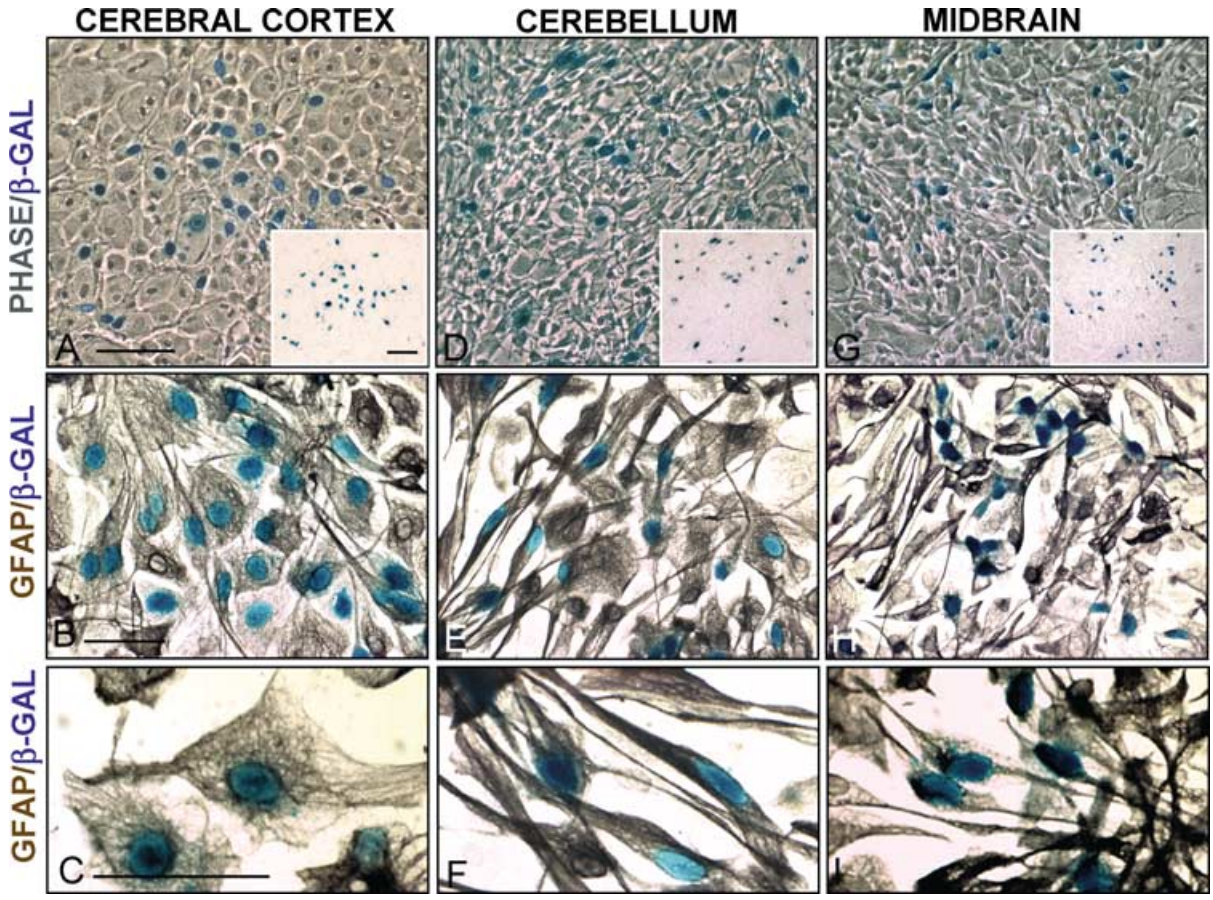

FIG. 1. Glial fibrillary acidic protein (GFAP) promoter-*lacZ* expression *in vitro*. (A–C) Cerebral cortex, (D–F) cerebellum and (G–I) midbrain astrocytes derived from newborn transgenic mice were kept for 12 days in culture (10 days in the presence of serum and 2 additional days in serum-free medium). GFAP promoter-directed expression of *lacZ* was revealed by 5-bromo-4-chloro-3-indolyl-β-D-galactoside (blue nuclei) prior to antiGFAP immunocytochemistry (brown staining). Most of the cells stain for GFAP whereas only a subpopulation of astrocytes express β-galactosidase (β-Gal) activity. While cortical astrocytes present a protoplasmic morphology, most of the cerebellar and midbrain astrocytes are process-bearing cells under these conditions. Insets in (A, D and G), brightfield photographs of the respective phase contrast images. Scale bars, 100 μm (A) and 50 μm (B and C).

GFAP-positive cells. However, the few GFAP-negative cells never exhibited such β-Gal activity (data not shown). Thus, these results provide evidence that GFAP promoter elements present in the transgene are specifically used by endogeneous GFAP-expressing cells of Cc, M and Cb *in vitro.*

Cortical neurons do not activate glial fibrillary acidic protein gene promoter from heterotypic astrocytes

We previously demonstrated that homotypic neuron–glia interaction [e.g. Cc neurons (or their CM) plated onto Cc glia] was able to induce GFAP gene promoter measured by a 60% increase in the number of β-Gal-positive astrocytes (Fig. 2; Gomes *et al.*, 1999a). Such CM presented a regional specificity as it failed to promote heterotypic astrocyte differentiation (Fig. 2; Gomes *et al.*, 1999a). As some interactions require membrane–membrane contact, in addition to soluble factors, we sought to investigate the involvement of cell contact on modulation of the GFAP gene in heterotypic coculture. To assess this question, neurons obtained from E14 Swiss mice Cc were directly plated onto transgenic newborn Cc, M or Cb astrocyte monolayers. The addition of cortical neurons onto the M astrocyte monolayer had no effect on the β-Gal cell number (Fig. 2) while it greatly increased this number in Cc monolayers (60%). Surprisingly, Cc neurons decreased Cb β-Gal-positive astrocytes by 30% (Fig. 2).

We previously showed that the ability to induce the GFAP gene promoter was not solely restricted to a specific neuronal population (Cc) as neurons derived from the midbrain or cerebella also increased the number of β-Gal-positive Cc astrocytes. However, heterotypic cocultures presented a smaller efficacy, revealed by a 25–30% increase in the number of β-Gal cells in comparison to 60% yielded by homotypic cultures (Fig. 2; Gomes *et al.*, 1999a). As modulation by neurons could be a characteristic of the GFAP gene of cortical astrocytes we next tested the ability of M and Cb glia to respond to homotypic neurons. The addition of M or Cb neurons had no effect on GFAP-*lacZ* astrocytes of midbrain or cerebellar monolayers, indicating that the GFAP gene of these astrocytes is probably not modulated by neuron–glia interactions (Fig. 2). Measurement of GFAP levels by western blot assays demonstrated a strict correlation between the GFAP-*lacZ* transgene and endogeneous GFAP gene in different brain regions, thus providing transgene as an useful tool to study GFAP regulation under the conditions used in this work (data not shown).

Our results demonstrate that Cc neurons do not promote astrocyte heterotypic differentiation, at least regarding GFAP expression, and might indicate the great complexity of neuron–glia interaction and regional preference of these interactions.

Cortical neurons induce transforming growth factor-beta 1 synthesis in heterotypic astrocytes

Induction of the GFAP gene of Cc astrocytes has been previously correlated by us to an increase in astrocytic TGF-β1 synthesis in response to neurons. We then investigated modulation of TGF-β1 synthesis by astrocytes in heterotypic cocultures. With this aim, total extracts of proteins of astrocytes cultured alone or with cortical neurons were analysed by western blot (Fig. 3). The TGF-β1 was identified in astrocytes derived from all brain structures studied (Cc, Cb and M). The expression of TGF-β1 was barely detected in astrocytes derived from M and Cc and its level was approximately 80% higher in Cb extracts (Fig. 3). The addition of neurons increased the level of TGF-β1 synthesis in all astrocyte cultures, although this increment was more drastically observed in Cc and M (approximately 200%). We confirmed that TGF-β1 detected in these cocultures was provided by astrocytic cells rather than neurons as incubation of Cb

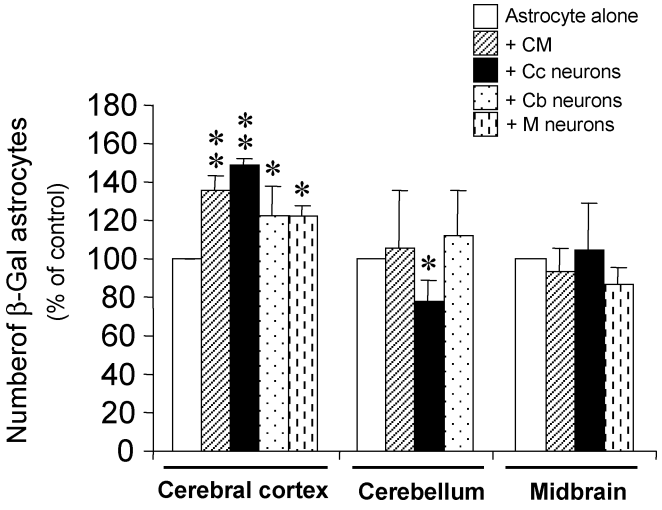

FIG. 2. Cortical neurons do not induce glial fibrillary acidic protein promoter-directed expression of *lacZ* in heterotypic cocultures. Cerebral cortex (Cc), cerebellum (Cb) and midbrain (M) astrocytes derived from newborn transgenic mice were cultured alone (open white bars) or in the presence of Cc conditioned medium (CM) or neurons derived from Cc, Cb and midbrain (see key). Cocultures were maintained for 24 h. After β-galactosidase (β-Gal) activity detection, the number of β-Gal-positive cells was quantified. Data represent the mean of three independent experiments, each done in triplicate. Cc neurons increase the number of β-Gal-positive cells in homotypic cultures but had a different effect on heterotypic cultures. While the number of β-Gal-positive midbrain astrocytes is not affected, that of Cb cultures is decreased by Cc neuron addition. $^{*}P < 0.05$; $^{**}P < 0.001$ (mean ± SD).

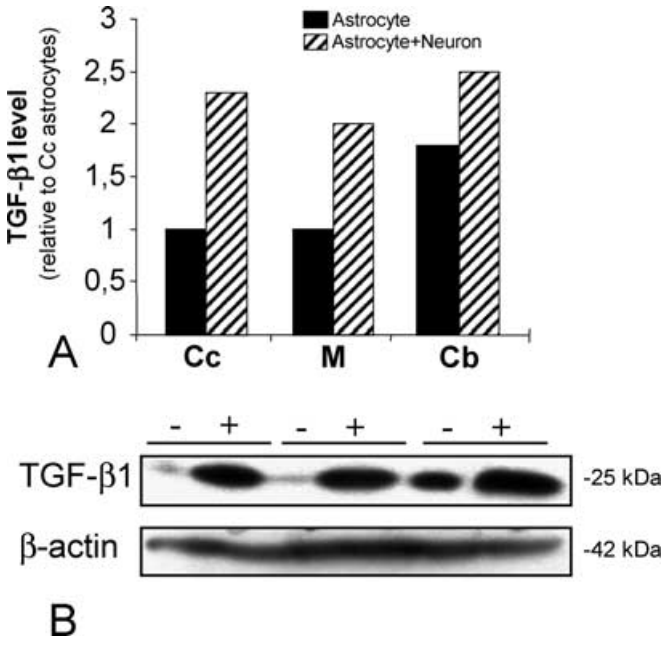

FIG. 3. Identification of transforming growth factor-beta 1 (TGF-β1) content in astrocytes. (A) Representative graphic analysis and (B) western blot showing TGF-β1 content: Equal amounts (10 μg/lane) of total protein of cellular extracts of astrocytes derived from cerebral cortex (Cc), midbrain (M) and cerebellum (Cb) of newborn transgenic mice cultured alone (−) or with 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 Cc astrocytes alone. Neurons greatly increased astrocytic TGF-β1 expression despite the brain region. Note increased expression of TGF-β1 in Cb.

and M astrocytes with cortical CM also induced TGF-β1 synthesis by those cells (data not shown). These results suggest that, in heterotypic cocultures, TGF-β1 synthesis is not necessarily closely correlated to GFAP gene promoter activation.

Unresponsiveness of glial fibrillary acidic protein gene of midbrain and cerebellar astrocytes to transforming growth factor-beta 1 does not correlate to transforming growth factor-beta receptor level

Although neurons do not activate midbrain and cerebellar GFAP gene promoter they induce TGF-β1 synthesis by these cells, which we have previously implicated in GFAP modulation (de Sampaio e Spohr *et al.*, 2002). We sought to investigate if such an apparent discrepancy might reflect differences in the expression of TGFRII. In order to address this, TGFRII levels were analysed by western blot and immunocytochemistry. Immunolabelling analysis did not reveal significant differences in the pattern of TGFRII expression in all subpopulations of astrocytes, the immunostaining was punctate and spread throughout the cellular membrane (Fig. 4A–D). Measurement of TGFRII protein content by western blot demonstrated similar levels of the receptor in cortex and midbrain astrocytes. Interestingly, cerebellar astrocytes, which have higher basal levels of TGF-β1 (Fig. 3), presented a twofold increase in TGFRII expression (Fig. 4C and E). These data indicate that unresponsiveness of midbrain and cerebellar GFAP to TGF-β1 is not due to the absence of functional TGFRII in these cells. Further, it might suggest a correlation between TGFRII and TGF-β1 levels in astrocytes.

Characterization of transforming growth factor-beta 1 synthesis and secretion by neurons from different regions

We previously demonstrated that cortical neurons induce GFAP gene promoter by secreting TGF-β1. This mechanism involves a paracrine effect in which neuronal TGF-β1 enhances astrocyte synthesis and secretion of this factor. An alternative explanation for the failure of cerebellar and midbrain neurons to induce GFAP gene promoter could be low levels or absence of TGF-β1 synthesis by these cells.

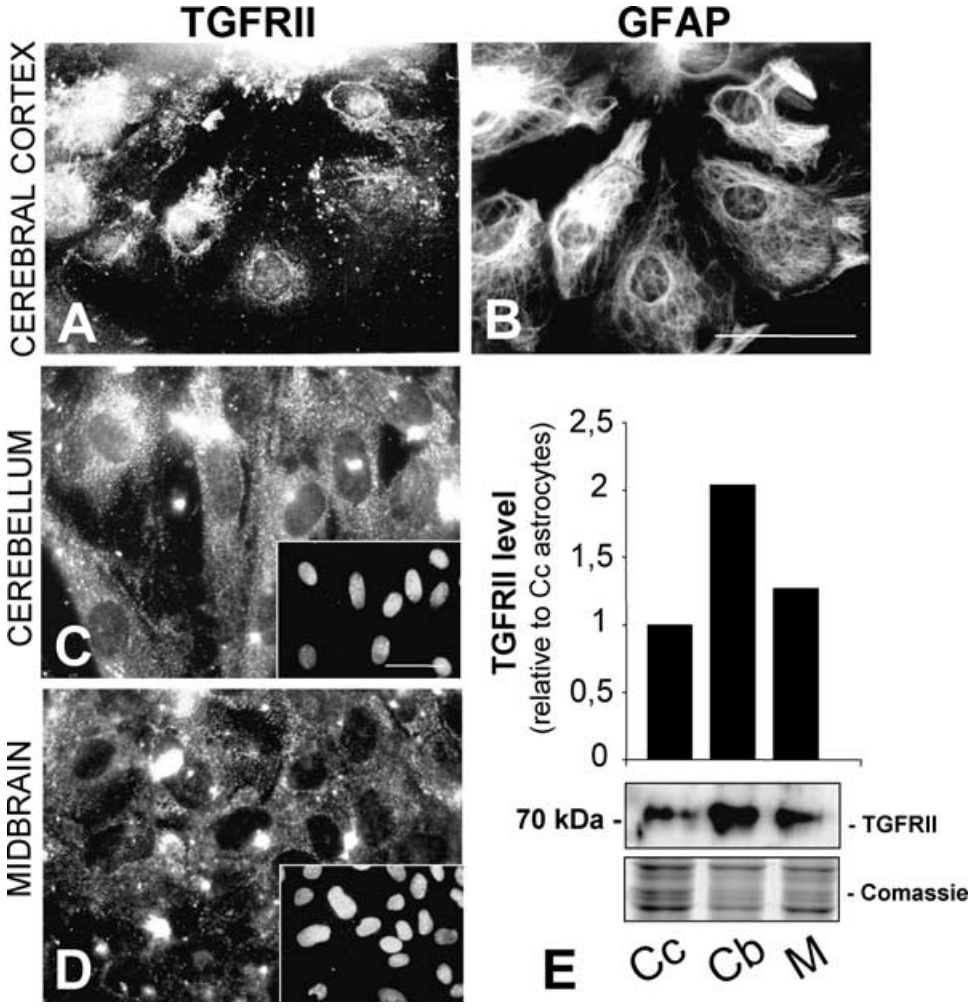

FIG. 4. Immunolocalization and western blot analyses of transforming growth factor-beta receptor (TGFR) II in primary cultured astrocytes. Astrocytes derived from newborn (A) cerebral cortex (Cc), (C) cerebellum (Cb) and (D) midbrain (M) transgenic mice were cultured for 10 days and immunostained for TGFRII as described in Materials and methods. Insets show DAPI (4′6-diamidino-2-phenylindole) nuclear labelling of the same field of the culture. (B) Glial fibrillary acidic protein (GFAP) immunostaining of Cc astrocytes (same field as in A) Scale bars, 50 μm. (E) Representative western blot and graphic analysis showing TGFRII content. Equal amounts (10 μg/lane) of total protein of cellular extracts of astrocytes derived from Cc, Cb and midbrain of newborn transgenic mice were resolved in 10% polyacrylamide gel and analysed by immunoblotting for TGFRII. Coomassie blue staining of the gel was used to monitor cellular protein loading. The levels of TGFRII protein immunoreactivity are expressed relative to the levels observed in cultures of Cc astrocytes. All astrocyte cultures present a punctate pattern of TGFRII expression spread all over the cellular membrane. A higher expression is observed in cerebellar astrocytes.

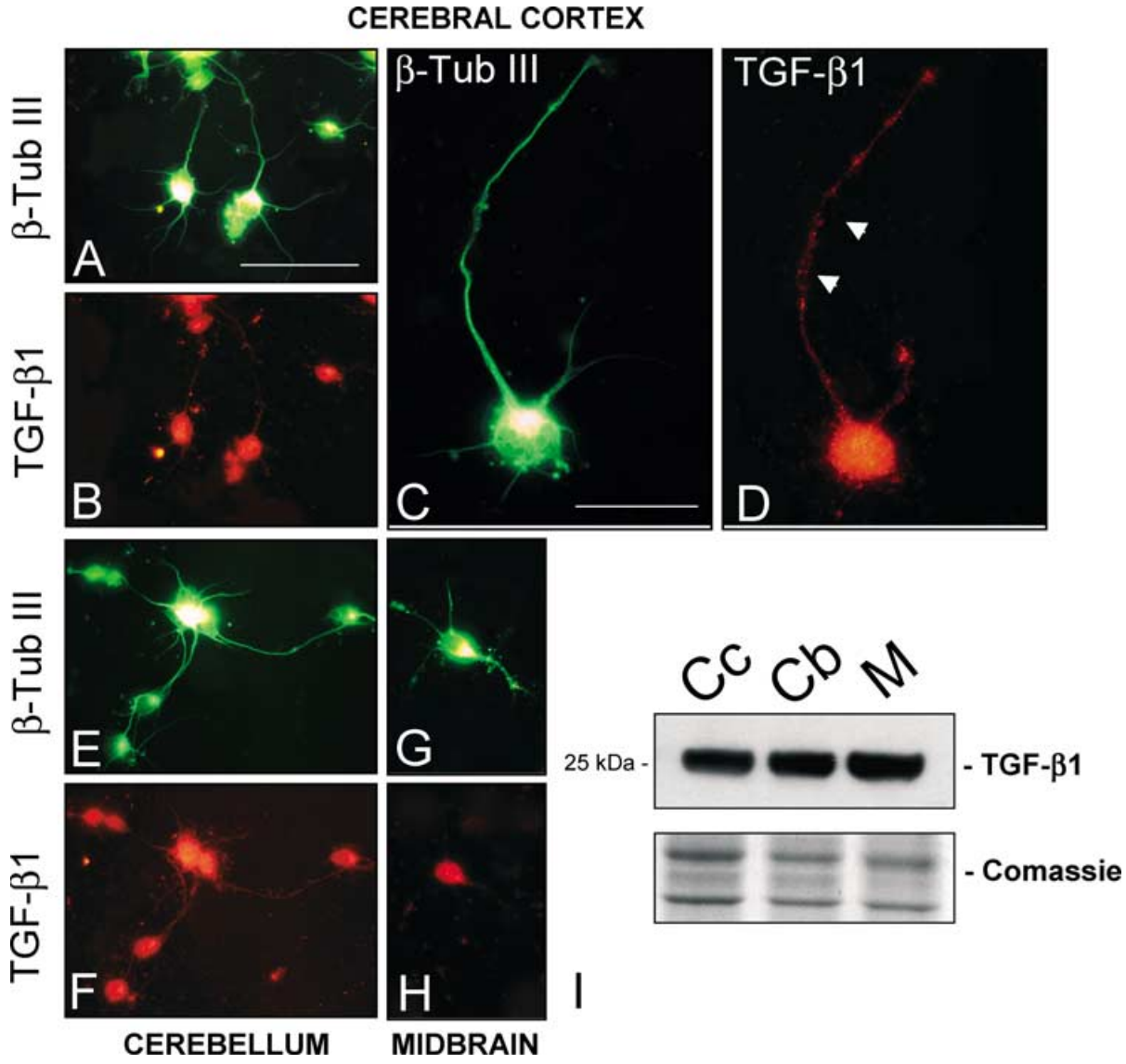

FIG. 5. Identification of transforming growth factor-beta (TGF-β) in primary cultured neurons. (A–H) Neurons derived from 14-day embryonic (E14) cerebral cortex (Cc) (A–D), newborn cerebellum (Cb) (E and F) and E14 midbrain (M) (G and H) Swiss mice were cultured for 24 h and immunostained for TGF-βs and β-tubulin III (β-Tub III) as described in Materials and methods. Scale bars: 50 μm (A) and 20 μm (C). Arrowheads in (D) depict punctate arrangement of TGF-β staining in the processes of cortical neurons. (I) Representative western blots showing TGF-β1 content. Equal amounts (10 μg/lane) of total protein of cellular extracts of neurons derived from Cc, Cb and midbrain were resolved in 10% polyacrylamide gel and analysed by immunoblotting for TGF-β1. Coomassie blue staining of the gel was used to monitor cellular protein loading.

In order to assess this, neuronal TGF-β1 expression was analysed by immunocytochemistry using an antibody against the three isoforms of TGF-β (TGF-β1, 2 and 3) (Fig. 5). Neurons derived from E14 cortex, E14 midbrain and P0 Cb were maintained for 24 h in serum-free medium and subsequently reacted with antibodies against the neuronal marker, β-tubulin III and TGF-β. Immunoreaction for TGF-βs was observed in all subpopulations of neurons although the pattern of labelling differed slightly. Whereas midbrain neurons presented a staining predominantly in neuronal soma, cerebellar and cortical neurons showed punctate labelling extending through the neuronal processes (arrowheads in Fig. 5D).

We next investigated TGF-β1 synthesis by neurons by western blot assay (Fig. 5I). For this, we have used an antibody specifically against isoform 1 of TGF-β. Analysis of total protein extracts from E14 cultured cortical and midbrain neurons and P0 cerebellar neurons identified TGF-β1 in all neuronal extracts (Fig. 5I).

Differential modulation of glial fibrillary acidic protein gene from distinct subpopulations of astrocytes is intrinsic to their promoter regions

Glial fibrillary acidic protein expression *in vivo* is modulated by several growth factors (for review see Laping *et al.*, 1994b; Gomes *et al.*, 1999a). We wondered whether, under our *in vitro* conditions, cerebellar and midbrain astrocytes might be somehow insensitive to GFAP inducers. In order to address this, we employed two well-known inducers of GFAP *in vivo*, bFGF and EGF (Laping *et al.*, 1994b). Astrocyte cultures were kept for 24 h in the presence of 10 ng/mL of bFGF, EGF and TGF-β1 (Fig. 6). Addition of TGF-β1 greatly increased the number of β-Gal cortical astrocytes (60% increase), whereas bFGF or EGF did not cause a significant increase (de Sampaio e Spohr *et al.*, 2002 and Fig. 6). Addition of both bFGF and EGF significantly increased the number of β-Gal cells in midbrain cultures

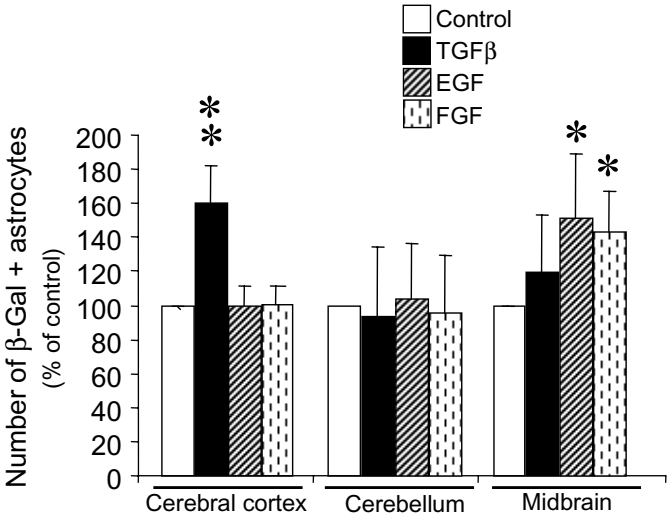

FIG. 6. Effect of transforming growth factor-beta 1 (TGF-β1), basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) on glial fibrillary acidic protein gene promoter-directed expression of *lacZ* of different brain regions. Cortical, cerebellar and midbrain astrocytes derived from newborn transgenic mice were cultured alone or in the presence of TGF-β1, EGF or bFGF. Factors were added at a final concentration of 10 ng/mL and kept for 24 h. After β-galactosidase (β-Gal) activity detection, β-Gal-positive astrocytes were quantified. Each point represents the average of three independent experiments done in triplicate. $^{*}P < 0.05$; $^{**}P < 0.00001$ (mean ± SD). Growth factors cause different effects on distinct subpopulations of astrocytes. Whereas TGF-β1 greatly increases the number of β-Gal cortical astrocytes, bFGF and EGF have no effect on this number. On the other hand, bFGF and EGF increase the number of midbrain β-Gal astrocytes but TGF-β does not. None of these factors has an effect on cerebellar β-Gal astrocyte number.

(EGF, 51% and bFGF, 43%), whereas neither of these factors had an effect on cerebellar astrocytes.

As all neurons failed to induce GFAP gene promoter from Cb and midbrain despite their synthesis of TGF-β1 we wondered whether the TGF-β1 concentration in coculture might be below the minimum necessary to achieve GFAP activation. To answer this, we directly added TGF-β1 to midbrain and cerebellar astrocytes and evaluated the number of β-Gal cells. As observed in Fig. 6, the addition of 10 ng/mL of TGF-β1 did not increase the β-Gal cell number either in cerebellar or midbrain cultures whereas it increased the number of cortical β-Gal astrocytes by 60%. Addition of a higher concentration of TGF-β1 (20 ng/mL) yielded similar results (data not shown). These results indicate that the GFAP gene promoter from different astrocyte subpopulations is distinctly modulated by growth factors.

Discussion

Recently, by using GFAP-*lacZ* transgenic mice we demonstrated that cortical neurons activate the GFAP gene promoter by inducing TGF-β1 secretion by astrocytes (de Sampaio e Spohr *et al.*, 2002). We now report that this event is regionally modulated. Cortical neurons increase the differentiation of cortical astrocytes; however, in midbrain, they have no effect on β-Gal expression and, unexpectedly, they decrease β-Gal astrocytes in cerebellar cultures. Surprisingly, although heterotypic cultures do not induce the GFAP gene, they yield an increase of TGF-β1 synthesis by astrocytes. Our data suggest that the mechanisms underlying GFAP gene activation and astrocyte differentiation might differ considerably throughout the CNS and indicate that GFAP gene modulation might not be strictly correlated to TGF-β1. In addition, our findings give strength to the current idea that astrocytes might comprise a much larger morphological and functional heterogeneity than neurobiologists previously thought, *in situ* as well as *in vitro*.

Transforming growth factor-beta pathway and glial fibrillary acidic protein regional specific modulation

It is intriguing that cortical neurons induce TGF-β1 secretion by cerebellar and midbrain astrocytes but they do not activate the GFAP gene in these cells. One explanation would be the lack or low levels of expression of TGF receptor (TGFRII) by cerebellar and midbrain astrocytes. This idea is supported by previous observations that the unresponsiveness of the GFAP gene promoter to TGF-β1 in late postnatal astrocytes is related to the scarce and diffuse expression pattern of TGFRII when compared with newborn astrocytes (de Sampaio e Spohr *et al.*, 2002). The TGFRII mRNA seems to be widely distributed in astrocytes and in neurons, *in vivo* as well as *in vitro*. However, available data on the localization of TGFRI and TGFRII are still conflicting (Böttner *et al.*, 1996, 2000; Tomoda *et al.*, 1996; Vivien *et al.*, 1998; Massagué, 2000; Perrilan *et al.*, 2002). We did not find significant differences between midbrain and cortical TGFR levels whereas cerebellar astrocytes presented a enhancement of TGFRII expression. Thus, our data are in agreement with those of Böttner *et al.* (1996) who detected, by reverse transcriptase–polymerase chain reaction, increased levels of TGFRII in the Cb in comparison to other brain regions.

The failure of TGF-β1 to affect the GFAP gene observed in cerebellar and midbrain astroglial cultures was not due to the absence of functional recognition or transduction machinery for this factor. This is supported by further observations that Cb and midbrain astrocytes increase TGF-β1 synthesis in response to cortical neurons which we previously demonstrated to secrete low amounts of TGF-β1 (de Sampaio e Spohr *et al.*, 2002). Such positive feedback of TGF-β1 expression has already been described for nervous and other systems where TGF-β1 modulates its own synthesis (Morgan *et al.*, 2000; Diez-Marques *et al.*, 2002). We observed that cerebellar astrocytes already presented a higher basal level of TGF-β1 when compared with midbrain and cortical cells. It is possible that such increased basal levels of cerebellar TGF-β1 could account for the increased TGFRII observed in cerebellar astrocytes as, besides regulation of its own synthesis, TGF-β1 might also modulate TGFRII levels (Norgaard *et al.*, 1996; Siegert *et al.*, 1999; Morgan *et al.*, 2000).

Our data on the effect of TGF-β1 on the GFAP-*lacZ* gene are in agreement with those reported by Baghdassarian *et al.* (1993), i.e. treatment of cerebellar astrocyte cultures with TGF-β1 did not increase the GFAP protein level and a slight increase was observed only after long-term culture in the presence of the factor.

Cortical neuronal CM had no effect on the cerebellar β-Gal astrocytic cell number although neurons themselves decreased this number. One possibility is that a contact-mediated mechanism is specifically involved in cerebellar decreased differentiation, as has been reported for inhibition of astrocyte proliferation and cell fate specification (Hatten, 1987; Krushel *et al.*, 1998; Tsai & McKay, 2000).

Another possibility for the unresponsiveness of Cb and midbrain astrocytes to cortical neurons could be that levels of TGF-β1 elicited by cortical neurons were not sufficient to activate the GFAP gene from these regions. This seems not to be the case as the addition of high concentrations of TGF-β1 to these astrocytes does not increase the β-Gal cell number but greatly increases the β-Gal cortex cell number. Here, we demonstrate by immunocytochemistry and western blot assays that neurons derived from the Cc, Cb and midbrain synthesize TGF-β1. This is the first time that TGF-β1 synthesis by cerebellar and midbrain neurons has been demonstrated. Other

members of the TGF-β family and their receptors have previously been detected in cortical and mesencephalic neurons (Flanders *et al.*, 1991; Dobbertin *et al.*, 1997; Unsicker & Strelau, 2000; Farkas *et al.*, 2003). *In vivo* and *in vitro* evidence of TGF-β1 synthesis by neurons has also been provided by other groups (Lefebvre *et al.*, 1992; Zhu *et al.*, 2000; Mittaud *et al.*, 2002). Mittaud *et al.* (2002) reported that TGF-β1 secretion by hypothalamic neurons modulated the oxytocin receptor in rat cultured astrocytes. *In vivo* data demonstrated that hippocampal neurons can express TGF-β1 under physiological conditions and up-regulate its expression after transient forebrain ischemia (Zhu *et al.*, 2000).

Although cerebellar and midbrain neurons synthesize TGF-β1, they failed to activate cortical GFAP gene promoter with the same efficacy as cortical neurons. As the concentration of TGF-β1 does not seem to be the main factor, the activation of the GFAP gene could depend on a cofactor acting in the TGF-β pathway. In fact, the biological effects of various extracellular factors have been demonstrated to depend on additional signals (Engele & Franke, 1996; Krieglstein *et al.*, 1998a,b; Abreu *et al.*, 2002). Thus, we cannot rule out the possibility of a yet unidentified molecule acting in synergism with TGF-β1 to ensure full GFAP gene promoter activation in cortical astrocytes.

Astrocytes are a regionally heterogeneous subgroup of glial cells in the CNS that greatly vary in responsiveness to several growth factors (Wilkin *et al.*, 1990; Schlüter *et al.*, 2002; Matthias *et al.*, 2003; Reuss *et al.*, 2003). Our data indicate that heterogeneity of GFAP regulation is not restricted to TGF-β. Epidermal growth factor, which has a widespread action in the CNS (Fricker-Gates *et al.*, 2000; Martinez & Gomes, 2002), has been demonstrated to regulate protein expression in a CNS region-specific manner (Schlüter *et al.*, 2002). In our culture conditions, EGF specifically induces the midbrain GFAP gene but had no effect on cortical and cerebellar astrocytes. In addition, FGF, a well-established modulator of cell differentiation and GFAP expression (Reuss *et al.*, 2000, 2003), also exhibited regional-specific regulation of the GFAP gene by specifically inducing the GFAP gene promoter from midbrain.

In summary, our data demonstrate that astrocytes from Cb, midbrain and Cc express functional active TGF-β1 and TGF-β receptors. We argue that differences in GFAP gene responsiveness to TGF-β1 are not related to levels of TGFRII or TGF-β1 expression by these cells but clearly reflect a distinct requirement for GFAP gene promoter activation in heterogeneous populations of astrocytes.

Glial heterogeneity and implications for nervous system development

Much evidence now supports the concept that astroglia isolated from a number of different brain regions vary markedly in their responsiveness to several agents, such as hormones, growth factors, neurotransmitters, gene regulation, pattern of cell interaction and even distinct progenitor potentials (Dennis-Donini *et al.*, 1984; Cholewinski & Wilkin, 1988; Garcia-Abreu *et al.*, 1995; Lima *et al.*, 1998; Perego *et al.*, 2000; Gomes *et al.*, 2001a,b; Schlüter *et al.*, 2002; Hall *et al.*, 2003; Matthias *et al.*, 2003; Reuss *et al.*, 2003). Our data contribute to this prevailing view by demonstrating that astrocytes derived from distinct brain regions modulate the GFAP gene differently in response to neurons and growth factors. The fact that the onset of the GFAP gene is part of the radial glia and astrocyte differentiation strengthens the hypothesis that the role of GFAP in nervous system development goes beyond its previously well-known cytoskeletal structural function. This idea fits well with recent findings that radial glia from different brain regions diverge considerably in their lineage progeny potential (Malatesta *et al.*, 2003). While cortical radial glia generate the vast majority of neurons in the Cc, radial glia in the ventral telencephalon generate very few neurons (Malatesta *et al.*, 2003). These results imply that in some regions of the brain, such as the Cc, the predominant radial glia progeny is neuronal and in others, such as the ganglionic eminence, it is glial. Which features, therefore, determine neuronal vs. glial progeny potential?

Regulation of cell type-specific genes, such as some of the intermediate filament proteins, is a key step for cell specification. Astrocytic-specific expression is regulated by a variety of *cis*- and *trans*-acting factors (Brenner *et al.*, 1994). Several putative growth factor-binding sites have already been identified in the GFAP gene promoter, including those involved in the TGF-β family pathway (Brenner *et al.*, 1994; Nakashima *et al.*, 1999). They seem to be differently used at least in the CNS and peripheral nervous system (Feinstein *et al.*, 1992). Such differences in GFAP gene modulation might also be widespread within the CNS itself. This idea is supported by the observation that expression of the transgene was restricted to some subpopulations of astrocytes as described for brain tissues by Galou *et al.* (1994) and by others who used distinct GFAP transgenic mice (Mucke *et al.*, 1991; Brenner *et al.*, 1994). It is conceivable that, *in vitro* as well as *in vivo*, heterogeneous subpopulations of astrocytes utilize different sets of GFAP regulatory regions. Brenner *et al.* (1994) have described critical differences in the GFAP promoter *lacZ* expression pattern depending on the length of GFAP promoter used in transgene construction, in some cases *lacZ* expression occurred throughout the brain whereas in others it was largely confined to the Cc and hippocampus. It is tempting to speculate that distinct *cis* and *trans* factors modulating the GFAP gene in Cb, midbrain and Cc, as observed in this work, might correlate with the radial glial fate potential observed in radial glia derived from these regions.

Transforming growth factor-beta 1 modulation of glial fibrillary acidic protein: considerations for brain injury

Glial fibrillary acidic protein expression shows brain region-specific responses to several growth factors and astrocyte–neuron interactions (Höke & Silver, 1994; Zhu *et al.*, 2000). During senescence, GFAP mRNA and protein levels tend to increase in the hippocampus, striatum and cortex and only later in other regions (for review Laping *et al.*, 1994b). Although it has been described that TGF-β1 increases GFAP *in vivo* and *in vitro* (Baghdassarian *et al.*, 1993; Laping *et al.*, 1994a; Reilly *et al.*, 1998), our work shows, for the first time, a TGF-β1 regional modulation of GFAP in astrocytes.

Moreover, TGF-β1 is up-regulated in brain lesions, which suggests that it plays an important role in organizing the response to degeneration of neurons and in mediating the anti-inflammatory reactions after brain injury (Vivien *et al.*, 1998; De Groot *et al.*, 1999; McTigue *et al.*, 2000). Activated glial cells have previously been suggested to be the major source of TGF-β1 in brain tissue. Astrocytes present distinct responsiveness to brain injury (Zhu *et al.*, 2000). Stewart (1994) reported that electroconvulsive seizures strongly induce GFAP mRNA levels in the dentate gyrus, whereas most other areas of the brain, including the Cc, show minimal if any changes in GFAP expression. Several findings also provide evidence that, although reactive gliosis is a hallmark of most CNS disease, the mechanism involved in GFAP gene activation may differ in distinct lesions (Titeux *et al.*, 2002). Our data of cortical neuronal synthesis of TGF-β1 and the findings that hippocampal neurons synthesize TGF-β1 after forebrain ischemia suggested an additional role for neurons in modulating astrocytic reaction. Thus, our data together with the fact that TGF-β1 mRNA and protein are highly enriched in glial scars might explain the different response of the GFAP gene in distinct brain regions during reactive gliosis.

Given the relevant role of GFAP during CNS development, as well as a factor in the reactive response to injury, the understanding of the mechanism of GFAP expression and its modulation should be useful in elucidating some steps of CNS physiology and pathology. These diverse alterations of astrocyte gene expression in response to neurons not only emphasize the astroglial heterogeneity but also imply that GFAP expression might be integrated within a more generalized transcriptional regulatory system that organizes neural cell generation and specification and astrocytic responses to neuronal activity.

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Abbreviations

bFGF, basic fibroblast growth factor; β-Gal, β-galactosidase; Cb, cerebellum; Cc, cerebral cortex; CM, conditioned medium; CNS, central nervous system; E14, 14-day embryonic; EGF, epidermal growth factor; GFAP, glial fibrillary acidic protein; M, midbrain; P0, newborn; PBS, phosphate-buffered saline; TGF-β1, transforming growth factor-beta 1; TGFR, TGF-β receptor.

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