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LUCIANA FERREIRA ROMÃO

INTERAÇÃO NEURÔNIO-GLIA: EFEITO DO FATOR DE CRESCIMENTO TRANSFORMANTE-β1 E FATOR DE CRESCIMENTO DE TECIDO CONJUNTIVO NA BIOLOGIA DA GLIA NORMAL E TUMORAL

TESE APRESENTADA À UNIVERSIDADE FEDERAL DO RIO DE JANEIRO COMO PRÉ-REQUISITO À OBTENÇÃO DO TÍTULO DE DOUTOR EM CIÊNCIAS MORFOLÓGICAS

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Tese submetida à Universidade Federal do Rio de Janeiro visando a obtenção do grau de Doutor em Ciências Morfológicas

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RESUMO

ROMÃO, Luciana Ferreira. **INTERAÇÃO NEURÔNIO-GLIA: EFEITO DO FATOR DE CRESCIMENTO TRANSFORMANTE-β1 E FATOR DE CRESCIMENTO DE TECIDO CONJUNTIVO NA BIOLOGIA DA GLIA NORMAL E TUMORAL.** Rio de Janeiro, 2007. Tese (Doutorado em Ciências Morfológicas) - Instituto de Ciências Biomédicas, Universidade Federal do Rio de Janeiro, Rio de Janeiro, 2007.

As interações celulares desempenham importante papel no desenvolvimento e patologias do sistema nervoso central (SNC). Neste trabalho, investigamos os mecanismos de interação neurônio-astrócito e neurônio-glioma mediados por fatores tróficos solúveis e moléculas de matriz extracelular (MEC) que pudessem interferir na diferenciação e controle da síntese de proteínas destas células. Para isso, estabelecemos 2 modelos *in vitro*: coculturas de astrócitos de roedores neonatos com neurônios embrionários de roedores e coculturas de células de gliomas humanos com neurônios de roedores.

No primeiro paradigma (interação neurônio-astrócito), demonstramos que neurônios de córtex cerebral ativam o promotor do gene de GFAP (proteína acídica fibrilar glial, marcador de astrócito maduro), através da indução da secreção do fator de crescimento transformante-beta 1 (TGF-β1) pelos astrócitos com ação autócrina sobre eles. Neurônios corticais ou meio condicionado por essas células não ativam o promotor do gene de GFAP de astrócitos derivados de mesencéfalo e cerebelo, sugerindo uma especificidade regional deste fenômeno. A síntese de TGF-β1 em astrócitos é induzida pelo neurotransmissor excitatório, glutamato. Estudamos o envolvimento de glutamato derivado de neurônios corticais na ativação do gene de GFAP e síntese de TGF-β1. Reportamos que glutamato ativa o promotor do gene de GFAP de astrócitos de diferentes regiões do cérebro através da ativação do receptor glial mGlu2/3R e conseqüente indução da síntese e secreção de TGF-β1. Ensaios utilizando anticorpos neutralizantes e antagonistas específicos demonstraram que a diferenciação astrocitária induzida pelos neurônios é mediada pelas vias de sinalização de MAPK e PI3K. Nosso trabalho é pioneiro em demonstrar que TGF-β1 é um mediador das ações do glutamato na diferenciação astrocitária.

Com o objetivo de estudar o segundo paradigma (interação neurônio-célula glial tumoral), utilizamos células derivadas de glioblastoma multiforme humano (Gbm), tumor cerebral maligno cuja adaptação ao microambiente do SNC envolve a produção e remodelamento da MEC. Para isso, neurônios de rato foram cultivados sobre astrócitos de rato ou sobre três linhagens de células de Gbms humanos. Observamos que os Gbms humanos mantêm as propriedades interativas com os neurônios, sendo capazes de sustentar a neuritogênese dessas células. A organização da laminina, uma das principais moléculas da MEC astrocitária, foi alterada de um padrão filamentoso para um mixto puntiforme/filamentoso, quando astrócitos e/ou Gbms foram cultivados com neurônios. Com o objetivo de analisar os mecanismos de interação entre neurônios e Gbms, focamos em 2 fatores de crescimento que agem em sinergismo em diversas patologias do SNC, CTGF (fator de crescimento do tecido conjuntivo) e TGF-β1. Demonstramos que neurônios inibem a síntese de CTGF em células de Gbm através da inibição da via de sinalização de MAPK. Esse evento independente da via de sinalização de TGF-β1.

Em conjunto, nossos dados demonstram que neurônios são capazes de induzir o programa de diferenciação astrocitária através da secreção de fatores tróficos de forma regionalmente específica. Mais ainda, apesar do processo de malignização, algumas das propriedades fundamentais interativas com os neurônios, são mantidas pela célula tumoral. Nosso trabalho para o entendimento do papel das interações celulares no desenvolvimento e no estabelecimento de patologias do SNC.

ABSTRACT

ROMÃO, Luciana Ferreira. **INTERAÇÃO NEURÔNIO-GLIA: EFEITO DO FATOR DE CRESCIMENTO TRANSFORMANTE-β1 E FATOR DE CRESCIMENTO DE TECIDO CONJUNTIVO NA BIOLOGIA DA GLIA NORMAL E TUMORAL.** Rio de Janeiro, 2007. Tese (Doutorado em Ciências Morfológicas) - Instituto de Ciências Biomédicas, Universidade Federal do Rio de Janeiro, Rio de Janeiro, 2007.

The cellular interactions play an important role in the central nervous system (CNS) development and pathologies. In this work, we investigate the neuron-astrocyte and neuron-glioma interaction mechanisms mediated by soluble trophic factors and molecules of the extracellular matrix (ECM). With this purpose, we established 2 *in vitro* models: cocultures of astrocytes derived from newborn rodents with neurons derived from embryonic rodents and cocultures of glioma human cells with neurons derived from rodents.

In the first paradigm (neuron-astrocyte interaction), we demonstrated that neurons derived from cerebral cortex activate the GFAP (glial fibrillar acidic protein, marker of mature astrocyte) gene promoter, through the induction of transforming growth factor-beta 1 (TGF-β1) secretion by astrocytes. Cortical neurons or conditioned medium by these cells do not activate the promoter of GFAP gene of astrocytes derived from mesencephalon and cerebellum, suggesting a regional specificity of this phenomenon. The synthesis of TGF-β1 by astrocytes is induced by the excitatory neurotransmitter, glutamate. We study the envolvement of glutamate derived from cortical neurons in the activation of GFAP gene and synthesis of TGF-β1. We report here that glutamate activates the GFAP promoter gene of astrocytes in differents regions of the brain through the activation of the mGlu2/3R glial receptor and consequent induction of the synthesis and secretion of TGF-β1. Assays using neutralizing antibodies and specific antagonists demonstrated that the astrocytic differentiation induced by neurons is mediated by the MAPK and PI3K pathways. Our work is pioneer in demonstrating that TGF-β1 is a mediator of the roles of glutamate in the astrocytic differentiation.

To study the second paradigm (neuron-tumoral glial cell interaction), we used cells derived from multiform glioblastoma (Gbm), malignant cerebral tumor which adaptation to the CNS environment involves the production and remodeling of the ECM. With this purpose, neurons derived from rats were cultivated over astrocytes also derived from rats or over three human Gbm cell lines. We observed that the human Gbms keep the interactive properties with neurons, being capable of supportting their neuritogenesis. The laminin organization, one of the main molecules of the astrocytic ECM, was altered from a fibrillar pattern to a punctiform/fibrillar pattern, when astrocytes and/or Gbms were cultivated with neurons. To analyse the interaction mechanisms between neurons and Gbms, we focused in 2 growth factors that act in synergism in several CNS pathologies, CTGF (connective tissue growth factor) and TGF-β1. We demonstrated that neurons inhibit the syntesis of CTGF in Gbm cells through the inhibition of the MAPK pathway. This event is independent of the TGF-β1 pathway.

In set, our data demonstrate that neurons are capable of inducing astrocytic differentiation through the secretion of trophic factors in a region-specific manner. It is interesting that, despite the malignization process, many of the interactive properties with the neurons are kept by the tumoral cell. Our work contributes to the understanding of the role of cell interactions in the development and establishment of the CNS pathologies.

LISTA DE ABREVIATURAS E SIGLAS

BMPs	Proteínas mofogenéticas ósseas, do inglês *Bone morphogenetic proteins*
β-Gal	β-Galactosidase
CTGF	Fator de crescimento do tecido conjuntivo, do inglês *Connective tissue growth factor*
E14	Camundongos embrionários de 14 dias
E18	Ratos embrionários de 18 dias
EGF	Fator de crescimento da epiderme, do inglês *Epidermal growth factor*
FGF-2	Fator de crescimento de fibroblasto 2, do inglês *Fibroblast growth factor- 2*
GABA	Ácido gama aminobutírico**,** do inglês *Gamma-aminobutyric acid*
GAGs	Glicosaminoglicanos
GBM	Glioblastoma multiforme
GDNF	Factor neurotrófico derivado da glia, do inglês *Glial cell line-derived neurotrophic factor*
GFAP	Proteína acídica fibrilar glial, do inglês *Glial fibrillary acidic protein*
GR	Glia radial
HUVEC	Células endoteliais de veia de cordão umbilical humano, do inglês *Human Umbilical Vein Endothelial Cells*
IL-3	Interleucina-3, do inglês *Interleukin-3*
LN	Laminina
MAPK	*Mitogen-activated protein kinase*
MCF7	Células de tumor de mama
MEC	Matriz extracelular
OMS	Organização Mundial de Saúde
P0	Ratos ou camundongos recém-natos
PI3K	*Phosphotidylinositol 3-kinase*
PGs	Proteoglicanos
PVDF	Fluoreto de polivinilideno, do inglês *Polyvinilidene fluoride*
RNA	Ácido ribonucléico
SFB	Soro fetal bovino
SN	Sistema nervoso
SNC	Sistema nervoso central
TGF-α	Fator de crescimento transformante-alfa, do inglês *Transforming growth factor-alpha*
TGF-β	Fator de crescimento transformante-beta, do inglês *Transforming growth factor-beta*
TNF-β	Fator de necrose tumoral-beta, do inglês *Tumor necrosis factor beta*
U79	Células de tumor de útero
VEGF	Fator de crescimento do epitélio vascular, do inglês *Vascular epidermal growth factor*
ZSV	Zona subventricular, do inglês S*ubventricular zone*

SUMÁRIO

INTRODUÇÃO

1. Estudo do desenvolvimento do sistema nervoso e sua aplicação na biologia dos tumores

O desenvolvimento harmônico do sistema nervoso (SN) depende fundamentalmente das relações entre as células que o compõem. Neurôr células da glia mantêm uma estreita relação, seja por contato celular, ou troca de fatores solúveis secretados por uma ou outra célula que paracrinamente agem nas demais células do organismo. Podem ainda estas células trocar metabólitos por junções comunicantes, o que nos permite pensar numa imensa rede celular, heterogênea, comunicante, capaz de responder pela organização e formação de um sistema tão complexo quanto o SN.

O Laboratório de Morfogênese Celular (LMC) e o Laboratório de Neurobiologia Celular (LNBC) têm dado especial atenção às interações celulares no Sistema Nervoso Central (SNC), particularmente por uma abordagem, *in vitro*, utilizando, sofisticados sistemas de cultura de células neurais. Para tanto, tomamos o conceito da relevância da glia como um importante substrato para o crescimento neurítico e o efeito das interações entre neurônios e células gliais em suas proteínas citoesqueletais, produzindo alterações morfológicas em ambos os tipos celulares. Desenvolvemos, então, uma série de investigações mostrando: (1) que a glia é heterogênea e produz moléculas que direcionam diferentemente a morfogênese celular (Trentin *et al*., 1995) e a neuritogênese (Garcia-Abreu *et al*., 1995; Martinez e Gomes, 2002); ou ainda, (2) que neurônios se comunicam intimamente com a glia astrocitária (Fróes *et al*., 1999) e que podem secretar moléculas indutoras da diferenciação glial (Gomes *et al*., 1999a; de Sampaio e Spohr *et al.*, 2002).

Demonstramos que a microglia participa deste sistema de interação celular, constituindo-se no terceiro elemento deste triângulo, sendo também, alvo de fatores

secretados pelas duas células (Lima *et al*., 2001, Mallat *et al*., 2002). Interagem, portanto, com neurônios e astrócitos, para a troca de fatores e sinalização que permitem, por exemplo, ao cérebro enfrentar algumas diversidades derivadas de patologias causadas por agentes infecciosos (Titeux *et al*., 2002; Rozenfeld *et al*., 2003).

Mais recentemente, demonstramos que fatores tróficos liberados por neurônios, especialmente membros da família TGF-β (fator de crescimento transformante beta), induzem a diferenciação de células de glia radial, consideradas hoje as principais progenitoras neurais do córtex cerebral (Stipursky e Gomes, 2007).

Se os estudos listados acima, realizados com astrócitos normais, indicavam uma sintonia entre neurônios e astrócitos na maturação de ambas as células e mais, de modo muito forte que neurônios modulam a diferenciação de astrócitos, tendo a família de fatores de crescimento TGF-β um papel preponderante neste fenômeno, nascia na nossa equipe a questão de se a glia tumoral teria propriedades interativas com neurônios, à maneira da glia normal. Uma simplificação seria dizer que estávamos interessados em saber em quanto estaria preservada a "memória" da célula glial, na passagem da normalidade ao câncer; se a célula glial reconhece o neurônio como seu parceiro na construção do encéfalo e se ele interage com ela, levando-a à proliferação ou diferenciação, e o que acontece na construção ou com a manutenção da harmonia do cérebro quando um dos elementos desta interação perde seu estado, dito, de normalidade e entra num processo de proliferação aparentemente incontrolável como o câncer.

Neste contexto desenvolveu-se este trabalho de tese. Que propriedades interativas poderiam ainda persistir quando a célula glial normal se torna uma célula

tumoral? Poderia ela, ao menos, reconhecer o neurônio e lhe garantir um bom substrato, oferecer-lhe moléculas ainda fundamentais à neuritogênese? Poderia o neurônio modular, ainda, a produção de moléculas implicadas na caracterização desta nova célula astrocitária-tumoral?

1.1 Aspectos históricos

O patologista alemão Rudolf Virchow descreveu em 1846, uma substância conectiva no cérebro e medula, que envolvia os elementos do SN (Somjen, 1988). Denominada em alemão *Nervenkitt* (cimento de nervo), foi traduzida como neuroglia, e a estas células foi atribuída a função de suporte dos neurônios (Kimelberg e Norenberg, 1989). Em 1860, Virchow concluiu que as células gliais corresponderiam ao tecido conjuntivo de outros órgãos (Peters *et al*., 1991).

Em 1885, o citologista Camillo Golgi, através do desenvolvimento de técnicas citoquímicas, observou que a neuroglia representava uma classe distinta de células não neuronais e adicionou o papel nutricional às células gliais, devido ao estreito contato dos prolongamentos gliais com vasos sanguíneos e células nervosas.

Mais tarde, o neurohistologista espanhol Santiago Ramón y Cajal descreveu morfologicamente, através da técnica de Golgi, por impregnação pela prata, os vários tipos celulares do SN (Kimelberg e Norenberg, 1989). Entre tantos tipos, descreveu os astrócitos em suas diferentes morfologias e distribuição nas distintas regiões do encéfalo.

1.2 As células gliais

O SN é composto basicamente por dois tipos celulares: os neurônios e as células da glia. As células gliais foram descritas inicialmente como um componente

passivo de função estruturante deste sistema. Uma rede celular de característica conjuntiva (Lent, 2001). Atualmente, propõe-se que as células gliais possam desempenhar, juntamente com os neurônios, uma série de funções relevantes no processamento das informações, garantindo o desenvolvimento e funcionalidade do SN. Neurônio e glia representam diferentes componentes de uma unidade funcional, sendo evidente que as funções cerebrais dependem de uma íntima sinalização do neurônio para a glia e da glia para o neurônio (Kimelberg e Norenberg, 1989; Gomes *et al*., 2001).

As células da glia são divididas em duas classes principais: microglia e macroglia. As células da microglia apresentam corpo celular pequeno e comportam-se como fagócitos que são normalmente mobilizados após infeções, lesões ou doenças degenerativas do SN (Cuadros e Navascues, 1998). Embora seja ainda objeto de intensa discussão, a hipótese atualmente mais aceita para a origem da microglia é de que provavelmente estas células provenham de monócitos que invadem o SN a partir de vasos sangüíneos no desenvolvimento. Como os astrócitos (Gomes *et al*., 2001; Trentin *et al*., 2001), a microglia também é capaz de secretar fatores de crescimento (Lima *et al*., 2001) que podem de modo autócrino modular seu processo de diferenciação. A microglia interage com neurônios e astrócitos, para a troca de fatores e sinalização (Rozenfeld *et al*., 2003).

As três classes funcionais principais da macroglia no SNC adulto são os oligodendrócitos, ependimócitos e astrócitos. As células da glia representam o grupo mais numeroso de células do cérebro e têm função estrutural, metabólica e de apoio trófico a neurônios. A glia regula a neurogênese, sobrevivência, proliferação, migração, crescimento e direcionamento neurítico, estabilidade sináptica e mielinização, sinalizando através de fatores solúveis e de contato celular (Garcia-

Abreu *et al*, 1995; Tessier-Lavigne e Goodman, 1996; Pfrieger e Barres, 1997; Lim e Alvarez-Buylla, 1999; Gomes *et al*, 1999b; Shu e Richards, 2001; Nagler *et al*, 2001; Ullian *et al*., 2001 e 2004; Song *et al*., 2002; Hatten, 2002; Martinez e Gomes, 2002; Nadarajah e Parnavelas, 2002; Volterra e Meldolesi, 2005).

Os oligodendrócitos são células com poucos prolongamentos responsáveis pela formação da bainha de mielina que envolve os axônios do sistema nervoso central (SNC) (Baumann e Pham-Dinh, 2001). Mais recentemente, a glia embainhante do bulbo olfatório também foi envolvida no processo de mielinização (da Silva *et al*., 2003). A mielinização é controlada por sinais de neurônios e astrócitos (Doyle e Colman, 1993). A incapacidade de regeneração neuronal no SNC adulto tem sido atribuída, pelo menos em parte, à presença de fatores inibidores do crescimento neuronal (Teng *et al*., 2004)

Os ependimócitos (células empendimárias) são células que revestem internamente os ventrículos encefálicos e o canal central da medula espinhal. Também participa da formação do plexo coróide, responsável pela produção do líquor. Os tanicitos são células alongadas que se estendem do lúmen ventricular até a superfície pial ou à barreira hematoencefálica. Johansson e colaboradores (1999) demonstraram que os ependimócitos podem ser potenciais células-tronco neurais, originando neurônios que migram para o bulbo olfatório ou se diferenciando em neurônios, astrócitos e oligodendrócitos *in vitro* (Mokrý e Karvanová, 2006). Embora esse trabalho tenha sido largamente contestado (Spassky *et al*., 2005), ele reforça a participação das células da glia na gênese do SN.

As células de glia radial foram descritas no encéfalo fetal de mamíferos no fim do século XIX por Giuseppe Magini (1888). Estas células possuem uma morfologia bipolar com seu corpo celular localizado na zona ventricular e seus prolongamentos

estendendo-se desde a superfície ventricular até a superfície pial. Inicialmente descritas e relacionadas aos eventos de migração neuronal e laminação do córtex cerebral (Hatten, 1999; Rakic, 1971; 1972), atualmente estas células têm sido foco de grandes estudos, uma vez que têm sido apontadas como potenciais células tronco neurais (Alvarez-Buylla *et al*., 2001; Noctor *et al.*, 2002; Malatesta *et al.*, 2003; Anthony *et al.*, 2004; Merkle *et al.*, 2004).

1.3 Astrócitos

As células da glia formam-se através de um processo denominado de gliogênese. Geralmente, esta segue-se à neurogênese, estendendo-se a ela em várias regiões do cérebro (Levitt e Rakic, 1980). A gliogênese é um processo controlado pelo balanço entre a ativação de alguns genes e fatores epigenéticos (Lee *et al*., 2000).

Os astrócitos se originam, em parte, das células de glia radial (GR) (Schmechel e Rakic, 1979; Levitt e Rakic, 1980; Munoz-Garcia e Ludwin, 1986; Voigt, 1989; Culican *et al.,* 1990; Bentivoglio e Mazzarello, 1999; Gotz e Barde, 2005; Mori *et al*., 2005). Presentes na maior parte do cérebro, a GR tem um papel crucial na construção do SN em roedores. Durante o desenvolvimento, estas longas células bipolares apresentam prolongamentos que se estendem radialmente por toda espessura da parede do tubo neural. No período de corticogênese e também no desenvolvimento do cerebelo, medula espinhal e hipocampo, para alcançar suas posições definitivas, populações de neurônios migram radialmente utilizando a GR como suporte (Rakic, 1990). Acreditava-se que uma vez terminada a migração, todas as células de GR transformavam-se em astrócitos por mecanismos ainda pouco esclarecidos (Schmechel e Rakic, 1979; Levitt e Rakic, 1980; Bentivoglio e

Mazzarello, 1999). Atualmente, sabe-se que a GR tem um papel fundamental na neurogênese como células precursoras, gerando neurônios e os direcionando aos seus destinos no córtex de murinos ou de humanos em desenvolvimento (Malatesta *et al.,* 2000; Hartfuss *et al*., 2001; Noctor *et al*., 2001; 2002; Parnavelas e Nadarajah, 2001; de Azevedo *et al*., 2003; Malatesta *et al.,* 2003; Kriegstein e Noctor, 2004; Gotz e Barde, 2005).

A transformação da GR em astrócitos em roedores é caracterizada, dentre outros fatores, pelo aparecimento de uma série de proteínas incluindo a proteína acídica fibrilar glial (GFAP), proteína de filamento intermediário abundante em astrócitos maduros (Eng *et al*., 1985; 2000). Outras proteínas como nestina e vimentina são predominantes em GR e astrócitos imaturos. Durante o desenvolvimento, ocorre uma troca da prevalência de nestina e vimentina para GFAP. O *switch* vimentina-GFAP normalmente começa no período embrionário final e estende-se até aproximadamente o fim da primeira semana pós-natal em roedores (Eng, 1985; Eng *et al*., 2000; Pekny, 2001).

Embora GFAP seja historicamente reconhecida como um marcador de maturação astrocitária, estudos mais recentes documentaram a expressão desta proteína em células progenitoras neuronais (Doetsch *et al*., 1999; Noctor *et al*., 2001; Campbell e Gotz, 2002; Imura *et al*., 2003). O fato de GFAP ser regulada ao longo do desenvolvimento, bem como em situações patológicas do SNC, torna o entendimento de sua modulação ferramenta importante não só para acompanhar o desenvolvimento astrocitário, mas também para estudar o papel de determinadas citocinas em processos patológicos (Laping *et al*., 1994; Menet *et al*., 2001; Kommers *et al*., 2002).

Os astrócitos desempenham uma série de funções importantes no SN: corrigem os níveis de potássio do meio extracelular alterados com a descarga de potenciais de ação dos neurônios (Frank *et al*., 1983); estão envolvidos na captação e liberação de diversos neurotransmissores, tendo um papel crítico no metabolismo de glutamato e GABA (Schousboe *et al*., 2004); são necessários para a formação, função e estabilidade das sinapses no SNC (Slezak e Pfrieger, 2003; Ullian *et al*., 2004); participam da formação da barreira hematoencefálica, interagindo com as células endoteliais, influenciando-as e sendo influenciados por elas (Abbott, 2002); secretam moléculas, fatores de crescimento e citocinas que modulam a sobrevivência e diferenciação neuronais (Muller *et al*., 1995); são componentes chaves da gliose reativa, característica da resposta do SN à injúria (Privat, 2003); podem contribuir para a gênese celular no SN adulto atuando como célula tronco (Doetsch *et al*., 1999; Steindler e Laywell, 2003) ou gerando microambientes favoráveis para a sobrevivência de progenitores neuronais e gliais (Horner e Palmer, 2003).

Mais recentemente, o papel dos astrócitos na sobrevida neuronal tem sido associado ao estabelecimento de uma série de patologias como a esclerose lateral amiotrófica, doença neurodegenerativa caracterizada por extensa morte de neurônios motores da medula espinhal (Vaccarino *et al*., 2007)

1.4 Interação neurônio-astrócito

Por muito tempo as células gliais foram consideradas passivas, mas hoje em dia, considera-se que neurônios e células gliais têm uma íntima relação de plasticidade morfológica e funcional, que aponta para uma influência mútua entre estes dois tipos de célula (Haydon, 2000).

Acredita-se que precursores neuronais possam proliferar somente em um microambiente restrito, em presença de determinados tipos celulares. Dentre esses tipos celulares, estão os astrócitos que produzem uma variedade de sinais celulares permissivos, tanto solúveis como associados à membrana, os quais propiciam um microambiente neurogênico para os precursores neuronais (Lim e Alvarez-Buylla, 1999; Song *et al*., 2002; Lie *et al*., 2005).

Lim e Alvarez-Buylla (1999) demontraram *in vitro* que o contato com astrócitos fornece suporte à proliferação de precursores neuronais da zona subventricular, assim como a sua diferenciação em neuroblastos, inclusive no SN adulto. A influência de astrócitos na neurogênese foi mostrada ainda mais recentemente pelo grupo de Fred Gage. Esses autores demonstraram que astrócitos adultos induzem a fomação de neurônios a partir de células tronco adultas (Song *et al*., 2002). Diversos fatores solúveis secretados por astrócitos como FGFb, EGF, TNF-β e IL-3, podem estar envolvidos na neurogênese (Gomes *et al*., 2001). Nosso grupo demonstrou que fatores secretados por astrócitos cerebelares modulam a proliferação e diferenciação de neurônios cerebelares (Gomes *et al*., 1999a; Martinez e Gomes, 2002; 2005). Nosso grupo, em colaboração com o grupo da Profa. Carla Tasca (UFSC), também demonstrou que derivados de guanina, secretados por astrócitos, promovem uma reorganização das proteínas da MEC (laminina e fibronectina) produzidas por astrócitos através da ativação da via de sinalização de MAPK, que poderia ser responsável por uma interação com neurônios em co-culturas **(Decker *et al*., 2007, anexo 5)**.

Nosso grupo vem estudando a heterogeneidade astrocitária no mesencéfalo e sua associação com a capacidade de promover o crescimento neurítico (Garcia-Abreu *et al*., 1995). Com o objetivo de entender porque somente alguns neurônios

cruzam a linha média do mesencéfalo enquanto outros não ou até mesmo são repelidos por ela, estudou-se a organização funcional desta região. Os autores demonstraram que os astrócitos da região lateral do mesencéfalo eram permissivos ao crescimento neurítico de neurônios cocultivados sobre eles, enquanto os astrócitos da linha média do mesencéfalo não eram permissivos ao crescimento neurítico, evidenciando que astrócitos de diferentes regiões do SN, ainda que muito próximas, são heterogêneos em função e podem modular diferencialmente o crescimento neuronal (Garcia-Abreu *et al*., 1995). Posteriormente, demonstraram que a produção e organização dos elementos da matriz extracelular (MEC) eram diferentes entre esses subtipos astrocitários. A laminina, por exemplo, se organiza na forma fibrilar na matriz dos astrócitos da região lateral e pontualmente na matriz dos astrócitos provenientes da linha média mesencefálica (Garcia-Abreu *et al*., 1995). Neste artigo, os autores chamam atenção para o efeito da cocultura e, possivelmente, dos neurônios sobre a laminina astrocitária. Ainda, a produção e distribuição compartimental dos glicosaminoglicanos (GAGs) sulfatados, como o heparan sulfato e condroitin sulfato, eram diferentes entre os subtipos astrocitários (Garcia-Abreu *et al*., 1996; 2000; De Felice *et al*., 2003; Cavalcante *et al.,* 2003; Mendes *et al*., 2003).

As células da glia desempenham um papel importante nas funções sinápticas. Está bem estabelecido que astrócitos que estão em contato íntimo com sinapses, sustentam a liberação de neurotransmissores no terminal pré-sináptico, fornecendo precursores de transmissores e substratos energéticos (Bacci *et al*., 1999; Carmignoto, 2000; Barres e Smith, 2001). No SN em desenvolvimento, células gliais guiam os axônios para seus alvos, e ajudam os neurônios a estabelecerem conexões sinápticas funcionais. Na ausência de glia, neurônios em cultura têm uma

habilidade limitada de formar sinapses (Pfrieger e Barres, 1997). Sinapses formadas na ausência de células gliais são normalmente imaturas, indicando que a funcionalidade das sinapses não é uma propriedade intrínseca, mas pode estar profundamente regulado por sinais extrínsecos (Nägler *et al.*, 2001; Pfrieger, 2002)**.** Astrócitos aumentam o número de sinapses funcionais dos neurônios, indicando que são necessários para manter a estabilidade sináptica (Pfrieger e Barres, 1997; Ullian *et al*., 2001). A ação dos astrócitos na sinaptogênese é mediado por sinais solúveis derivados de astrócitos que desempenham um papel crucial na formação da sinapse *in vivo*, onde ativam um processo de maturação que aumenta a eficácia das transmissões sinápticas (Nägler *et al*., 2001). Astrócitos secretam colesterol que é interiorizado pelos neurônios, levando ao aumento de colesterol nas membranas neuronais, o que estimula um aumento da eficácia das sinapses (Mauch *et al*., 2001; Slezak *et al*., 2006). Astrócitos também secretam trombospondina, que promove a sinaptogênese (Allen e Barres, 2005; Christopherson *et al*., 2005). Atualmente se propõe, então, que a glia devia ser somada ao esquema típico da sinapse, os elementos pré e pós-sinápticos, como um terceiro elemento (**Figura 1**) (Bacci *et al*., 1999; Haydon, 2001; Ullian *et al*., 2001).

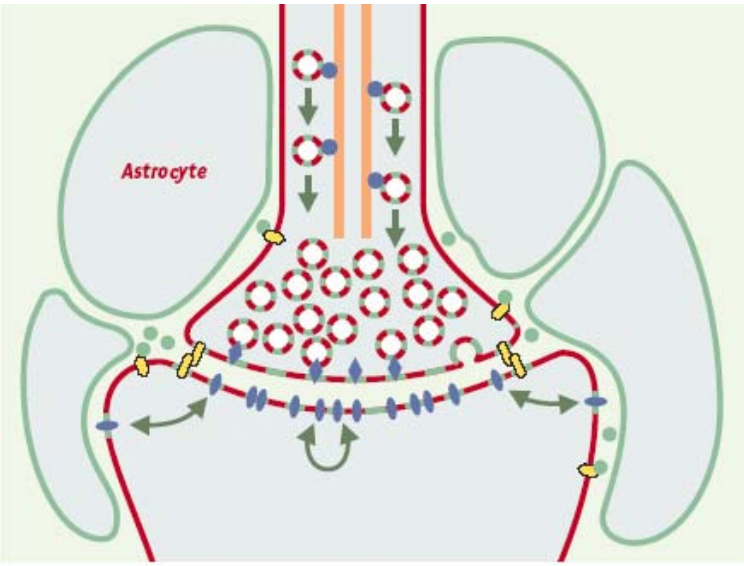

Figura 1. *A sinapse tríplice.* A sinapse é formada pelo elemento pré-sináptico, pós-sináptico e o terceiro elemento: a célula glial perisináptica. A célula glial tem um envolvimento ativo na sinaptogênese, função e estabilidade da plasticidade sináptica. A glia modula à atividade sináptica secretando neurotransmissores, deste modo participando ativamente do funcionamento do SNC (modificado de Pfrieger, 2003).

Estes dados relatam o papel dos astrócitos no desenvolvimento neuronal, mas estudos sobre a influência dos neurônios na biologia da célula glial tiveram início com os trabalhos do grupo de Mary Hatten. Esses relatos iniciais demonstraram que os neurônios são capazes de influenciar a morfologia de astrócitos cerebelares, os quais quando em contato com neurônios, mudam de uma morfologia protoplasmática para uma mais alongada, mais eficaz para suportar a migração neuronal. Além disso, astrócitos diminuem sua taxa de proliferação quando cocultivados com neurônios (Hatten *et al*., 1984; Hatten, 1985).

Outro exemplo, mais recente, demonstrativo da influência dos neurônios na morfogênese astrocitária é o da determinação do padrão de expressão de subtipos de transportadores astrocitários de glutamato. Na ausência de neurônios, os astrócitos expressam transportadores de glutamato do tipo GLAST, característicos

de astrócitos imaturos, enquanto quando cocultivados com neurônios passam a expressar também transportadores do tipo GLT-1, característicos de astrócitos diferenciados. Estes dados sugerem que a diferenciação astrocitária pode sofrer influência neuronal (Swanson *et al*., 1997).

Mais recentemente ainda, nosso grupo investigou o papel de fatores solúveis neuronais na diferenciação astrocitária. Focamos uma parte dos nossos trabalhos na avaliação da expressão de GFAP como um sinal desta diferenciação. Para tanto, utilizamos como modelo experimental um camundongo transgênico que possui parte do promotor murino do gene de GFAP ligado ao gene codificante da enzima bacteriana β-Galactosidase (β-Gal) (Galou *et al*., 1994).

Neste estudo, ficou evidente que neurônios embrionários eram capazes de ativar o promotor do gene de GFAP do astrócito transgênico, representado por um aumento de 60% no número de células β-Gal positivas (Gomes *et al*., 1999b). Estes dados evidenciam a capacidade neuronal de induzir a diferenciação de astrócitos. A adição de meio condicionado derivado de neurônios corticais embrionários apresentou um efeito similar, sugerindo a participação de um fator solúvel neste evento (Gomes *et al*., 1999b). Posteriormente, nosso grupo mostrou que o principal mediador deste fenômeno é o fator de crescimento transformante-beta 1 (TGF-β1) (de Sampaio e Spohr *et al*., 2002), um dos alvos de estudo desta tese.

Se a célula glial reconhece o neurônio como seu parceiro na construção da citoarquitetura do SNC e se ele interage com ela, levando-a a proliferação ou diferenciação, o que acontece na construção ou com a manutenção da harmonia do cérebro quando um dos elementos desta interação perde seu estado de normalidade e entra num processo de proliferação aparentemente incontrolável como o câncer?

1.5 Gliomas

A maioria dos óbitos decorrentes de tumores cerebrais se deve às metástases e aos gliomas. Estes são neoplasias neuroepiteliais que se originam de células gliais e correspondem a 50-60% dos tumores intracranianos primários (Walker *et al*., 1985).

De acordo com a última classificação da Organização Mundial de Saúde (OMS) (Kleihues e Cavenee, 2000), os tumores do tecido neuroepitelial podem ser: astrocíticos, oligodendrogliais, mistos, ependimários, do plexo coróide, gliais de origem incerta, neuroblásticos, do parênquima da pineal e embrionários. Os tumores astrocíticos incluem o astrocitoma difuso, o astrocitoma anaplásico, o glioblastoma multiforme (Gbm), o xantoastrocitoma pleomórfico e o astrocitoma subependimário de células gigantes. Os gliomas representam tumores cerebrais primários de grande importância clínica.

Os astrocitomas difusos correspondem ao grau II da classificação de gliomas pela OMS e são caracterizados por um alto grau de diferenciação celular, crescimento lento e infiltração difusa das estruturas cerebrais vizinhas. Estas lesões afetam adultos jovens e têm uma tendência à progressão maligna para astrocitoma anaplásico e, finalmente, glioblastoma multiforme (Gbm) (Kleihues *et al*., 1993). Esses tumores são referidos também como astrocitoma de baixo grau. Os astrocitomas anaplásicos, correspondentes ao grau III da OMS, são definidos como astrocitoma difusamente infiltrante com anaplasia focal ou dispersa e um acentuado potencial proliferativo. São referidos como astrocitoma de alto grau, que podem tanto se originar de um de baixo grau como não terem indicação de uma lesão precursora. Apresentam uma tendência de progressão para Gbm. Os Gbm correspondem ao grau IV da OMS e são os tumores astrocíticos mais malignos. São compostos por

astrócitos neoplásicos pobremente diferenciados. Afetam principalmente adultos e podem se desenvolver a partir de um astrocitoma difuso grau II ou III (Gbm secundário) ou podem se originar “de novo”, sem uma lesão precursora menos maligna (Gbm primário) (Kleihues e Cavenee, 2000) (**Tabela 1**).

	Astrocitomas de baixo grau	Astrocitoma anaplásico	Glioblastoma
Sobrevida	5-10 anos	2-3 anos	9-12 meses
Proliferação	+/-	++	+++
Invasividade	++	++	+++
Angiogênese	–	–	+++
Necrose	–	–	+++
Sensibilidade à radioterapia	–	++	–
	•Mutações de p53 •Superexpressão de PDGF/R	•Mutação de RB •Amplificação de CDK4 •Deleção de INK4a/ARF •Deleção de PTEN •Deleção de DMBT1/mxl •Deleção de 19q •Deleção de 11p	•Amplificação de EGFR •Mutação de EGFR •Deleção de INK4a/ARF •Deleção de PTEN •Mutação de RB

Tabela 1. *Relação entre sobrevivência, características histológicas e principais mutações associadas com cada tumor* (modificado de Maher *et al*., 2001).

A classificação atual da OMS apresenta avanços nas áreas de imuno-histoquímica e genética molecular. Atualmente, a escala de malignidade da OMS é a mais amplamente aceita (Kleihues *et al*., 1993). Além disso, para os astrocitomas difusos, o sistema de gradação St Anne/Mayo, baseado em quatro critérios (atipia nuclear, mitose, proliferação microvascular e/ou necrose), pode ser reprodutível e preditivo para a sobrevida do paciente (Daumas-Duport *et al*., 1988; Kim *et al*., 1991). Mesmo assim, essas classificações de malignidade dos gliomas, atualmente, têm sido questionadas (Daumas-Duport *et al*., 2000; Kleihues *et al*., 2002).

Enquanto os sintomas neurológicos resultantes do desenvolvimento dos gliomas dependem basicamente do sítio do tumor no SNC, a evolução clinica do paciente e a chance de uma sobrevida mais longa e sem recorrências estão associadas à biologia intrínseca da neoplasia. Indicadores significativos de anaplasia incluem atipia nuclear, atividade mitótica, celularidade, proliferação vascular e necrose (Kleihues e Cavenee, 2000). A sobrevida também depende de vários fatores, como idade e condição clínica do paciente, avaliada pela escala de Karnofsky, localização do tumor e estratégia terapêutica utilizada, como extensão da ressecção do tumor, uso de radioterapia ou quimioterapia (Burger *et al*., 1987; Berger *et al*., 1994; Sneed *et al*., 1995). Apesar dessas variáveis, as médias de sobrevida são maiores que cinco anos para os astrocitomas difusos grau III, de dois a cinco anos para os astrocitomas anaplásicos grau III e menores que um ano para os Gbm grau IV (Burger *et al*., 1985; Daumas-Duport *et al*., 1988; Kim *et al*., 1991).

Os Gbm correspondem a 50% dos tumores gliais e a 25% de todos os tumores intracranianos do adulto (Helseth *et al*., 1989). Manifestam-se em qualquer idade, mas afetam principalmente adultos entre 45 e 70 anos (Kleihues e Cavenee, 2000). O quadro clínico depende da localização e do tamanho da lesão, podendo ocorrer cefaléia, crise convulsiva, distúrbio cognitivo, déficit neurológico focal e coma. O diagnóstico é facilmente estabelecido por tomografia computadorizada ou ressonância nuclear magnética. Astrocitomas de alto grau e Gbm apresentam captação do meio de contraste nos exames de imagem, ao contrário do que ocorre nos tumores de baixo grau (DeAngelis, 2001). Em tomografias computadorizadas, o Gbm se apresenta como uma lesão de formato irregular, com uma zona periférica em anel que capta contraste, ao redor de uma área central de necrose, a qual geralmente é hipodensa (Kleihues e Cavenee, 2000). Análises de cortes de cérebro

de Gbm não tratados mostram que essa estrutura em anel não representa a borda tumoral mais externa, e as células do glioma podem ser encontradas 2 cm além da margem (Burger *et al*., 1988; Nagashima *et al*., 1999).

O arsenal terapêutico atualmente disponível para tratamento dos Gbm inclui cirurgia, radioterapia e, às vezes, quimioterapia. Entretanto, a mortalidade no primeiro ano após o tratamento permanece alta (DeAngelis, 2001).

Os glioblastomas ocorrem mais freqüentemente na substância branca subcortical e podem se espalhar através das comissuras e corpo caloso para ambos os hemisférios cerebrais. Infiltração tumoral geralmente se estende ao córtex adjacente e aos núcleos da base. Gbm intraventricular, de cerebelo e medula espinhal são raros, assim como no tronco encefálico. Estes últimos são mais comuns em crianças (Dohrmann *et al*., 1976). Óbito ocorre por crescimento tumoral local e invasão cerebral (Kleihues e Cavenee, 2000; DeAngelis, 2001).

Microscopicamente, o Gbm é composto de células tumorais astrocíticas, pobremente diferenciadas, geralmente pleomórficas, com acentuada atipia nuclear e alta atividade mitótica. Proliferação microvascular proeminente e/ou necrose são fatores diagnósticos essenciais. Como o termo glioblastoma multiforme sugere, a histopatologia desses tumores é variável. Algumas lesões mostram alto grau de polimorfismo celular e nuclear e outras são mais monótonas. A natureza astrocítica pode ser facilmente identificada em alguns tumores, sendo mais difícil em outros, devido ao alto grau de anaplasia. Essa heterogeneidade regional dos Gbm torna-se um desafio quando o diagnóstico histopatológico é baseado em biopsia por agulha guiada estereotaxicamente (Burger *et al*., 1989). Imunorreatividade para GFAP é extremamente variável nesses tumores e sua expressão tende a diminuir durante a

progressão do glioma. A expressão de vimentina e nestina é comum, por serem marcadores de células indiferenciadas (Kleihues e Cavenee, 2000).

Os mecanismos de invasão e progressão dos gliomas ainda permanecem por serem elucidados. Sabe-se que a invasividade da célula neoplásica está relacionada com sua motilidade, capacidade de adesão e de proteólise da MEC (Pilkington, 1994). O crescimento tumoral, além disso, requer a indução de angiogênese. Esse processo é regulado por fatores indutores e inibidores de proliferação e migração de células endoteliais (Pilkington, 1994; Vajkczy *et al*., 1999; Hamel *et al*., 2000). Esses dois aspectos cruciais no desenvolvimento dos gliomas, invasividade e angiogênese, constituem atualmente alvos para novas terapias. A proliferação microvascular acentuada é uma das principais características do glioblastoma e é traduzida principalmente por proliferação de células endoteliais (Wesseling *et al*., 1997). Considerando que os tumores sólidos dependem da angiogênese para manter seu crescimento, várias estratégias de tratamento envolvendo o bloqueio da angiogênese têm sido estudadas (Kirsch *et al*., 2000).

Um padrão estabelecido de invasividade de células tumorais malignas ocorre com células tumorais se soltando da massa primária tumoral crescente e aderindo à MEC por receptores específicos. Logo após, ocorre a degradação dos componentes da MEC, permitindo às células tumorais migrarem para tecidos subjacentes. Ocorre um aumento na expressão de componentes da MEC no estroma do tumor e nos locais de invasão do parênquima cerebral. Sendo assim, o crescimento do tumor, a proliferação e a invasão poderiam estar sendo modulados pelos componentes da MEC, com ênfase em algumas de suas glicoproteínas como laminina e fibronectina e seus proteoglicanos (Bellail *et al*., 2004).

1.6 Laminina

É uma glicoproteína (850 kDa) com multidomínios, presente de forma organizada na lâmina basal. Reveste epitélios, vasos sanguíneos, nervos e encontra-se subjacente às meninges. A laminina é sintetizada e secretada por diversos tipos celulares e interage com receptores de superfície celular. Está envolvida em adesão celular, migração e regeneração (Timpl e Dziadel, 1986; Beck *et al*., 1990).

A rede molecular de laminina diretamente em contato com a superfície celular consiste de laminina polimerizada que se auto-organiza mesmo na ausência de outros elementos da membrana basal (Yurchenco *et al*., 1992). Além de seu papel estrutural, a laminina associada à membrana basal modula várias funções celulares como a adesão celular, proliferação, migração e a metástase tumoral (Kleinman *et al*., 1993; Aumailley e Smyth, 1998; Freire e Coelho-Sampaio, 2000).

Aparentemente, a laminina adsorvida ao substrato forma uma rede molecular com as propriedades da matriz de laminina *in vivo*. Entretanto, tem-se observado que a organização e a arquitetura da laminina sofrem variações em astrócitos de regiões distintas do SN. Alguns destes arranjos, como uma arquitetura de laminina disposta pontualmente ou de maneira filamentosa na superfície celular, parecem ser determinantes para assegurar certas funções da glicoproteína (Garcia-Abreu *et al*., 1995). De todo modo, no cérebro de rato em desenvolvimento, *in vivo*, a distribuição dos polímeros de laminina tem arranjos variados (Zhou, 1990).

A polimerização de laminina *in vitro* pode ocorrer tanto em condições de pH neutro ou acídico, mas os polímeros resultantes nestas duas condições têm arranjos estruturais diferentes, e estas matrizes apresentam propriedades distintas, por exemplo, podem favorecer a proliferação de células neuronais crescidas sobre elas,

ou favorecer a neuritogênese destes neurônios (Freire *et al*., 2002). Mais ainda, esta organização de matrizes de laminina pontuada ou fibrosa (filamentosa) parece ser modulada por resíduos de ácidos siálicos associados a lípidios de domínios da membrana celular (Freire *et al*., 2004).

A laminina está envolvida em diversos eventos da morfogênese do SN, como diferenciação e direcionamento axonal *in vitro* (Chamak e Prochiantz, 1989; Hunter e Brucken, 1997; Colognato e Yuchenko, 2000). Os astrócitos são a principal fonte de moléculas de MEC no SNC (Powell e Kleinman, 1997) e *in vitro* eles também produzem laminina. *In vivo*, a expressão de laminina diminui durante a maturação astrocitária e no SNC adulto aparece restrita à lâmina basal e algumas regiões específicas (Liesi, 1985; Jucker *et al*., 1996).

A expressão de laminina na MEC astrocitária parece ser controlada por neurônios em regime de cocultura. É o que sugere o trabalho de Garcia-Abreu e colaboradores (1995), que analisou o arranjo de laminina glial em células utilizadas como tapetes de neurônios em crescimento. Esses autores observaram uma maior quantidade de laminina na superfície glial quando os astrócitos estavam em contato com neurônios. Demonstraram ainda que astrócitos tratados com EGF (fator de crescimento da epiderme) aumentam a expressão de laminina e fibronectina, tornando-se um substrato mais permissivo ao crescimento neurítico (Martinez e Gomes, 2002).

Embora a laminina seja muito expressa em astrócitos durante a vida embrionária e sua expressão diminua nos astrócitos na vida adulta (Pindzola *et al*., 1993; Powell e Kleinmam, 1997), ela reaparece como uma das proteínas mais abundantes nos tumores em geral e nos gliomas em especial (Hunt e Sherbert, 1989; Zamecnik *et al*., 2004).

1.7 Fator de crescimento transformante-beta

A família de fatores de crescimento transformantes-β (TGF-β: *transforming growth factor-β*) compreende um grande número de fatores de crescimento polipeptídicos relacionados, capazes de regular diversos processos celulares como proliferação, determinação de linhagem, diferenciação, motilidade, adesão e morte (Massagué, 1998). Todas estas funções atribuem a esta família um papel essencial no desenvolvimento, homeostase, e reparo de virtualmente todos os tecidos em diversos organismos. De acordo com a similaridade da seqüência primária, estes fatores podem ser divididos em grupos, incluindo as subfamílias de proteínas morfogenéticas ósseas (BMP: *bone morphogenetic protein*), Nodal, Activinas, TGF-β e outras. A subfamília TGF-β é contituída por três membros em roedores: TGF-β1, TGF-β2 e TGF-β3 (Massagué, 1998).

Os TGF-βs sinalizam através de uma família de glicoproteínas transmembrana serina/treonina cinases que baseadas nas suas propriedades estruturais e funcionais podem ser divididas em duas subfamílias: os receptores tipo I (TGFR-I) e os receptores tipo II (TGFR-II), responsáveis pela transdução do sinal (Massagué, 1992; 1998; Böttner *et al*., 2000; Massagué e Chen, 2000).

A ativação da cascata de sinalização pela subfamília TGF-β é dada pela conexão do ligante ao receptor tipo II, que promove uma auto-fosforilação e fosforila o receptor tipo I (Massagué, 1998). Wrana e colaboradores (1992) mostraram uma interdependência entre os receptores: o receptor I requer o receptor II para ligar-se ao TGF-β, e o receptor II requer o receptor I para sinalizar. Uma vez fosforilado, o receptor tipo I dá início a uma cascata de sinalização que envolve os membros da família SMAD.

Na via de sinalização canônica do TGF-β1, o fator liga-se ao TGFRII, recrutando o TGFRI e há a formação de um complexo. O TGFRII fosforila e ativa o TGFRI. Uma vez ativado, o TGFRI fosforila as proteínas SMAD 2 e/ou 3, que se associam com a SMAD 4, formando um complexo que transloca-se para o núcleo da célula. No núcleo, com a cooperação de fatores de transcrição e co-ativadores, as proteínas SMADs fazem com que os genes alvos específicos sejam transcritos (**Figura 2**) (Massagué, 1998; Miyazono, 2000; Wrana e Attisano, 2000; Moustakas *et al*., 2001; **Gomes *et al*., 2005, Anexo 1**; Massagué e Gomis, 2006).

Existem poucos trabalhos mostrando a via de sinalização de SMADs no SNC e a maioria dos dados é sobre a sinalização de BMPs em neurônios (Lein *et al*., 2002; Angley *et al*., 2003; Farkas *et al*., 2003; Rios *et al*., 2004). Recentemente, um grupo gerou um animal *knock out* com deficiência na expressão de SMAD 4 no SN. Embora o desenvolvimento destes animais seja aparentemente normal, há um decréscimo no número de neurônios de Purkinje no cerebelo e uma alteração no controle motor (Zhou *et al*., 2003).

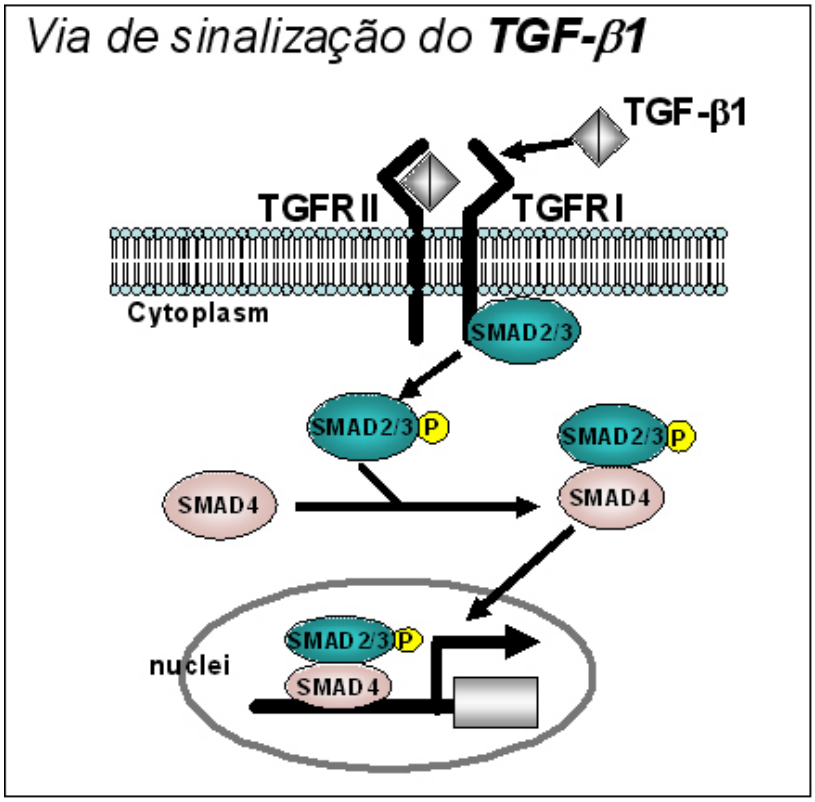

Figura 2. *Sinalização canônica de TGF-β1.* O TGF-β1 se liga diretamente ao receptor tipo II (TGFRII), e este forma um comlexo com o receptor tipo I (TGFRI). O TGFRI ativado fosforila as proteínas citoplasmática SMAD 2 e/ou 3. As SMADs 2/3 podem associar-se com a proteína SMAD 4, formando um complexo que transloca para o núcleo. Este complexo citoplasmático promove a transcrição de genes alvos específicos com o auxílio de co-fatores e fatores ligadores de DNA.

As crescentes evidências do papel de TGF-β no desenvolvimento do SN têm levado mais recentemente ao estudo das vias de sinalização desta molécula em astrócitos (Baghdassarian *et al*., 1993; Laping *et al*., 1994; Wyss-Coray *et al*., 1995; Burton *et al.,* 2002; de Sampaio e Spohr *et al*., 2002; Brionne *et al*., 2003; Garcia *et al*., 2004; Siegenthaler e Miller, 2004; **Sousa e Romão *et al*., 2004;** Stipursky e Gomes, 2007).

A participação do TGF-β em processos de injúria do SN é bem conhecida. O TGF-β1 pode induzir sua própria expressão, mantendo alto o nível do fator no local, onde vai induzir a angiogênese e a produção de MEC colaborando no reparo tecidual (Flanders *et al*., 1998). No SNC, a expressão de TGF-β é aumentada em

áreas de disfunção neuronal (Brionne *et al*., 2003; Buisson *et al*., 2003; **Gomes *et al*., 2005, Anexo 1**; Tesseur e Wyss-Coray, 2006; Vivien e Ali, 2006). Astrócitos e microglia migram para o local da injúria, provavelmente devido à atividade quimiotática do fator, fazendo com que estas células sejam ativadas e expressem um fenótipo reativo, aumentando a síntese de citocinas (Flanders *et al*., 1998; Buckwalter e Wyss-Coray, 2004).

Experimentos *in vitro* e *in vivo* mostraram que o TGF-β1, liberado por astrócitos, possui um papel importante na proteção dos neurônios contra a toxicidade do glutamato (Bruno *et al*., 1998). O glutamato, ativando o seu receptor metabotrópico mGluR2/3, induz a síntese de TGF-β1. D'Onofrio e colaboradores (2001) demonstraram a necessidade da ativação das vias de sinalização de MAPK e PI3K para a síntese de TGF-β1.

Além dos processos de injúria aguda, membros da família TGF-βs estão envolvidos em patologias degenerativas, como doença de Alzheimer e doença de Parkinson. O TGF-β1 pode ser encontrado em placas senis na doença de Alzheimer (Pratt e McPherson, 1997; Tesseur e Wyss-Coray T, 2006). Lesné e colaboradores (2003) mostraram que o TGF-β1 potencializa a produção de amilóide-β (peptídeo crucial para o desenvolvimento da doença de Alzheimer em astrócitos), levando ao aumento da formação de placas senis. Em pacientes parkinsonianos, há um aumento de TGF-β1 nos terminais dopaminérgicos estriatais. Contraditoriamente, *in vitro*, TGF-βs aumentam a sobrevida de neurônios dopaminérgicos (Pratta e McPherson, 1997). A doença de Parkinson é caracterizada pela morte dos neurônios dopaminérgicos na substância nigra, presente na base do mesencéfalo, e pela presença citoplasmática de corpos de Lewy, compostos principalmente por proteína α-sinucleina. Demostramos juntamente com a equipe da Profa. Débora Foguel

(UFRJ) que agregados de α-sinucleina em culturas de neurônios mesencefálicos diminuem o número e comprimento neurítico e aumentam o número de neurônios apoptóticos **(Follmer *et al*., 2007, anexo 3)**.

Embora a participação do TGF-β1 em situações patológicas seja relativamente conhecida, pouco se sabe sobre a atuação deste fator na fisiologia do SN. Nosso laboratório tem contribuído para entender essa questão e tem relacionado esta citocina com as interações neurônio-astrócito, importantes não só para o desenvolvimento como para o funcionamento adequado do SN adulto. Mostramos que, no córtex cerebral, fatores solúveis neuronais induzem a secreção de TGF-β1 por astrócitos induzindo a expressão de GFAP e a diferenciação astrocitária (**Figura 3**) (**Gomes *et al*., 2005, Anexo 1**).

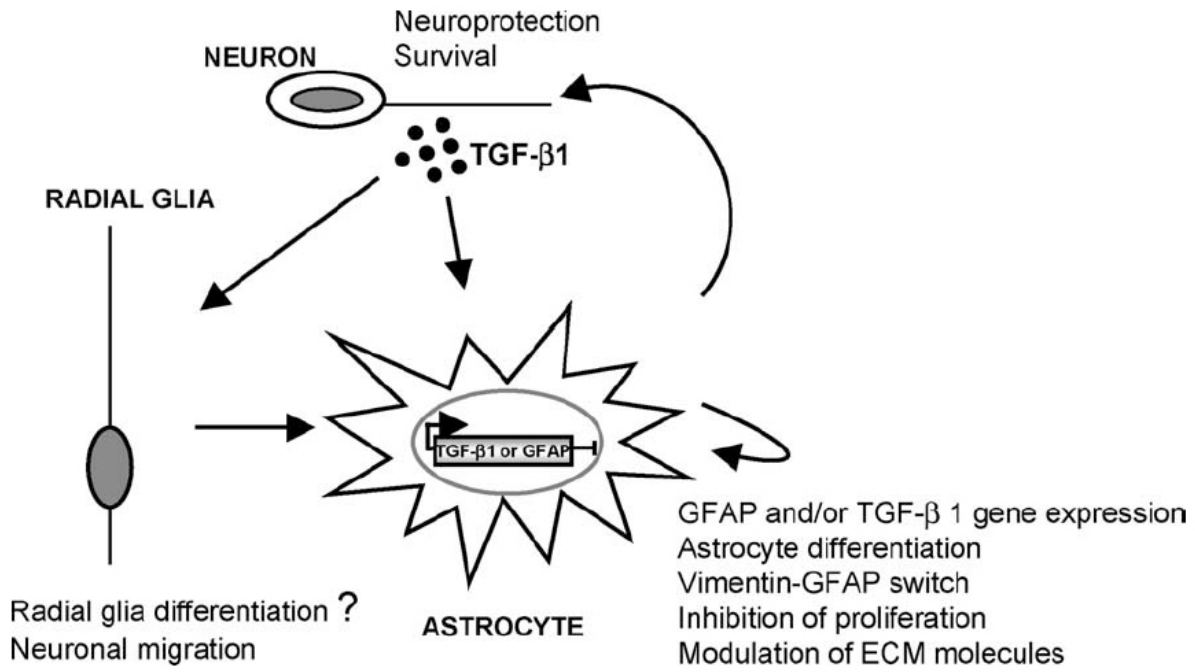

Figura 3. *TGF-β1 como um mediador das interações astrocitárias.* Os neurônios induzem o aumento da síntese e liberação de TGF-β1 por astrócitos, que protegem os neurônios contra a toxicidade. TGF-β1 secretado por neurônios atua na glia radial, modulando a migração neuronal e a diferenciação da glia radial. Nos astrócitos, TGF-β1 modula vários eventos celulares: induz a expressão do gene de

GFAP e a diferenciação; inibe a proliferação e modula os níveis das proteínas da matriz extracelular (retirado de **Gomes *et al*., 2005, Anexo 1**).

Recentemente, Miller (2003) identificou imunomarcação para TGF-β1 em vários compartimentos da parede cerebral em ratos embrionários, recém-natos e adultos. A expressão dos TGF-βs ao longo do desenvolvimento e no adulto, em diversas áreas, sugere que estes fatores tenham ação ampla no SN. Brionne e colaboradores (2003) utilizando camundongos *knock out* para TGF-β1 mostraram que este fator possui um papel crítico na manutenção da integridade e sobrevivência neuronal e na ativação microglial, e surgere ainda que alguns destes efeitos podem ser resultados da modulação na expressão de MEC. Mais recentemente, Siegenthaler e Miller (2004) demonstraram que o TGF-β1 modula a migração neuronal em culturas organotípicas de cérebros de ratos embrionários.

Como dito anteriormente, membros da família TGF-β desempenham uma importante função como inibidores da proliferação glial. *In vitro*, TGF-βs inibe a síntese de DNA em astrócitos normais de ratos jovens e adultos, além de reverter os efeitos mitogênicos de fatores de crescimento como FGF (fator de crescimento de fibroblasto), EGF e PDGF (fator de crescimento derivado de plaquetas) (Jennings e Pietenpol, 1998).

Contrariamente a sua ação anti-mitogênica bem estabelecida para astrócitos normais, a ação de TGF-β em tumores gliais é controversa e de difícil entendimento. TGF-β exerce múltiplos efeitos nos gliomas incluindo inibição e/ou estimulação da proliferação, imunossupressão local e indução de angiogênese, entre outros (Jennings e Pietenpol, 1998). Aparentemente, TGFβ age como um supressor tumoral em estágios precoces da tumorigênese e como estimulador tardiamente. Algumas evidências demonstram que a perda da capacidade de responder ao efeito

inibitório de TGF-β sobre a proliferação é acompanhada da progressão do estágio de malignidade. O mecanismo pelo qual astrócitos tumorais escapam à influência supressora deste fator ainda não está totalmente elucidado. Há quatro propostas para explicar esta falta de resposta: 1) incapacidade dos tumores de ativarem o TGF-β latente (TGF-β é secretado numa forma inativa, dependente de ativação após sua secreção); 2) mutações que levem à perda de função ou não expressão dos receptores de TGF-β, TGFRI ou TGFRII; 3) diferenças de ligação e ativação dos receptores pelas diferentes isoformas de TGF-β; 4) alterações nas vias de sinalização desencadeadas pelos astrócitos normais e tumorais.

1.8 Fator de crescimento do tecido conjuntivo

O termo "connective tissue growth factor" (CTGF) foi usado pela primeira vez em 1991 por Bradham e colaboradores para descrever um novo fator de crescimento, secretado por células endoteliais humanas em cultura e que estimulou a síntese de DNA e a quimiotaxia em fibroblastos. CTGF é uma proteína secretada de 38 KDa, membro da família CCN, formada pelas proteínas *cysteine-rich 61* (Cyr61/CCN1), CTGF (CCN2), *nephroblastoma overexpressed* (NOV/CCN3), *WNT-inducible signaling pathway protein 1* (WISP1/CCN4), WISP2 (CCN5) e WISP3 (CCN6). Os membros da família CCN apresentam estrutura semelhante e participam do controle de diversos processos celulares, exercendo, dentre outras funções, um importante papel no desenvolvimento vascular e esquelético (Perbal *et al*., 2003).

Coletivamente, essas proteínas estimulam mitose, adesão, apoptose, produção de MEC, parada de crescimento e migração, e regulam angiogênese, placentação, implantação, embriogênese e ossificação endocondral. As células alvo

incluem fibroblastos, células epiteliais, células endoteliais, células musculares lisa e células neuronais (Moussad e Brigstock, 2000).

O CTGF foi detectado em várias lesões fibróticas (pele, pulmão, rim e fígado) (Moussad e Brigstock, 2000). Ele pode agir como um cofator, responsável por indução de fibrogênese. Foi observado que o CTGF é induzido pelo TGF-β, estando, então, envolvido na regulação de migração, proliferação celular, formação de MEC e cicatrização (Grotendorst, 1997). Embora muitos papéis fisiológicos tenham sido propostos para o CTGF, seu mecanismo de ação permanece desconhecido. Abreu e colaboradores (2002) mostraram que o CTGF age, em parte, através da ligação no espaço extracelular com BMP4, inibindo-a, e ao TGF-β1, ativando-o. O TGFβ-1 é o primeiro coefetor de CTGF a ser descoberto e um dos mais importantes fatores de crescimento que atua com sua colaboração (Grotendorst, 1997).

CYR62 (CCN1) e CTGF promovem crescimento, migração, adesão e sobrevivência da células endoteliais *in vitro* e sua ação na angiogênese é mediada, pelo menos em parte, através de interações com integrinas na superfície da célula, induzindo efeitos de sinalização intracelular que incluem ativação de cinase e transcrição de genes (Lau e Lam, 1999). Ambos regulam a atividade e produção de outras proteínas angiogênicas. Shimo e colaboradores (1999) demonstraram funções angiogênicas do CTGF *in vitro* e *in vivo*, indicando essa proteína como um potente fator angiogênico, com participação em vários estágios desse processo, como proliferação, adesão, migração e quimiotaxia de células endoteliais. A hipóxia estimula células de câncer de mama a liberar CTGF, iniciando a cascata de angiogênese, modulando o balanço entre síntese e degradação de MEC via metaloproteinases secretadas por células endoteliais em resposta ao CTGF. Essa cascata deve exercer um papel crítico na neovascularização induzida por hipóxia

que acompanha a invasão tumoral (Kondo *et al*., 2002). De acordo com isso, o CTGF tem sido relacionado à função de mediar a adesão e migração da célula endotelial (Babic *et al*., 1999 e Shimo *et al*., 1999) e de se ligar ao VEGF (Inoki *et al*., 2002), afetando, desse modo, a sobrevida da célula endotelial e angiogênese *in vivo*.

Surveyor e Brigstock (1999) mostraram que vários tecidos e órgãos produzem CTGF em um padrão espaço-temporal específico durante a embriogênese, suportando um papel do CTGF na diferenciação celular e no desenvolvimento embrionário. O padrão de expressão do CTGF tem sido extensivamente descrito. A expressão de CTGF é fortemente encontrada no mesênquima, em condrócitos hiperplásicos, no glomérulo em desenvolvimento e no tecido conjuntivo ao redor do músculo em desenvolvimento (Yanamoto *et al*., 2002; Friedrichsen *et al*., 2003). No adulto, o CTGF é encontrado no endotélio e em neurônios do córtex cerebral (Bradham *et al*., 1991; Heuer *et al*., 2003).

A deleção do gene de CTGF é letal em camundongos. A falta do CTGF induz defeitos esqueléticos importantes como incapacidade de ossificação da caixa torácica, devido à alteração na proliferação de condrócitos e no remodelamento da matriz durante condrogênese (Ivkovic *et al*., 2003).

Em amostras de tumores, a expressão anormal de proteínas da família CCN está associada ao desenvolvimento tumoral (Planque *et al*., 2003). Em muitos casos, proteínas CCN, cuja expressão encontra-se aumentada, estão associadas à acentuada proliferação e/ou inibição do crescimento tumoral. Elevada expressão de CTGF tem sido detectada em carcinomas ductais invasivos de mama, dermatofibromas, granulomas piogênicos, células endoteliais de angiolipomas e angioleiomiomas, e em tumores pancreáticos (Perbal, 2001). Além disso, o CTGF foi

relacionado, juntamente com outros fatores, ao desenvolvimento de metástase óssea em câncer de mama, através de sua ação na angiogênese tumoral (Kang *et al*., 2003).

De acordo com estudos prévios por imunocitoquímica no tecido cerebral de rato (Kondo *et al*., 1999), a astroglia expressa CTGF, principalmente nas regiões do córtex cerebral e da substância branca da medula espinhal. Entre os neurônios, o CTGF foi detectado em uma subpopulação de neurônios piramidais, assim como nos ependimócitos dos ventrículos, plexo corióide e tanicitos ao redor do canal central da medula espinhal. Os autores desse estudo (Kondo *et al*., 1999) concluíram que a maior parte do CTGF foi observada em astrócitos, sugerindo que essa proteína possa participar de processos de cicatrização após lesão neuronal, via regulação da gliose reativa. A ampla distribuição e variável intensidade da marcação do CTGF em diferentes regiões e componentes celulares observadas nesse estudo sugere que o CTGF é um fator multifuncional no SNC. Schwab e colaboradores (2000; 2001) mostraram que o CTGF está implicado na reconstrução pós-lesional do tecido do SNC, promovendo a formação de cicatriz glial.

Expressão aumentada de CTGF na medula espinhal de pacientes com esclerose lateral amiotrófica foi detectada, principalmente nos astrócitos reativos (Spliet *et al*., 2003), apoiando a participação do CTGF na progressão e persistência de astrogliose em doenças neurodegenerativas. Além disso, em pacientes com doença de Alzheimer, Ueberham e colaboradores (2003) encontraram positividade para CTGF no córtex entorrinal, hipocampo, córtex temporal, assim como nos neurônios e astrócitos associados com placas.

O CTGF foi detectado em glioma e em algumas linhagens de tumores humanos derivados do SNC (Xin *et al*., 1996; Inoki *et al*., 2002). Pan e

colaboradores (2002) demonstraram a expressão e localização do CTGF em um grande número de células endoteliais em proliferação e células neoplásicas em Gbm humano. Esses autores sugerem que células tumorais e endoteliais expressam CTGF, podendo ser especulado que células de Gbm produzem CTGF, o qual se difunde na proximidade de vasos pequenos. É sugerido que o CTGF possa exercer um papel na angiogênese do Gbm, uma vez que o TGF-β, proteína envolvida no processo de angiogênese, é um grande indutor do CTGF e age, em algumas de suas funções, através do CTGF (Grotendorst, 1997). Recentemente, Xie e colaboradores (2004) quantificaram a expressão de genes da família CCN em gliomas e a correlacionaram com parâmetros clínicos e patológicos desses tumores. Os autores observaram que o aumento da expressão de CTGF em tumores estava associado ao estágio do tumor e aos aspectos patológicos tumorais.

OBJETIVOS

2 OBJETIVOS

2.1 Objetivos gerais

Esse trabalho tem especial interesse no estudo das interações neurônio-glia durante o desenvolvimento do SNC e durante a formação de processos tumorigênicos, com ênfase especial naquelas mediadas pelos fatores solúveis, TGF-β1 e CTGF, e pela proteína de MEC, laminina. As seguintes questões foram formuladas: Quais os efeitos de TGF-β1 como mediador das interações neuro-astrocitárias durante o desenvolvimento do SNC? As células gliais normais quando adquirem comportamento tumoral, guardam as mesmas propriedades astrocitárias de interação com neurônios? A interação é mediada por contato celular e/ou fatores solúveis? Há efeito na neuritogênese?

2.2 Objetivos específicos:

- Analisar o papel das interações neuro-gliais mediadas por TGF-β1, no processo de diferenciação astrocitária, em diferentes regiões do encéfalo em desenvolvimento;
- Analisar o papel de glutamato na diferenciação astrocitária através da ativação do promotor do gene de GFAP e suas vias de sinalização;
- Analisar o potencial neuritogênico de glioblastomas humanos *in vitro*;
- Analisar as vias de sinalização, especificamente de TGF-β1 e CTGF, na interação neurônio-glioblastoma *in vitro*.

RESULTADOS

3 RESULTADOS

3.1 Promotor do gene de GFAP é modulado diferencialmente por TGF-β1 em astrócitos de regiões diferentes do cérebro

A expressão de GFAP, proteína majoritária de filamento intermediário de astrócitos maduros, é regulada no SN em desenvolvimento e em condições patológicas. Durante a maturação astrocitária, nestina e vimentina são gradualmente substituídas por GFAP. Recentemente, TGF-β1 foi apontado como novo mediador das interações neuro-gliais. Através do cultivo de neurônios sobre células gliais provenientes do córtex cerebral de camundongos transgênicos contendo o promotor do gene de GFAP ligado ao gene codificante da enzima bacteriana β-galactosidase (β-Gal), foi demonstrado que neurônios ativam o promotor de GFAP e induzem a diferenciação astrocitária em córtex cerebral (Gomes *et al*., 1999b). Ensaios com meios condicionados de neurônios identificaram TGF-β1 como principal mediador deste evento (de Sampaio e Spohr *et al*., 2002). Entretanto, muitas evidências sustentam o conceito de que astrócitos isolados de diferentes regiões de cérebro variam notavelmente sua responsividade a vários agentes, como hormônios, fatores de crescimento, neurotransmissores, regulação gênica e padrão de interação celular. Nesse trabalho investigamos o papel da interação neuro-glial na ativação do promotor de GFAP e na diferenciação astrocitária em outras regiões encefálicas. Demonstramos que embora neurônios ativem o promotor do gene de GFAP de astrócitos do córtex cerebral, esse evento não é observado em astrócitos derivados de mesencéfalo e cerebelo, sugerindo uma especificidade regional desse fenômeno. Surpreendentemente, apesar de não induzirem a diferenciação desses astrócitos, os neurônios corticais induzem a síntese e secreção de TGF-β1 por essas células.

Ensaios de *Western blot* e imunocitoquímica identificaram o receptor de TGF-β1 em todas as subpopulações astrocitárias e revelaram a expressão de TGF-β1 em neurônios derivados de todas as regiões, indicando que a não responsividade do promotor de GFAP em astrócitos de mesencéfalo e cerebelo por TGF-β1 não é um defeito da sinalização de TGF-β1. Nossos dados destacam a alta complexidade da interação neurônio-glia e sugerem um mecanismo distinto de modulação do gene de GFAP em populações heterogêneas de astrócitos no SNC.

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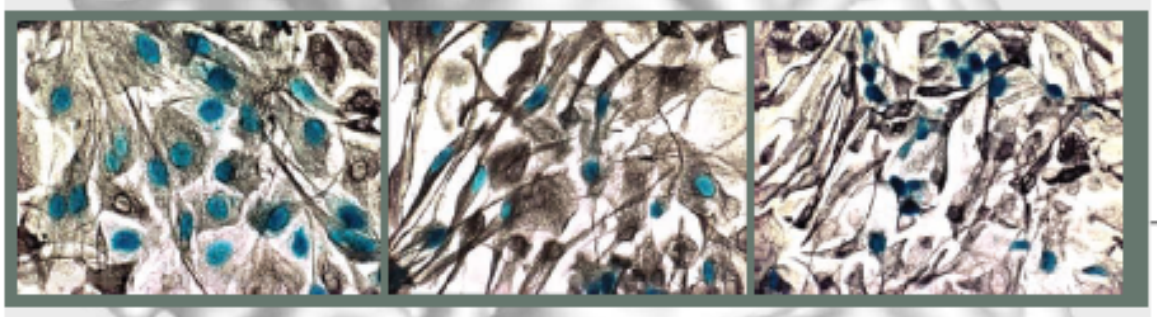

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Glial fibrillary acidic protein gene promoter is differently modulated by transforming growth factor-beta 1 in astrocytes from distinct brain regions

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Keywords: astrocyte differentiation, central nervous system development, glial heterogeneity, neuron–glia interaction, transforming growth factor-beta 1, transgenic mice

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,

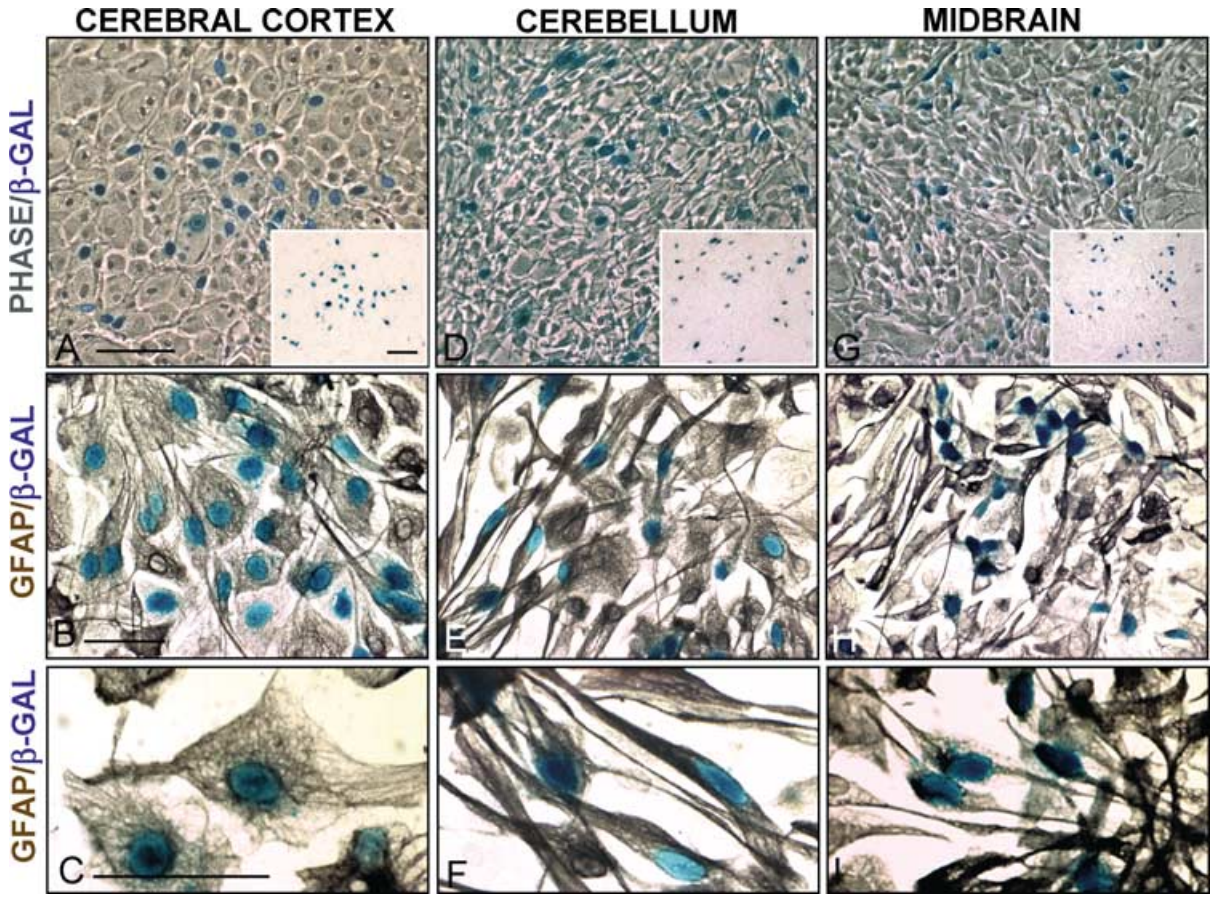

FIG. 1. Glial fibrillary acidic protein (GFAP) promoter-*lacZ* expression *in vitro*. (A–C) Cerebral cortex, (D–F) cerebellum and (G–I) midbrain astrocytes derived from newborn transgenic mice were kept for 12 days in culture (10 days in the presence of serum and 2 additional days in serum-free medium). 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

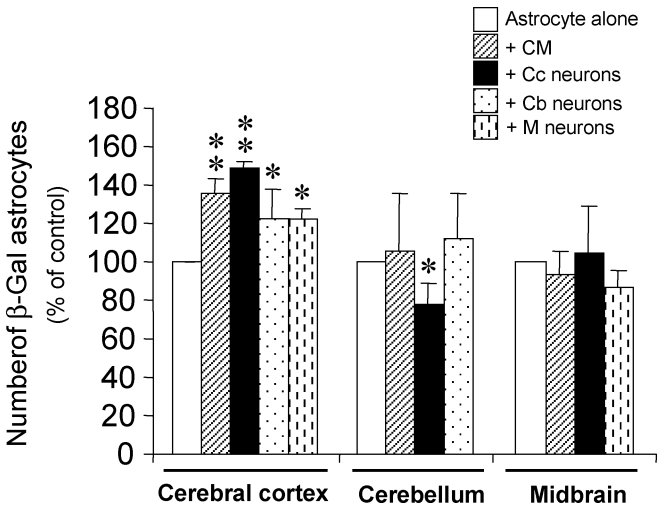

FIG. 2. Cortical neurons do not induce glial fibrillary acidic protein promoter-directed expression of *lacZ* in heterotypic cocultures. Cerebral cortex (Cc), cerebellum (Cb) and midbrain (M) astrocytes derived from newborn transgenic mice were cultured alone (open white bars) or in the presence of Cc 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).

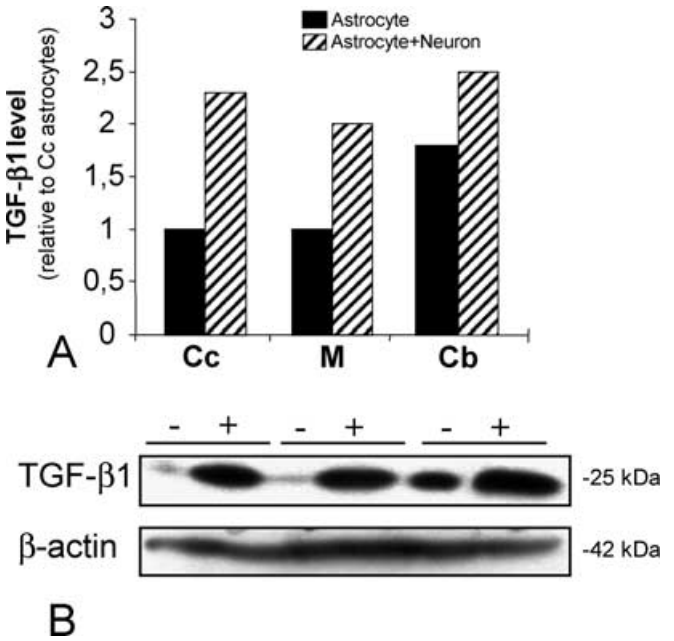

FIG. 3. Identification of transforming growth factor-beta 1 (TGF-β1) content in astrocytes. (A) Representative graphic analysis and (B) western blot showing TGF-β1 content: Equal amounts (10 μg/lane) of total protein of cellular extracts of astrocytes derived from cerebral cortex (Cc), midbrain (M) and 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.

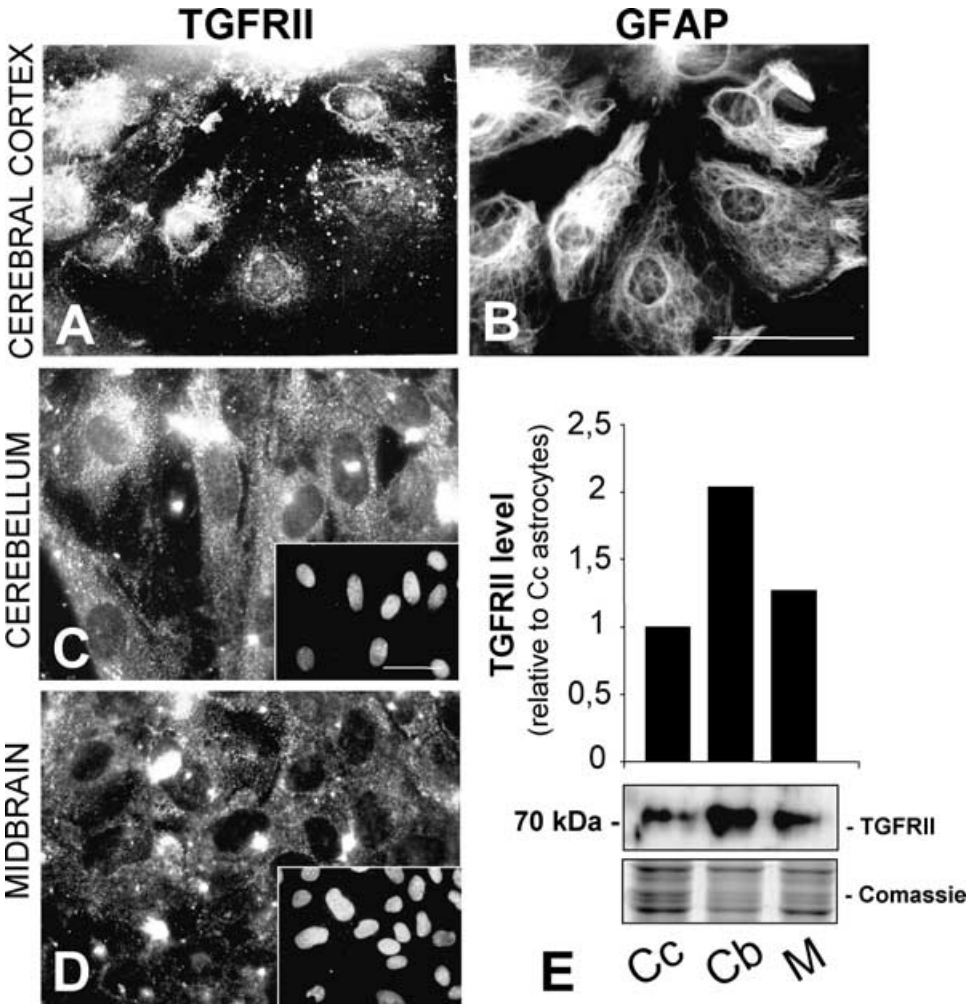

FIG. 4. Immunolocalization and western blot analyses of transforming growth factor-beta receptor (TGFR) II in primary cultured astrocytes. Astrocytes derived from newborn (A) cerebral cortex (Cc), (C) cerebellum (Cb) and (D) midbrain (M) transgenic mice were cultured for 10 days and immunostained for 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.

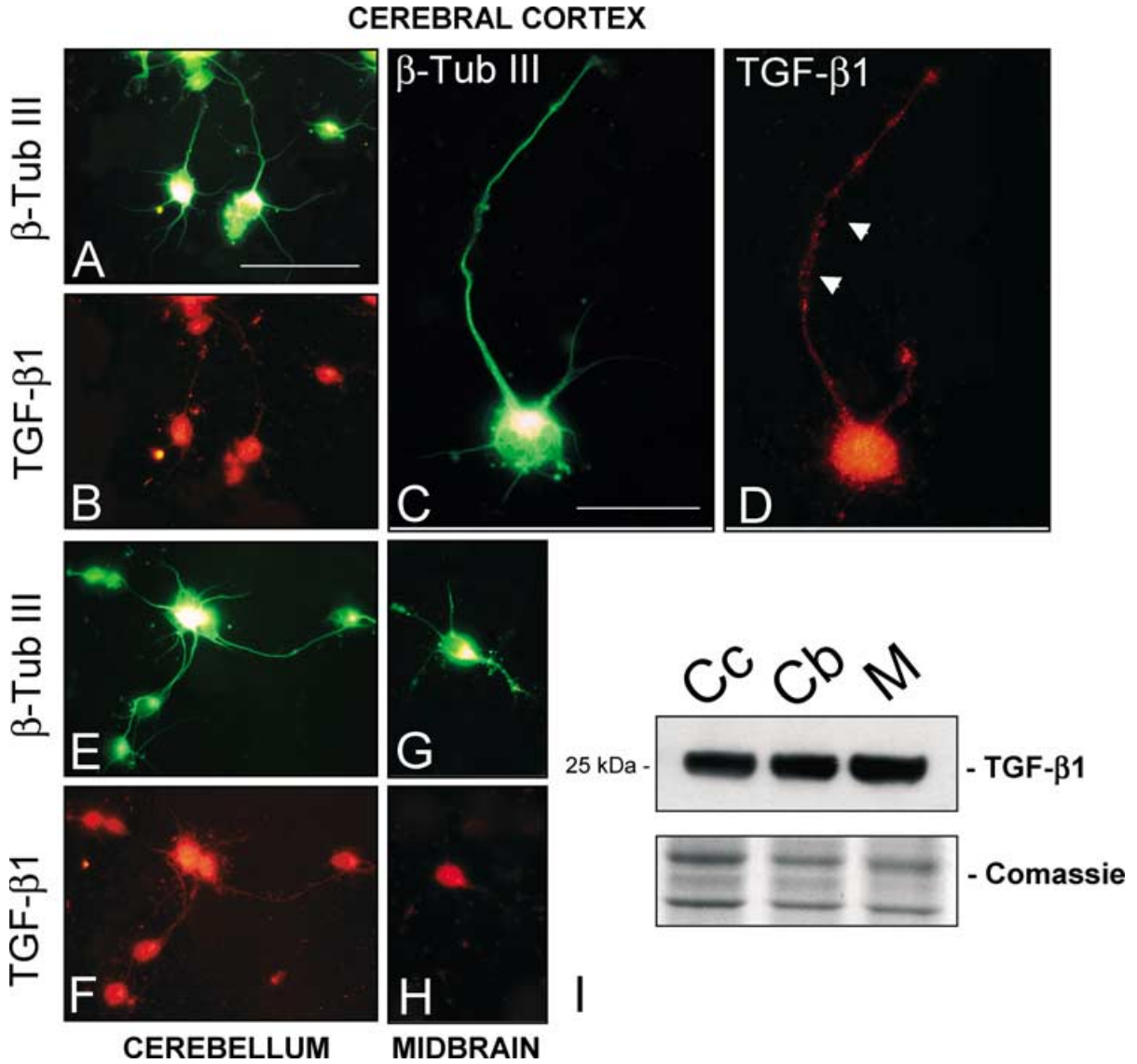

FIG. 5. Identification of transforming growth factor-beta (TGF-β) in primary cultured neurons. (A–H) Neurons derived from 14-day embryonic (E14) cerebral cortex (Cc) (A–D), newborn cerebellum (Cb) (E and F) and E14 midbrain (M) (G and H) Swiss mice were cultured for 24 h and immunostained for TGF-βs 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

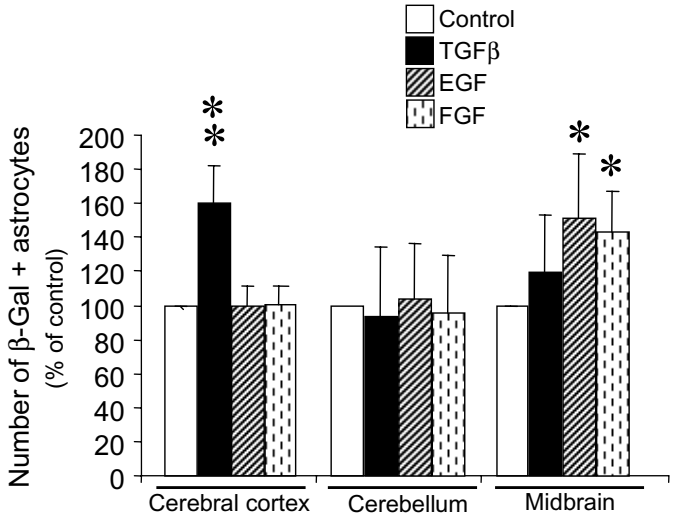

FIG. 6. Effect of transforming growth factor-beta 1 (TGF-β1), basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) on glial fibrillary acidic protein gene promoter-directed expression of *lacZ* of different brain regions. Cortical, cerebellar and midbrain astrocytes derived from newborn 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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3.2 Ativação do promotor do gene de GFAP por glutamato é mediado por TGF-β1 e envolve as vias de sinalização de MAPK e PI3K

Demonstramos recentemente que neurônios corticais induzem a ativação do gene de GFAP em astrócitos de córtex cerebral através da secreção de TGF-β1. Dados prévios demonstraram que glutamato, principal neurotrasmissor excitatório do SNC, induz a síntese e secreção de membros da família TGF-β por astrócitos. Recentemente, evidências demonstrando o envolvimento dos astrócitos na transmissão sinpatica vêm crescendo substancialmente. Experimentos *in vitro* e *in vivo* demonstraram que TGF-β1, liberado por astrócitos, possui um papel importante na proteção dos neurônios contra a toxicidade do glutamato. Neste trabalho investigamos o envolvimento de elementos sinápticos e neurotransmissores na diferenciação astrocitária *in vitro* mediada por TGF-β1. Para isso, utilizamos um camundongo transgênico contendo o gene codificante da proteína bacteriana β-Galactosidase sob regulação do promotor de GFAP. A adição de sinaptossomas, meio condicionado sinaptossomal ou glutamato aumenta consideravelmente o número de células β-Gal positivas. Esse efeito é revertido pela adição de anticorpo neutralizante contra TGF-β1 ou antagonista do receptor metabotrópico de glutamato, sugerindo que a ativação do gene de GFAP em resposta a glutamato/neurônio seja mediada por TGF-β1. Ensaios utilizando inibidores específicos das vias de sinalização de MAPK e PI3K implicaram ambas as vias na diferenciação astrocitária induzida por neurônios. Estes dados indicam que o glutamato ativa o promotor do gene de GFAP através do receptor astrocitário mGlu2/3 e que este evento é mediado pela secreção de TGF-β1 envolvendo as vias de MAPK e PI3K. Nosso trabalho revela um papel importante para interação neurônio-glia no

desenvolvimento astrocitário e demonstra fortemente o envolvimento de neurotransmissores e fatores de crescimento neste evento.

TITLE: GLUTAMATE ACTIVATES GFAP GENE PROMOTER THROUGH TGF-β1 SMAD-DEPENDENT AND -INDEPENDENT PATHWAYS

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ABSTRACT (330 words)

Glial cells are currently viewed as active partners of neurons on synapse formation. The close proximity of astrocytes in the synaptic cleft suggests that these cells might be potential targets for neuronal-released molecules although this issue has been less addressed. In the present work, we evaluated the role of the excitatory neurotransmitter, glutamate, in astrocyte differentiation. We recently demonstrated that cortical neurons activate the gene promoter of the astrocyte maturation marker, GFAP (glial fibrillary acidic protein) of cerebral cortex astrocytes by inducing TGF-β1 (transforming growth factor beta 1) secretion *in vitro*. To access the effect of glutamate on GFAP gene, we used transgenic mice bearing the β-Galactosidase (β-Gal) reporter gene under the regulation of the GFAP gene promoter. We report that 100μM glutamate activates the GFAP gene promoter of astrocytes from cerebral cortex and midbrain revealed by a significant increase in the number of β-Gal positive astrocytes. Addition of neutralizing antibody against TGF-β fully prevented induction of GFAP gene in response to neurons or glutamate indicating that this event is mediated by TGF-β1. Further, induction of GFAP gene in response to glutamate was followed by nuclear translocation of the Smad transcription factor, a hallmark of TGF-β1 pathway activation. Addition of MCPG, an antagonist of the metabotropic glutamate receptor, inhibited neuronal effect suggesting that neuronal activation of GFAP gene promoter involves glutamate metabotropic receptors. MAPK (PD98059) and PI3K (LY294002) inhibitors fully prevented activation of GFAP gene promoter by all treatments. Surprisingly, these inhibitors also abrogated TGF-β1 direct action on GFAP gene although they did not inhibit Smad-2 phosphorylation, suggesting that TGF-β1-induced GFAP gene activation is triggered by cooperation between the canonic and non-canonic TGF-pathways. Together, our results suggest that glial mGlu2/3R activation by neurons induces TGF-β1 secretion, leading to GFAP gene activation and astrocyte differentiation and involves Smad and MAPK/PI3K

pathways. Our work provides evidence that synaptically associated astrocytes are target of neuronal activity and might shed light into the role of glial cells into neurological disorders associated with glutamate neurotoxicity.

Key Words: neuron-glia interaction, GFAP gene, CNS development, TGF-β1, astrocyte, glutamate, synapse.

Running title: Glutamate-TGF-β1 mediated effects on GFAP gene.

INTRODUCTION (676 words)

Glial cells have traditionally been considered supportive, satellite cells of the nervous system. Works over the past decade however have revealed that neuron-glia interactions play key roles in several events of brain development, such as neuronal proliferation and differentiation (Lim and Alvarez-Buylla 1999; Song *et al.* 2002; Lie *et al.* 2005), neuronal migration (Hatten 2002); axonal guidance (Garcia-Abreu *et al.* 1995; Tessier-Lavigne and Goodman 1996; Martinez and Gomes 2002; 2005); synapse formation (Cristopherson *et al.* 2005) and glial maturation (Gomes *et al.* 1999a; de Sampaio e Spohr *et al.* 2002; Schmid *et al.* 2003; Sousa *et al.* 2004; Barnabé-Heider *et al.* 2005; Patten *et al.* 2006; Stipurksy and Gomes 2007).

Recently, astrocytes have been viewed as active partners of the presynaptic and postsynaptic terminals in the elaboration of final synaptic structures (Araque *et al.* 1999). Astrocytes derived signals induce the formation of new synapses and modulate the number and strength of synapses in several systems in rodents and humans (Pfrieger and Barres 1997; Mauch *et al.* 2001; Nagler *et al.* 2001; Ullian *et al.* 2001; 2004; Christopherson *et al.* 2005; Elmariah *et al.* 2005; Goritz *et al.* 2005; Johnson et al., 2007). The intimate relationship between astrocytes and synaptic terminals *in vivo* (Peters *et al.* 1991; Ventura and Harris 1999) suggests that besides affecting synapse formation, astrocytes are also potential targets for neuronal-derived molecules such as neurotransmitters. This question, however, has been less addressed. In the present work, we analyzed the role of glutamate, the major central nervous system (CNS) excitatory neurotransmitter, in astrocyte differentiation.

Astrocytes express functional ionotropic and metabotropic glutamate receptors (mGluRs) (Bruno *et al.* 2001), and regulate glutamate uptake and inactivation (Rosenberg *et al.* 1992). The mGluRs form a family of at least eight subtypes that regulate a variety of intracellular signaling systems via activation of GTP binding proteins (Nakanishi 1994). They have been classified into three groups on the basis of their sequence homology,

pharmacological profile and transduction pathways (Pin and Duvoisin 1995; Schoepp *et al.* 1999). Group-I includes mGlu1 and -5 receptors, which are coupled to polyphosphoinositide hydrolysis; group-II (mGlu2 and -3) and group-III (mGlu4, -6, -7, and -8) receptors are negatively coupled to adenylyl cyclase activity in heterologous expression systems (Bruno *et al.* 2001; Flor *et al.* 2002). Activation of group II metabotropic glutamate receptor in astrocytes is associated with a neuroprotective action provided by synthesis and secretion of TGF-β1 (Glowinski *et al.* 1994; Ciccarelli *et al.* 1997; Bruno *et al.* 1998; Corti *et al.* 2007).

The TGF-β superfamily is constituted by multifunctional polypeptide members, which perform critical functions in tissue repair and development (Shi and Massague, 2003). TGF-β1 is involved in several CNS pathologies, where it has been implicated in organization of the glial scar in response to injury and several neurodegenerative diseases (Moon and Fawcett 2001; Zhu *et al.* 2002; Vivien and Ali 2006). Emerging evidences have suggested a key role for this factor in several CNS developmental processes such as cell adhesion, neuronal migration, glial differentiation and synaptogenesis (Bottner *et al.* 2000; de Sampaio e Spohr *et al.* 2002; Brionne *et al.* 2003; Miller *et al.* 2003; Sanyal et al., 2004; Sousa *et al.* 2004; Gomes *et al.* 2005; Massague and Gomis 2006; Stipursky and Gomes 2007).

By using transgenic mice bearing 2 kbp of the 5′ flanking region of the GFAP gene linked to the β-Galactosidase (β-Gal) reporter gene, we previously demonstrated that cortical neurons activated the GFAP gene promoter and induced astrocyte differentiation *in vitro* (Gomes *et al.* 1999). This event was mediated by synthesis and secretion of TGF-β1 by astrocytes in response to neurons (de Sampaio e Spohr *et al.* 2002). The identity of the neuronal-derived molecule responsible for induction of TGF-β1 secretion remained unidentified. In the present paper, we investigated the role of glutamate in GFAP gene promoter activation and astrocyte differentiation *in vitro*. We now report that activation of glial group-II mGlu receptors enhances the synthesis of TGF-β1 and induces GFAP gene

promoter through the activation of three signaling pathways: the mitogen-activated protein (MAP) kinase, the phosphatidylinositol (PI)-3-K and the Smad pathways.

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-Galactosidase (β-Gal) reporter gene (lacZ) as previously described (Sousa *et al.* 2004). Cultures were prepared from cerebral cortex (Cc), midbrain (M) and cerebellum (Cb) derived from newborn transgenic mice. All animals were kept under standard laboratory conditions according to NIH guidelines. Briefly, 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), previously coated with polyornithine (1.5 µg/mL, molecular weight 41,000; Sigma Chemical Co., St Louis, MO), in Dulbecco's modified Eagle's medium/F12 (DMEM-F12) medium supplemented with 10% fetal calf serum (Invitrogen, Carlsbad, CA). 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 (2 x 10^6 cells/well) freshly dissociated from 14-days (E14) embryonic Swiss mice were plated onto the transgenic glial monolayer carpets and cocultures kept for 24 h according to Gomes *et al.* 1999b.

Synaptosomal preparations

Synaptosomes were prepared on a discontinuous Percoll gradient according to the method of Dunkley *et al.* (1988). In brief, cerebral structures pooled from four mice were weighed and homogenized in 0.32 M sucrose, 1 mM EDTA, and 0.25 mM DTT, pH 7.4 (SED), using a glass homogenizer. The preparation was centrifuged at 1,000 *g* for 10 min. The resultant

pellet was discarded, and the supernatant was gently transferred to a four-step gradient of 3%, 7%, 15%, and 23% Percoll in SED solution. Tubes were centrifuged at 30,000 *g* for 13 min. The synaptosome fractions were collected from the interface of the 15% and 23% Percoll steps. The fraction was washed twice by centrifugation at 30,000 *g* for 18 min and resuspended in Dulbecco's modified Eagle's medium/F12 (DMEM-F12). All synaptosomes were used on the same day of preparation. Protein concentration was measured by the method of Bradford *et al.* (1988). Synaptosomes conditioned medium was prepared in DMEM-F12 serum-free for 16 h at 37°C in a humidified 5% CO_2, 95% air chamber. The medium was then recovered, centrifuged at 1500g for 5 min to get rid of eventual debris and used immediately.

Detection of β-Gal activity and quantitative analysis of β-Gal 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 (X-Gal, U.S. Biochemical Corp., Cleveland, OH) 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 analyzed for β-Gal-positive astrocytes under a Nikon Eclipse TE300 microscope. At least five fields were counted per well. Approximately 5 x 10^3 cells were counted per experiment. The experiments were done in triplicate and each result represents the mean of three independent experiments. Statistical analyses were performed by ANOVA.

Conditioned medium preparation

Neuronal conditioned medium (CM) was prepared as previously described (Gomes *et al.* 1999b; de Sampaio e Spohr *et al.* 2002). Briefly, neurons derived from E14 mice in DMEM-

F12 serum-free for 24 h at 37°C in a humidified 5% CO_2, 95% air chamber. The culture medium was then recovered, centrifuged at 1500g 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. CM from cocultures was prepared by cultivating 14 days embryonic (E14) neurons with newborn astrocytes for 24 h. After recovering of coculture CM, the same procedure was followed as described for neuronal CM. Use of coculture or neuronal CM yielded similar results.

Treatment of astrocyte monolayers with conditioned medium, TGF-β1 and glutamate

Astrocyte monolayers derived from newborn transgenic mice cortices were prepared as previously described. After reaching the confluence, the cultures were incubated for an additional day with serum-free medium and then incubated in the following conditions: a) in the presence of TGF-β1 (10 ng/mL), (R&D Systems, Buckinghamshire, UK), or b) glutamate (Amersham, UK) (10 μM, 100 μM and 1 mM), or c) neuronal conditioned medium. Cultures were kept for an additional 24 h at 37°C in a humidified 5% CO_2, 95% air chamber and then stained with X-Gal as described.

Inhibition and antibody blocking assays

Astrocyte monolayers were concomitantly incubated in the presence of TGF-β1 (10 ng/mL), glutamate (100 μM), neurons, or CM and specific signaling pathway inhibitors for 24 h, accordingly to the previously described protocol (de Sampaio e Sporh *et al.* 2002). The

following inhibitors were used: PD98059, MAPK-specific inhibitor (50 μM); LY294002, PI3K-specific inhibitor (5 μM); *(S)*-Methyl-4-carboxyphenylglycine, group I/II metabotropic glutamate receptor antagonist (MCPG-500 μM). All inhibitors were purchased from Calbiochem (La Jolla, CA) and diluted in dimethylsulfoxide (DMSO, Sigma Chemical Co., St Louis, MO). For neutralization assays, astrocyte monolayers were cultivated simultaneously in the presence of glutamate (100 μM) or neurons and 10 μg/mL of a neutralizing antibody against transforming growth factor-β1 (TGF-β1) (chicken anti-human TGF-β1 antibody; R&D Systems) or a control unrelated immunoglobulin.

Immunocytochemistry

Immunocytochemistry was performed as previously described (Sousa *et al.* 2004). Briefly, 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) in PBS (blocking solution) for 1h and incubated overnight at room temperature with the anti-rabbit GFAP antiserum (Dako, Carpinteria, CA; 1/500) diluted in blocking solution. 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. After primary antibody incubation, cells were extensively washed with PBS/10% normal goat serum and incubated with secondary antibody conjugated with horseradish peroxidase (goat anti-rabbit; Sigma Chemical Co., St Louis, MO; 1/200) for 1 h at room temperature. Peroxidase activity was revealed with 3,3'-diaminobenzidine (DAB peroxidase substrate kit; Vector Laboratories, Inc.). Analysis of Smad nuclear translocation was performed by immunofluorescence as previously described by Stipursky and Gomes 2007. Primary antibody was mouse anti-Smad 4 (Santa Cruz Biotechnology; Santa Cruz, CA; 1:5) and secondary antibody was Cy3 conjugated-goat anti-mouse IgG, (Molecular Probes,

1:300). Nuclei were counterstained with DAPI (4',6-Diamidino-2-phenyindole, dilactate; (Sigma Chemical Co., St Louis, MO). After immunostaining, cells were visualized using a TE 300 Nikon microscope. 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 blotting analysis

Biochemical characterization of proteins was done according to Stipursky and Gomes 2007. Astrocyte cultures were lysed in 2X loading buffer (100 mM Tris-Cl [pH6.8]; 4% of SDS; 0.2% of bromophenol blue; 20% of glycerol; 200 mM of dithiotreitol (DTT)) and then boiled for 5 minutes before loading in the gel. Approximately 20 µg of protein per lane were submitted to eletrophoresis in a 10% SDS-PAGE mini gel and electrically transferred onto a Hybond-ECL transfer membrane nitrocellulose (Amersham Pharmacia Biotech, Miami, FL) for 1 h. Membranes were blocked in PBS-milk 5% and primary antibodies were added for 1 h at room temperature followed by peroxidase-conjugated secondary antibody incubation for 1 h at room temperature. Proteins were visualized using the enhancing chemiluminescence's detection system (SuperSignal West Pico Chemiluminescent Substrate – Pierce). The following primary antibodies were used: rabbit anti-Phospho-Smad2 (Cell Signaling) and mouse anti-alpha-Tubulin (Sigma; 1:1000). The following secondary peroxidase-conjugated antibodies were used: goat anti-rabbit IgG and goat anti-mouse IgG (Amersham Pharmacia Biotech; 1:3000).

RESULTS

Glutamate activates GFAP gene promoter of transgenic astrocytes

We previously demonstrated that neurons activated GFAP gene promoter from cortical astrocytes by secreting soluble molecules (Gomes *et al.* 1999). In order to evaluate the involvement of neuronal-derived neurotransmitters in this event we employed synaptosome assays, which have been considered a useful tool to study neurotransmitter molecules (Dunkley *et al.* 1988). Cortical astrocytes derived from transgenic mice bearing de *lacZ* reporter gene under the control of the GFAP gene promoter were cultivated in the presence of synaptosome preparations or conditioned medium derived from them for 24 h (**Fig. 1A**). In all situations, there was an increase in the number of β-Gal positive astrocytes similarly to the one observed when astrocytes were cultivated with cortical neurons themselves. Since synaptosome preparations are known to be mainly constituted by neurotransmitter vesicles we sought to analyze the involvement of glutamate, the main excitatory neurotransmitter secreted by central neurons.

Treatment of astrocyte monolayers with increasing concentrations of glutamate (10 μM, 100 μM and 1 mM) increased the number of β-Gal positive astrocytes by 150% (**Fig. 1B,C**) suggesting that this neurotransmitter might be a positive modulator of GFAP gene and thus of astrocyte differentiation in the cerebral cortex.

Activation of GFAP gene by glutamate is mediated by TGF-β1

Activation of group II metabotropic glutamate receptor (mGlu2/3) induces synthesis and secretion of TGF-β1 in astrocyte (Glowinski *et al.* 1994; Ciccarelli *et al.* 1997; Bruno *et al.* 1998; Corti *et al.* 2007). We previously demonstrated that induction of the GFAP gene promoter by cortical neurons is mediated by TGF-β1 secreted by astrocytes in response to

neuronal soluble factors. Here, we sought to analyze the role of TGF-β1 as mediator of glutamate effect on GFAP gene promoter.

We thus, cultivated cerebral cortex astrocytes derived from newborn transgenic mice alone or with 100 μM of glutamate, cortical neurons or 10 ng/ml of TGF-β1, in the presence or absence of 10 ng/ml of TGF-β1 neutralizing antibody (anti-TGF-β1) or 500 μM of mGlu2/3 receptor antagonist (MCPG). After 24 h, β-Gal positive cells were analyzed. Glutamate, neurons or TGF-β1 greatly increased the number of β-Gal positive astrocytes (**Fig. 2A**). Addition of neutralizing antibody against TGF-β1 fully prevented this increase (**Fig. 2A**), whereas addition of a control unrelated immunoglobulin had no effect (data not shown). Likewise, addition of the glutamate receptor antagonist, MCPG, fully prevented glutamate and neurons effects on GFAP gene, whereas it had no effect on TGF-β1 activation of the GFAP gene promoter (**Fig. 2A**).

To fully demonstrate that TGF-β1 is a mediator of glutamate actions on GFAP gene we analyzed Smad 4 nuclear translocation, a hallmark of TGF-β1 pathway activation, in response to glutamate. As observed in **Fig. 2**, control astrocytes present Smad 4 predominantly in the cytoplasm **(Fig. 2B,B')**. Treatment of these cells with TGF-β1 **(Fig. 2C,C')** or glutamate **(Fig. 2D,D')** for 24 h induced nuclear translocation of this transcription factor demonstrating that glutamate activates the TGF-β1/Smad pathway in cortical astrocytes. These data together suggest a key role for TGF-β1 as a downstream mediator of glutamate and neurons effect on GFAP gene promoter.

GFAP genes from distinct brain regions are differently modulated by glutamate

We previously demonstrated that GFAP gene promoter of astrocytes from different brain regions are distinctly modulated by growth factors and cell interactions (Sousa *et al.* 2004). Here, we investigated glutamate influence on GFAP gene from astrocytes derived from different structures. To assess this question, cerebral cortex, midbrain and cerebellum astrocytes from newborn transgenic mice were cultured alone and in the presence of 100 μM of glutamate or 10 ng/ml of TGF-β1 for 24 h. Whereas glutamate and TGF-β1 greatly increased the number of β-Gal positive astrocytes in cerebral cortex monolayers, neither of these molecules have effect on cerebellar astrocytes (**Fig. 3**). In midbrain astrocyte monolayers, addition of TGF-β1 had no effect on the number of β-Gal positive cells as previously shown by us (Sousa *et al.* 2004). However, glutamate increased by 75% the number of β-Gal midbrain positive astrocytes. Thus, taken together, these results provide evidence that GFAP gene is differently modulated by glutamate in distinct brain regions.

Activation of the GFAP gene promoter involves MAPK/PI3K pathways

Activation of metabotropic glutamate receptor has been associated with induction of MAPK/PI3K pathways in cortical astrocytes leading to TGF-β1 secretion (D'Onofrio *et al.* 2001). In order to investigate the signaling pathways involved in GFAP gene activation, we employed MAP and PI3 kinase inhibitors. Cerebral cortex astrocytes derived from newborn transgenic mice were cultivated alone or with 100 μM of glutamate, neurons, conditioned medium (CM) from cocultures or 10 ng/ml of TGF-β1, in the presence or absence of 50 μM of PD98059 (MAPK inhibitor), 5 μM of LY294002 (PI3K inhibitor) or with both. As showed before, neurons, CM, glutamate and TGF-β1 greatly increased the number of β-Gal astrocyte **(Fig. 1,2)**. Addition of MAPK or/and PI3K inhibitors fully prevented such increase in all conditions studied **(Fig. 4,5)**, consistent with previous data that glutamate induction of

synthesis and secretion of TGF-β1 involves MAP/PI3K pathways (D'Onofrio *et al.* 2001). Surprisingly, addition of MAPK and PI3K inhibitors also abrogated TGF-β1 direct action on GFAP gene promoter. Since MAP/PI3K pathways have been reported to modulate the Smad pathway we sought to investigate if the observed effect was due to secondary blockage of the canonic pathway by the inhibitors (Derynck and Zhang 2003; Javelaud and Mauviel 2005; Dziembowska *et al.* 2007). We thus analyzed the levels of Smad 2 phosphorylation by Western blotting assays. Treatment of astrocytes with 10 ng/ml of TGF-β1 for 30 min increased the levels of P-Smad 2 2,8 times. Such increase was sustained even in the presence of 50 μM of PD98059 (MAPK inhibitor) and 5 μM of LY294002 (PI3K inhibitor) suggesting that MAPK and PI3K do not modulate Smad 2 phospholylation **(Fig. 6)**. Addition of PD98059 and LY294002 to astrocytes cultures in the absence of TGF-β1 had no effect on the levels of P-Smad 2.

Together, these data show that glutamate/TGF-β1 induction of the GFAP gene promoter involves either the Smad-canonic pathway and the non-canonic MAPK/PI3K pathways.

DISCUSSION (2394 words)

In the present work, we provide the first evidence that the excitatory neurotransmitter glutamate activates the GFAP gene promoter of astrocytes through induction of TGF-β1 signaling pathway. This event was CNS region-specific since astrocytes derived from cerebellum, midbrain and cerebral cortex distinctly respond to glutamate challenge. Our work provides strong evidence that synaptically associated astrocytes besides controlling synaptic function are also target of neuronal activity. Further, our data provide new insights into the role of glial cells in neurodegenerative disorders associated with glutamate neurotoxicity.

TGF-β as mediator of glutamate action in the nervous system

Metabotropic glutamate (mGlu) receptors form a family of eight subtypes (mGlu1-8) subdivided into three groups (I-III) on the basis of their amino acids sequence identity, pharmacological profiles and signal transduction pathways (Flor *et al.* 2002). The individual mGluR-subtypes show a wide distribution through the mammalian nervous system. Subtypes of all three groups are highly expressed in neocortical layers, hippocampus, basal ganglia, thalamus/hypothalamus, cerebellum and spinal cord. Glial and neuronal cells highly express different isoforms of mGluR Activation of group-II mGlu receptors in astrocytes leads to increased formation and release of TGF-β, which is associated with neuroprotective action either *in vitro* and *in vivo* (Bruno *et al.* 1998; Corti *et al.* 2007). We previously demonstrated that neurons activate GFAP gene promoter and induce astrocyte differentiation *in vitro* by inducing synthesis and secretion of TGF-β1 by astrocytes (de Sampaio e Spohr *et al.* 2002; Sousa *et al.* 2004). In the present work, we extend this observation by demonstrating that GFAP expression and astrocyte differentiation induced by cortical neurons is triggered by activation of mGluR in astrocytes, which enhances TGF-β1 synthesis.

Increasing evidence accumulated showing that perisynaptically glia augments synaptogenesis and synaptic transmission (Slezac and Prifeger 2003; Christopherson *et al.* 2005; Johnson *et al.* 2007). Conversely, the possibility of astrocytes being target for neuronal signaling has been less investigated. Previous data demonstrated that the inhibitory neurotransmitter GABA (gamma amino-butyric acid) modulate astrocyte morphology and GFAP expression *in vitro* and *in vivo* (Matsutani and Yamamoto 1997; Mong *et al.* 2002; Runquist and Alonso 2003). Our work is the first to report that the excitatory neurotransmitter glutamate activates the GFAP gene promoter and triggers astrocyte differentiation.

Most of the astrocytes from the cerebral cortex arise from a population of specialized progenitor cells, called radial glia cells (RG), which act as a scaffold for newborn migrating neurons (Rakic 1971). After neurogenesis and neuronal migration are completed, most of RG cells transform into astrocytes (Culican *et al.* 1990; Schmechel and Rakic 1979; de Azevedo *et al.* 2003). The transition from RG phenotype to astrocytes in the rodent CNS is characterized by replacement of RG-markers such as the astrocyte-specific glutamate transporter (GLAST), the brain lipid binding protein (BLBP) and the intermediate filament proteins nestin and vimentin by the astrocyte differentiation marker, GFAP (Dahl 1981; Pixley and De Vellis 1984).

An interesting cross talk between GFAP/astrocyte differentiation and glutamate signaling has been recently proposed. Expression of GFAP was shown to be essential to anchor the glutamate transporter GLAST in the astrocyte plasma membrane thus enhancing GLAST-mediated transport (Sullivan *et al.* 2007). This work, together with ours, suggests that changes in GFAP gene expression and glutamate homeostasis might mutually influence each other. This hypothesis is supported by the observation that GFAP knockout mice exhibit reduced glutamate clearance (Hughes *et al.* 2004). This cross talk might be a key step to ensure that astrocyte differentiation temporally fit synapse establishment period.

Our data clearly show that TGF-β1 is a mediator of glutamate-GFAP gene activation. Addition of neutralizing antibody against TGF-β1 completely inhibited neuron and/or glutamate effect on GFAP gene.

Transforming growth factor betas (TGF-βs) are known as multifunctional growth factors, which participate in the regulation of key events of development, disease and tissue repair (Shi and Massague 2003). In CNS, TGF-β1 has been widely recognized as an injury-related cytokine, specially associated with astrocyte-scar formation in response to brain injury (Pratt and McPherson 1997). Recently, emerging data revealed that TGF-β signaling is a crucial regulator of several steps of CNS development including glial differentiation, neuronal migration and synapse formation (de Sampaio e Spohr *et al.* 2002; Brionne *et al.* 2003; Packard *et al.* 2003; Siegenthaler and Miller 2004; Sousa *et al.* 2004; 2006; Gomes *et al.* 2005; Stipursky and Gomes 2007).

The expression of mGlu receptor subtypes is differently regulated during nervous system development, showing distinct regional and temporal profiles. Whereas mGlu2 mRNA expression is low at birth and increases during postnatal development, mGlu3 is highly expressed at birth and decreases during maturation to adult levels (Catania *et al.* 1994; Luján *et al.* 2005). Although glutamate receptors have not been undoubtedly identified in RG cells, the fact that glutamate antagonists impair their development and neuronal migration, together with well-described expression of glutamate receptors isoforms in the embryonic brain greatly suggest that these cells might be target for glutamate action (Nacher *et al.* 2003; Luján *et al.* 2005; Suzuki *et al.* 2006). Recently, activation of mGlu3, but not mGlu2, was related to synthesis and secretion of TGF-β1 by astrocytes (Corti *et al.* 2007). We recently demonstrated that TGF-β1/smad signaling is involved in RG-astrocyte commitment (Stipursky and Gomes 2007). We showed that neurons activate the GFAP gene promoter in RG cells by activating the TGF-β1 pathway (Stipursky and Gomes 2007). Increased expression of mGlu3 in the late

embryonic, gliogenic period, together with the fact that RG expresses TGFR (Miller *et al.* 2003; Sousa *et al.* 2006; Stipurksy and Gomes 2007) opens the question that glutamate/TGF-β homeostase might be key elements in RG-astrocyte transition during cerebral cortex development.

TGF-β signaling is mediated mainly by 2 serine threonine kinase receptors, TGFRI and TGFRII, which activate Smad 2/3 and Smad 4 transcription factors. Phosphorylation and activation of these proteins is followed by formation of Smads 2/3-4 complex, which translocates to the nucleus regulating transcriptional responses to TGF-β (Shi and Massague 2003). In the present work, we demonstrated that glutamate priming of cerebral cortical astrocytes induced Smad nuclear translocation, a hallmark of TGF-β1 pathway activation. Further, it also involves the MAP and PI3K pathways. Addition of the specific inhibitor of MEK1/2 kinase, PD98059, or the PI3K inhibitor, LY294002, fully prevented activation of GFAP gene promoter in response to glutamate. This is in close agreement with previous data that astrocyte TGF-β1 synthesis in response to activation of glial metabotropic receptors is mediated by MAP and PI3 kinases (D'Onofrio *et al.* 2001; Corti *et al.* 2007). Surprisingly, these inhibitors also abrogated TGF-β1 direct action on GFAP gene promoter suggesting that besides the Smad pathway, non-canonic pathways are also implicated in TGF-β1-induced GFAP gene activation.

Our data are supported by recent evidence that TGF receptors might act through multiple intracellular pathways such as protein kinase A, JNK and p38 kinases, and MAP and PI3 kinases, besides the classical Smad-mediated pathway (Zhu *et al.* 2002; 2004). Pharmacological inhibitors of PI3K were found to strongly interfere with MAPK pathways in several systems (Carballada *et al.* 2001; Yart *et al.* 2001; 2002; Kim *et al.* 2002). In agreement with these data, addition of the PI3K pathway inhibitor, LY294002, completely abolished TGF-β effect on GFAP gene activation. A functional cross-talking between PI3K

and MAP kinase pathways has already been demonstrated (Fang *et al.* 1999; Carballada *et al.* 2001; Kim *et al.* 2002; Yart *et al.* 2002; Yu *et al.* 2002). As pharmacological inhibition of MAPK or PI3K completely abrogated the effect of TGF-β1 in GFAP gene promoter, it is conceivable that transactivation of TGFR leads to 1) induction of PI3K followed by MAPK pathway activation (either PI3K upstream or downstream) or 2) alternatively, TGFR may activate two separate cascades, a PI3K dependent pathway and the classical MAPK pathway (**Fig. 7**). The fact that administration of LY294002 and PD98059 alone is sufficient to completely inhibit TGF-β effect and concomitant addition does not yield additive inhibition (data not shown) calls in favor of converging rather than independent pathways.

We previously demonstrated that activation of GFAP gene promoter by TGF-β1 in RG-like cells and astrocytes is triggered by the Smad-dependent pathway (Stipursky and Gomes 2007). Although this pathway is the central mediator of TGF-β signaling, TGF responses have been showed to be more complex than previously thought, including interactions of Smad signaling within a complex network of other intracellular signaling pathways that largely contribute to modify the initial rather linear TGF-β/Smad pathway (Javelaud and Mauviel 2005). An alternative possibility would be that TGF-β-induced GFAP gene activation, as reported here, involves interaction between Smad-dependent and -independent pathways (Derynck and Zhang 2003; Javelaud and Mauviel 2005). Such cross talk has been observed for example, in human glioblastoma cells, in which Smad 2 phosphorylation is decreased by p38/MAPK inhibitor (Dziembowska *et al.* 2007). Our data contrast with those since MAPK and PI3K inhibitors did not interfere with Smad 2 phosphorylation. The fact that inhibition of non-canonical pathway completely inhibited TGF effect on GFAP suggests that the Smad canonic pathway is not sufficient to assure GFAP gene activation in astrocytes in response to TGF-β1. Our data shows that TGF-β1-activation of GFAP gene involves 2 separate cascades: the canonic and non-canonic pathways.

Elucidation of the molecular mechanisms implicating PI3/MAP kinase and Smad pathways in GFAP gene activation and astrocyte differentiation await further experiments.

Astrocyte heterogeneity and glutamate signaling

We reported here that astrocytes derived from different brain regions present distinct responses to the glutamatergic signaling: whereas either glutamate and TGF activated the GFAP gene promoter from cerebral cortex glia, these molecules had no effect on cerebellar astrocytes and only glutamate exerted an effect on midbrain astrocytes. Many evidence now support the concept that astroglia is formed by a heterogeneous population of cells that markedly vary in their responsiveness to several agents such as hormone, growth factors, neurotransmitters, pattern of cell interaction and even progenitor potentials (Dennis-Donini *et al.* 1984; Cholewinski and Wilking 1988; Garcia-Abreu *et al.* 1995; Lima *et al.* 1998; Perego *et al.* 2000; Gomes *et al.* 2001a;b; de Sampaio e Spohr *et al.* 2002; Schlüter *et al.* 2002; Hall *et al.* 2003; Matthias *et al.* 2003; Reuss *et al.* 2003; Sousa *et al.* 2004).

In situ hybridization studies suggest that glial cells from many different brain regions express glutamate receptors (Porter and Mccarthy 1997). However, such expression exhibits regional and intraregional heterogeneity, which might account for the diversity of glutamatergic signaling found during nervous system development and in response to injury. The fact that cerebellar astrocytes do not respond to glutamate activating *lacZ*-GFAP gene calls in favor of TGF-β as a mediator of glutamate effects on GFAP gene since TGF-β1 has been already shown not to activate GFAP gene from these cells (Sousa *et al.* 2004). It is intriguing, however, that although TGF-β1 does not activate the *lacZ*-GFAP gene from midbrain astrocytes (Sousa *et al.* 2004; data from the present paper), addition of glutamate activated the GFAP gene from midbrain astrocytes. A possibility is that activation of group-II mGluRs might be associated with modulation of other factors, rather than TGF-β in midbrain,

as suggested for other systems (Ciccarelli *et al.* 1999). This is supported by evidences that although pharmacological activation of mGlu2/3 receptors in cultured astrocytes induced de novo synthesis of TGF-β1, *in vivo*, a similar induction is observed in cerebral cortex and caudate nucleus, but not in the hippocampus (D'Onofrio *et al.* 2001).

Glutamate signaling is a balance between their receptors and transporters, which untimely determines the extracellular concentration of the neurotransmitter. Previous work provided evidence that glutamate signaling are under local CNS region-specific modulation (Schlüler *et al.* 2002; Regan *et al.* 2007). Whereas PACAP, TGF-α and EGF act as potent regulators of the expression of glutamate transporters in striatal glia, these factors completely failed to affect GLT-1 and GLAST protein levels in astroglial cultures from cerebellum, mesencephalon and spinal cord (Schlüler *et al.* 2002). The fact that cerebellar, midbrain and cerebral cortical astrocytes differently activate GFAP gene in response either to glutamate or TGF-β suggest that the link between GFAP gene and glutamatergic signaling considerably differ through out CNS. Our data contribute to the prevailing view that astrocytes are heterogeneous and shows that mechanisms underlying astrocyte differentiation might be more complex than previously thought.

TGF-β/Glutamate cross talk: implications for synapse function and dysfunction

Assembly of synapses requires coordination between pre and pos synaptic neurons and the surround glial cells. Cross talk between these cells is essential for the proper apposition of synaptic components. Astrocytes play a key role in several events of synaptogenesis including modulation of the number and strength of synapses (Allen and Barres 2005). TGF-β signaling has recently been implicated in synapse formation and stabilization in invertebrate models (Sanyal *et al*. 2004). Mutant *Drosophila* flies deficient for the Wit protein, a type II BMP receptor exhibit reduced glutamatergic neuromuscular junctions, with downregulation of the

cell adhesion molecule fascilin II (FasII), mislocalization of the presynaptic protein synaptogamin, associated with impair activation of the BMP/TGF-β signaling (Aberle *et al.* 2002; Marques *et al.* 2002; Eaton and Davis 2005). Although this mechanism has not been demonstrated for vertebrate synapses, modulation of TGF-β1 synthesis by glutamate might be one of the feedback mechanism by which astrocytes modulate synapse strength. This hypothesis is supported by the demonstration that the extracellular matrix protein, trombospondin, a TGF-β1 binding protein mediates astrocyte-induced synaptogenesis in rodents (Christopher *et al.* 2005). The data presented here address as an attractive issue, the investigation of the role of TGF-β1 in development of glutamatergic synapses.

It has been suggested that dysfunctional glial glutamate transporters and receptors are associated with several neurodegenerative diseases such as amyotrophic lateral sclerosis, Alzheimer's disease as well as hypoxic/ischaemic conditions and traumatic brain injuries (Seifert *et al.* 2006). Coincidently, high levels of TGF-β1 are observed in several diseases where glutamate metabolism dysfunctions are described (Tesseur and Wyss-Coray 2006). The cross talk between TGF-β1 and glutamate signaling might offer a new strategy to increase the local production of neurotrophic factors in the CNS. This is highlighted by the fact that mGlu3 is localized on the vascular side of the astrocytes, in proximity of endothelial cells, thus easily reached by drugs present in the blood stream and able to cross the blood brain barrier formation (Flor *et al.* 2002). Understanding the cellular components (astrocytes and neurons) and molecules (TGF-β and neurotransmitters) underlying the synapse functions not only contribute to go deeper into neuro-glia interactions during CNS development but might create new strategies towards clinical neuroprotection trials with mGluR-selective compounds.

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LEGENDS

Figure 1: Glutamate activates GFAP gene promoter of transgenic astrocytes. Cerebral cortex astrocytes derived from newborn transgenic mice were cultured for 24h in the following conditions: alone **(Control; A,B)**; in the presence of increasing concentrations of synaptosome **(Syn 2; Syn 20; Syn 50; A)**, embryonic cortical neurons **(Neuron)**, conditioned medium derived from neurons **(CM Neuron)** or from synaptosome **(CM Syn)**; in the presence of increasing concentrations of glutamate **(10µM; 100µM; 1mM; A-B)** After β-Gal activity detection, the number of β-Gal positive cells was quantified **(A;C)**. Each point represents the average of 3 independent experiments done in triplicate. Addition of glutamate greatly increased the number of β-Gal positive cells. *p<0.001 (in comparison to the control). Scale bar: 100 µm.

Figure 2: Activation of GFAP gene by glutamate is mediated by TGF-β1/Smad pathway. Cerebral cortex astrocytes derived from newborn transgenic mice were cultured alone or in the presence of glutamate (100µM), E14 neurons or TGF-β1 (10ng/mL), in presence or absence of 10µg/mL of neutralizing antibodies against TGF-β1 or 500µM of the mGlu2/3 receptor antagonist, MCPG. After 24h of culture, β-Gal astrocyte number was analyzed **(A)**. Each point represents the average of 3 independent experiments carried out in triplicate *p<0.001 (in comparison to the control). Glutamate, neurons and TGF-β1 dramatically increase β-Gal astrocyte number whereas addition of anti-TGF-β1 or MCPG prevented this effect. Simultaneous addition of MCPG and TGF-β1 did not inhibit TGF-β1 induction of GFAP gene. Immunolabeling for Smad 4 **(B-D)** revealed a cytoplasmic localization in control cultures **(B)** and nuclear distribution in TGF-β1 **(C)** and glutamate-treated **(D)** cultures. **B'**, **C'** and **D'** represent higher magnification of the field. Scale bars: 50 µm.

Figure 3: GFAP genes from distinct brain regions are differently modulated by glutamate. Cerebral cortex, cerebellum and midbrain astrocytes derived from newborn transgenic mice were cultured alone, in the presence of glutamate (100 μM) or TGF-β1 (10ng/mL) for 24h. After β-Gal activity detection, β-Gal-positive astrocytes were quantified. Each point represents the average of three independent experiments done in triplicate.* $p<0.05$; ** $p<0.001$ (in comparison to the control) (mean ± SD). Glutamate and TGF-β1 different by affect distinct subpopulations of astrocytes. Whereas both neuromodulators have effect on cortical astrocytes, neither of them activates cerebellar GFAP gene and only glutamate induces GFAP gene from midbrain.

Figure 4: Activation of the GFAP gene promoter by glutamate or TGF-β1 itself is mediated by MAPK/PI3K pathways. Cerebral cortex astrocytes derived from newborn transgenic mice were cultured alone or in the presence of 100μM glutamate, E14 neurons, conditioned medium (CM), or 10ng/mL TGF-β1 in presence or absence of the MAPK inhibitor, PD98059 (50μM), the PI3K inhibitor, LY294002 (5μM) or with both. After 24h of culture, β-Gal astrocyte number was analyzed. Each point represents the average of 3 independent experiments carried out in triplicate * $p<0.001$ (in comparison to the control). Glutamate, neurons, CM and TGF-β1 increased β-Gal astrocyte number. Addition of MAPK or PI3K inhibitors fully prevented effect of glutamate neurons (or CM) and TGF-β1 on GFAP gene.

Figure 5: GFAP promoter-lacZ expression in vitro. Cerebral cortex astrocytes derived from newborn transgenic mice were cultured alone **(A)**, in the presence of glutamate 100μM **(B,E,H)** or TGF-β1 (10ng/mL) **(C,F,I)**; either in the presence or absence of PD98059 (50μM) + LY294002 (5μM) **(G-I)** or 10μg/mL of neutralizing antibodies against TGF-β1 **(D-E)**.

GFAP promoter-directed expression of lacz was revealed by X-Gal (blue nuclei) prior to anti-GFAP immunocytochemistry (brown staining). Scale bar corresponds to 50 μm.

Figure 6: TGF-β1 phospholylation of Smad 2 is independent of MAPK/PI3K pathways in astrocytes. Cerebral cortex astrocytes derived from newborn mice were cultured alone **(Control)**, in the presence of TGF-β1 (10ng/mL) **(TGF-β1)**; PD98059 (50μM) + LY294002 (5μM) **(PD + LY);** or simultaneously, in the present of TGF-β1 and both inhibitors **(TGF+PD+LY)** for 30 minutes. **A)** Representative Western blot and **B)** graphic analysis showing levels of phosphorylated Smad 2 (P-Smad 2) content: Equal amounts (20 μg/lane) of total protein of astrocytes cultures were resolved in 10% poliacrylamide gel and analyzed by immunobloting for P-Smad 2. The levels of the Smad protein immunoreactivity are expressed relative to the levels of the housekeeping gene protein, α-Tubulin **(B)**. Inibition of the non-canonic TGF-β1 pathways, MAPK and PI3K pathways, did not block Smad 2 phosphorylation in response to TGF-β1. * $p<0.05$ (in comparison to the control).

Figure 7: Cross-talking between glutamate and TGF-β1 signaling pathways. Astrocyte differentiation induced by the neurotransmitter glutamate is mediated by group-II glial metabotropic receptors 2 and 3 (mGlu2/3) and requires glial production of transforming growth factor-β1 (TGF-β1). Through activation of MAPK and PI3K pathways, glutamate increases production and release of TGF-β1 by astrocytes, which in turn protects neighboring neurons against excitotoxic death. Additionally, TGF-β1 modulates several events in astrocytes biology such as: activation of the GFAP gene and induction of differentiation through the canonical Smad pathway and non-canonical MAPK/PI3K pathways.

FIG. 1 - Romão *et al*., 2007

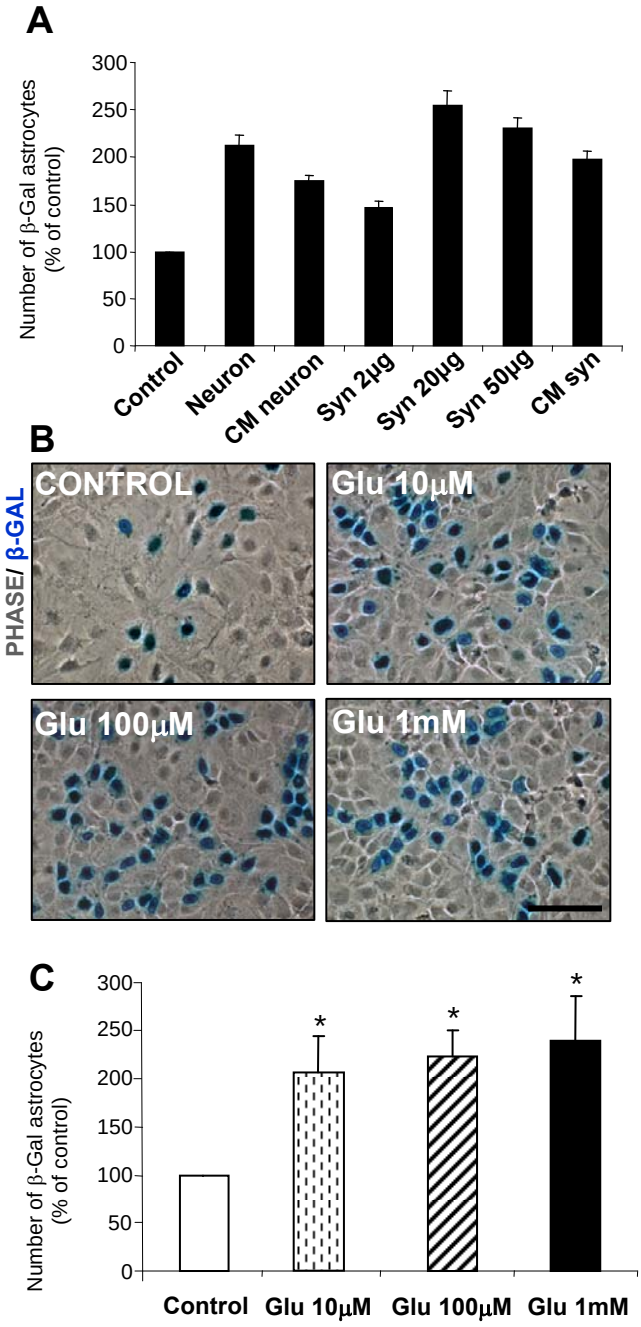


FIG. 2 - Romão *et al*., 2007

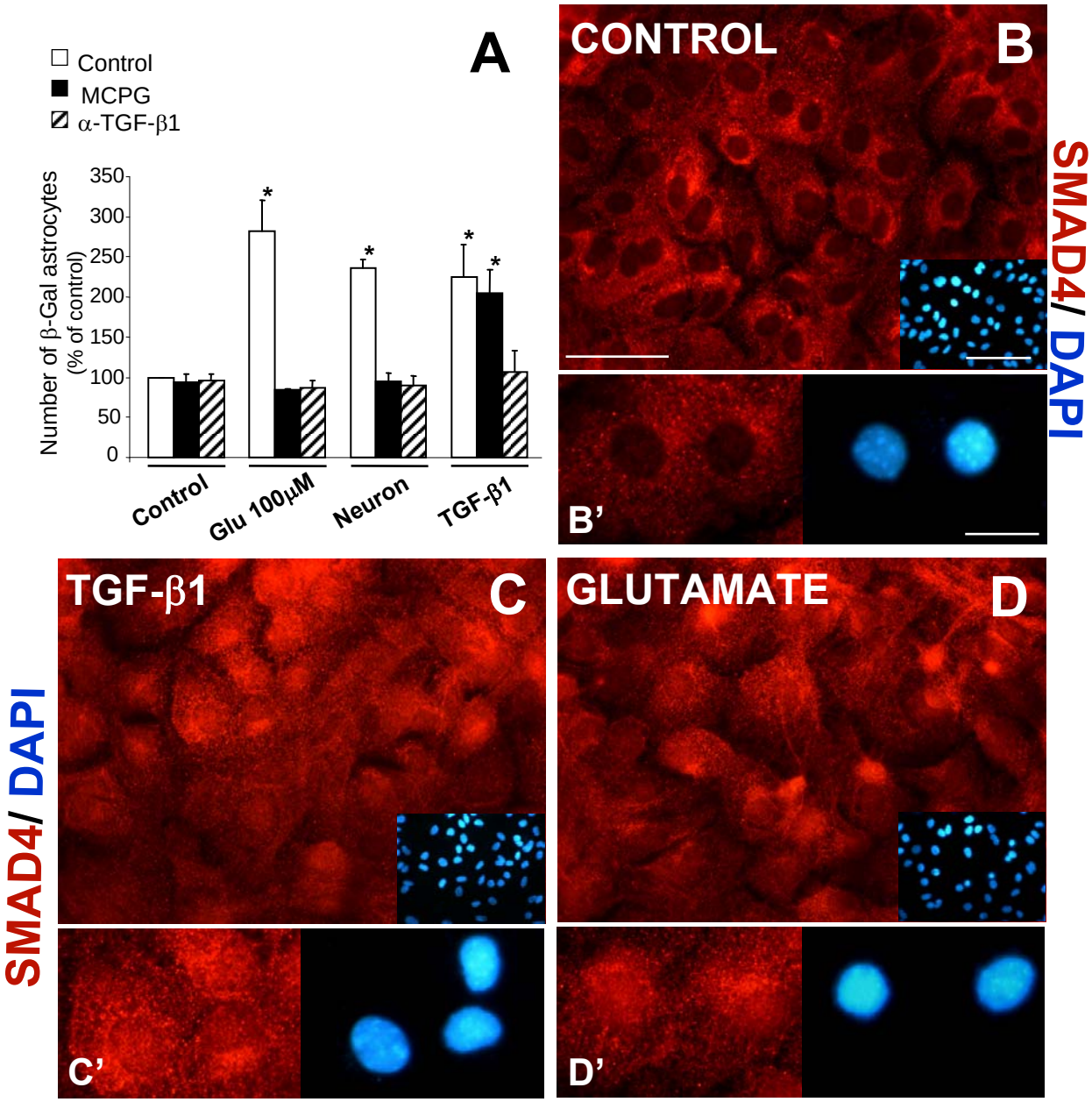
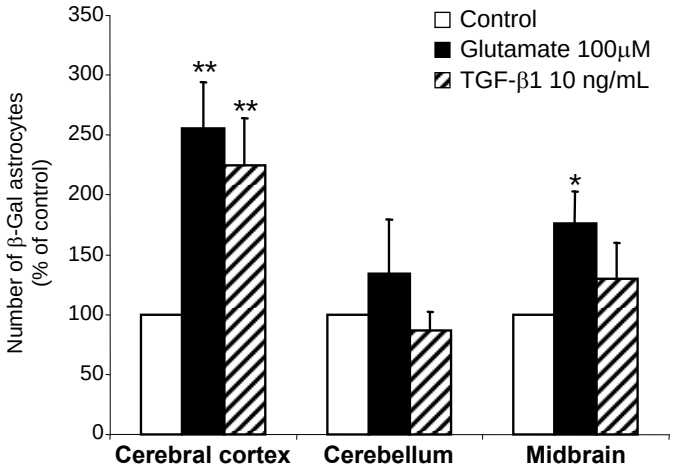


FIG. 3 - Romão *et al*., 2007



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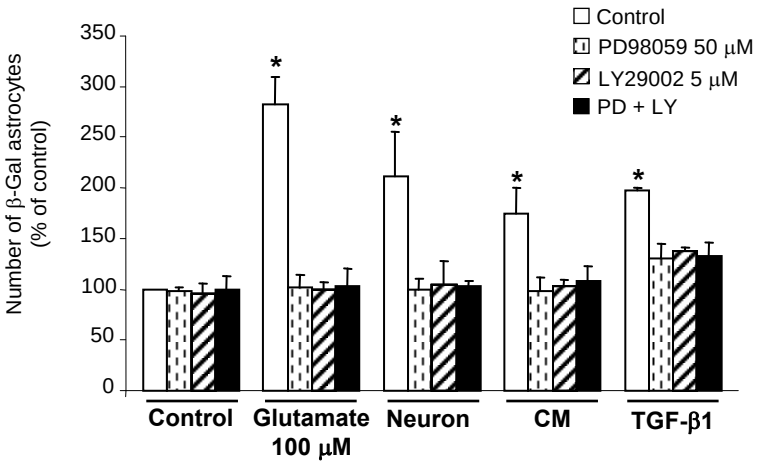


FIG. 5 - Romão *et al*., 2007

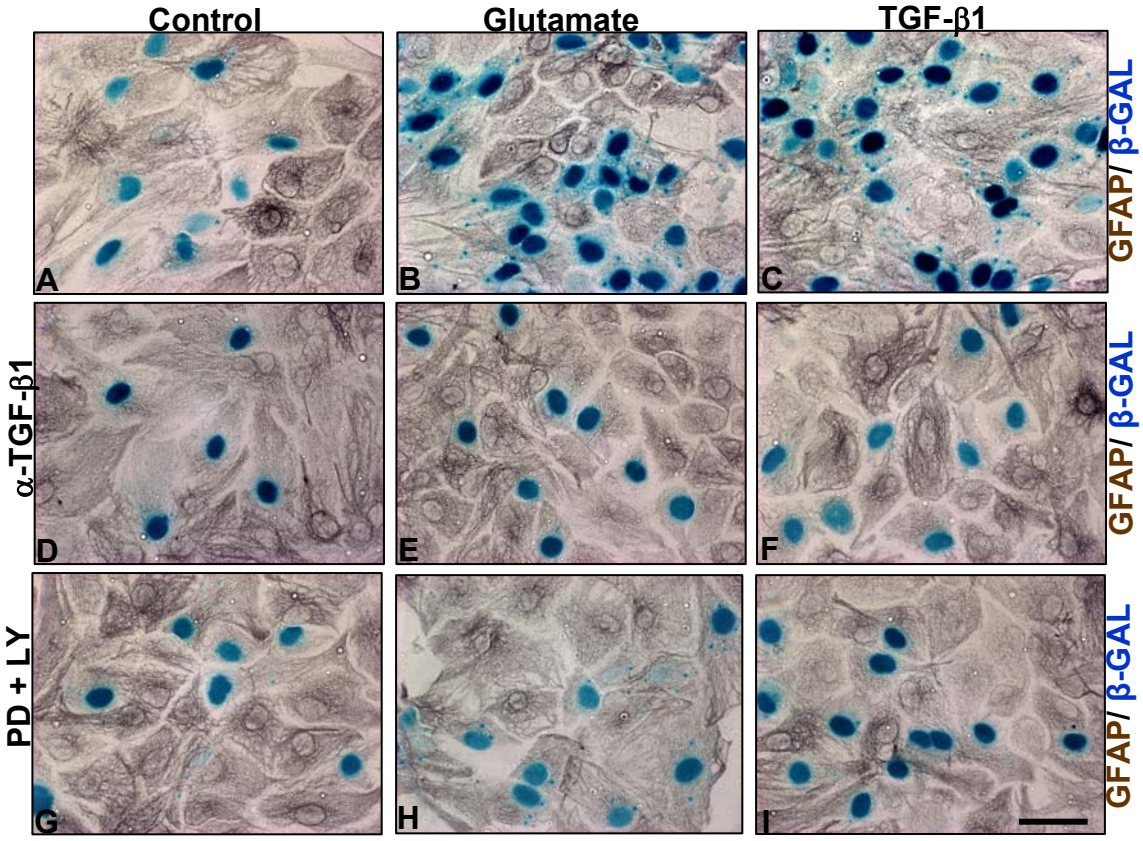
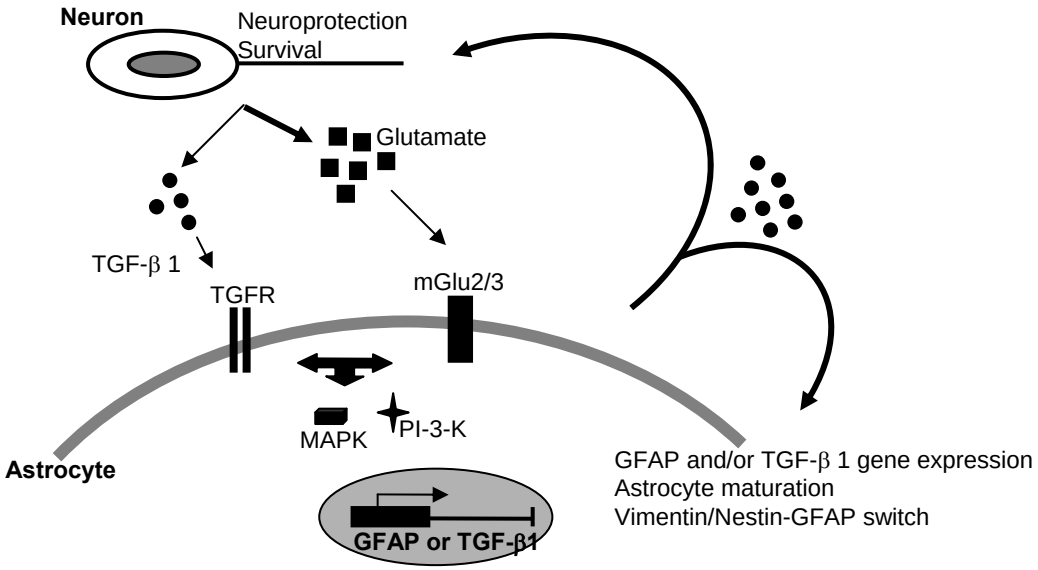


FIG. 6 - Romão *et al*., 2007



3.3 Propriedades interativas das células de glioma humano com neurônios numa abordagem *in vitro*

Os astrócitos representam a principal fonte de fatores de crescimento e moléculas de MEC, essenciais para o desenvolvimento de neurônios tanto *in vitro* quanto *in vivo*. Por sua vez, neurônios também secretam moléculas que modulam a organização da MEC das células gliais e, sobretudo controlam sua proliferação e diferenciação. Pouco se sabe sobre a interação de neurônios com células astrocitárias, que sofreram transformação tumoral. O objetivo deste trabalho foi investigar a interação entre neurônios e células de glioblastoma multiforme, o mais maligno tumor astrocitário. Neste trabalho, estabelecemos linhagens de glioblastoma humano provenientes imediatamente de ato neurocirúrgico. As células foram submetidas a regimes de coculturas com neurônios provenientes de cérebros de ratos embrionários ou recém-nascidos. Demonstramos que as células tumorais preservam a propriedade de substratos permissivos para o crescimento de neurônios e arborização de seus neuritos. Demonstramos que as células tumorais secretam fatores capazes de induzir complexa neuritogênese. O exame da composição e arranjo de laminina na MEC destes tumores gliais mostrou que células de glioblastoma têm uma concentração de laminina mais exuberante que os astrócitos normais. Mais ainda, os resultados sugerem que neurônios interferem na organização de laminina na superfície da célula tumoral: enquanto na cocultura tumor-neurônio essa organização é completamente fibrilar, culturas de glioblastoma apresentam um padrão filamentoso de laminina. Esses dados sugerem que, apesar do estado tumoral da glia, células de glioblastoma mantêm as propriedades interativas com os neurônios.

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ORIGINAL ARTICLE

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Interactive properties of human glioblastoma cells with brain neurons in culture and neuronal modulation of glial laminin organization

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Abstract: The harmonious development of the central nervous system depends on the interactions of the neuronal and glial cells. Extracellular matrix elements play important roles in these interactions, especially laminin produced by astrocytes, which has been shown to be a good substrate for neuron growth and axonal guidance. Glioblastomas are the most common subtypes of primary brain tumors and may be astrocytes in origin. As normal laminin-producing glial cells are the preferential substrate for neurons, and glial tumors have been shown to produce laminin, we questioned whether glioblastoma retained the same normal glial–neuron interactive properties with respect to neuronal growth and differentiation. Then, rat neurons were co-cultured onto rat normal astrocytes or onto three human glioblastoma cell lines obtained from neurosurgery. The co-culture confirmed that human glioblastoma cells as well as astrocytes maintained the ability to support neuritogenesis, but non-neural normal or tumoral cells failed to do so. However, glioblastoma cells did not distinguish embryonic from post-natal neurons in relation to neurite pattern in the co-cultures, as normal astrocytes did. Further, the laminin organization on both normal and tumoral glial cells was altered from a filamentous arrangement to a mixed punctuate/filamentous pattern when in co-culture with neurons. Together, these results suggest that glioblastoma cells could identify neuronal cells as partners, to support their growth and induce complex neurites, but they lost the normal glia property to distinguish neuronal age. In addition, our results show for the first time that neurons modulate the organization of astrocytes and glioblastoma laminin on the extracellular matrix.

Key words Glioma · neuron–glia interactions · extracellular matrix · brain tumors

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Introduction

The harmonious development of the central nervous system (CNS) depends on the interactions of the neuronal and glial cells. These interactions are established early on during brain development and may be

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controlled by growth factors released in the extracellular space, by extracellular matrix (ECM) elements, or gap-junctions, in order to achieve CNS construction. During neurogenesis, astrocytes, the larger glial cell population in the brain, play a pivotal role in both embryonic and adult neural cell development (Lim and Alvarez-Buylla, 1999; Song et al., 2002; Nakayama et al., 2003). It has been shown that astrocytes promote neuronal proliferation, axonal guidance, and synapse formation *in vitro* (Noble et al., 1984; Mallat et al., 1986; Nedergaard, 1994; Garcia-Abreu et al., 1995b; Tessier-Lavigne and Goodman, 1996; Gomes et al., 1999b) and also *in vivo* (Rakic, 1972, 1981; Snow et al., 1990; Pires-Neto et al., 1998). These interactions can also be seen by functional coupling of neurons to glia (Fróes et al., 1999) or by the capacity of neurons to control gap-junctional communication in astrocytes (Rouach et al., 2000, 2002) and modulate oxytocin receptor expression in rat cultured astrocytes with involvement of transforming growth factor β (TGF β) (Mittaud et al., 2002). In fact, an increasing amount of evidence points toward neurons as modulators of glial development. For instance, neuron-derived signals induce proliferation and morphological changes in cerebellar glia (Hatten, 1985, 1987), cortical glial maturation with an increasing expression of the astrocyte marker GFAP through TGFβ1 secretion and dependent on neuronal brain region (Gomes et al., 1999a; De Sampaio e Spohr et al., 2002; Sousa et al., 2004). These data suggest that neuronal and glial cells' interactions may be differentially regulated during development of the brain according to brain-specific regions. In fact, it has been proved that astrocytes can define the three-dimensional shape of mouse mesencephalic neurons based on its regionally specific heterogeneity (Denis-Donini et al., 1984; Garcia-Abreu et al., 1995b). We have demonstrated that astrocytes derived from distinct sub-regions of the midbrain can differently modulate neurite extension and arborization. Astrocytes from the lateral region of the midbrain are permissive to neurite outgrowth of midbrain neurons, whereas those derived from the midline are non-permissive to neuritic growth (Garcia-Abreu et al., 1995b). This difference of midbrain astrocytes property could be partially due to the concentration of sulfated proteoglycans distributed in the astrocyte ECM or delivered in the medium by the cells from the distinct midbrain sub-regions (Garcia-Abreu et al., 1996, 2000) as well as by their abilities to deposit laminin differentially in their own ECM, showing a different organization on each of the astrocyte types: filamentous on lateral astrocytes ECM or mixed, filamentous, and punctuate shape, on medial astrocytes (Garcia-Abreu et al., 1995a).

Among several ECM components, laminin has been implicated in the morphogenesis of the nervous system with abundant evidence of its ability to promote cell migration, differentiation, and axonal guidance *in vitro* (Chamak and Prochiantz, 1989; Liesi, 1990; Hunter and Brunken, 1997; Colognato and Yurchenco, 2000). Astrocytes are thought to be a major source of ECM molecules in the CNS (Pindzola et al., 1993; Powell and Kleinman, 1997) even when differentiated *in vitro,* and they also produce laminin. *In vivo*, its laminin expression decreases during normal astroglial maturation and in the injured adult CNS, laminin is produced only in some reactive astrocytes near lesions (Liesi, 1985; Giftochristos and David, 1988; McKeon et al., 1991).

Glioblastomas are the most common subtype of primary brain tumors and are characterized by its highly proliferative index, aggressive invasiveness, and short survival, being considered one of the deadliest of human cancers (Kleihues and Cavenee, 2000). As normal laminin-producing glial cells and laminin itself from an exogenous source are the preferential substrate for neuron growth, and glial tumors have been shown to produce laminin *in vitro* and *in vivo* (Giese et al., 1995; Chintala et al., 1996; Ljubimova et al., 2006), we questioned whether glioblastoma retained the normal glial properties and behavior with respect to supporting neuronal growth. To approach these questions, rat neurons were co-cultured onto rat normal astrocytes or non-neural cells or onto three human glioblastoma cell lines obtained from neurosurgery. We verified that human glioblastoma cells as well as astrocytes supported neuritogenesis, but non-neural cells failed to do so. Different from astrocytes, glioblastoma cells did not distinguish embryonic from neonatal neurons in relation to neurite pattern in the co-cultures. Laminin organization on both normal and tumoral glial cells was changed from a filamentous arrangement to a mixed punctuate/filamentous pattern when in co-culture with neurons. Our data strongly suggest that like astrocytes, Gbm cells recognize neurons as partners to support growth of complex neurite and that neurons modify glioblastoma laminin organization.

Materials and methods

Animals

All animals were kept under standard laboratory conditions according to NIH guidelines.

Primary astrocyte, neuronal, and endothelial cell cultures

Astrocyte primary cultures were prepared from newborn (0–1-day old; P0) Wistar rat cerebral cortices, as described previously (Trentin and Moura Neto, 1995). Dissociated cells were plated in Dulbecco's modified Eagle's medium (DMEM)/F12/10% FBS into a 15.5 mm diameter well (24-well plate) and/or 25 cm^2 tissue culture flasks (Corning Inc., New York, New York), previously coated with polyornithine (1.5 μg/ml, Mr 41,000; Sigma, St. Louis, MO). 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 near confluence.

Primary neuronal cell cultures were prepared from 18 day embryonic (E18) and newborn (0–1 day old; P0) Wistar rats cerebral cortices as previously described (Moura Neto et al., 1983). Briefly, single-cell suspensions were obtained by dissociating cells of cerebral cortices in DMEM/F12 medium supplemented with glucose (33 mM), glutamine (2 mM), and sodium bicarbonate (3 mM). 5×10^4 cells were plated either on polyornithine-treated coverslips placed on a 24-well plate to single neuronal cultures or plated onto normal or tumor cell monolayers to a co-culture, as indicated below. The neuronal cultures or the co-cultures were kept in DMEM/F12 medium without serum or supplements for up to 24 or 48 hr.

Human umbilical vein endothelial cells (HUVECs) were obtained by treatment of umbilical veins with a 0.1% collagenase IV solution (Sigma) as described previously (Jaffe et al., 1973) and adapted by Ferrari de Outeiro-Berstein et al. (2002). Dissociated cells were plated in plates previously treated with porcine skin gelatin and grown in M199 supplemented with 2 mM glutamine, 2.5 mg/ml amphotericin B, 100 mg/ml penicillin, 100 mg/ml gentamycin, 0.13% sodium bicarbonate, and 20% fetal calf serum (FCS). Cells were maintained as indicated for astroglial cells.

Human glioblastoma (Gbm) cell culture

Primary human glioblastoma cell cultures (Gbm) were obtained, by surgical biopsy, from patients who had given written consent to the study, and the procedures were in agreement with the Brazilian Ministry of Health Ethic Committee (CONEP No. 2340). The tumor samples were analyzed histologically by the Pathology Service of the Federal University of Rio de Janeiro Hospital. The glial tumor biopsies were washed in DMEM medium, mechanically dissociated, plated onto glass coverslips previously coated with polyornithine, or directly plated on a 24-well plate and/or 25 cm^2 tissue culture flasks (Corning) in growth medium DMEM/F12 supplemented with 10% FCS. The cell cultures were kept as described above. The medium was changed every 3 days until the culture was near confluence, in around 7 days. Then, cell cultures were either fixed and processed for characterization as described below, or split, or frozen in media containing 50% glycerol, 50% growth medium in cryotubes, and conserved in liquid N_2. The tumor cells were named Gbm95, Gbm02, and Gbm03.

Cell line cultures

RAT (Fibroblast cell line) was a generous gift from Dr. Ulisses Gazos Lopes (Lopes et al., 1997); MCF7 (breast cancer cell line) and U79 (uterus cancer cell line) were obtained from Rio de Janeiro Cell Bank (http://www.bcrj.hucff.ufrj.br/home.html). These cell lineages were cultured as described for Gbm cells.

Co-cultures

Primary astrocytes, Gbm, and cell line monolayer plated on coverslips were used as a carpet to grow, in co-culture, neurons from E18 and P0 rat cortices as described in Garcia-Abreu et al. (1995b). Briefly, cell monolayers near confluence were washed three times with serum-free DMEM/F12. Cells (5×10^5) freshly dissociated from E18 or P0 rat brain hemispheres obtained as indicated above were plated onto those monolayers. The co-cultures were maintained 24 or 48 hr in serum-free DMEM/F12 and then, the media were discarded and the co-cultures were fixed with paraformaldehyde for immunocytochemistry, morphometry, and statistical analyses of the neuronal morphology.

Histological analyses and immunocytochemistry

Biotin–streptavidin–peroxidase immunohistochemistry was performed in 3–4 μm thickness paraffin tumor sections. Sections were immunostained with anti-GFAP monoclonal primary antibody (1:50, Dako, CA). The Universal Immunostaining System Streptavidin–Peroxidase kit (Coulter, Fullerton, CA) was used to develop the reaction. Hematoxylin was used to stain and counterstain paraffin tumor sections and mounted with PERMOUNT. The primary antibody was omitted to provide negative controls.

All samples were examined under a light microscope Nikon (Tokyo, Japan) TE 300 and some were selected for image documentation on a CoolSNAP-Procf, ROPER Scientific Photometrics (Tokyo, Japan).

Immunocytochemistry was performed as described previously (Garcia-Abreu et al., 1995b and Gomes et al., 1999b). Briefly, cultured or co-cultured cells plated on glass coverslips were fixed with 4% paraformaldehyde for 30 min and permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 5 min at room temperature, except by laminin staining. After blocking, cells were incubated with primary antibodies for 1 hr at room temperature, followed by PBS washes and incubation with specific secondary antibody conjugated with Cy3, fluorescent isothiocyanate, or horseradish peroxidase. To perform morphometry analysis, neurites were visualized using the VIP kit (Vector, Burlingham, CA) reaction. Primary antibodies used and dilutions were as follows: anti-GFAP (1:500, Dako), anti-nestin, anti-vimentin and anti-β-tubulin III (1:200, Sigma), and anti-laminin (1:30, Sigma). In all immunostaining-negative controls, reactions were performed by omitting the primary antibody. No reactivity was observed when the primary antibody was absent.

Morphometry and statistical analyses

Neurons immunostained for β tubulin III were analyzed by the total neurite length using the Sigma Scan Pro Software (Jandel Scientific, Linslade, UK). In each experiment, at least five independent experiments were performed and each in triplicate, about 100 neurons per well, encompassing three to four fields randomly chosen on the coverslips, were scored per condition. Statistical significance was evaluated using the Mann–Whitney test (Siegel, 1956) from the statistical package of Microsoft Excel version 7.0.

Electrophoresis on polyacrylamide gel and Western blot analysis

Protein concentration on cell extracts was measured in triplicate by the Bradford method (Bio-Rad, Hercules, CA). Fifty micrograms of protein per lane was loaded to electrophoresis in a 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were electrically transferred onto a Hybond-P polyvinylidene difluoride membrane (Amersham Biosciences, Miami, FL) for 1 hr. Membranes were blocked in buffer containing non-fat milk 5%, and incubated with the polyclonal rabbit anti-Laminin (1:300, Sigma), 4°C, overnight. Peroxidase-conjugated secondary antibody (1:2,000, Amersham) was added to the membrane and incubated for 1 hr at room temperature. Proteins were visualized using the enhancing chemiluminescence detection system (ECL-Plus; Amersham Pharmacia Biotech, Miami, FL). Coomassie blue staining of the gel was used to monitor protein loading.

Results

Human glioblastoma characterization

Surgical biopsies from three glial tumors diagnosed as glioblastoma were used in this study. The computed tomography (CT) and magnetic resonance (MR) imaging showed a typical ring-shaped aspect with a hypodense center because of necrosis and peripheral contrast

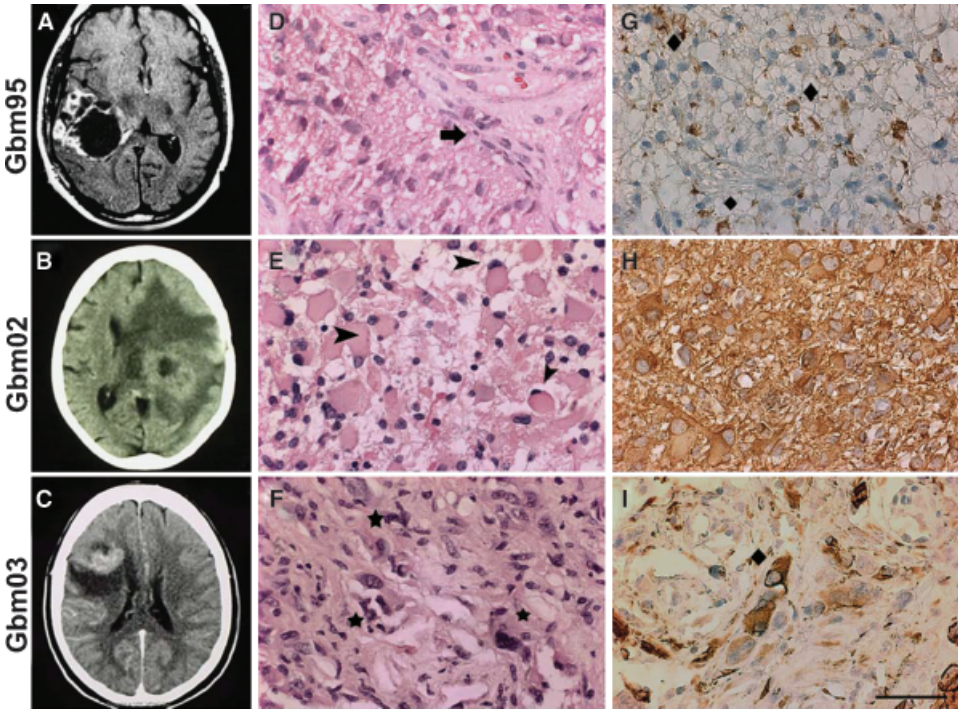

Fig. 1 Magnetic resonance, computed tomography image, and histopathology of human glioblastoma. (**A**) Gbm 95, magnetic resonance depicting a temporal lobe-enhancing lesion with central necrosis and mass effect. Brain computed tomography scans of Gbm 02 (**B**) and Gbm 03 (**C**). Note the hypodense area in the lesion area suggesting an edema. Hematoxylin–eosin staining of Gbm 95, Gbm 02, and Gbm 03 (**D–F**) showing vascular endothelial proliferation (black arrow) gemistocystic figures (arrowhead) and cellular pleomorphism (stars). GFAP immunoreaction of Gbm 95, 02, and 03 (**G–I**). Note that some neoplasic cells were GFAP-positive (diamonds), although this antigen was primarily identified in the reactive astrocytes that intermingled with tumor cells Scale bar: 50 μm.

enhancement, plus the peritumoral hypodense area due to edema (Figs. 1A–1C). The contrast enhancement was a criterion to suspect a glioblastoma. Hematoxylin–eosin (HE) staining of 4 μm histological sections of each tumor sample is shown in Figures 1D–1F. The temporal lobe tumor Gbm95 (Fig. 1A) corresponds to a glial neoplasia with diffuse proliferation of fibrillary astrocytes, moderate nuclear pleomorphism, occasional multinucleated cells with many mitoses, and necrosis as a feature of glioblastoma. In addition, vascular proliferation can be observed in the tumor area (Fig. 1D). Gbm 02 also shows temporal localization (Fig. 1B) and the HE staining permits observation of glial neoplasia with cellular pleomorphism and many gemistocystic figures (Fig. 1E). The frontal lobe neoplasia, Gbm 03 (Fig. 1C), is characterized by pleomorphic astrocytic proliferation and evident hypercromatic nuclei. In all three tumors, we found focal areas of necrosis, microvascular, and endothelial proliferation (data not shown).

Gbm02 and Gbm 03 were GFAP-positive in many of the neoplasic cells (Figs. 1H,1I). However, in Gbm95, some neoplasic cells were GFAP-positive, although this antigen was primarily identified in the reactive astrocytes that intermingled with cells (Fig. 1G). Together, these observations in combination allowed these three tumors to be classified as glioblastoma.

Glioblastoma cell cultures

At phase contrast, the glioblastoma cell cultures, Gbm95, Gbm02, and Gbm03, did not show significant morphological difference (Figs. 2A–2C). However, Gbm cells were morphologically different from the flat-shaped primary astrocytes and non-neural tumors cell lines (data not shown). All these types of glioblastoma cells are positive to vimentine (Figs. 2D–2F), exhibiting the filament network of this protein in the cytoplasm and concentrated around the cellular nuclei. In addition, all Gbm cells were also positive to the anti-GFAP antibody, but the cytoplasm staining has a more punctuate distribution than that of the filamentous network usually observed in normal rat astrocytes (Figs. 2G–2I). Although all cells were stained to these glial-filaments, some of them were more clearly labeled than others (Figs. 2G–2I). Nestin, a precocious intermediate filament cell marker, was present in glioblastoma cells in culture as well (Figs. 2J–2L). Interestingly, we identified many cells that did not react with anti-nestin antibody (Figs. 2J–2L). These results show that in culture Gbm cells express neural markers, thus retaining astroglial identity.

The normal glial and glioblastoma cells are preferential substrates for neurons

In order to evaluate whether human glioblastoma Gbm95, Gbm02, and Gbm03 cells could sustain neurite outgrowth, freshly dissociated cells obtained from 18 day embryonic (E18) or neonatal (P0) rat brain were cultured, under serum-free conditions, onto monolayers of each three types of glioblastoma for 24 hr. The neuron growth supporting properties of glioblastoma

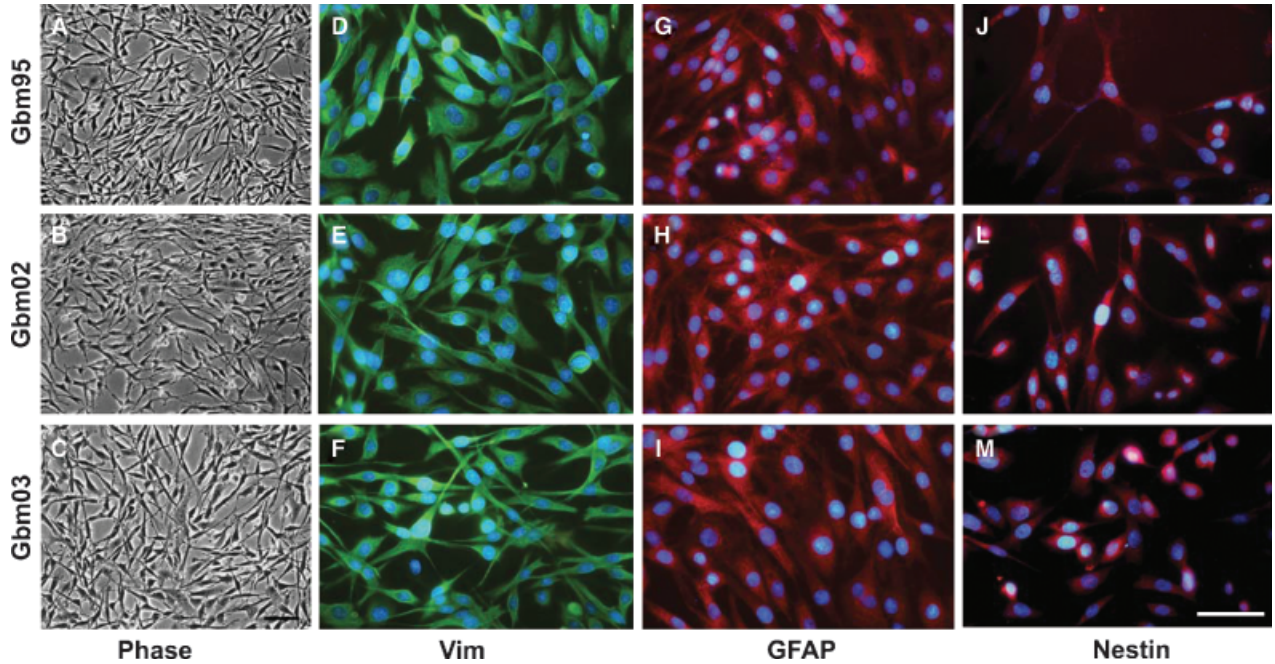

Fig. 2 Human glioblastoma cell characterization. Phase contrast photomicrography of Gbm 95 (**A**), Gbm 02/02 (**B**), and Gbm 03/02 (**C**). Vimentin, GFAP, and nestin immunostaining of Gbm 95 (**D, G, J**), Gbm 02/02 (**E, H, L**), and Gbm 03/02 (**F, I, M**), respectively. Scale bar: 50 μm. Panels D to M show blue nuclei 4′,6-diamidino-2-phenylindole staining. Scale bar: 50 μm.

monolayers was compared with that of normal primary astrocytes from newborn rat brain (Figs. 3A–3D). During the first 24 hr, E18 neurons extended neurite with very complex branches when co-cultured onto normal astrocytes as well as onto glioblastoma cells, although we observed that the neurites were shortened on Gbm than in astrocyte carpets (Figs. 3A–3D). The Gbm02 seemed to be a slightly better substrate for neurite extension than the other two glioblastoma, Gbm95 and Gbm03 (compare Figs. 3C with 3B, 3D).

In order to observe the progression of neurites, we maintained the co-cultures for one more day (Figs. 3E–3H) under the same condition. Neurons co-cultured onto normal glial or onto Gbm cell carpet showed very long and similar complex neurites (Figs. 3E–3H). We noticed that in both glial and Gbm carpets, neurons sometimes aggregate their cell bodies and extent neurites (Figs. 3E–3H). In order to investigate whether glial and tumor carpets are capable of supporting the growth of aged neurons, we co-cultured neonatal cells onto these carpets (Figs. 3I–3Q). As we observed previously, neurons exhibited similar pictures of complex neurites when co-cultured onto glial or tumor carpets for one or two days (Figs. 3I–3Q). On the second day of co-culture, neurons exhibited a very similar profile, extending normally branched neurites (Figs. 3N–3Q), although it seemed that both P0 neurites were shorter on the second day of co-culture than E18 neurites co-cultured onto the glial substrates (compare Figs. 3E,3N).

Monolayers of a lineage of rat fibroblast were used as non-neural cell carpet to co-culture neurons under the same conditions, but in this case neurons exhibited a paucity of neurites (not shown). Despite the scarcity of neurites on the neurons co-cultured onto fibroblast, under this condition no neuron was able to survival more than 6 hr. To verify whether another human cell, but non-neural cell, could support neurite growth of neurons, we co-cultured freshly prepared newborn neurons on monolayers of MCF7, a breast tumor lineage, a U79 human uterus tumor lineage, and on HUVEC, a normal human cell prepared from a human umbilical vein. It was observed that neurons did not survive, and died during the first 6 hr of co-cultures on all these cell carpets tested (not shown).

These observations led to the conclusion that human glioblastoma cells retain the property of normal glia to recognize neurons as a partner and favor their development and neurite growth in culture.

Does neurite outgrowth show age-related differences?

In order to compare the neurite patterning of embryonic neurons with that of newborn neurons in glial and tumor substrates, we used a morphometry study of the neuronal complexity in co-cultures described above (Fig. 4). The longest neurite (Fig. 4A) growing on normal glia carpet from embryonic E18 neurons were 400 μm in the first 24 hr. After 48 hr in co-culture, the longest neurite increased 50% of growth, reaching 600 μm in length (Fig. 4B). E18 neurons cultured onto human glioblastoma cells developed shorter neurites,

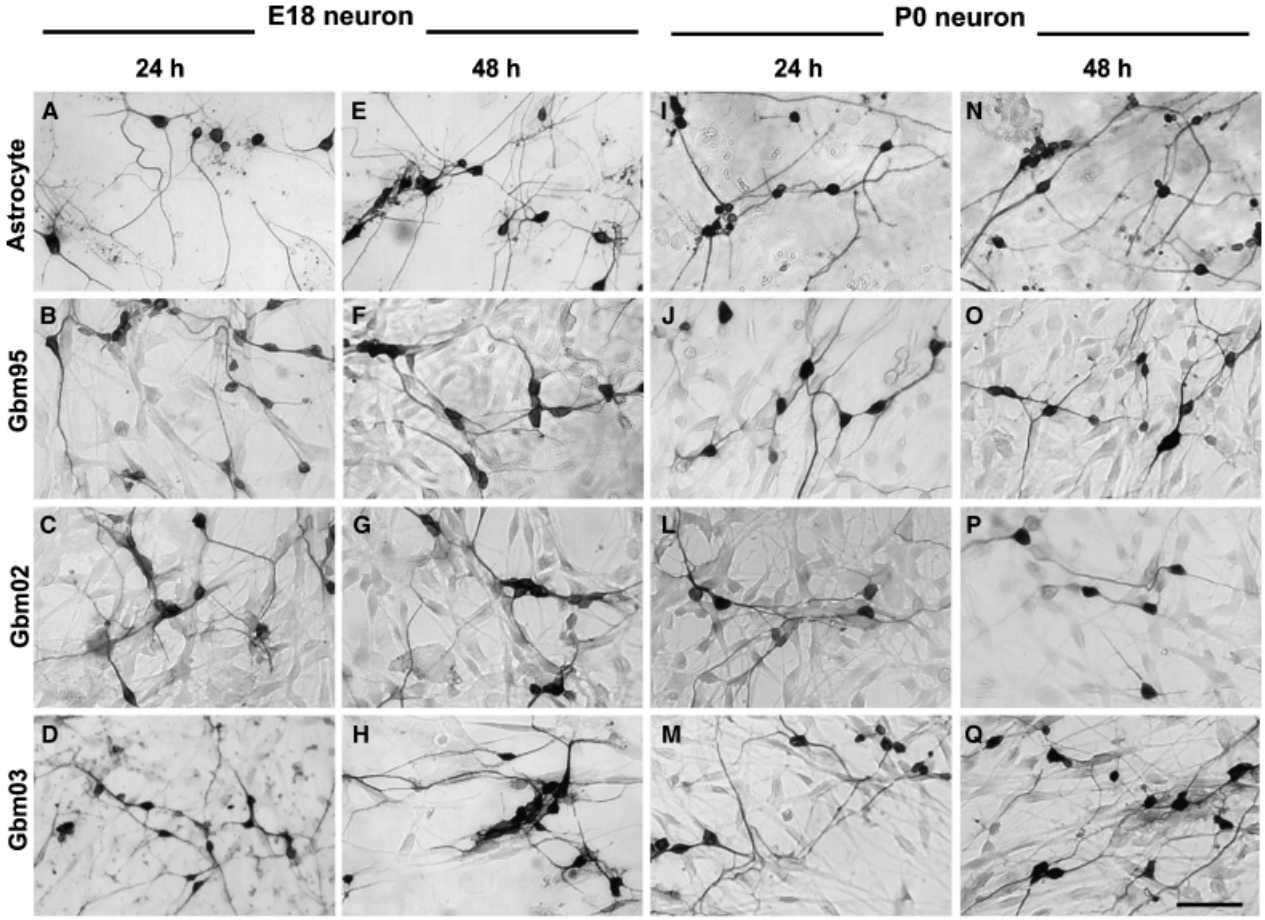

Fig. 3 Representative morphology of β tubulin III-positive neurons co-cultured onto astrocytes and Gbm cells in different times. E18 neurons were co-cultured for 24 and 48 hr onto astrocytes (**A, E**), Gbm 95 (**B, F**), Gbm 02 (**C, G**), and Gbm 03 (**D, H**). P0 neurons were co-cultured for 24 and 48 hr onto astrocytes (**I, N**), Gbm 95 (**J, O**), Gbm 02 (**L, P**), and Gbm 03 9 (**M, Q**). Scale bar: 50 μm.

the longest being 200 μm (Figs. 4A,4B). These differences between neurite lengths of the two types of substrates, normal versus tumoral glia, were around three to four times in the first 24 hr and increased to four to five times during the next day of co-culture, favoring the growth of neurites onto normal glia carpet. Although the longest neurite of E18 neurons on normal glia carpet was four times longer than those found on tumor glia, when we analyzed the median of total length we found an increase of only 1.5 times on the first day and up to two times for the second day (Figs. 4A,4B). This could then explain the apparent similarity shown in Figure 3.

The normal astrocytes, during the first 24 hr of co-culture, supported P0 neurite growth up to 240 μm and it reached nearly 400 μm on the second day of co-culture, which was lower than the corresponding length displayed by E18 neurons (Figs. 4A–4D). The median of total length neurite was nearly a half of the neurite length from embryonic E18 neurons, even after 48 hr in co-culture (Figs. 4C,4D). Thus, astrocytes were a better substrate, although the total lengths of the longest neurites in P0 neurons were shorter than those observed from the E18 neurons (Figs. 4A–4D), suggesting that astrocytes can distinguish embryonic neurons developing from newborn rat neurons by inducing different neuritic patterning. On the other hand, it was noticeable that embryonic and neonatal neurons growing onto glioblastoma cell carpets showed total length of neurites and median lengths that were similar during 24 and 48 hr of co-culture (Figs. 4A–4D). These results led us to conclude that astrocytes are able to distinguish the different neurite growth potential characteristic of embryonic and neonatal neurons. Further, Gbm cells are not able to distinguish these different neuronal potentials. Therefore, Gbm cells cannot recapitulate some aspects of astrocyte–neuron interaction, but they at least retain the permissive properties of neurite growth.

Neurons modulate laminin pattern on glial and Gbm cells

In order to verify whether laminin expressed by Gbm and glial cells in co-culture shows a particular organi-

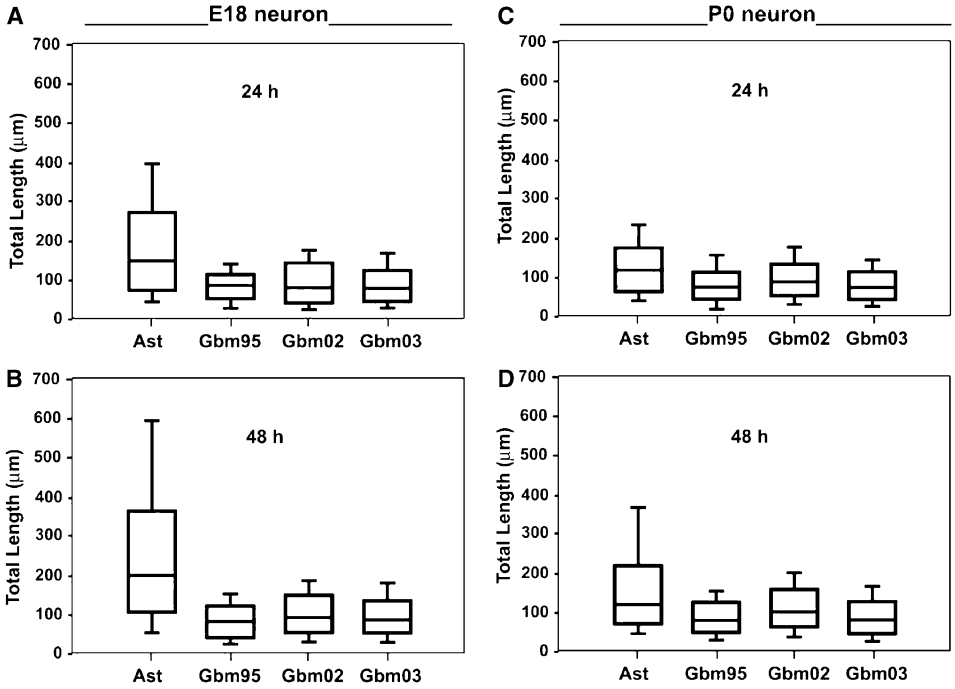

Fig. 4 Morphometry analysis of total length of neurites from β tubulin III-positive neurons co-cultured onto astrocytes and Gbm cells in different times. Graphs showing the total length of neurites from β tubulin III-positive neurons. E18 neurons were co-cultured for 24 hr (**A**) and 48 hr (**B**) onto astrocytes and Gbm 95, 02, 03 cells. P0 neurons were co-cultured for 24 hr (**C**) and 48 hr (**D**) onto astrocytes and Gbm 95, 02, 03 cells. Box and whisker plots show distribution of the total length of neurites on each cellular type. Boxes enclose 25th and 75th percentiles of each distribution and are bisected by the median; whiskers indicate 5th and 95th percentiles.

zation related to permition and sustaining neurite outgrowth, a polyclonal anti-laminin antibody was used to identify this glycoprotein deposition on normal astrocytes and Gbm cells under the same experimental conditions used previously (Fig. 5). Laminin was stained as a mesh-network (hypodense) on the surface of astrocyte cultures (Fig. 5A). On Gbm cells, we observed an intense fluorescence of laminin staining on its surface that seemed to be arranged in a manner similar to normal astrocytes, but with a highly dense staining following cell shape and indicating a higher laminin density in tumor ECM (Fig. 5C). In fact, protein extracts from the three Gbm cell lines seemed to be at least twice the amount of laminin if compared with those of normal astrocytes (Fig. 5E). In an attempt to evaluate the laminin organization in both glia and tumor cells under the co-cultures described above, a comparative immunostaining of laminin was performed (Figs. 5B,5D). Surprisingly, it was observed that the fibrillar meshwork of laminin on the surface of glial and tumor cells was changed when both embryonic and neonatal neurons were present on top of the carpets (Figs. 5B,5D). Fibrillar ECM laminin changed to a mixed arrangement, slightly fibrillar, but with a densely distributed punctuate laminin organization in both astrocytes and glioblastoma cells. In addition, we observed laminin patches more clearly present in neuron-Gbm co-cultures than in Gbm cultures. Interestingly, we rarely observed these laminin patches in astrocyte cultures. These results suggest that the laminin reorganization was due to the neuron–glia co-culture condition, suggesting that neurons could control the organization of normal or glioblastoma ECM cells, at least regarding laminin patterning.

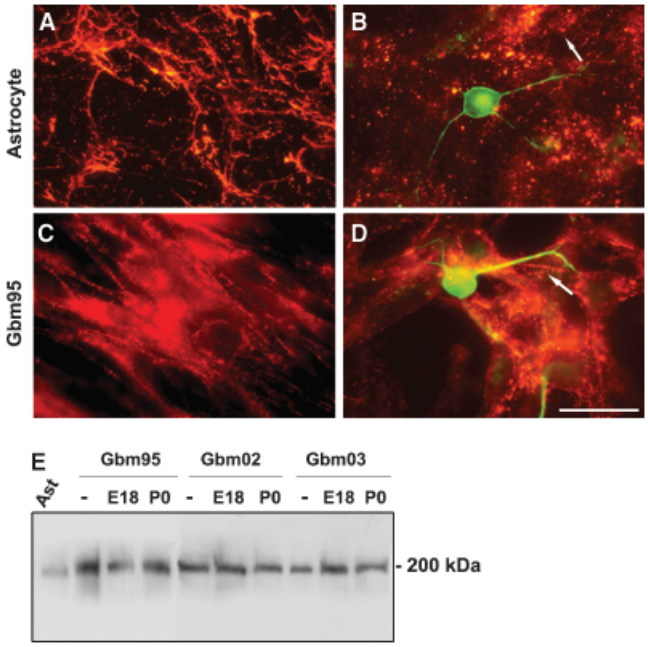

Fig. 5 Neurons modulate laminin pattern on glial and Gbm cells. Laminin immunoreaction (red) of Astrocyte (**A**) and Gbm 95 cells (**C**) showing the predominant fibrillar pattern on the cell surface. E18 β tubulin III-positive neurons (green) co-cultured onto astrocytes (**B**) and Gbm 95 cells (**D**) altered the fibrillar pattern to a mixed fibrillar and punctuate pattern (Arrows). The same results were found when P0 neurons were used (not shown). (**E**) Immunoblot analysis of the 200 KDa laminin content in the cell extracts from astrocytes, Gbm cells co-cultured or not with E18 or P0 neurons. Scale bar: 50 μm.

Discussion

In this report, we investigated the interactive properties of neuron and glial cells, looking for similarities and differences between normal and tumor glial cells, with respect to survival and support of neuritic growth from embryonic and neonatal neurons. Our experiments point out that the interactivity characteristic of glial cells with neurons is well preserved, even when malignant transformation of glia is occurring. The following conclusions can be arrived at from our experiments: (1) rat astrocytes distinguish the neuritic growth potential of embryonic and newborn neurons; (2) although supportive to neuritic growth, glioblastoma cells lose their characteristic of normal glia to distinguish this potential; (3) non-neural cells tested here cannot support neuronal growth; (4) glioblastoma cell produces laminin as an exuberant density deposited on its ECM; and finally, in a pioneer experiment to our knowledge, (5) it was demonstrated that neurons modulate laminin organization on ECM from normal or tumoral glial cells.

Among neuroectodermal neoplasias, glioblastoma is a highly malignant tumor. This tumor entity (Russell and Rubinstein, 1989) of astrocytic lineage can arise through a process of anaplasia from astrocytoma, by progressive steps of pre-existent lower grade astrocytic tumor (Schiffer, 1997). Glioblastoma are mainly localized in cerebral hemispheres, preferentially arising in the subcortical white matter (Kleihues and Cavenee, 2000) and rarely present in the gray matter. Three samples from different patients were investigated and showed glioblastoma patterns as indicated by tomography diagnosis, staining with HE in paraffin sections, and GFAP immunostaining. Although GFAP has been considered a specific marker to normal or tumor glial cells from astrocytic lineage (Eng et al., 1971) and is currently used for diagnosis (Deck et al., 1978), investigation of the GFAP presence in these three tumors showed varied expression, with some positive cells to astrocytic antigen and other negative ones, and sometimes the overall GFAP levels were lower than normal glia. A similar picture has been described for SV-40T antigen immortalized glial cells (Moura Neto et al., 1986; Frisa et al., 1994) or for another human glioblastoma (Gomès et al., 1997). In fact, these differences of GFAP expression in glioblastoma could account for possible highly proliferating tumor cells negative for GFAP (Schiffer, 1997) and because of an increasing astrocytic anaplasia, causing a progressive loss of GFAP production (Duffy et al., 1982). Thus, these differences of GFAP expression between glioblastoma cells could be explained by the cellular heterogeneity of the tumor (Singh et al., 2003) or perhaps because of a transformation of stem cells by asymmetrical division (Reya et al., 2001; Berger et al., 2004). Then, the expression of GFAP could be preceded by another protein expression, for instance nestin before the conversion of immature glial cells and GFAP astrocytic tumoral cells.

After tumor removal, the biopsies of glioblastoma used here were cultured for several generations, frozen, and re-plated, and they always retained the same morphology and growth rate in culture. Three Gbm cells were stained for GFAP and vimentin, but nestin immunoreaction was not found in all cells. In fact, it has been demonstrated that established astrocytoma cell lines, with enhanced motility and invasive potential, are decorated with nestin (Rutka et al., 1999). Although we could not double immunostain for intermediate filaments in our experiments, it is possible that nestin-positive cells are also GFAP positive. This co-expression could either point to a close relationship between proliferating glioblastoma cells and precursor cells or proliferating reactive astrocytes present in the tumor mass (Lin et al., 1995). Our work is more in accordance with the first possibility above, as it would be difficult to believe in the presence of reactive astrocytes in the culture after more than 15 splits.

Astroglia is a unique substrate for the *in vitro* growth of central nervous system neurons (Noble et al., 1984). In our experiments, normal glia or human Gbm cell supported neuron survival and neurite growth with complex arborization. Neither rat fibroblast and HUVEC, nor non-neural human tumor cell tested in this work supported neuron. Moura Neto et al. (1986) reported that mesencephalic rodent glial cells, normal or transformed by SV-40T antigen (SV-40 Tag), support the development of rodent neurons (Mallat et al., 1986). These SV-40-transformed mesencephalic astrocytes could induce the same degree of growth complexity in mesencephalic or striatal neurons. It has also been shown that SV-40 Tag-immortalized astrocytes that expressed lower GFAP levels supported neurite extension, but less efficiently than normal astrocytes (Frisa et al., 1994). Our results from the astroglial and Gbm cells are in agreement with these reports, and show for the first time that despite being able to support neurite growth, Gbm cells different from astrocytes are not able to distinguish the distinct neuritic growth potential characteristic of embryonic and newborn neurons. Although we cannot rule out the possibility that these differences are due to homotypes versus heterotypes cells, rat neuron/rat glia versus rat neuron/human glia, respectively, these findings strongly suggest that changes in the glial cells ECM, or in the glial membrane components, or glial-soluble molecules recognizing the surface of the neurons are playing a pivotal role in this neuron–glia interaction.

Laminin is one of the ECM components implicated in neurite growth and is expressed by glial cells. Four distinct immunoreactive patterns can be organized in developing rat brain: small and large punctiform laminin, sheath laminin, and somal laminin on the ECM (Zhou, 1990) with unique spatial and temporal distributions. Our previous study demonstrated that fibrillar or punc-

tuate glial-ECM laminin organization is related to different degrees of neurite extension (Garcia-Abreu et al., 1995a; Freire et al., 2002; for a review, see Cavalcante et al., 2002). Here, we verified that levels of laminin expression seem to be higher in Gbm than in glial cells. In fact, laminin isoforms change from normal astrocytes to glioblastoma and it is overexpressed in glial tumor cells (Ljubimova et al., 2004, 2006). Interestingly, in our experiments the presence of neurons on glial or Gbm cell surface changed the laminin organization from fibrillar to a predominant punctuate array. Differences in laminin organization may be due to different isoforms expressed (Ljubimova et al., 2004) or as we have published, due to a focal altered pH on cell surface (Freire and Coelho-Sampaio, 2000) that could be induced by sialic acid present on the membrane surface of the glial cells (Freire et al., 2004), and by consequence favors neuritogenesis or cell proliferation. In these reports, the implications of protein kinases A or C were demonstrated. As laminin is involved both in β1 integrin-dependent and independent signaling mechanisms during neurogenesis (Andressen et al., 2005), our results suggest that the interconversion of laminin forms described here could also be dependent on a possible role of neurons or their signalizing molecules acting on the glial membrane surface and ECM similar to Reelin, Disabled 1, and β1 integrins acting on radial glial (Dulabon et al., 2000; Förster et al., 2002), for instance. Experiments are in progress to understand this mechanism more clearly. Our results show that neuronal control of laminin might be relevant to understanding the biological properties of glial and tumor cells *in vivo*. If malignant glia cells recognize signals provided by normal neurons, then perhaps migration and invasion of glioblastoma cells in the brain are supported by cross-talk between migrating tumoral cells and the neighboring neurons.

In conclusion, our results show for the first time that human glioblastoma cells, in spite of their malignant progression, maintain glial characteristics to support neuronal survival and neurite outgrowth, although they did not retain normal glial properties of distinguishing young neurons from aged ones. In addition, we also concluded that neurons modulate laminin organization in glioblastoma cells as well as in normal glia.

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3.4. A interação neurônio-glioblastoma modula a expressão do fator de crescimento de tecido conjuntivo (CTGF) através de inibição da via de sinalização de MAPK

Apesar do crescente avanço no entendimento do estudo da interação neurônio-glia, ainda é desconhecida a interação entre neurônios e tumores de origem glial, como os gliomas. Glioblastomas multiforme (Gbm) são os subtipos mais comuns de tumores cerebrais primários e são caracterizados por seu alto índice proliferativo, agressividade e invasividade, sendo considerado o mais mortal dos cânceres humanos. Sua adaptação ao microambiente do SNC exige a produção e remodelamento de matriz extracelular (MEC). Neste trabalho, analisamos se neurônios podem influenciar a expressão de proteínas da matriz extracelular e fatores de crescimento como CTGF e TGF-β1. Demonstramos por imunocitoquímca e RT-PCR que células de Gbm e astrócitos expressam TGF-β1 e CTGF. Constatamos que a síntese e expressão de CTGF foram inibidas quando neurônios neonatais foram cocultivados sobre monocamadas de Gbm ou astrócitos. Entretanto, o meio condicionado de neurônios embrionários e neonatais não afetou a expressão de CTGF em células de Gbm. A inibição de CTGF em co-culturas de Gbm com neurônios neonatais não está relacionada a uma inibição da fosforilação de SMAD2/3 (característica da via TGF-β1), no entanto, a adição de TGF-β1 impediu a inibição da expressão de CTGF nos ensaios de co-cultura. Observamos que a fosforilação de MAPK é diminuída em Gbm cocultivados com neurônios, sugerindo que a expressão de CTGF em Gbm é regulada negativamente por neurônios neonatais através da inibição das vias de sinalização de MAPK e independe da sinalização TGF-β/SMAD.

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Professor Martin Schwab, Editor-in-chief
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Dear Professor Schwab

I would like to submit the manuscript entitled "**CONNECTIVE TISSUE GROWTH FACTOR (CTGF) IS NEGATIVELY REGULATED DURING NEURON-GLIOBLASTOMA INTERACTION**" by Romão *et al* to be published in Cancer Letters.

Although we have devoted great interest in the understanding of neuron-glial interactions in the last 10 years, it is not clear yet, the results from the interaction between normal neurons and abnormal cells, such as tumors cells. In this respect, we developed a system to coculture normal neurons onto glioblastoma (Gbm) cells and showed that, like astrocytes, Gbm can support neurite growth (Faria et al., 2006, Differentiation 74:562-572).

In the present manuscript we showed that connective tissue growth factor (CTGF) mRNA and protein is inhibited when neonatal normal rat neurons were cocultured onto glioblastoma cells. This inhibition can not be mimicked by conditioned medium from embryonic and neonatal neurons. Furthermore, we performed biochemical experiments to uncover the signaling pathway involved in this inhibition, thereby we investigated the two major signaling pathway that modulate CTGF: TGFβ and MAPK. We found that CTGF inhibition on Gbm cocultured with P0 neurons seems not to be related to TGFβ/SMAD pathway. However, phospho-p44/42MAPK (Erk1/2) is decreased in Gbm cocultured with P0 neurons.

We believe these data will be of general interest for oncology field, particularly if we take in account the scarcity of studies on the cell and molecular level about the role of CTGF in brain tumors.

Our experiments were carried out in accordance to the international guidance and rules for animal care in research and followed the guidelines of the Institute of Biomedical Science of the Federal University of Rio e Janeiro.

Sincerely yours,

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Title: CONNECTIVE TISSUE GROWTH FACTOR (CTGF) IS NEGATIVELY REGULATED DURING NEURON-GLIOBLASTOMA INTERACTION.

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ABSTRACT

Connective-tissue growth factor (CTGF) is a matricellular secreted protein involved in complex processes such as wound healing, angiogenesis, fibrosis and metastasis by regulating cell proliferation, migration and extracellular matrix remodeling. Glioblastoma Multiform (Gbm) is the major malignant primary brain tumor and its adaptation to the central nervous system (CNS) microenvironment requires production and remodelling of the extracellular matrix (ECM). We developed an *in vitro* approach to test if neurons can influence expression of the extracellular matrix. We showed that neurons remodeled glioma cells laminin. Here we show that neurons are also able to modulate CTGF expression in glioblastoma. RT-PCR shows that Gbm95 cells express CTGF as well as the astrocyte marker GFAP. CTGF immnoreactivity and mRNA levels in Gbm95 cells are dramatically decreased when these cells are co-cultured with neonatal neurons. Increasing the number of neonatal neurons cocultured onto Gbm95 cells inhibit the reporter luciferase activity under control of the CTGF promoter suggesting inhibition at the transcription level. This inhibition seems to be contact-mediated since conditioned medium from embryonic and neonatal neurons do not affect CTGF expression in Gbm95 cells. The inhibition of CTGF expression in Gbm95/neuronal co-cultures is not related to an inhibition of SMAD2/3 phosphorylation, but levels of phospho-p44/42 MAPK are decreased in co-cultured Gbm95 cells. Previous data of laminin and these results showing that CTGF is down-regulated in glioblastoma cocultured with neonatal neurons point out an interesting view to understand interactions of the tumor and cerebral microenviroment.

INTRODUCTION

Neuron-glia interactions play fundamental roles during development of the Central Nervous System (CNS). These interactions occur reciprocally from early to late stages of neurogenesis and gliogenesis as well as during synapse establishment. Several lines of evidence illustrate the key participation of glial cells during neuronal network formation, in neurogenesis [1, 2], neuroblast proliferation [3], neuron migration [4, 5], neurite growth and guidance [6, 7, 8, 9, 10], as well as in myelination and synapse establishment [11, 12, 13]. Neuronal cells can also control glial cell events such as survival and proliferation by cell contact-mediated signaling or by growth factor secretion as shown in the interaction between axons and oligodendrocytes or Schwann cells (see [14] for review). *In vitro*, it has been demonstrated that cell contact between astrocytes and neurons modulates astrocyte proliferation and differentiation through two distinct mechanisms [15, 16]. Neuronal membranes are sufficient to trigger inhibition of astrocyte proliferation, whereas astrocyte differentiation requires cell contact with living neurons [15, 16] and/or using TGFβ-1 as signaling [17, 18].

Despite this growing knowledge on normal neuronal-glial interactions, the effects of the interaction between normal neurons and tumors of glial origin, such as gliomas, are still unclear.

Glioblastomas are the most common subtype of primary brain tumors and are characterized by their highly proliferative index, aggressiveness, invasiveness, and short patient survival, being considered the deadliest of human cancers [19]. Glial cells as well as glioblastoma cells can produce and modulate the synthesis of extracellular matrix (ECM) molecules in the brain [20, 21, 22], as laminin which may affect tumor aggressiveness and invasiveness. Indeed, our

previous report shows that glioblastoma express laminin and that neurons co-cultured with these tumor cells remodeled the laminin architecture on the glioblastoma surface [23].

More recently, much interest has been devoted to CTGF [24, 25]. CTGF belongs to a family of secreted ECM-associated proteins that are involved in the regulation of cellular functions such as adhesion, migration, mitogenesis, differentiation and survival [26]. CTGF mRNA has been detected in glioma and several human tumors cell lines derived from nervous system and in tissue reorganization after brain injury, suggesting that it may play a role in the regulation of tumor invasiveness in the brain [27, 28, 29]. CTGF contains four different structural modules: an amino terminal insulin-like growth factor binding domain (IGFB), followed by the CR/vwc domain, a thrombospondin type 1 repeat (TSP-1), and a carboxyl terminal cystine knot (CT) domain [30, 31]. In the developing CNS of rodents, CTGF is expressed in the olfactory bulb, choroid plexus and dorsal root ganglia [32, 33]. In the adult CNS, CTGF is expressed in cortical astrocytes, cortical pyramidal cells, hippocampus, ependymal cells, tanicytes and in the white matter of spinal cord [28].

CTGF is involved in many biological events signaled by TGFβ, such as the control of collagen deposition and anchorage-independent growth induced by TGF-β in fibroblasts [34, 35]. CTGF promoter contains a TGFβ responsive element [36, 37], and the CTGF molecule can positively modulate TGFβ1 interaction with its cognate receptor and thereby enhance signaling at low concentrations of TGFβ [38].

CTGF gene expression and function can also be controlled by mitogen-activated protein (MAP) kinase pathways [39, 40]. The three major MAP kinase pathways, extracellular signal-regulated kinases (ERK), p38 and jun N-terminal kinase (JNK) serve as mediators and cross-talk links for a variety of signalling molecules and growth factors, and have been

implicated in diverse cellular processes including cell growth, migration, proliferation, differentiation, survival and development. ERK is classically activated via the sequential activation of Ras G proteins, Raf kinases and MEK1 and 2. MEKs, in turn, phosphorylate and activate their only known substrates, ERK1 and 2. ERK1/2, also know as p44/42 MAPK, are proline-directed serine or threonine kinases which phosphorylate P-X-S/T-P sequences in a large number of intracellular substrates, leading to diverse cellular outcomes [41] . ERK activates CTGF expression in different cell types including fibroblasts, mesangial cells and smooth muscle cells [42, 43, 44].

Despite of the expression of CTGF in different CNS cell types and also in glioma derived cells, the role of CTGF in the interactions between normal and pathological CNS cells remains unknown.

The present study was undertaken to investigate whether neurons can modulate CTGF expression during their interaction with glioblastoma cells. To do this, we analyzed the expression of glial markers and CTGF in glioblastoma cell lines cocultured with embryonic or neonatal neurons. We found that Gbm95 cells cultured alone express CTGF and this expression is slightly changed in the presence of E18 neurons, but is not modified by treatment with conditioned medium from E18 and P0 neurons. However, CTGF immunoreactivity and mRNA level decrease dramatically when Gbm95 cells are cultured in contact with P0 neurons. Smad2/3 phosphorylation is not affected in Gbm95 cells cocultured with P0 neurons, suggesting that CTGF inhibition induced by coculture with P0 neurons is independent from TGFβ/Smad2/3 actions. Interestingly, we detect a decrease in phospho P44/P42-MAPK, indicating that MAPK signalling is inhibited in the cocultures. These data show that neonatal neurons are able to downregulate CTGF expression in Gbm95 cells; that

this modulation seems not to depend on neuron-secreted soluble factors; and that downregulation may involve modulation of MAPK signalling pathway.

MATERIAL AND METHODS

All cell culture reagents and oligonucleotides were purchased from Invitrogen, and cDNA synthesis and PCR reagents were from Promega, unless specified in the text below. Anti-GFAP was purchased from Dako, anti-β tubulin III and anti-CNPase from Sigma, anti-CTGF from Torrey Pines, anti-P-SMAD2/3 and anti-P44/42MAPK from Cell Signaling. All solvents and reagents were of analytical grade.

Primary cultures of rat neural cells

Virtually pure astrocyte cultures were obtained as described by Trentin et al. [45]. These cultures were used for GFAP expression analysis by RT-PCR and immunocytochemistry. Cultures enriched in rat neurons were obtained from embryonic 18-day-old and neonatal hemispheres dissected as above. Cells were plated in dishes or onto coverslips treated with poly-L-ornithine and mantained in serum-free DMEM. These cultures were kept in 37°C at 5%/95% CO_2/H_2O atmosphere for no longer than 24 h. Both glial and neuronal cell cultures were assayed by immunocytochemistry with anti-GFAP, anti-βtubulin III antibodies to identify astrocytes and neurons respectively.

Human tumor cell culture

Human Glioblastoma cell line (Gbm95) was obtained according to Faria et al. [23], following procedures established by the Brazilian Ministry of Health Ethic Committee (CONEP No.

2340). Cells were plated in 35 mm or 16 mm well plates and had the medium changed every 3 days until confluence, when cells were usually split or frozen. The glioblastoma U-87 and A-172, the uterus U79 and the lung GLC4 cell lines are described in the cell line collection of ATCC.

Cocultures

Gbm95 monolayers were used to grow neurons from E18 and P0 rat brains as described by Abreu and his collaborators [6]. Cells ($5x10^4$) dissociated from E18 and P0 rat hemispheres as described above were plated on top of a Gbm95 monolayer previously grown on 16mm coverslips. For luciferase reporter assays, either 10^4 or $2x10^5$ cells were plated onto the Gbm95 monolayer. After 24 hours the medium was discarded and the cocultures were fixed with 4% paraformaldehyde for immunocytochemistry or lysed with TRIZOL for RT-PCR.

Conditioned medium from E18 and P0 neurons

Cells ($3x10^5$) dissociated from E18 and P0 hemispheres were prepared as described above and gently washed three times with PBS containing glucose. The cells were then incubated with the smallest possible volume of serum-free medium DMEM-F12 for 24 h. The conditioned medium was collected, centrifuged at 1000 g to discard cell debris and the supernatant was used immediately or stored at -80°C.

Immunocytochemistry

Immunostaining reaction was performed as described by Abreu et al. [6, 46]. Briefly, fixed cultures or cocultures were washed with PBS, permeabilized with 0.1% Triton X-100 in PBS for 5 min when necessary, and blocked for 1 hour with PBS containing 5% bovine serum albumin. Cultures were then incubated with rabbit anti-GFAP, Rabbit anti-CTGF or mouse anti-βtubulin III primary antibody for 1 hour at room temperature. Incubation with specific secondary antibodies conjugated either with fluorochromes Cy3 or FITC ensued for 1 hour at room temperature. After PBS washes, slides were mounted and observed in a Nikon TE 2000 inverted microscope. Images were captured using a CoolSNAP-Pro (Media Cybernetics) digital camera.

Western blot analysis of the cell lysates

Gbm95 monolayers or Gbm95 cells cocultured with neurons maintained in 35 mm plates were lysed in 50 µl RIPA (0.05M Tris-HCl pH 7.4, 0.15M NaCl, 1% NP-40, 2 mM EDTA, 1 mg/mL pepstatin, 1 mM PMSF, 1 mM NaF, 1 mM Na_3VO_4) buffer. Samples were taken to measure protein concentration by the Bradford method [47]. Before loading, samples were mixed with 5X sample buffer containing 200mM DTT 4% SDS, 125 mM Tris pH 6.8, 20% glycerol and 0.02% bromophenol blue. DNA was sheared in a 22-gauge needle syringe. Samples were heated during 5 minutes at 95°C and the proteins were separated in 9% SDS-PAGE. Proteins were transferred to nitrocellulose membranes (Amersham Biosciences) in transfer buffer containing 25 mM Tris, 192 mM glycine, 0.1% SDS and 20% methanol. Membranes were blocked in 5% polyvinyl-pirrolidone (Sigma) for phospho-SMAD2/3

reaction or non-fat dry milk in 20mM tris pH 7.6, 137 mM sodium chloride and 0.1% Tween 20 (Merk) for phospho-p44/42 MAP reaction. Primary antibodies anti-Phospho-SMAD2/3 (Cell Signaling Technology), phospho-p44/42 MAPK (Cell Signaling Technology), total p44/42 MAPK (Cell Signaling Technology) or Anti-tubulin diluted 1:1000 in blocking solution were incubated with the membranes overnight at 4°C, followed by incubation with HRP-labeled secondary antibodies. The immunoblot reaction was developed with Super Signal West Pico chemiluminescent substrate (Pierce) and exposed in Kodak X-OMAT film.

Gene expression analysis by RT-PCR

Gbm95, astrocyte, neuron cultures and cocultures of Gbm95 and neurons were lysed in TRIZOL and RNA extraction was performed according to the manufacturer directions. Before cDNA synthesis, RNA concentration was measured at 260 nm. For cDNA synthesis, 1μg of RNA was reverse-transcribed using an ImProm II kit following manufacturer directions for 1 h at 42°C. For semi-quantitative PCR, 3 μl of cDNA was mixed with PCR buffer containing 1.5 mM MgCl2, 0.2 mM dNTP, 0.2 μM oligonucleotide sense or anti-sense primers, 0.25 U Taq polymerase and nuclease-free water. The oligonucleotide sequences used were GAPDH (571 bp, 25 cycles) forward 5' ATC ACC ATC TTC CAG GAG CG 3' and reverse 5' CCT GCT TCA CCA CCT TCT TG 3', CTGF (236 bp, 30 cycles) forward 5' GAA GGG CAA AAA GTG CAT CC 3' and reverse 5' GAC AGT TGT AAT GGC AGG CA 3' [48], TGFβ1 (161 bp, 30 cycles) forward 5' GCC CTG GAC ACC AAC TAT TGC T 3' and reverse 5' AGG CTC CAA ATG TAG GGG CAG G 3' [49]. cDNA fragments were

separated by eletrophoresis in a 2% agarose gel pre-stained with ethidium bromide (0.2 μg/ml).

Cell transfection and luciferase activity assay

Glioblastoma ($1.5x10^4$) cells plated in 96-well plate were transfected, immediately before attaching to the substrate, with plasmids CTGF-lux (luciferase reporter containing the entire CTGF promoter in pGL2) and renilla luciferase for efficiency control, using the FuGENE 6 transfection reagent (Roche). DNA/FuGENE mixture was maintained at room temperature for 20 min in DMEM-F12 (Invitrogen) without serum. This mixture was added to the cells at the moment of plating with DMEM-F12 with 10% fetal bovine serum (Invitrogen). CTGF-lux/renilla transfected Gbm95 cells were cocultured 24h after the transfection with $1x10^4$, $5x10^4$ or $2x10^5$ freshly dissociated neurons from P0 rat cortex. Another 24h later, all cells (cocultured or not) were lysed with passive lysis buffer (Promega) and luciferase activity was detected by adding the enzyme substrate provided in the Dual-Glo™ luciferase assay system (Promega) according to the manufacture´s directions. The samples were read in a Veritas microplate luminometer (Turner BioSystems Inc., California, US). In order to normalize the data, the luciferase activity index was calculated by dividing firefly luciferase activity by renilla luciferase activity. Three independent experiments were made and statistical analysis was performed in GraphPad Prism 4.0 software.

RESULTS

CTGF expression in Glioblastoma

In order to investigate CTGF modulation in glioblastoma cells we cultured Gbm95 cells previously generated in our laboratory [23] and compared with other commercial glioma and non-glioma cells in respect to CTGF expression. Gbm95 cultures had a similar aspect to cultures of the commercial cell lines A172 or U87. All cultures showed fibrous morphology at conventional light microscopy (not shown). We combined RT-PCR and immunocytochemistry to investigate CTGF expression in Gbm95, A172 and U87 glioblastoma cell lines. Immunostaining of Gbm95 with anti-CTGF showed expression of the protein on the surface of the cells in a punctate/aggregated pattern (Fig. 1A and B). In order to evidence cell morphology we double-stained Gbm95 with antibodies against CTGF and the intermediate filament Vimentin. We observed an interesting pattern of CTGF expression at the tip of cell processes or borders (Fig. 1A and B). The glial origin of Gbm95 cells was confirmed by the pattern of expression of GFAP mostly around the nuclei, distributed in the cytoplasm in a punctate pattern, different from the typical fiber-like organization found in rat astrocytes [50, 51]. *GFAP* expression was detected by RT-PCR in Gbm95, A172 and U87 cell lines, although an increased number of PCR cycles was required to obtain levels of expression similar to those detected in astrocytes (Fig. 1D, lanes 1-4). We found that astrocytes as well as Gbm95, A172 and U87 express similar levels of *CTGF* transcripts, but the non-glial-derived tumor cell lines U79 and GLC4 did not express *CTGF* (Fig. 1 D). These data show that Gbm95 express the glial marker GFAP as well as CTGF, and is therefore a suitable model for the study of CTGF modulation in neuron-glioblastoma interaction.

CTGF expression is downregulated in glioblastoma cells cocultured with rat neurons

In order to investigate if normal embryonic or neonatal rat neurons could modulate the expression of CTGF in glioblastoma cells, we cocultured E18 and P0 onto Gbm95 cell carpets and performed double immunocytochemistry with anti-CTGF antibody and anti-β tubulin III (Fig. 2).

We observed a dramatic decrease in CTGF staining of Gbm95 cells when cocultured with either embryonic or neonatal neurons (Fig. 2C-F). Surprisingly, we found a strong CTGF staining in embryonic neurons, mostly in the cell body (Fig. 2C and D). Such staining was also found when embryonic neurons were cultured in a tumor-free condition (data not shown). However, CTGF staining in P0 neurons was very weak suggesting that these neurons had lost CTGF expression (Fig. 2E and F).

RT-PCR analysis showed that the level of *CTGF* mRNA was decreased in cocultures of P0 neurons and Gbm95 cells in comparison to pure cultures of Gbm95 cells (Fig. 3A, lanes 1 and 3, and 3B), and slightly decreased when E18 neurons were cocultured onto Gbm95 cells (Fig. 3A, lanes 1 and 2, and 3B). Although we did not find *CTGF* mRNA expression in P0 neurons, it was detectable in E18 neurons (Fig. 3A, lanes 4 and 5, and B). In order to address the total RNA dilution between culture and coculture samples, we counted the number of rat neurons onto Gbm95 cells and found that they represent 5% to 8% of the total cell population in the plate (not shown). We also stained these rat brain cells cocultured with Gbm95 and found that they consisted of 95% β-tubulin III-positive and 5% GFAP-positive cells (not shown). In order to investigate if the CTGF modulation promoted by P0 neurons was a specific feature of Gbm95 cells, we performed coculture experiments using astroglial

monolayers as substrate for E18 and P0 Neurons. As seen in glioblastoma cells, CTGF expression was decreased in astrocytes cocultured with both E18 and P0 neurons (not shown). These results show that CTGF protein and mRNA expression is inhibited in glioblastoma cells cocultured with neonatal neurons.

Neuron-Glioblastoma interaction inhibits CTGF promoter activation

Because our results suggested that neuron-Gbm95 interaction negatively regulates CTGF transcription in Gbm cells, we performed a luciferase reporter assay to test if neurons could inhibit the activation of the CTGF promoter (Figure 4). First, we transfected Gbm95 cells with a reporter luciferase sequence controlled by a full CTGF promoter (CTGF-lux). Transfected Gbm95 cells were cocultured with $1x10^4$, $5x10^4$ or $2x10^5$ freshly dissociated neurons from P0 rat cortex and luciferase activity was measured after 24 h. Inhibition of luciferase activity seemed to be proportional to the number of neurons since cocultures with $5x10^4$ neurons inhibit more than 40% luciferase activity but $2x10^5$ neurons led to more than 50% inhibition (Fig 4). As a positive control for the luciferase reporter assay, we incubated Gbm95-transfected cells with 0.5 nM TGFβ-1 and noticed activation of the CTGF promoter (Fig 4). These results show that neuron-Gbm95 interaction negatively regulates transcription via the CTGF promoter.

Conditioned medium (cm) from E18 and P0 neurons does not affect CTGF expression in glioblastoma cells

In order to test if neuron-secreted factors modulate CTGF expression in gliobastoma cells, we analyzed the effect of conditioned medium from E18 (cmE18) and P0 (cmP0) neurons on

Gbm95 cultures (Fig. 5). Gbm95 monolayers were incubated for 24 hours with cmE18 or cmP0 and we assayed CTGF by immunocytochemistry and RT-PCR. As seen in figure 1A, CTGF immunostaining revealed a punctate/aggregated pattern in both Gbm95 and Gbm95 treated with cmE18 or cmP0 (Fig. 5A, B and C). RT-PCR analysis showed that the level of expression of CTGF transcripts was not altered when Gbm95 cells were incubated with cmE18 or cmP0 (Fig. 5D).

Inhibition of CTGF in Gbm95 cells by P0 neurons does not affect TGFβ/SMAD2/3

We tested whether the TGFß1/SMAD2/3 signalling pathway was involved in CTGF inhibition by P0 neurons by analyzing Smad2/3 phosphorylation in western blots of Gbm95 and Gbm95/P0 cocultures (Fig. 6A and B). In this assay, neurons were maintained in coculture with Gbm95 cells for 30 min in order to detect SMAD 2/3 phosphorilation. As expected, addition of 500 pM TGFβ1 to Gbm95 cultures for 30 min dramatically increased the levels of SMAD2/3 phosphorylation (Fig. 6A, lane 3, and 6B) showing that these cells contain a functioning TGFβ signaling machinery. However, we did not detect differences in SMAD2/3 phosphorylation between Gbm95 and Gbm95 cocultured with P0 neurons (Fig. 6, lanes 1 and 2; 6B). CTGF protein levels were also decreased when P0 neurons were cocultured onto Gbm95 cells for 30min (Fig. 6, lanes 1-2). Inhibition of CTGF expression in Gbm95 cells by P0 neurons seems not to depend on TGFβ1/SMAD2/3 signalling cascade since cocultures of Gbm95/P0 neurons did not have altered basal levels of SMAD2/3 phosphorylation relative to Gbm95 pure cultures (Fig. 6, lane 2; 6B).

p44/42-MAPK phosphorylation decreases during Gbm95/P0 interaction

Since the MAPK signaling pathway has been also shown to modulate CTGF expression, we next measured the levels of phospho p44/42-MAPK by western blot (Fig. 6C and D). We detected p44/42-MAPK and its phosphorylated form in both Gbm95 and Gbm95/P0 cocultures, but not in cultures of P0 neurons alone (Fig. 6C, lanes 1-3 and D). However, the level of phospho p44/42-MAPK was decreased in Gbm95 cocultured with P0 neurons for 30min compared to Gbm95 cells alone (Fig. 6C, lane 2 and D). Altogether, these results indicate that the inhibition of CTGF expression in Gpm95 cells by P0 neurons may not involve the TGFβ1/SMAD2/3 pathway, but might be mediated by negative regulation of MAPK/ERK signaling.

DISCUSSION

In the present study we investigated the modulation of the expression of the matricellular molecule CTGF during neuron-glioblastoma *in vitro*. Our results show that CTGF transcription and transduction is negatively regulated in Gbm95 cells in the presence of normal neonatal neurons. This downregulation of CTGF seems to require Gbm95/neuron contact and seems not to involve the TGFβ/SMAD2/3 pathway, a major regulator of CTGF expression, but may involve at least one effector of MAPK signaling, p44/42 MAPK (ERK1/2), which is negatively affected during this interaction.

In our previous work, we demonstrated that human glioblastoma cells (Gbm95) can recapitulate neuron-astrocyte interaction by supporting neurite growth. We also showed that laminin has its distribution remodeled from a fiber-like to an aggregate pattern in Gbm95 cells or astrocytes cocultured with neonatal neurons [23]. Here we describe further the neuron-Gbm95 interaction. First, we show that Gbm95 cells synthesize the secreted ECM-associated protein CTGF and its RNA. Indeed, it has been demonstrated *in vivo* that not only neoplastic GFAP-positive astrocyte-like cells express CTGF, but even that GFAP-negative cells in the tumor can also express CTGF [25]. Despite the differences between *in vivo* and *in vitro* microenvironments, it seems that glioblastoma cells can maintain their ability to express glial phenotypes and keep some functional properties of non-neoplasic glia.

It has been shown that CTGF contains a unique TGFβ-inducible element on its promoter, pointing to TGFβ1 as a stronger inducer of CTGF [52]. In addition, we demonstrated more recently that the same protein domain of CTGF binds TGFβ1 and BMP2/4, but these interactions result in different modulations of these signalling pathways

[31, 38]. CTGF enhances the binding of TGFβ1 to its cognate receptors and increases SMAD2/3 phosphorylation [31]. Gbm95 cells are responsive to TGFβ1 leading to expression of high levels of *ctgf* transcripts. Although *ctgf* transcription in Gbm95 cultures was decreased in the presence of P0 neurons, we found that P0 neurons did not affect TGFβ/SMAD signalling, suggesting that another signalling pathway must be involved, probably one that is contact-mediated, since increasing numbers of neurons cultured over Gbm95 cells enhanced inhibition of the *ctgf* promoter, but neuronal conditioned medium had no effect.

MAPK signaling pathways are known to be involved with CTGF actions such as proliferation and differentiation and in CTGF-mediated adhesion [42, 43, 53, 54]. These studies have focused mainly on cytoskeletal rearrangement/integrity and focal adhesion induced by CTGF that is triggered by its interaction with ECM-related molecules [44, 54, 55]. It is believed that TGFβ is able to signal via Ras and Rac proteins and activate MAP kinases, including ERK1 and 2 and JNK [39]. Chen and coworkers showed that maximal TGFβ induction of CTGF expression requires synergy between SMAD and Ras/MEK/ERK signalling [40]. Recently, Shimo and coworkers [56] showed that CTGF is important for chondrocyte maturation and that its expression during this process was regulated positively by ERK1/2 and negatively by p38 MAP kinases. We show that contact with P0 neurons downregulates CTGF expression in Gbm95 cells concomitantly with a decrease in ERK1/2 phosphorylation. Although we could not detect activation of TGFβ/SMAD2/3 during neuron-Gbm95 interaction, the inhibition of Erk1/2/MAPK could still be related to an involvement of non-canonical TGFβ signaling.

It can be argued that the heterotypic (human *vs*. rat) coculture conditions weaken our findings. However, similar neurite growth and regulation of CTGF expression was observed when rat astrocyte-neuron cocultures were employed (not shown), suggesting conservation of this interaction. It is striking that the strongest CTGF inhibition observed in Gbm95 cells was induced by neonatal, but not by embryonic neurons. One possible explanation would be that contact-mediated interactions with embryonic versus neonatal neurons involve different molecules that the tumor cells can not distinguish. Indeed, our previous work showed that astrocytes are better substrate for embryonic than for post-natal neurons [23] and have also shown that neurons can secrete specific soluble factors which induce GFAP promoter gene suggesting that neuron-glia interaction can induce the astrocytic differentiation program [51]. However, Gbm95 cells support neurite outgrowth of both embryonic and post-natal neurons [23] supporting the idea that Gbm95 cells can not interpret all the signals produced by embryonic or post-natal neurons.

Glioma cell invasion is a complex and multistep mechanism involving a large array of molecules and cell-cell and cell-ECM interactions. Gbm95 is usually located in one of the cerebral hemispheres, with an epicenter in the white mater, but frequently invades bilaterally the subcortical white matter through the corpus callosum. Rapid spread is observed also into the white matter of the internal capsule, fornix and anterior commissure [19]. Thus, the modulation of signalling pathways in tumor-normal brain interactions is likely to be of great importance in explaining specific invasion patterns of gliomas, including along white matter tracts, into the subpial space and along the external walls of blood vessels.

It has been shown that breast cancer cell subpopulation with highly metastatic activity overexpress CTGF gene and other secreted and cell surface molecules that act cooperatively

in a multigenic osteolytic metastasis [57]. Although very little is known regarding CTGF function on glioblastoma cells it has been found that CTGF mRNA is 58% increased in primary gliomas compared to normal brain samples [24]. Furthermore, there was significant correlation between CTGF mRNA levels in these tumors with tumor grade and pathology suggesting that CTGF may play a role in the progression of gliomas [24]. Thus, our results pointing CTGF inhibition in glioblastoma through neuronal contact highlight the importance of the interactions between tumor and normal cells for progression and invasiveness properties of glioblastoma multiform.

In summary, our present results show that neuron-Gbm95 interaction negatively modulates CTGF. This inhibition occurs at the transcriptional level, seems to depend on cell contact and may involve MAPK signaling. Together these data could contribute to a better knowledge of the glioblastoma behavior in the brain microenvironment, and strongly suggest that glial tumors can maintain fundamental properties of glial cell interaction with neurons.

ACKNOWLEDGEMENTS

We thank Dr. Suzana Herculano Houzel for discussions and comments on the manuscript and Adiel Batista for animal care. CTGF-lux plasmide reporter was a gift from Dr. Karen Lyons's laboratory (UCLA). This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ), Programa de Núcleos de Excelência (PRONEX).

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FIGURE LEGENDS

Figure 1. **CTGF is expressed by glioblastoma cells.** Double immunostaining showing CTGF (A, B red) and vimentin (B green) expression. GFAP immunostaining of Gbm95 cells (C). Nuclei-DAPI staining is shown in B and C. Scale bars (A, B, C) 50 μm. RT-PCR analysis of CTGF and GFAP expression of astrocytes, Gbm95, A172, U87 MG, U79 and GLC4 cells (lanes 1-6). Base pair numbers appear on the right side of each gel. GAPDH was used as loading control.

Figure 2. **CTGF immunoreactivity is decreased in cocultures of glioblastoma cells with neurons.** Immunocytochemistry showing CTGF expression (red) in Gbm 95 cultures (A, B) and cocultures of Gbm95 with E18 (C, D) or P0 neurons (E, F). β-tubulin III stained (green) of neurons are shown in (D, F). Arrowhead shows a CTGF stained E18 neuron (C). Notice that CTGF staining decreased in cocultures (compare A with C, E). Bar 50 μm.

Figure 3. **CTGF mRNA is downregulated in cocultures of glioblastoma cells with neurons**. RT-PCR analysis of CTGF expression in Gbm95 cocultured with E18 or P0 neurons (A). Base pairs numbers appears on the right side of each gel. GAPDH was used as loading control. Histogram expressing arbitrary units (A.U) product obtained from CTGF over GAPDH of the PCR bands (B). Gbm95 cocultured with P0 neurons CTGF expression was decreased when compared with Gbm.

Figure 4. **CTGF promoter activity is inhibited when neurons are cocultured with Gbm95 cells.** Graph shows Firefly/Renilla luciferase activity of Gbm95 cells transfected with CTGF-lux and Renilla plasmids cocultured with different amounts of freashly dissociated neurons. Note that $5x10^4$ neurons were able to inhibit more than 40% of luciferase activity (compare first to third bar, $p<0.05$) and $2x10^5$ neurons were able to inhibit more than 50% of luciferase activity controlled by CTGF promoter (compare first to fourth bar, $p<0.01$). As a positive 0.5nM of TGFβ-1 was used and we could observed an increase of almost 40% of luciferase activity (compare first to fifth bar, $p<0.05$).

Figure 5. **Condiotioned medium from E18 and P0 neurons does not affect CTGF expression in glioblastoma**. CTGF immunocytochemistry of Gbm95 cells (A) treated with conditioned medium (cm) from E18 (B) or P0 neurons (C). Bar 50 μm. (D) RT-PCR Analysis of CTGF expression in Gbm95 cultured for 24 h with cmE18 and cmP0 neurons. Base pairs number appears on the right side of each gel. GAPDH was used as loading control. CTGF expression was not changed in Gbm95 cells cultured in neuronal conditioned medium.

Figure 6. **Smad2/3 phosphorylation is not changed, but phospho-p44/42 MAPK decreases in Gbm cells cocultured with P0 neurons**. (A) Western blot analysis of the cell extracts from Gbm95 (lane 1) and Gbm95 cocultured with P0 neurons (lane 2) and Gbm95 in presence of 500 pM TGFβ1 recombinant protein (lane 3). From top to bottom, the pictures show membranes reacted with anti-phospho SMAD2/3, anti-CTGF and anti-αTubulin antibodies. Histogram expressing the arbitrary units (A.U) product obtained from SMAD2/3

over α-Tubulin bands (B). Notice that P0 neurons did not affect SMAD2/3 phosphorylation and that Gbm cells are responsive to TGFβ1 protein. (C) From top to bottom, membranes were reacted with anti-phospho-p44/42 MAPK and anti-p44/42 MAPK. Histogram expressing the AU product from p44/42 MAPK over MAPK bands (D). Levels of total p44/42 MAPK did not change, but phospho p44/42 decreased in Gbm95/P0 cocultures. Molecular weight in KDa is showed on the left side of the picture.

Figure1

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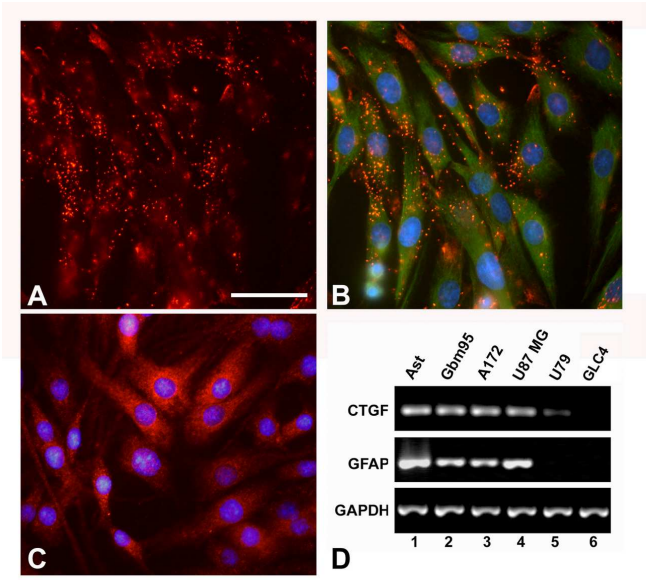

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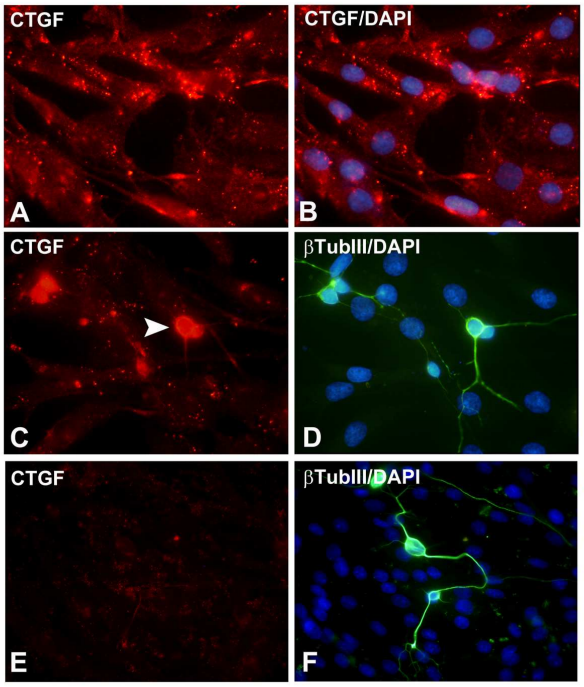

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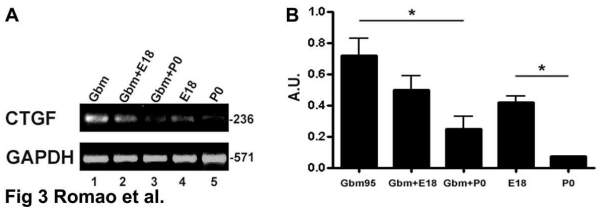

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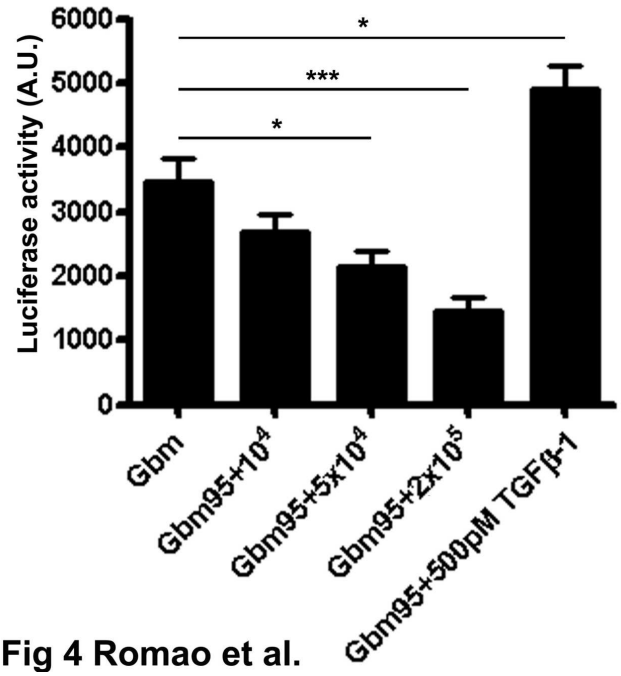

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Figure5

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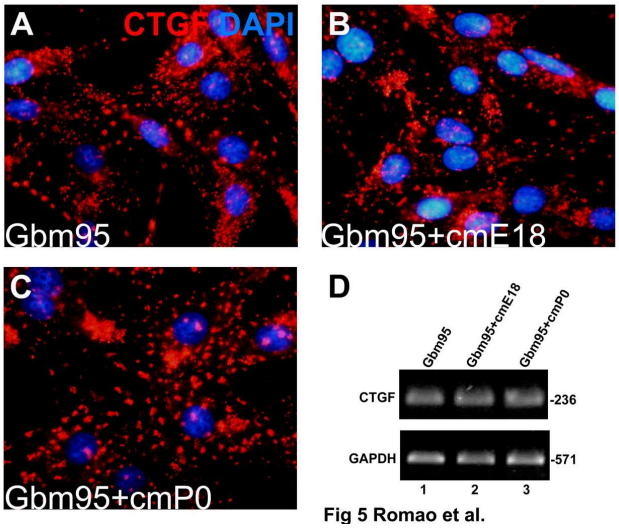

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Figure6

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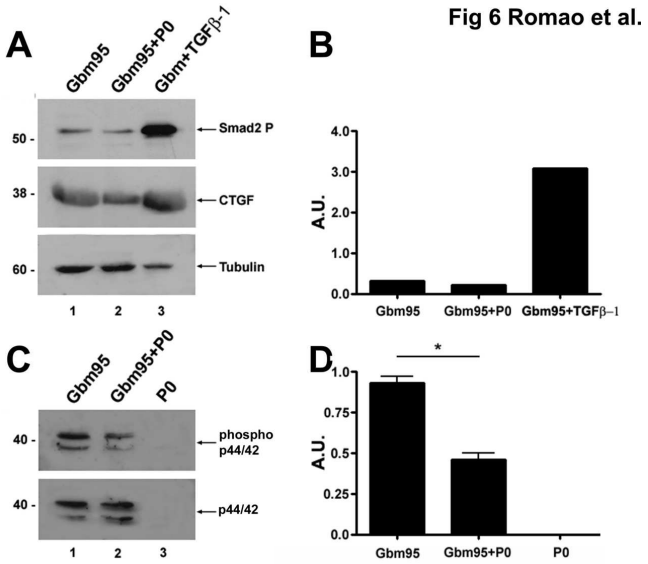

*** Reviewer suggestions**

September, 18th 2007.

Professor Martin Schwab, Editor-in-chief
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Dear Professor Schwab

Bellow you will find some names suggested as reviewers for the manuscript entitled "**CONNECTIVE TISSUE GROWTH FACTOR (CTGF) IS NEGATIVELY REGULATED DURING NEURON-GLIOBLASTOMA INTERACTION**" by Romão *et al*

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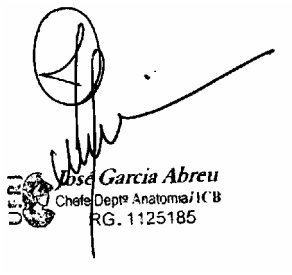

DISCUSSÃO

4. DISCUSSÃO

Este trabalho de tese analisou as propriedades interativas entre células gliais normais e tumorais com neurônios. Vamos discutir aqui nossos resultados no contexto da literatura, tomando, parte à parte, a **interação neurônio-glia**, que contribui para entender a **diferenciação astrocitária** durante o **desenvolvimento** do SNC e em seguida, discutiremos a **interação neurônio-glia tumoral,** evento crucial para o entendimento de **processos patológicos** do sistema nervoso.

Através da nteração neurônio-astrócito, demonstramos que neurônios de córtex cerebral ativam o promotor do gene de GFAP, através da indução da secreção do fator de crescimento transformante-beta 1 (TGF-β1) pelos neurônios e astrócitos. Neurônios corticais ou meio condicionado por essas células não ativam o promotor do gene de GFAP de astrócitos derivados de mesencéfalo e cerebelo, sugerindo uma especificidade regional deste fenômeno. A síntese de TGF-β1 em astrócitos é induzida pelo neurotransmissor excitatório, glutamato. Demonstramos que a ativação do gene de GFAP por glutamato é mediada pela síntese de TGF-β1, através da ativação do receptor glial mGlu2/3R e ativação das vias de sinalização MAPK e PI3K. Nosso trabalho é pioneiro em demonstrar que TGF-β1 é um mediador das ações do glutamato na diferenciação astrocitária.

Na interação neurônio-célula glial tumoral, utilizamos células derivadas de glioblastoma multiforme humano (Gbm), tumor cerebral maligno cuja adaptação ao microambiente do SNC envolve a produção e remodelamento da MEC. Para isso, neurônios de rato foram cultivados sobre astrócitos de rato ou sobre três linhagens de células de Gbms humanos. Observamos que os Gbms humanos mantêm as propriedades interativas com os neurônios, sendo capazes de sustentar a neuritogênese dessas células. A organização da laminina, uma das principais

moléculas da MEC astrocitária, foi alterada de um padrão filamentoso para um mixto puntiforme/filamentoso, quando astrócitos e/ou Gbms foram cultivados com neurônios. Com o objetivo de analisar os mecanismos de interação entre neurônios e Gbms, focamos em 2 fatores de crescimento que agem em sinergismo em diversas patologias do SNC, CTGF e TGF-β1. Demonstramos que neurônios inibem a síntese de CTGF em células de Gbm através da inibição da via de sinalização de MAPK. Esse evento independente da via de sinalização de TGF-β1.

Em conjunto, nossos dados demonstram que neurônios são capazes de influenciar as células gliais normais e tumorais induzir através da secreção de fatores.

4.1 Interação neurônio-glia: diferenciação astrocitária

Uma característica fundamental no desenvolvimento neural em vertebrados é que a grande diversidade celular é gerada em uma seqüência; primeiros neurônios, seguidos por astrócitos e oligodendrócitos (Levitt e Rakic, 1980). Os mecanismos utilizados na determinação do destino dos progenitores durante o desenvolvimento ainda não estão bem entendidos, mas inclui mudanças nas propriedades intrínsecas dos progenitores neurais e em seu microambiente (Guillemot, 2007).

A especificação celular neuronal e glial representam um modelo complexo de interações entre múltiplos mecanismos de sinalização, fatores de transcrição e mecanismos epigenéticos no controle do destino celular (Guillemot, 2007). A diferenciação astrocitária é um processo controlado pelo balanço entre a ativação de alguns genes e por fatores epigenéticos (Lee *et al.*, 2000). Dentre esses fatores, os principais são representados pelas interações que as células farão ao longo do desenvolvimento.

A maior parte dos astrócitos do córtex cerebral de diversas espécies origina-se de células de glia radial (Levitt e Rakic, 1980; Culican *et al.,* 1990; Bentivoglio e Mazzarello, 1999; Gotz e Barde, 2005; Mori *et al*., 2005). Após o fim da migração neuronal, essas células retraem seus prolongamentos e diferenciam-se em astrócitos por processos ainda não completamente estabelecidos (Schmechel e Rakic, 1979; Munoz-Garcia e Ludwin, 1986; Voigt, 1989). Atualmente, o termo "glia" radial tem sido discutido uma vez que hoje essa célula é considerada, além de progenitor glial, o principal progenitor de neurônios do córtex cerebral (Alvarez-Buylla *et al*., 2001; Noctor *et al.*, 2002; Malatesta *et al.*, 2003; Anthony *et al.*, 2004; Merkle *et al.*, 2004).

O fato de neurônios e células de GR e/ou astrócitos apresentarem um íntimo contato ao longo do desenvolvimento do SNC sugere que sinais provenientes de neurônios possam modular a diferenciação astrocitária. De fato diversas moléculas e vias de sinalização induzidas por neurônios têm sido envolvidas no controle da determinação de progenitores com o destino astroglial. Aparentemente, as vias de sinalização notch e neuregulina 1-Erb2 têm um papel relevante na manutenção de progenitores em um estado não diferenciado durante a fase neurogênica do desenvolvimento do SNC (Mizutani e Saito, 2005). A inibição dessas vias durante o processo de migração neuronal e laminação no córtex cerebral resulta na transformação prematura de glia radial em astrócitos e conseqüentes deficiências na formação das camadas corticais (Schmid *et al*., 2003; Patten *et al*., 2003; 2006).

Mais recentemente, a função de Notch no desenvolvimento neural mostrou-se mais complexa dependendo do estágio de desenvolvimento e da estrutura cerebral estudada. Através de ensaios utilizando a forma ativada de Notch *in vivo*, Gaiano e colaboradores demonstraram que a sinalização de Notch promove astrogênese no

cérebro de adulto (Gaiano *et al.,* 2000), e análise da função de Notch em progenitores hipocampais adultos indicou que Notch promove a diferenciação de progenitores multipotentes em astrócitos (Tanigaki *et al.,* 2001). Diversas outras moléculas têm sido apontadas como astrocitogênicas como LIF, CNTF e BMPs (Gross *et al.,* 1996; Nakashima *et al.*, 2001).

A diferenciação astrocitária em roedores é caracterizada *in vivo* e *in vitro* pela expressão de marcadores específicos como a proteína de filamento intermediário, GFAP. Seu promotor tem sido amplamente utilizado por nosso grupo como ferramenta para examinar os mecanismos moleculares que controlam a especificação e diferenciação do destino astrocitário (Gomes *et al.,* 1999; de Sampaio e Spohr *et al.,* 2002; **Sousa e Romão *et al.,* 2004**). Com o objetivo de estudar o papel de moléculas solúveis derivadas de neurônios (fatores de crescimento e neurotransmissores) na diferenciação astrocitária *in vitro*, estabelecemos um modelo de cultura de astrócitos derivados de animais transgênicos contendo o gene da enzima bacteriana β-galactosidase sob a regulação do promotor do gene da proteína GFAP murina (Galou *et al.,* 1994). Demonstramos previamente que neurônios corticais ativam o promotor de GFAP e induzem a diferenciação de astrócitos de córtex cerebral *in vitro* através da secreção de TGF-β1 (Gomes *et al.,* 1999; de Sampaio e Spohr *et al*., 2002). Neste trabalho de tese, investigamos a especificidade regional deste evento. Relatamos que a influência neuronal na diferenciação astrocitária, no que diz respeito à expressão de GFAP, é dependente da estrutura encefálica estudada. Embora neurônios corticais induzam a síntese de TGF-β1 por astrócitos de cerebelo e mesencéfalo, esse fator não ativa o promotor do gene de GFAP em mesencéfalo e o reprime em cerebelo.

Nossos dados sugerem que os mecanismos de ativação do gene de GFAP e da diferenciação astrocitária diferem nas diferentes regiões do SNC.

Neste trabalho, também analisamos o efeito do principal neurotransmissor excitatório do SNC, glutamato, na diferenciação astrocitária. Dados prévios de outros grupos demonstraram que glutamato induz a síntese e secreção de membros da família TGF-β por astrócitos, representando um papel importante na proteção dos neurônios contra a toxicidade do glutamato (Bruno *et al*., 1998; D´Onofrio *et al*., 2001). Aliado a esse fato recentemente, diversas evidências vêm apontando significativamente para o envolvimento dos astrócitos na transmissão sináptica (Pfrieger e Barres, 1997; Araque *et al*., 1999; Mauch *et al*., 2001; Nägler *et al*., 2001, Ullian *et al*., 2001; 2004; Christopherson *et al*., 2005; Elmariah *et al*., 2005; Goritz *et al*., 2005; Nishida e Okabe, 2007). Desta forma, investigamos o envolvimento de elementos sinápticos e neurotransmissores na diferenciação astrocitária *in vitro*. Demonstramos que o neurotransmissor glutamato induz a diferenciação astrocitária, de forma regionalmente específica, através da modulação da via de sinalização de TGF-β1.

4.2 TGF-β1 na diferenciação astrocitária

A família TGF-β é representada por três isoformas: TGF-β1, -β2 e -β3, todos produzidos por células gliais e neuronais. A sinalização de TGF-β é principalmente mediada por dois receptores serina treonina cinase, TGFRI e TGFRII, que ativam os fatores de transcrição SMAD 2/3 e SMAD 4. Fosforilação e ativação destas proteínas são seguidas pela formação do complexo Smad 2/3-4, que transloca-se para o núcleo regulando os genes alvo de TGF-β (Massagué e Gomis, 2006).

Membros da família TGF-β1 estão envolvidos em uma série de eventos cruciais para o desenvolvimento dos metazoários como proliferação e diferenciação celular, migração e cicatrização (Massagué, 2000; Massagué e Gomis, 2006). No SNC, a maior parte dos dados a cerca dos membros dessa família relaciona-se às lesões e patologias cerebrais. A isoforma TGF-β1 está relacionada à injúria, associada à formação da cicatriz astrocitária em resposta a danos no cérebro (da Cunha *et al*., 1993; Pratt e McPherson, 1997; Flanders *et al.*, 1998; Moon e Fawcett, 2001). Recentemente, diversas evidências vêm demonstrando um papel crucial para a via de sinalização de TGF-β1 no desenvolvimento de SNC (**Gomes *et al.,* 2005; Anexo 1**).

Diversos processos da biologia astrocitária são potenciais alvos de TGF-β1 como motilidade, morfologia e proliferação astrocitárias (Labourdette *et al.,* 1990; Gagelin *et al.,* 1995). *In vitro*, TGF-β1 causa mudanças na morfologia astrocitária incluindo indução da formação de ramificações e hipertrofia astrocitária (Labourdette *et al.,* 1990). Além disso, TGF-β1 estimula a produção de laminina e fibronectina e a incorporação destas na MEC (Baghdassarian *et al.,* 1993; Wyss-Coray *et al.,* 1995). Mais recentemente, o papel de TGF-β1 na produção da MEC foi corroborado pela demonstração de que animais *knockout* para TGF-β1 apresentam reduzida expressão de laminina (Brionne *et al*., 2003).

Além dos efeitos mencionados sobre a diferenciação e produção de MEC dos astrócitos, TGF-βs são descritos como inibidores da proliferação astrocitária, podendo inibir a proliferação diretamente, aumentando ou antagonizando a ação de outros fatores de crescimento (Toru-Delbauffe *et al.,* 1990; Baghdassarian *et al.,* 1993; Krieglstein *et al*., 1998a;1998b; 1998c).

Evidências apontaram recentemente, TGF-β1 como um novo mediador das interações neurônio-glia, especialmente durante a diferenciação astrocitária (**Gomes *et al*., 2005, Anexo 1**). Um caminho para estudar a diferenciação astrocitária é avaliando os níveis de proteínas que variam durante o desenvolvimento astrocitário como GFAP (Eng *et al.,* 1971; Bignami *et al.*, 1972), a enzima glutamina sintetase (Kvamme *et al.,* 1982) e transportadores de glutamato (Wurdig e Kugler, 1991). Para estudar a diferenciação astrocitária induzida por neurônios, nosso grupo tem analisado a ativação do promotor de GFAP de camundongos transgênicos contendo uma parte do promotor do gene de GFAP murino ligado a um gene repórter, β-galactosidase (β-Gal). Trabalhos prévios demonstraram que neurônios corticais ativam o promotor do gene de GFAP seguido por uma diferenciação astrocitária *in vitro*. Este evento é dependente da origem dos neurônios no cérebro, seguido por uma diminuição na proliferação dos astrócitos e indução da diferenciação glial. A adição de meio condicionado (MC) derivado de neurônios corticais resultou em efeito semelhante, sugerindo que um fator solúvel derivado de neurônios podia ser responsável pela indução do promotor do gene de GFAP (Gomes *et al.*, 1999a). Posteriormente, identificamos TGF-β1 como o principal mediador deste evento (de Sampaio e Spohr *et al.,* 2002). Ambos os tipos celulares, neurônios e astrócitos, sintetizam e secretam este fator, porém, a adição de neurônios à monocamada astrocitária aumenta a síntese e secreção de TGF-β1 por astrócitos (de Sampaio e Spohr *et al.*, 2002).

Nesta tese, demonstramos que esse evento apresenta uma especificidade regional. A ausência de efeito do TGF-β1 sobre os astrócitos cerebelares e mesencefálicos está de acordo com o reportado na literatura. Sabe-se que TGF-β1 nao é capaz de aumentar os níveis de GFAP em astrócitos cerebelares em cultura;

um pequeno aumento foi observado somente depois de um longo período na presença do fator (Baghdassarian *et al*., 1993). Embora o MC de neurônios corticais não tenha ação sobre a expressão de GFAP em astrócitos cerebelares, o contato destas células com os neurônios corticais promove um decréscimo no número de células β-Gal positivas. Uma possível explicação para a ação neuronal, é que este mecanismo seja mediado pelo contato celular. O contato entre as células foi descrito como relevante em processos como inibição da proliferação astrocitária. Experimentos *in vitro* mostraram que o contato neurônio-glia inibe a síntese de DNA de astrócitos derivados do cerebelo de camundongos pós-natais (Hatten, 1987). Posteriormente, a molécula neural de adesão celular, N-CAM, expressa por neurônios e astrócitos, foi identificada como inibidora da proliferação celular mediada por contato entre as células (Krushel *et al*., 1998). É possível que uma molécula de contato esteja envolvida na inibição nos níveis de expressão de GFAP no cerebelo.

Verificamos que neurônios corticais ativam o promotor do gene de GFAP de astrócitos de córtex cerebral, mas não de cerebelo e mesencéfalo. Três possibilidades foram analisadas para explicar este evento: 1) ausência do receptor tipo II para TGF-β1 nesses astrócitos; 2) incapacidade dos neurônios corticais induzirem a síntese e secreção de TGF-β1 por astrócitos cerebelares e mesencefálicos; 3) concentração de TGF-β1 sub-ótima para ativação do promotor do gene de GFAP de cerebelo e mesencéfalo (**Sousa e Romão *et al*., 2004**).

Wrana e colaboradores (1992) mostraram a importância da interação entre os receptores tipo I e tipo II para a ativação da via de sinalização de TGF-β1: o TGFRI requer o TGFRII para se ligar ao fator, enquanto o TGFRII precisa do TGFRI para sinalizar. Esta interdependência revela a importância da presença do receptor tipo II, sendo o primeiro membro da sinalização, necessário para que o receptor tipo I

propague o sinal. A presença da proteína TGFRII no SNC tem sido relatada por diversos grupos (Tomoda *et al*., 1996; Vivien *et al*., 1998; Böttner *et al*., 1996, 2000; Massagué, 2000; Sousa *et al.,* 2006). Surpreendentemente, todos os tipos astrocitários estudados no presente trabalho apresentam o receptor. Não observamos diferenças significativas nos níveis de TGFRII em astrócitos corticais e mesencefálicos, enquanto em astrócitos cerebelares apresentam uma expressão maior deste receptor. Estes dados estão de acordo com os mostrados por Böttner e colaboradores (1996) que descreveram níveis maiores de TGFRII em cerebelo, por RT-PCR, quando comparado com outras regiões encefálicas.

Mostramos previamente que o nível de expressão de TGF-β1 em astrócitos corticais é aumentado em presença de neurônios e este aumento resulta na indução da expressão de GFAP (de Sampaio e Spohr *et al*., 2002). Esta retroalimentação positiva, onde o TGF-β1 é capaz de regular sua própria síntese, já foi descrita no SN e em outros sistemas (Morgan *et al*., 2000; Diez-Marques *et al*., 2002). A não indução da síntese de TGF-β1 poderia ser responsável pela incapacidade de astrócitos cerebelares e mesencefálicos responderem aos neurônios corticais (possibilidade 2, citada na página acima). No entanto, os neurônios do córtex cerebral são capazes de aumentar a expressão de TGF-β1 nos astrócitos das diferentes regiões. Observamos que astrócitos cerebelares já apresentavam um nível basal mais elevado de TGF-β1 que os astrócitos corticais e mesencefálicos. Além de regular sua própria síntese, o TGF-β1 pode regular positivamente a síntese de seu receptor (Morgan *et al*., 2000). Assim, é possível que este nível mais elevado de TGF-β1 seja responsável pelos altos níveis de TGFRII em astrócitos cerebelares.

Além de caracterizarmos a expressão de TGF-β1 em astrócitos, mostramos a expressão desse fator em neurônios cerebelares e mesencefálicos. A distribuição

dos membros da subfamília TGF-β1 no SNC foi caracterizada em embriões de camundongos. Outros autores observaram marcação para TGF-β2 e TGF-β3 ao longo de todo SNC, como encéfalo e medula espinhal (Flanders *et al*., 1991; Unsicker e Strelau, 2000). No SN adulto, a marcação para TGF-β2 e 3 também está presente (Flanders *et al*., 1991). Recentemente, Miller (2003) mostrou células imunomarcadas para TGF-β1 na parede cerebral de ratos embrionários e adultos. Previamente, nosso grupo descreveu a expressão e secreção de TGF-β1 em astrócitos e neurônios do córtex cerebral (de Sampaio e Spohr *et al*., 2002). Nessa tese, estendemos a identificação da expressão dessa citocina para outras regiões do SNC (córtex cerebral, mesencéfalo e cerebelo). Demonstramos por imunocitoquímica e *western blot* que neurônios do córtex, cerebelo e mesencéfalo mantidos *in vitro* sintetizam TGF-β1.

Em conjunto, nossos dados sugerem que a não responsividade do promotor de GFAP de cerebelo e mesencéfalo ao TGF-β1 não esteja relacionada a ausência de expressão de membros dessa via de sinalização. Em termos funcionais, análises futuras nos níveis de fosforilação e translocação nuclear das proteínas SMAD deverão contribuir para a elucidação da via TGF-β-SMAD em outras regiões encefálicas.

Outra possível explicação para o fato de neurônios corticais não estarem induzindo a expressão de GFAP em astrócitos cerebelares e mesencefálicos seria a necessidade de uma concentração maior de TGF-β1 nestas células (possibilidade 3). Este não parece ser o caso, uma vez que mesmo a adição de altas concentrações de fator não ativa o promotor de GFAP em cerebelo e mesencéfalo.

Desta forma, apesar de astrócitos cerebelares e mesencefálicos apresentarem a maquinaria celular e molecular, TGF-β1 e TGFRII, para

responderem aos neurônios, a atividade do promotor do gene de GFAP não é estimulada por neurônios e/ou pelo fator.

Diversas evidências sugerem que astrócitos isolados de várias regiões de cérebro diferem nas repostas a vários agentes, como hormônios, fatores de crescimento e neurotransmissores (Dennis-Donini *et al.*, 1984; Cholewinski e Wilkin, 1988; Garcia-Abreu *et al.*, 1995; Lima *et al.*, 1998; Perego *et al.*, 2000; Gomes *et al.*, 2001a,b; Schluter *et al.*, 2002; Matthias *et al.*, 2003; Reuss *et al.*, 2003). Nossos dados contribuem para esta visão, demonstrando que astrócitos derivados de regiões distintas do cérebro modulam o promotor do gene de GFAP diferentemente em resposta a fatores secretados por neurônios. Resultados semelhantes referentes à heterogeneidades astrocitária foram obtidos em reposta a outros fatores de crescimento utilizados no nosso trabalho, como EGF e FGF (Reuss *et al.*, 2000; 2003; Schluter *et al.*, 2003).

O EGF tem ação ao longo de todo o SNC no desenvolvimento (Fricker-Gates *et al.*, 2000; Martinez e Gomes, 2002), e também possui uma ação região-específica descrita, podendo estimular a expressão de transportadores de glutamato em astrócitos derivados do corpo estriado, mas falhando em induzir este fenômeno em astrócitos de cerebelo, mesencéfalo e medula espinhal (Schluter *et al.*, 2003). Da mesma forma que o FGF, no nosso sistema, somente astrócitos mesencefálicos, e não corticais e cerebelares, responderam ao EGF (**Sousa e Romão *et al.*, 2004**).

Como já discutido anteriormente, as células gliais apresentam uma grande heterogeneidade morfológica e funcional (Emsley e Macklis, 2007). Astrócitos derivados de diferentes regiões do cérebro e de diferentes idades exibem propriedades bioquímicas, farmacológicas e, portanto funcionais diferentes (Moura Neto *et al.*, 1983). Astrócitos de hipocampo e medula espinhal diferem drasticamente

na sua capacidade de induzir neurogênese a partir de células tronco adultas (Song *et al*., 2002). Além das diferenças entre as regiões encefálicas, há ainda diferenças dentro de uma mesma região. Enquanto astrócitos derivados da porção lateral do mesencéfalo embrionário são permissivos ao crescimento neurítico, aqueles derivados da porção medial são inibitórios (Garcia-Abreu *et al*., 1995). Nosso grupo sugere que esta diferença no suporte do crescimento neurítico seja devida à expressão distinta e balanceada das concentrações de glicosaminoglicanos por estas células, sendo a glia mesencefálica lateral mais permissiva ao crescimento neurítico por apresentar maior expressão da molécula de MEC, condroitim sulfato (Mendes *et al*., 2003). Corroborando a idéia de heterogeneidade astrocitária dentro de uma mesma região encefálica, Matthias e colaboradores (2003) descreveram a coexistência de tipos astrocitários no hipocampo de camundongos com propriedades morfológicas, moleculares e funcionais distintas.

Recentemente, nosso grupo demonstrou o papel da interação neurônio-glia mediado por TGF-β1 na determinação do fenótipo astrocitário (Stipursky e Gomes, 2007). Demonstramos que culturas de progenitores corticais, enriquecidas em células com marcadores de glia radial, adotam um fenótipo astrocitário quando tratadas por TGF-β1 ou meio condicionado de neurônios.

É importante considerar que as estruturas cerebrais estudadas nessa tese apresentam estágios de desenvolvimento diferentes, mas ainda, a gliogênese e modulação da expressão de GFAP ocorre *in vivo* de forma diferente nas regiões estudadas. No cerebelo, por exemplo, GFAP é expresso na glia de Bergmann, considerada morfologicamente semelhante à glia radial do córtex cerebral. A expressão de GFAP na glia de Bergmann ocorre mesmo no período embrionário quando não se observa GFAP na maior parte da glia radial de roedores (Bignami e

Bahl, 1974; Ajtai e Kálmán, 1998; Shu e Richards, 2001). Além da questão relacionada ao desenvolvimento, como discutido e mostrado por nós e outros autores, existe uma grande heterogeneidade do padrão da resposta astrócitária a fatores tróficos (Cholewiski e Wilkin, 1988; Laping *et al.*, 1994; Miller *et al.*, 1994; Shao *et al.,* 1994). Assim, outra possibilidade é que astrócitos cerebelares e mesencefálicos respondam ao TGF-β1, não com ativação do promotor de GFAP, mas com outro tipo de resposta funcional, por nós não analisada e muito provavelmente dependendente da presença de outros fatores que contrabalancem a expressão de TGF-β1.

Deste modo, nós não podemos eliminar a possibilidade de uma molécula ainda não identificada agindo em sinergismo com TGF-β1 para a ativação do promotor do gene de GFAP nos astrócitos. Um bom candidato para esta função é o glutamato, o principal neurotransmissor excitatório do SNC, por estar envolvido na interação neurônio-glia e em modelos de neuroproteção através da síntese de TGF-β1.

Em resumo, nossos dados demonstram que astrócitos de córtex cerebral, cerebelo e mesencéfalo expressam TGF-β1 e seu receptor. Nós discutimos que as diferenças na responsividade do promotor do gene de GFAP para TGF-β1 não estão relacionadas aos níveis de expressão de membros da via deste fator (TGFRII ou TGF-β1) por estas células, mas refletem claramente diferenças funcionais e/ou estruturais em populações heterogêneas de astrócitos.

Nossos resultados contribuem fortemente para o conceito de heterogeneidade astrocitária ao longo do SNC, na capacidade de responder a fatores de crescimento secretados ou não por neurônios. Nossos dados ajudam a entender o papel dos fatores epigenéticos, no desenvolvimento do cérebro. Mais

ainda, nossos resultados contribuem para o entedimento da intimidade entre neurônios e células da glia, astrócitos em particular, na construção do SN.

4.3 Glutamato na diferenciação astrocitária

A íntima relação que existe entre astrócitos e o terminal sináptico *in vivo* (Peters *et al*., 1991; Ventura e Harris, 1999) tem sugerido nos últimos anos um importante papel para os astrócitos na formação e função sináptica. Recentemente, o astrócito tem sido considerado o terceiro elemento da estrutura sináptica, um elemento ativo juntamente com os elementos pré e pós-sináptico (Araque *et al*., 1999). Estudos *in vitro* de neurônios do SNC purificados mostraram que fatores solúveis derivados de astrócitos induzem formação de novas sinapses, mais eficientes (Pfrieger e Barres, 1997; Nägler *et al*., 2001; Ullian *et al*., 2001; 2004). Dois fatores sinaptogênicos foram identificados: colesterol, transportado via apolipoproteína-E (Mauch *et al*., 2001; Goritz *et al*., 2005) e a proteína da MEC, trombospondina (Christopherson *et al*., 2005). No entanto, é possível que os astrócitos secretem outros fatores com atividade sinaptogênica, ainda não identificados (Elmariah *et al*., 2005). Recentemente, foi demonstrado que astrócitos induzem formação de sinapses funcionais a partir de células-tronco embrionárias humanas (Johnson *et al*., 2007). O fator responsável permanece desconhecido.

Além de afetar a formação sináptica, secretando várias moléculas solúveis, os astrócitos também são alvos de moléculas neurais como o glutamato, principal neurotransmissor excitatório. Trabalhos do grupo de Nicoletti demonstraram previamente que a ativação de receptores glutamatérgicos *in vivo* e *in vitro* induzem a síntese e secreção de TGF-β1 e -β2 por esses astrócitos (Bruno *et al*., 1998; D'Onofrio *et al*., 2001). Com o objetivo de investigar a cooperação entre o

neurotransmissor glutamato e TGF-β1 na diferenciação astrocitária, utilizamos como modelo experimental preparação de sinaptossomas de cérebro de ratos e culturas de astrócitos de córtex cerebral.

Sinaptossomas são terminais pré-sinápticos íntegros, fechados hermeticamente, obtidos por fracionamento celular e separados por densidade em um gradiente descontínuo de Percoll (Dunkley *et al*., 1986; 1988). Essas estruturas foram e têm sido extensivamente usadas para estudar o mecanismo de liberação de neurotransmissores *in vitro,* pois preservam a bioquímica, morfologia e propriedades eletrofisiológicas da sinapse (Garcia-Sanz *et al*., 2001). Observada à microscopia eletrônica, a fração sinaptossomal revela a presença de mitocôndrias e de vesículas sinápticas (Romão *et al.*, resultados não publicados), onde se encontram estocados neurotransmissores como o glutamato, o GABA, a acetilcolina e a dopamina (Romão *et al.*, dados não publicados e ver também Somers e Clemente, 2002). Os sinaptossomas são utilizados como um modelo vantajoso em estudos envolvendo a transmissão sináptica devido à sua viabilidade, funcionalidade e integridade após isolamento.

Nesta tese, demonstramos que sinaptossoma e meio condicionado sinaptossomal ativam o promotor do gene de GFAP de astrócitos de córtex cerebral, induzindo a diferenciação astrocitária (**Romão *et al.*, 2007a, artigo em preparação**). Os astrócitos estão intimamente ligados à estrutura sináptica e secretam fatores que promovem a sinaptogênese (Mauch *et al*., 2001; Christopherson *et al*., 2005; Allen e Barres, 2005; Slezak *et al*., 2006). Provavelmente, as sinapses estão secretando fatores e/ou neurotransmissores que podem atuar na diferenciação (ou proliferação) astrocitária. Essa interação é biunívoca, sinapse-astrócito, astrócito-sinapse.

As primeiras evidências do papel dos neurônios e/ou metabolismo glutamatérgico na diferenciação astrocitária vieram com os trabalhos de Swanson e colaboradores (1997), demonstrando que a influência neuronal deve ser importante na determinação do padrão de expressão de subtipos de transportadores de glutamato. Este trabalho demonstra que na ausência dos neurônios, os astrócitos expressam os transportadores de glutamato do tipo GLAST, característicos de astrócitos imaturos, enquanto que quando cocultivados com neurônios passam a expressar também transportadores do tipo GLT-1, característicos de astrócitos diferenciados. Estes dados sugerem que a diferenciação astrocitária pode sofrer influência neuronal e quem sabe, sináptica.

Demonstramos com nosso modelo de cocultivo sinaptossoma-astrócitos que diferentes concentrações de glutamato ativam o promotor de GFAP, sugerindo fortemente que os astrócitos sejam alvos de moléculas neurais como o glutamato. Nosso trabalho é pioneiro em demonstrar o papel de um neurotransmissor na modulação dos níveis de expressão de GFAP. Trabalhos anteriores demonstraram a fosforilação de GFAP por glutamato via receptor NMDA em culturas organotípicas de córtex cerebral, hipocampo e culturas mistas de cerebelo (Kommers *et al.*, 1998; 1999; 2002). Ressalte-se que a fosforilação de GFAP *in vivo* é alvo de estudos desde longa data por diferentes grupos, como por exemplo, Wofchuk e Rodnight em Porto Alegre, UFRGS (Wofchuk e Rodnight, 1994; 1995; Kommers *et al.*, 1998; 1999; 2002).

A ativação do promotor do gene de GFAP por glutamato observada nessa tese é diferentemente modulada em astrócitos de diversas regiões, corroborando com o conceito de heterogeneidade astrocitária discutido acima (Garcia-Abreu *et al.,* 1995; Schluter *et al*., 2002; Song *et al*., 2002; Matthias *et al*., 2003). Da mesma

forma, D'Onofrio e colaboradores (2001) demonstraram *in vitro* e *in vivo* que glutamato induz síntese de TGF-β1 no núcleo caudado e córtex cerebral, mas não no hipocampo. Isto poderia refletir uma heterogeneidade funcional das células gliais ou uma sensibilidade baixa dos receptores glutamatérgicos nestas células.

Os astrócitos removem, através de transportadores específicos, o glutamato do espaço sináptico, o qual é convertido em glutamina, que por sua vez é lançada no meio extracelular e capturada por neurônios como precursora para síntese de glutamato neuronal (Bergles *et al*., 1999). Neste contexto, dados preliminares do nosso laboratório, através de ensaios usando inibidores de transportadores glutamatérgicos, demonstraram que a ativação do promotor do gene de GFAP independe de transportadores de glutamato (dados não mostrados).

Astrócitos expressam receptores glutamatérgicos de ambos os tipos, ionotrópicos e metabotrópicos (Bruno *et al*., 2001). Os receptores metabotrópicos (mGluR) formam uma família de pelo menos oito subtipos de receptores acoplados à proteína G que regulam uma variedade de vias de sinalização intracelular (Nakanishi, 1994). A ativação do grupo de receptores mGluR II, que inclui os receptores mGlu-2 e –3 em astrócitos, é associada à ação neuroprotetora fornecida pela síntese e secreção de TGF-β1 (Glowinski *et al*., 1994; Ciccarelli *et al*., 1997; Bruno *et al*., 1998).

Demonstramos que a ativação do promotor do gene de GFAP por glutamato é dependente do receptor mGluR II, através de ensaios utilizando antagonista específico para este receptor. No entanto, a ativação do promotor do gene de GFAP por TGF-β1 não depende da ativação do mGluR II. Bruno e colaboradores (1998) demonstraram que astrócitos liberam uma quantidade baixa de TGF-β1, e quando estes astrócitos são expostos ao glutamato ocorre um aumento de sete vezes na

concentração de TGF-β1 liberada por estes astrócitos. A adição de anticorpos neutralizantes contra TGF-β1 juntamente com glutamato inibiu a ação deste neurotransmissor na ativação do promotor de GFAP. Ao contrário, a adição do antagonista metabotrópico de glutamato não teve efeito na ativação do promotor de GFAP induzida por TGF-β1. Esses dados sugerem que TGF-β1 seja um mediador das ações de glutamato na diferenciação astrocitária (**Romão *et al.*, 2007a, artigo em preparação**).

Diversas evidências indicam que TGF-β1 ativa múltiplos mecanismos, incluindo as vias de MAPK, PI3K e NF-κβ (Xiao *et al*., 2002; Yu *et al*., 2002; Zhu *et al*., 2004). D'Onofrio e colaboradores (2001) sugeriram que a ativação do receptor glial mGlu2/3 induz síntese de TGF-β1 através da ativação das vias de MAPK e PI3K necessárias para a neuroproteção. Aqui examinamos os mecanismos intracelulares mediadores da ativação do promotor do gene de GFAP em resposta à ativação do receptor mGlu2/3. Para isso, utilizamos inibidores específicos das vias de sinalização em questão. A adição de PD98059, inibidor de MAPK kinase (Dudley *et al*., 1995) e LY294002, inibidor de PI3K (Vlahos *et al*., 1994), a culturas de astrócitos corticais, isoladamente ou em combinação, reverteu a ativação do promotor do gene de GFAP em resposta a TGF-β1 e glutamato (**Romão *et al.*, 2007a, artigo em preparação**).

Em conjunto, nossos dados nos permitem concluir que a ativação de receptores gliais do grupo-II mGlu2/3 promove a síntese de TGF-β1 levando à ativação do promotor do gene de GFAP. A falta do efeito aditivo entre PD98059 e LY294002 em reverter o estímulo do glutamato à síntese de TGF-β1 pode sugerir que as vias de MAPK e PI3K sejam interdependentes.

O TGF-β1 está associado a diversas patologias, injúria e isquemia do SNC, onde foi implicado na organização da cicatriz glial (Moon e Fawcett, 2001; Zhu *et al*., 2002; Dhandapani e Brann, 2003; Vivien e Ali, 2006). TGF-β1 tem um importante papel na neuroproteção induzida por hipóxia/isquemia, neurotoxicidade glutamatérgica, beta-amilóide e danos oxidativos. O mecanismo de neuroproteção mediado por TGF-β1 não é completamente entendido, mas evidências sugerem que TGF-β1 regule a expressão e relação de proteínas apoptóticas e antiapoptóticas, criando um ambiente favorável para a sobrevivência destas células nos locais de injúria (Dhandapani e Brann, 2003).

A toxicidade neuronal por excesso de glutamato no meio extracelular parece ser parcialmente contrabalançada pela liberação de TGF-β1 pelos astrócitos (Bruno *et al*., 1998; D'Onofrio *et al*., 2001). Entender os mecanismos que regulem a secreção de fatores neuroprotetores pelos astrócitos, poderá ter no futuro conseqüências importantes em patologias do SNC em que o glutamato aparece preponderantemente. É o caso, por exemplo, de tumores gliais. Supõe-se que a liberação de glutamato pelos gliomas seja um dos mecanismos de toxicidade importante para geração de morte neuronal com conseqüente expansão da área tumoral (Takano *et al*., 2001). Saber manipular essa contraditória situação entre toxicidade glutamatérgica versus proteção por TGF-β1 poderá no futuro contribuir para o controle do crescimento de tumores astrocitários.

4.4 Interação neurônio-glia tumoral

Até então demonstramos interações importantes entre neurônios e células gliais astrocitárias. Propriedades interativas que induzem a maturidade da célula glial. A competência dos neurônios seja através da produção de TGF-β1 ou ainda

através da liberação de glutamato poderem interferir na maturidade e diferenciação astrocitária. Se de fato esta interação se passa numa janela do desenvolvimento, definida por propriedades regionais do SNC, nossos resultados corroboram com a literatura, uma interação célula à célula num sistema saudável, num cérebro que se desenvolve normalmente. Porém uma pergunta que surge deste conjunto de resultados é se tais propriedades interativas persistem num quadro do desenvolvimento de uma patologia, envolvendo células gliais. Por exemplo, se a glia é tumoral, um astrocitoma, guarda ela propriedades interativas de sua contrapartida normal?

Pode a célula tumoral glial continuar como um sustrato ideal para o crescimento neurítico como faz a glia normal (Garcia-Abreu *et al.,* 1995)? Pode o neurônio agir sobre a morfogênese e maturação da glia tumoral como o faz sobre a glia normal (Gomes *et al*., 1999)? Secretaria o neurônio TGF-β1 competente para agir sobre a glia tumoral como o faz sobre a glia normal (de Sampaio e Spohr *et al*., 2002; **Sousa e Romão *et al*., 2004**)?

Para responder a algumas destas perguntas, na segunda parte deste trabalho de tese, analisamos as propriedades interativas entre células gliais tumorais e neurônios. Desenvolvemos culturas primárias de células tumorais, estabelecemos linhagens celulares e cultivamos linhagens já estabelecidas para comparar diferentes tipos celulares com as células gliais normais, no que concerne sua competência para dar suporte ao crescimento de neurônios. Nossos resultados mostram claramente que células gliais tumorais, embora no processo de malignização, ainda guardam a capacidade de reconhecer o neurônio como seu parceiro na construção de ambiente harmônico na formação do cérebro. Esta propriedade de parceria parece não poder ser exercida por nenhum outro tipo de

célula não neural, tumoral ou não. Entretanto, também é notável que embora gliomas sejam eficientes substratos para neurônios, eles não reconhecem toda a potencialidade de diferenciação neuronal como a glia normal o faz. Apresentamos a evidência de que neurônios podem influenciar a célula glial nas suas propriedades de organizar sua MEC, atuando, por exemplo, na redistribuição da laminina, e também influenciar a capacidade glial de produzir fatores de crescimento, como o CTGF. De fato, parece que a célula tumoral entende o sinal neuronal para organizar sua MEC ou controlar a expressão de CTGF.

4.5 Caracterização das células de glioblastomas humanos

Os tumores gliais de origem astrocitária são os tumores cerebrais primários mais comuns, constituindo cerca de 40% de todas as neoplasias do SNC. Os tumores malignos do cérebro estão entre as mais graves formas de câncer. O mais comum destes tumores, que são incuráveis, é o glioblastoma multiforme (GBM), responsável por 50% de todos os gliomas intracranianos e 25% dos tumores intracranianos em adultos (Curran *et al*., 1993; Davis *et al*., 2001).

A origem de um tumor é ainda um fato inexplicável. Hoje, o grande interesse e estudo nas células tronco trouxe para estas uma possível responsabilidade sobre a origem dos tumores. No sistema nervoso em desenvolvimento, uma correlação entre células tronco neurais e tumores cerebrais tem sido sugerida (Al-Hajj e Clarke, 2004; Berger *et al*., 2004; Singh *et al*., 2004b; Pilkington, 2005; Nicolis, 2006; Morrison e Kimble, 2006; Vescovi *et al*., 2006; Beier *et al*., 2007; Pisati *et al*., 2007).

A classificação destes tumores obedece às regras estabelecidas pela comissão da Organização Mundial de Saúde (OMS) que reformulou esta caracterização dos tumores menos infiltrantes, até os mais agressivos e fatais, como

o glioblastoma multiforme. Entretanto, embora esta classificação venha sendo utilizada amplamente pelos patologistas de todo o mundo, há controvérsias sérias ao seu emprego. A equipe do Hospital Sainte-Anne (Paris, França), liderada pela Professora Catherine Daumas-Duport, propôs outros critérios de classificação dos tumores que, basicamente, pudessem considerar a heterogeneidade celular do tumor. Esta classificação reconhece que o microambiente pode conter células tumorais e células normais, reativas ao tumor como os astrócitos e que por vezes podem dificultar uma classificação mais precisa.

O diagnóstico de tumores, particular de tumores gliais é ainda uma questão de pouca convergência dos patologistas. Para gliomas a classificação da OMS encontra críticas da classificação Saint-Anne. Isto se deve provavelmente à heterogeneidade celular da massa tumoral implicando algumas vezes divergências de diagnóstico. Por isto a necessidade da busca de novos marcadores. Mas também se torna importante desenvolver tecnologias que melhorem a precisão, a resolução do sistema de análise de tumores em geral, não só os cerebrais e ainda possam diminuir o custo do diagnóstico. Com este interesse nosso laboratório se associou ao de Patrícia M.A. Farias da UFPE e Carlos César Lenz da UNICAMP para desenvolvimento de processos nanotecnológicos que pudessem estabelecer novas sondas para diagnóstico em câncer. Usando o sistema de *quantum dots* temos desenvolvido marcadores celulares que poderão ser usados para este fim **(Farias *et al*., 2006, anexo 2).**

Seguiremos aqui a classificação do serviço de Anatomia Patológica do HUCFF que, por enquanto, baseia seus critérios mais próximos daqueles difundidos pela OMS.

Assim, um possível potencial de oncogenicidade para as células tronco vem ganhando espaço na literatura (Al-Hajj e Clarke, 2004; Berger *et al*., 2004; Singh *et al*., 2004b; Pilkington, 2005; Nicolis, 2006; Morrison e Kimble, 2006; Vescovi *et al*., 2006; Beier *et al*., 2007; Pisati *et al*., 2007). Células tronco de glioblastoma multiforme e tumores pediátricos (DNT) têm marcadores precoces e crescem em suspensão em forma de esferas, chamadas de oncoesferas (Hemmati *et al*., 2003; Galli *et al*., 2004; Singh *et al*., 2004; Yuan *et al*., 2004). Nestes procedimentos de isolamento de oncoesferas, pude colaborar com a equipe do Dr. H. Chneiweiss do Collège de France, Paris, durante estágio PDEE-CAPES, onde isolamos oncoesferas a partir de glioblastomas multiforme e fizemos uma análise proteômica (manuscrito em preparação). Estas células tem um potencial multipotente e podem se diferencias em células com fenótipo de neurônios, astrócitos e oligodendrócitos.

Propõe-se que o desenvolvimento e proliferação destes tumores se sucedem por etapas de desdiferenciação progressiva, desde um tumor raramente infiltrante e pouco proliferativo, como o astrocitoma de grau I, até o glioblastoma multiforme, altamente maligno e sempre invasivo (Vandenberg, 1992). A formação de tumores cerebrais e seus mecanismos de progressão ainda são desconhecido. Sugerindo que ocorra uma instabilidade nas células maduras para a formação de gliomas, tumores cerebrais primários derivandos de astrocytes e/ou células troco neurais (Wechsler-Reya and Scott, 2001). Nós demonstramos que TGF-α, um fator epigenético, promove regressão progressiva de astrócitos maduros em progenitores neuronais e células tronco **(Sharif *et al*., 2007, anexo 4).**

Células tronco têm um tropismo por glioblastomas *in vivo* e *in vitro* (Aboody *et al*., 2000; Tang *et al*., 2003; Glass *et al*., 2005). As células tronco migram em direção ao tumor e podem se diferenciar em neurônios ou astrócitos e têm o potencial de

induzir apoptose nestes tumores (Glass *et al*., 2005). Muitos grupos vêm estudando o potencial terapêutico destas células tronco, com vistas à terapia celular (Ehtesham *et al*., 2002; Glass *et al*., 2005; Shah *et al*., 2005).

Em se tratando de tumores gliais, poder-se-ia de imediato supor que deveriam expressar a proteína específica de filamentos intermediários de células gliais-astrocitárias, GFAP. De fato, os 3 tipos de tumores (Gbm95, 02, 03) expressam GFAP com intensidades diferentes. Embora GFAP seja frequentemente usada como marcador de diagnóstico de origem destes tumores (Chen e Zhang, 1989; McLendon e Bigner, 1994), nem sempre sua expressão é identificada nestes tumores, com algumas células positivas e outras negativas (Schiffer, 1997), ou se apresentando em diferentes intensidades de marcação pelo anticorpo (Liberski e Kordek, 1997), que corresponderiam ao grau de diferenciação ou ao grau de malignidade do tumor (Tascos *et al*., 1982).

Glioblastomas são gliomas polimorfos e sua característica astrocítica nem sempre está presente. Eles apresentam uma grande densidade celular, um pleomorfismo nuclear e, às vezes, algumas células são multinucleadas ou apresentam núcleos aberrantes (Schiffer, 1997). Entretanto, a caracterização inicial dos filamentos de GFAP se fez pela primeira vez por Eng e colaboradores (1971) em astrócitos fibrosos e seu emprego para a caracterização de tumores tem sido rotineiramente usado (Deck *et al*., 1978). Ao longo dos anos, diferentes laboratórios têm confirmado que GFAP é um marcador de tumores gliais, astrocitários de origem. A identificação por imunocitoquímica desta proteína de diferentes tecidos de tumores cerebrais parece ser de grande importância diagnóstica.

Nossas secções de tumor em parafina foram marcadas somente com GFAP. A idéia de realizar esta marcação era simplesmente de ter a informação (da

presença ou não) de GFAP no tumor, que geraria em seguida as células em cultura, também testadas para GFAP e confirmando sua marcação como veremos adiante. As marcações eram heterogêneas, comparados os 3 tumores e não pareciam mesmo homogêneas no interior de uma mesma massa tumoral. Células não expressando GFAP, na massa tumoral, podem sugerir uma heterogeneidade do tumor, uma variação da progressão aleatória do tumor, derivando algumas células para a perda de expressão, ou ainda células em estágio precoce de sua diferenciação, perdendo a expressão ao longo da progressão tumoral.

Para minimizar uma possivel derivação das células tumorais com inúmeras passagens ou repiques, procuramos trabalhar com o cultivo de células em número definido de passagens. O Gbm95 foi utilizado sempre por culturas que vinham da 15ª até a 20ª passagem, enquanto os outros dois tumores (Gbm02 e Gbm03) foram sempre empregados entre a 5ª e 10ª passagem. Com passagens mais elevadas, por exemplo, em 20ª passagem, excluíamos a possibilidade de que as células em cultura não fossem as imortalizadas pela tumoração.

De fato, nenhuma diferença de comportamento realmente distinto em sua interação com neurônios, que era nosso padrão de observação, foi constatada entre as 3 linhagens, fossem quais fossem as passagens em que se encontravam.

As marcações das células tumorais com GFAP confirmavam o caráter glial do tumor de origem e era comparativamente positiva em relação ao padrão de marcação verificada com os astrócitos em cultura primária. Entretanto, a marcação com GFAP das células dos tumorais gliais não seguia o padrão de distribuição totalmente fibrilar, filamentosa, verificada nos astrócitos em cultura primária, se apresentava pontilhada e difusa no citoplasma astrocitário. É difícil interpretar esta diferença; podendo-se argumentar que se trata de um arranjo mais típico de células

gliais tumorais. A marcação apresentava, porém outra característica interessante: as células não tinham a mesma intensidade de marcação, o que poderia indicar que a cultura não era sincrônica e as células não estavam todas na mesma fase de ciclo celular, ou ainda, poderíamos supor que a cultura não era necessariamente clonal e encontrávamos células em diferentes estágios de “diferenciação”. Esta diferença de intensidade de marcação era também encontrada para duas outras proteínas de filamento intermediário, vimentina e nestina. Com anti-nestina, as diferenças de intencidade de marcação eram mais evidentes ainda. O conjunto das três marcações, diferentes em intensidade na população de células em cultura, poderia sugerir que a cultura de células tumorais era heterogênea. Agora, nós estamos clonando as células tumorais, através de diluição clonal, e encontramos clones de células mais diferenciadas, isto é, com uma maior intensidade de marcação para GFAP e clones indiferenciados com marcação para nestina. Como perspectivas futuras desta tese pretendemos analisar o potencial de formação de oncoesferas destes clones de células indiferenciadas.

De fato, esta diferença na expressão de GFAP em glioblastoma está relacionada ao alto índice de proliferação das células tumorais negativas para GFAP (Schiffer, 1997). Vescovi e colaboradores (2006) sugerem que exista uma sub-população de células tronco com um alto índice de proliferação que sustenta a progressão e crescimento tumoral. Esta heterogeneidade dos glioblastomas pode estar relacionada a sua origem, podendo se desenvolver de células tronco neurais ou células que adquiriram um potencial multipotente por reprogramação ou desdiferenciação em resposta a mutações oncogenéticas (Kondo e Raff, 2000; Bachoo *et al*., 2002; Zhu e Parada, 2002).

4.6 Interação neurônio-glioblastoma

As células gliais representam um elemento importante no desenvolvimento neural. Os astrócitos contribuem em vários processos do desenvolvimento do SNC, como neurogênese (Lim e Alvarez-Buylla, 1999 e Song *et al.*, 2002), migração neuronal (Rakic, 1972; Nadarajah e Parnavelas, 2002), proliferação e diferenciação (Gomes *et al.*, 1999b; Martinez e Gomes, 2002), sinalização neural (Fróes *et al.*, 1999), formação de sinapse (Pfrieger e Barres, 1997; Nägler *et al.*, 2001; Ullian *et al.*, 2001), organização de barreira hematoencefálica (Abbott, 2002; Garcia *et al.*, 2004), direcionamento axonal (Garcia-Abreu *et al.*, 1995; Goodman e Tessier-Lavigne, 1997) e como células precursoras (Doetsch *et al.*, 1999). Decidimos analisar se as células gliais normais quando adquirem comportamento tumoral, guardam algumas das propriedades astrocitárias de interação com neurônios.

O conjunto de nossos resultados com neurônios primários expostos ao contato com o tapete celular glial normal ou tumoral é fortemente a favor de que as células gliais tumorais, apesar da progressão tumoral, e apesar de constituírem um tumor de alto grau de malignidade, guardam a “memória” da propriedade interativa com os neurônios. Neurônios emitem seus neuritos e se arborizam. O glioblastoma ainda é um substrato glial. Nossos resultados mostram que eles são melhor que qualquer outro substrato celular não neural testado, como os fibroblastos, as linhagens humanas de tumor de mama, de tumor de útero e as células humanas não tumorais, como as HUVEC (**Faria e Romão *et al*., 2006**).

Os tumores gliais, *in vivo,* podem crescer e invadir o parênquima, apresentando no seu interior, em profundidade, neurônios, reconhecíveis pela presença de grânulos de Nissl, atividades enzimáticas e marcadores imunohistoquímicos. Com a progressão do tumor, neurônios poderão mostrar sinais

de atrofia e progressivamente desaparecerem (Schiffer, 1997). A progressão tumoral, passa por uma complexa atividade interativa, no parênquima, por exemplo, com o suprimento de vasos e oxigenação das células tumorais ou daquelas vizinhas a elas. O crescimento do tumor gera também um crescimento da pressão intracraniana pela massa do tumor. Tudo isto, em conjunto pode levar à falência da rede celular cerebral (Maher *et al*., 2001). Alguns autores relacionam a progressão tumoral pela destruição do parênquima através da citotoxicidade causada pela liberação de glutamato (Ye e Sontheimer, 1999; Takano *et al*., 2001). Outros grupos sugerem que a microglia presente nos gliomas pode promover e suportar o fenótipo invasivo tumoral, através de fatores secretados, incluindo proteases de matriz extracelular e citocinas que podem influenciar a migração, invasividade e proliferação (Bettinger *et al*., 2002; Rao, 2003; Markovic *et al*., 2005; Watters *et al*., 2005; Sliwa *et al*., 2006)

Nossos resultados demonstram a eficiência do substrato glial tumoral para a neuritogênese:

a) célula glial tumoral humana suporta o crescimento de neurônios de ratos, mesmo com origem de espécies diferentes, uma humana, outra roedor. Isto sugere que algumas destas propriedades interativas podem ser evolutivamente preservadas, ao menos entre mamíferos. É Importante ressaltar que fibroblastos originados de rato, não são um substrato ideal para neurônio de rato, o que nos faz sugerir que, independente da espécie, o comprometimento da célula com a diferenciação numa linhagem de sistema nervoso é importante para o reconhecimento, interação e suporte entre células.

Nós também analisamos a interação celular na mesma espécie, onde utilizamos glioma murino, C6, com neurônios de rato. Observamos um crescimento

neurítico comparável aos resultados obtidos com os gliomas humanos (dados não mostrados). Nós pretendemos no futuro próximo, analisar a interação de glioblastomas humanos com neuroesferas humanas, fonte de células tronco. Nossos resultados preliminares com neuroesferas de camundongo demonstraram que o tumor suporta a sobrevida e diferenciação em células com fenótipo neuronal ou glial, a partir das neuroesferas, analisadas através de imunomarcação para marcadores neuronais (β-tubulina III) e marcadores astrocitário (GFAP) (dados não mostrados).

Estes resultados embora preliminares são prometedores e etimulantes a um aprofundamento do estudo em etapa seguinte a esta tese.

b) outras células tumorais humanas, não de sistema nervoso, como as de tumor de útero ou de mama, não são um substrato ideal para o crescimento de neurônios. Isto reforça nossa sugestão de que, sendo células comprometidas com linhagens, não neurais, elas não exibiram competência interativa para reconhecer os neurônios como células do sistema nervoso, ou perderam estas propriedades no "momento" do comprometimento com a sua diferenciação em linhagem.

É interessante constatar que a célula tumoral secreta moléculas que permitem o crescimento de neuritos. Quando utilizamos um meio condicionado proveniente de células de glioblastoma humano para crescer neurônios, verificamos que há crescimento de neuritos, que se arborizam e apresentam cones de crescimento (resultados não mostrado; Fonseca, monografia 2007). Não se conhecem exatamente as moléculas que estão presentes neste meio condicionado, mas fatores de crescimento como TGF-α, TGF-β e EGF têm sido apontados como fatores implicados na diferenciação e/ou proliferação celular, quer seja de células de glioma (Brockmann *et al.*, 2003; Held-Feindt *et al.*, 2003) ou neurônios como nossas equipes puderam demonstrar (Gomes *et al.,* 1999; Martinez e Gomes, 2002). Outros

fatores solúveis, como semaforinas e netrinas, também, têm sido implicados como suporte celular para o crescimento e direcionamento neurítico (Bagnard *et al*., 1998; Koeberle e Bahr, 2004). Estudos recentes demonstram que gliomas humanos expressam semaforinas e seus receptores (Rieger *et al*., 2003) e talvez estes elementos, semaforinas e netrinas, estejam presentes no meio condicionado dos glioblastomas estudados nesta tese.

4.7 Interação neurônio-glioblastoma: modulação da laminina

Laminina, uma proteína de matriz extracelular tem sido implicada como um substrato permissivo para o crescimento neurítico, a migração e diferenciação de neurônios (Denis-Donini *et al*., 1994; Martinez e Gomes, 2002). Também está implicada como um substrato na migração de tumores cerebrais primários, sendo produzida por estas células (Pindzola *et al.*, 1993, Powell *et al.*, 1997, Ziu *et al*., 2006). Em nosso modelo, a laminina está presente na glia primária, os astrócitos corticais cerebrais. Na MEC destas células, ela aparece com um arranjo predominantemente fibrilar, filamentoso, semelhante aquele que Garcia-Abreu e colaboradores (1995) descreveram para a glia mesencefálica da sub-região lateral do mesencéfalo, e que permitia o crescimento de neurônios com longos neuritos. Entretanto, este padrão de organização da laminina sofre pequenas alterações, em função da cocultura. A laminina apresenta em diferentes campos da cocultura, um padrão misto de organização, filamentar e pontiforme. Isto parece sugerir que neurônios poderiam estar induzindo um rearranjo de laminina na superfície da célula glial. Talvez o contato celular seja responsável por estas alterações. Freire e colaboradores (2002; 2004) demonstraram que a laminina pode ter estas duas formas de padrão de expressão, dependendo do pH da solução em que laminina é

posta polimerizar. Pudemos verificar que a glia tumoral densamente rica em laminina na sua superfície, também mostra sinais de sensibilidade à presença dos neurônios. Observamos que ocorre um rearranjo da disposição da expressão de laminina.

Ensaios preliminares através de ELISA demonstraram que ocorre uma diminuição na expressão de laminina quando os glioblastomas são cocultivados com neurônios (dados não mostrados).

Laminina puntiforme tem sido descrita, também *in vivo*, no sistema nervoso. Liesi e Silver (1988) descreveram laminina puntiforme durante o desenvolvimento embrionário do nervo óptico de camundongo e no corpo caloso e no fórnix. É interessante que as observações dos autores indicam que este arranjo de laminina puntiforme em astrócitos, na fase embrionária está, sobretudo, em íntima associação com os axônios em crescimento. O que sugere que estas alterações de configuração da rede de laminina que descrevemos nesta tese, poderiam também, estar relacionadas à interação da glia normal ou tumoral com neurônios (**Faria e Romão *et al*., 2006**). Freire e colaboradores (2004) propõem que as configurações como as descritas aqui sejam frutos de alterações focais de pH, por exemplo, por alteração dos resíduos siálicos da membrana celular. Não seria exagero pressupor que neurônios pudessem induzir flutuações focais de ácido sialico na membrana da célula glial, criando assim zonas de pH mais ácido ou menos ácido fora destes focos, causando então rearranjo da laminina como o descrito nos nossos resultados.

4.8 Interação neurônio-glioblastoma: modulação de CTGF

CTGF, fator de crescimento de tecido conjuntivo, foi detectado em várias lesões fibróticas (pele, pulmão, rim e fígado) (Moussad e Brigstock, 2000) e parece poder agir na indução de fibrogênese. Sua expressão é induzida por TGF-β,

envolvido na regulação de migração, proliferação celular, formação de MEC e cicatrização (Grotendorst, 1997), embora seu mecanismo de ação permaneça desconhecido.

Muito recentemente, Abreu e colaboradores (2003) mostraram que o CTGF age, em parte, através da ligação no espaço extracelular com BMP4, inibindo-o, e com TGF-β1, ativando-o.

Recentemente ainda, os achados de Kondo e colaboradores (2002) sugerem que a hipóxia estimula células de câncer de mama a liberar CTGF, a qual inicia a cascata de angiogênese, modulando o balanço entre síntese e degradação de MEC, via metaloproteinases, secretadas por células endoteliais em resposta ao CTGF. Essa cascata deve exercer um papel crítico na neovascularização, induzida por hipóxia, que acompanha a invasão tumoral. Essa atividade pró-angiogênica apóia seu papel no estabelecimento e função da vascularização e em doenças vasculares.

Em amostras de tumores, a expressão anormal de proteínas da família CCN está associada ao desenvolvimento do câncer (Planque e Perbal, 2003), talvez associada à proliferação acentuada do crescimento tumoral

De acordo com estudos prévios de imunocitoquímica no tecido cerebral de rato (Kondo *et al*., 1999), a astroglia expressa CTGF, principalmente nas regiões do córtex cerebral e da substância branca da medula espinhal. Entre os neurônios, o CTGF foi detectado em uma subpopulação de células piramidais nas camadas III e V do córtex cerebral, assim como nas células ependimárias dos ventrículos, plexo corióide e tanicitos ao redor do canal central da medula espinhal (Kondo *et al*., 1999). A maior parte do CTGF foi observada em astrócitos, sugerindo que essa proteína possa participar de processos de cicatrização após lesão neuronal, via regulação da gliose astrocitária. CTGF também foi detectado em gliomas e em

algumas linhagens de tumores humanos derivados do SNC (Xin *et al*., 1996; Inoki *et al*., 2002).

Nós demonstramos a capacidade de neurônios interferirem na MEC de glioblastoma, através da reorganização da proteína laminina (**Faria e Romão *et al.*, 2006**). Com estes resultados podemos sugerir que neurônios podem controlar a morfogênese molecular de astrócitos e, particularmente, controlar a morfogênese de tumores gliais.

Demonstramos, ainda, esta influência neuronal em células gliais malignizadas. Nossos últimos resultados mostram com clareza que neurônios modulam a expressão de CTGF (**Romão *et al.*, 2007b, artigo em preparação**).

Será que neurônios podem influenciar expressão de proteínas da matriz extracelular e fatores de crescimento como CTGF e TGF-β1 pelas células da glia normal e tumoral?.

Demonstramos por imunocitoquímica e RT-PCR evidenciam que células de Gbm humanos e astrócitos normais de rato expressam CTGF (Xin *et al*., 1996; Pan *et al*., 2002) e também expressam TGF-β1(Gomes *et al.*, 1999; Piek *et al.*, 1999; Kjellman *et al.*, 2000; Pan *et al.*, 2005; Schneider *et al*., 2006).

A síntese e expressão de CTGF pelas células astrocitárias normais e tumorais foram parcialmente inibidas quando neurônios neonatais foram cocultivados sobre monocamadas destas células (**Romão *et al.*, 2007b, artigo em preparação**). Entretanto, surpreendentemente, o meio condicionado de neurônios embrionários e neonatais não afetaram a expressão de CTGF em células de Gbm sugerindo a necessidade do contato celular, como proposto por Hatten (1987) em estudo de proliferação astrocitária em cerebelo. No nosso exemplo parece haver de fato a

necessidade do contato celular para que a inibição da síntese de CTGF via neurônio se manifeste.

CTGF pode se ligar e interagir com uma série de proteínas, modulando, desde fenômenos a nível celular como: proliferação, migração e diferenciação celular, até fenômenos no tecido, como: cicatrização, angiogênese, odontogênese, condrogênese, osteogênese e carcinogênese (Perbal, 2001; 2004; Planque e Perbal, 2003). O primeiro co-efetor de CTGF a ser descoberto e um dos mais importantes fatores de crescimento que atua com sua interveniência, é TGF-β1 (Grotendorst, 1997).

A sinalização clássica começa quando TGFβ-1 se liga ao seu receptor II (TGFRII), promovendo a aproximação e ligação ao receptor I (TGFRI), formando um complexo ternário. O receptor II fosforila a seqüência Gly/ser do receptor I o qual, através da atividade serina/treonina quinase, fosforilará proteínas intracelulares chamadas SMADs. A fosforilação de SMAD é específica para cada membro da superfamília TGF-β. No caso do TGF-β1 as SMADs fosforiladas serão SMAD 2 e 3. SMADs fosforiladas se associarão as co-SMADs, formando um complexo que se translocará para o núcleo onde se ligarão ao DNA, ativando a transcrição gênica (Massagué e Chen, 2000). Apesar da via clássica simples, o controle da sinalização de TGFβs é muito mais complexo. A combinação entre os diferentes receptores I e II e diferentes ligantes promoverá ativação diferenciada de SMADs e conseqüentemente a transcrição gênica será regulada de forma diferenciada (Massagué, 1998; Miyazono, 2000; Wrana e Attisano, 2000; Moustakas *et al*., 2001; **Gomes *et al*., 2005, Anexo 1**; Massagué e Gomis, 2006).

A inibição de CTGF em coculturas de Gbm com neurônios P0 não está relacionada a uma inibição de SMAD2/3 fosforilada, mas a adição de TGF-β1

impediu a inibição da expressão de CTGF nos ensaios de cocultura. Dziembowska e colaboradores (2007) demonstraram a interação dos mecanismos de sinalização de SMAD e MAPK por TGF-β1 em glioblastomas. Gbms não respondem ao TGF-β1, modificando sua viabilidade e proliferação, mas são capazes de responder na molulando a síntese de proteínas da matriz. Através de ensaios com inibidores de MAPK, este grupo demonstrou uma diminuição da via de SMAD, sugerindo a interdependencia de SMAD e MAPK (Dziembowska *et al*., 2007).

Porém, a fosforilação de MAPK é diminuída em Gbm cocultivados com neurônios P0, sugerindo que a expressão de CTGF em Gbm é regulado negativamente por neurônios neonatais através da inibição das vias de sinalização de MAPK e independente da sinalização TGF-β/SMAD (**Romão *et al*, 2007b; artigo em preparação**). Nossos dados corroboram com Pannu e colaboradores (2007) que demonstraram que TGF-β induz síntese de CTGF através da ativação da via de ERK1/2 e SMAD1 e esta indução é independente da via de SMAD2/3.

Astrócitos e glioblastomas expressam CTGF e seu principal indutor, TGF-β1. Alguns grupos sugerem que ocorre um aumento na expressão de CTGF na progressão tumoral (Xin *et al*., 1996; Xie *et al*., 2004).

CTGF foi descrito como um dos principais genes envolvidos na progressão da metástase do câncer de mama (Kang *et al.*, 2003). A maioria das funções de CTGF podem ser moduladas através de interações com células e matriz extracelular e são essenciais para a progressão de tumor. O mecanismo de ação de CTGF ainda não foi proposto (Abreu *et al*., 2002), e em células gliais normais e tumorais sua função sequer é conhecida.

Em nosso modelo a modulação de CTGF parece não ser dependente de TGF-β1 produzido pelos neurônios, pois não descobrimos mudanças na transcrição

de TGF-β1 medida por RT-PCR. Ainda mais, como vimos a adição de TGF-β1 recombinante recupera a expressão de CTGF pelo Gbm em cocultura com neurônios.

Neurônios embrionários (E18) ou de cérebros de animias recé-nascidos (P0) não parecem expressar TGF-β1. Estes dados confirmam aqueles obtidos previamente no laboratório que demonstrava que TGF-β1 é expresso, *in vitro,* por neurônios mais jovens, neurônios de camundongo de 14 dias embrionários (de Sampaio e Spohr *et al*., 2002).

CTGF contém um elemento de resposta ao TGF-β1 no seu promotor, sugerindo que TGF-β1 é o principal indutor de CTGF (Grotendorst, 1997). Recentemente foi demonstrado que CTGF e TGF-β1 têm domínios de ligação, e estas interações resultam na modulação de mecanismos de sinalização (Abreu *et al.*, 2002). CTGF aumenta a ligação de TGF-β1 ao seu receptor e aumenta a fosforilação de SMAD2/3 (Abreu *et al.*, 2002). Gbms respondem ao TGF-β1 aumentando a expressão de CTGF. Entretanto, nas nossas condições, de trabalho demonstramos que neurônios não afetaram a via de sinalização de TGF-β (SMAD) quando cocultivados com Gbm, sugerindo que um outro mecanismo de sinalização poderia estar envolvido; talvez a via de sinalização de MAPK.

A via de sinalização de MAPK está envolvida na proliferação e diferenciação induzida por CTGF (Yosimichi *et al*., 2001; Yang *et al*., 2004; Yosimichi *et al*., 2006). Além disso, TGF-β1 também pode sinalizar através da via de MAPK (Xiao *et al*., 2002; Yu *et al*., 2002; Zhu *et al*., 2004).

Chen e colaboradores (2002) mostraram que a indução de CTGF por TGF-β1 exige um sinergismo estre as vias de sinalização de SMAD e Ras/MEK/ERK.

Recentemente, Shimo e colaboradores (2005) mostraram que CTGF é importante para a maturação de condrócitos e que sua expressão durante este processo era positivamente regulada por ERK1/2 e negativamente por p38 MAPK. Nós demonstramos que a cocultura de neurônios com células de Gbm induz uma diminuição da expressão de CTGF acompanhada por uma diminuição da fosforilação de ERK1/2. Também observamos que a adição de TGF-β1 ao sistema de cocultura neurônio-glioblastoma pode recuperar a expressão de CTGF ativando as vias de sinalização de SMAD e ERK1/2. Deste modo, nossos dados corroboram os resultados recentes da literatura de análise em outros modelos celulares.

Para tentar esclarecer o mecanismo envolvido na inibição da expressão de CTGF durante a interação celular, nós testamos se fatores secretados de cultura de neurônios virtualmente puras e coculturas de neurônio-glioblastoma (meio condicionado neuronal e meio condicionado de cocultura) podiam modular esta expressão. Os resultados não mostraram nenhuma variação da expressão de CTGF, analisada por RT-PCR e imunocitoquímica, sugerindo que o contato entre neurônios e glioblastomas é definitivamente importante para a inibição de CTGF.

Assim, nesta tese pudemos abrir uma nova janela sobre as interações neurônio-glia, particularmente envolvendo a célula astrocitária normal e ainda a tumoral.

Esta abordagem sobre a interação neurônio-glia permite melhor entender a contribuição de cada uma destas duas células à construção do cérebro, focalizando sobre duas populações celulares que parecem ter origem comum e, portanto, teriam alguma facilidade de reconhecimento entre si. Esta tese nos mostra o efeito dos fatores de crescimento, como TGF-β1 ou até mesmo CTGF, mas também o efeito de um neuromediador tão importante como glutamato no caminho da diferenciação

celular, denunciando o papel importante que tem o neurônio na definição do comprometimento glial. Olhada a interação agora sob ótica da glia tumoral, a tese contribue para o entendimento da biologia da célula glial tumoral e talvez assim aponta para uma nova estratégia no combate ao tumor, através dos sinais de controle tumoral que o neurônio foi capaz de evidenciar nos nossos experimentos. Ainda é necessário estudar mais profundamente este fenômeno, pois ele abre novas perspectivas para o enfrentamento dos tumores cerebrais, trazendo, quem sabe, a possibilidade de novas abordagens terapêuticas.

CONCLUSÕES

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Do conjunto de nossos resultados, quer seja da interação do neurônio com a glia normal ou com a glia tumoral, podemos considerar que:

1 – A ativação do promotor de GFAP pode ser modulada diferentemente por neurônios de acordo com a região encefálica, sugerindo uma heterogeneidade astrocitária;

2 – A ativação do receptor mGluR2/3 induz a síntese e secreção de TGF-β1 pelos astrócitos e envolve as vias de sinalização de MAPK e PI3K;

3 – O glioblastoma humano suporta o crescimento neurítico e a sobrevivência dos neurônios. A organização de laminina de Gbm é modulada pela interação com neurônios;

4 – A expressão de CTGF em Gbm é independente da sinalização de TGF-β1 e pode ser regulada negativamente por neurônios através da inibição das vias de sinalização de MAPK e.

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ANEXO 1

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Review

Emerging roles for TGF-β1 in nervous system development

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Abstract

Transforming growth factor betas (TGF-βs) are known as multifunctional growth factors, which participate in the regulation of key events of development, disease and tissue repair. In central nervous system (CNS), TGF-β1 has been widely recognized as an injury-related cytokine, specially associated with astrocyte scar formation in response to brain injury. TGF-βs family is represented by three isoforms: TGF-β1, -β2 and -β3, all produced by both glial and neuronal cells. They are involved in essential tissue functions, including cell-cycle control, regulation of early development and differentiation, neuron survival and astrocyte differentiation. TGF-β signaling is mediated mainly by two serine threonine kinase receptors, TGFRI and TGFRII, which activate Smad 2/3 and Smad 4 transcription factors. Phosphorylation and activation of these proteins is followed by formation of Smad 2/3–4 complex, which translocates to the nucleus regulating transcriptional responses to TGF-β. Very few data are available concerning the intracellular pathway required for the effect of TGF-β in brain cells. Recently, emerging data on TGF-β1 and its signaling molecules have been suggesting that besides its role in brain injury, TGF-β1 might be a crucial regulator of CNS development. In this review, we will focus on TGF-βs members, specially TGF-β1, in neuron and astrocyte development. We will discuss some advances concerning the emerging scenario of TGF-β1 and its signaling pathways as putative modulators of astrocyte biology and their implications as a novel mediator of cellular interactions in the CNS.

Keywords: TGF-β1; Neuron–glia interaction; Astrocyte; Development; Central nervous system; Smads

Contents

Abbreviations: APP, Alzheimer β amyloid precursor protein; BMPs, bone morphogenetic proteins; CNS, central nervous system; ECM, extracellular matrix molecules; EGF, epidermal growth factor; FGF, fibroblast growth factor; GFAP, glial fibrillary acidic protein; GDNF, glial cell line-derived neurotrophic factor; Ilα, interleucin alpha; IL2, interleucin 2; NMDA, *N*-methyl-D-aspartate; PAI-1, type-1 plasminogen activator inhibitor; PDGF, platelet-derived growth factor; Shh, sonic hedgehog; TGF-β1, transforming growth factor beta type 1; TGF-β2, transforming growth factor beta type 2; TGF-β3, transforming growth factor beta type 3; TGFR, TGF-β receptor; t-PA, tissue-type plasminogen activator

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1. Introduction

Transforming growth factor betas (TGF-βs) constitute a large superfamily, which plays pleiotropic roles in the growth of many organs and systems (Böttner et al., 2000; Massagué, 2000; Unsicker and Strelau, 2000; Abreu et al., 2002). Expressed in complex temporal and tissue-specific patterns, TGF-β and related factors play roles in numerous cells and tissues, including cell-cycle control, differentiation, regulation of early development, differentiation, extracellular matrix formation, angiogenesis, hematopoiesis and immune functions (Massagué, 1998; Böttner et al., 2000). At the present time, the TGF-β superfamily comprises as many as 100 distinct proteins, including nearly 30 proteins in mammals, e.g. TGF-βs (isoforms 1, 2 and 3), activins and inhibins, nodal, myostatin, bone morphogenetic proteins (BMPs), growth/differentiation factors (GDFs) and others (Massagué, 1998; Böttner et al., 2000). In this review, we will focus on TGF-βs (isoforms 1, 2 and 3), specially TGF-β1, on astrocyte development and interactions.

TGF-β1 has been widely considered an injury-related cytokine, however, over the past 3 years, this cytokine has emerged as crucial regulator of nervous system physiology. The distribution of TGF-β1 and receptors in the developing nervous system in vivo is consistent with the notion that it might be involved in brain development (Böttner et al., 2000). This idea is strongly supported by the recent finding that TGF-β1 knockout mice present severe impairment in cortical development with widespread increased neuronal cell death and microgliosis (Brionne et al., 2003).

Although TGF-β1 has been exhaustively related to brain diseases, it will not be the scope of the present report to discuss this implication (for this subject refer to Pratt and McPherson, 1997; Masliah et al., 2001; Akhurst, 2004; Buckwalter and Wyss-Coray, 2004). In this review, we will focus on advances in the understanding of TGF-βs members, specially TGF-β1, in neuron and astrocyte development. Firstly, we present distribution and function of TGF-β ligands through out CNS. Secondly, we briefly describe TGF-β receptors and signaling and what we know about molecules involved in TGF-β pathway in the nervous system. Finally, we discuss some advances concerning the emerging scenario of TGF-β1 as a putative modulator of astrocyte biology and their implications as a novel mediator of cellular interactions in the CNS.

2. Members of the TGF-β family and their expression in CNS

TGF-βs family is represented by a small group of multifunctional cytokines, consisting of three isoforms: TGF-β1, -β2 and -β3. Each isoform has a different distribution in vivo (Pelton et al., 1991) and they are considered to be independent regulatory molecules.

Within the CNS, all three isoforms of TGF-β are produced by both glial and neuronal cells (Dobbertin et al., 1997; Jones et al., 1998; Vivien et al., 1998; Zhu et al., 2000; de Sampaio e Spohr et al., 2002; Mittaud et al., 2002; Sousa et al., 2004). They are involved in essential cell and tissue functions, including cell-cycle control, regulation of early development and differentiation and neuron cell survival (Pratt and McPherson, 1997; Böttner et al., 2000; Unsicker and Strelau, 2000; Unsicker and Krieglstein, 2002; Table 1).

TGF-β2, -β3 and their receptors are expressed in early embryonic structures such as notochord and floor plate, as

Table 1
TGF-βs actions in nervous system

Growth factor	Function
TGF-β1	Neuroprotection against glutamate cytotoxity (Bruno et al., 1998; Buisson et al., 1998; Docagne et al., 2002)
	Control of astrocytic cytoskeleton, morphology and motility (Labourdette et al., 1990; Toru-Delbauffe et al., 1990; Baghdassarian et al., 1993; Flanders et al., 1993; Gagelin et al., 1995; Laping et al., 1994)
	Extracellular matrix production (Baghdassarian et al., 1993; Wyss-Coray et al., 1995; Buisson et al., 1998; Docagne et al., 1999; Docagne et al., 2002; Brionne et al., 2003)
	Astrocyte differentiation (de Sampaio e Spohr et al., 2002; Sousa et al., 2004)
	Wound healing and immunosuppression (Pratt and McPherson, 1997)
	Cell migration in the cerebral cortex (Siegenthaler and Miller, 2004)
	Control of neuronal death and microgliosis (Brionne et al., 2003)
	Inhibition of astrocyte proliferation (Johns et al., 1992; Lindholm et al., 1992; Morganti-Kossmann et al., 1992; Baghdassarian et al., 1993; Hunter et al., 1993; Vergeli et al., 1995; Rich et al., 1999)
	Organization of glial scar (Moon and Fawcett, 2001)
	Induction of blood brain barrier characteristics in endothelial cells (Garcia et al., 2004)
	Survival of neurons (Krieglstein et al., 1998a,b,c; Roussa et al., 2004)
TGF-β2	Inhibition of mitogen-induced astrocyte proliferation (Hunter et al., 1993)
	Neuroprotection against glutamate cytotoxity (Bruno et al., 1998)
	Induction of microglia proliferation (Dobbertin et al., 1997)
	Survival of neurons (Krieglstein et al., 1998a,b,c; Poulsen et al., 1994)
TGF-β3	Inhibition of mitogen-induced astrocyte proliferation (Hunter et al., 1993)
	Induction of midbrain dopaminergic phenotype (Farkas et al., 2003)
	Survival of neurons (Krieglstein et al., 1998a,b,c; Poulsen et al., 1994)

well as in the area where midbrain dopaminergic neurons are developing, suggesting that TGF-β is required for the promotion of survival of ventral midbrain dopaminergic neurons. Recently, several works identified these molecules as essential inducers of midbrain dopaminergic neuronal survival (Poulsen et al., 1994; Farkas et al., 2003; Roussa et al., 2004). Poulsen et al. (1994) demonstrated that TGF-β2 and -β3, but not TGF-β1, are potent survival factors for midbrain dopaminergic neurons in vitro. Several evidences suggest that TGF-βs are not neurotrophic by themselves but act in concert with other molecules such as FGF2, FGF8 and glial cell line-derived neurotrophic factor (GDNF) to induce neuronal survival (Krieglstein et al., 1998a,b; Roussa et al., 2004; Roussa and Krieglstein, 2004). Recently, the group of Krieglstein reported that TGF-βs induce midbrain neuronal specification and survival in synergism with sonic hedgehog (Shh) (Farkas et al., 2003). They showed that TGF-β2 and -β3, and TGFR-II are expressed in the ventral mesencephalon of E12.5 rat embryos at locations where the first tyrosine hydroxylase (TH)-positive neurons are born. This work provide in vitro and in vivo evidences that TGF-βs are essential for Shh induction of dopaminergic neurons. Together all these data raise the possibility that TGF-βs may be useful as therapeutic agents in neurological disorders specially Parkinson's disease, whose primary neurochemical features are degeneration of dopaminergic neurons of the substantia nigra.

Immunohistochemical studies show widespread expression of immunoreactive TGF-β2 and -β3 in the developing CNS. Staining is present in radial glial cells, as well as in neuronal cell bodies in the telencephalic cortex and cerebellum suggesting that TGF-βs 2 and 3 may play a role in regulation of neuronal migration and differentiation, as well as in glial cell proliferation and differentiation (Flanders et al., 1991; Pelton et al., 1991; Miller, 2003). The expression of TGF-βs 2 and 3 persists in the adult rat CNS. TGF-βs 2 and 3 mRNAs are found in all areas of the CNS including cortex, hippocampus, striatum, brainstem and cerebellum. Immunoreactive TGF-βs 2 and 3 are seen in white matter astrocytes, as well as neurons within the hippocampal pyramidal neurons, dentate gyrus granule cells, large cortical neurons within layers II, III and V, and subpopulations of cerebellar Purkinje cells (Flanders et al., 1998; Böttner et al., 2000; Unsicker and Strelau, 2000).

There are very few anatomical studies of the distribution of TGF-β1 in the brain. Data available are contradictory. Flanders et al. (1991) found TGF-β1 restricted to meninges and choroid plexus in the intact brain and upon lesioning it is induced in neurons, astrocytes and microglia (Flanders et al., 1998). Recently, Miller (2003) described TGF-β1 expression in proliferative zones and distributed all over the cortical plate. TGF-β1 immunoreactive cells were evident in the cerebral wall from E16 to P30 (Miller, 2003). TGF-β1 is expressed by cells in the proliferative zone and TGF-β receptors are expressed by migrating neurons and radial glia suggesting that this growth factor might play a role in neuronal migration. These apparent contradictory data from Flanders and Miller studies might be due to specificity of the antibodies used by different studies. Another argument, which calls in favor of TGF-β1 expression in normal brain, is that, although Flanders et al. (1991) did not find widespread localization of TGF-β1 in brain, mRNA and bioactive TGF-β1 could be extracted from E15 brain (Flanders et al., 1991). In vivo role for TGF-β1 in unlesioned brain is also supported by evidences provided by in vitro studies. Several groups have already identified TGF-β1 expression in neuron and astrocyte in culture (Lindholm et al., 1992; Morganti-Kossmann et al., 1992; Vivien et al., 1998; de Sampaio e Spohr et al., 2002; Mittaud et al., 2002; Sousa et al., 2004). In vitro immunoreaction for TGF-β1 was identified in neurons of different brain regions, although the pattern of labeling differed slightly. Whereas midbrain neurons presented a staining predominantly in neuronal soma, cerebellar and cortical neurons showed punctate labeling extending through the neuronal processes (Sousa et al., 2004).

Additional evidence on TGF-β role in brain development came recently with generation of TGF-β1 knockout mice. Those animals showed increased number of apoptotic neurons, a reduction in neocortical presynaptic integrity, reduced expression of the extracellular matrix protein laminin, and widespread microgliosis (Brionne et al., 2003). Thus, these emerging data point the endogenous TGF-β1 system as a key modulator of the developing cerebral cortex.

3. TGF-βs receptors distribution in CNS

The first molecular analysis of TGF-β signaling pathways was the identification and cloning of type II receptor for activin, and soon after for TGF-β (Mathews and Vale, 1991; Lin et al., 1992). Studies revealed that TGF-β binds to cell-surface proteins termed type I (TGFRI), type II (TGFRII) and type III (TGFRIII) (Massagué, 1990). In mammals, a diversity of cell type specific receptors mediates a qualitatively diverse set of TGF-β signaling (Shi and Massagué, 2003). Receptors II and I are glycoproteins and belong to the class of transmembrane protein serine/threonine kinase receptors. Both are composed of a short extracellular ligand-binding region, a single transmembrane segment, and an intracellular region with serine/threonine activity (Massagué, 1992, 1998; Wrana et al., 1994; Böttner et al., 2000; Massagué and Chen, 2000; Shi and Massagué, 2003). Ligand binding induces the formation of a complex in which the type II receptor phosphorylates and activates the type I receptor which then propagates the signal. Phosphorylation of the type I receptor occurs at a cluster of serine and threonine residues within a highly conserved region known as the GS domain and located immediately upstream of the kinase domain (Böttner et al., 2000; Chang et al., 2002; Lutz and Knaus, 2002). Active type I receptor phosphorylates members of a family of transcriptional regulators, the

Smads, which transduce the TGF-β signal into the cell nucleus (Heldin et al., 1997; Attisano and Wrana, 2002).

Types I and II receptors are present in different brain regions in the developing and adult nervous system (Böttner et al., 1996, 2000; Tomoda et al., 1996; Vivien et al., 1998). TGFRII mRNA is apparently widely distributed in the CNS. It was detected in cerebral cortex, midbrain, cerebellum, brain stem and hippocampus (Böttner et al., 1996). Localization by in situ hybridization has revealed TGFRII mRNA in the embryonic rat hindbrain at E14 along radially extending structures, which may represent radial glial cells (Galter et al., 1999). Recently, TGFRI and TGFRII expression in radial glia-like cells was confirmed by Miller (2003). Neurons and astrocytes express TGFRI and TGFRII in vitro and in vivo; these receptors are regulated spatially and temporally in the development (Tomoda et al., 1996; Vivien et al., 1998; de Sampaio e Spohr et al., 2002; Sousa et al., 2004).

Type III receptors correspond to two related proteins called betaglycan or endoglin (Cheifetz et al., 1988; Lopez-Casillas et al., 1991). These receptors are frequently involved in formation of receptor complexes and modulate ligand access to the signaling receptors (Letamendía et al., 1998; Eickelberg et al., 2002). Betaglycan is a membrane-anchored proteoglycan, with a large extracellular domain, a single transmembrane domain and a short cytoplasmic region lacking apparent signaling function (Cheifetz et al., 1988; Massagué, 1998). It is capable of fine-tuning the availability of TGF-β to the signaling receptors, thereby determining the outcome of the TGF-stimulation. In the brain, betaglycan mRNA is localized in discrete regions of the forebrain and brain stem, including olfactory, septal and hypothalamic nuclei (MacConell et al., 2002).

4. TGF-β signal transduction pathways

The central event in TGF-β1-mediated receptor activation is phosphorylation of serine and threonine residues of TGFRI by TGFRII. This results in activation of the TGFRI kinase and allows receptor I to propagate the signal to downstream elements (Massagué, 1992, 1998; Wrana et al., 1994; Böttner et al., 2000; Massagué and Chen, 2000). The first identified substrates of TGF-β receptors kinases were the proteins of the Smad family, initially identified in *Drosophila* (as Mad, mothers against dpp; Raftery et al., 1995) and *C. elegans* (Sma, similar to Mad; Savage et al., 1996) and now presenting many homologues in vertebrates (Smads; Sma/Mad-related).

Smads are subdivided into three subclasses based on their structure and function. Receptor-regulated Smads (R-Smads) are directly phosphorylated by the type I receptor kinases. Upon phosphorylation, R-Smads form complexes with common-partner Smads (Co-Smads) and translocate into the nucleus, where they regulate the transcription of target genes. In addition to these positively acting Smads, the inhibitory Smads (I-Smads) interfere with the receptor activation or complex formation of R-Smads (Massagué, 1998; Miyazono, 2000; Wrana and Attisano, 2000; Moustakas et al., 2001). Smads 2 and 3 are phosphorylated by TGF-βs and activin receptors and while Smads 1, 5 and 8 are activated by BMP receptors. In mammals, a single co-Smad, Smad 4, has been identified. The antagonistic Smads are Smads 6 and 7 (Wrana and Attisano, 2000).

In the CNS, there is very scarce evidence about Smad pathway. Most of data concerning Smad-mediated pathways in nervous system refer to BMPs signaling in neurons (Lein et al., 2002; Angley et al., 2003; Farkas et al., 2003; Rios et al., 2004). TGF-βs/Smad activated pathways in glial cells represent an emergent field.

One compelling evidence of the role of Smad signaling in nervous system development came recently with the generation of mice lacking Smad 4 in the brain (Zhou et al., 2003). Because Smad 4 is the central mediator of the signal transduction of all TGF-βs in mammals, mutant mice carrying a target disruption of Smad 4 died at embryonic period (Sirard et al., 1998; Yang et al., 1998). Zhou et al. (2003) recently addressed the role of Smad 4 in nervous system by producing a Smad 4 knockout mice with specifically disruption of this protein in the brain. Although the brains of these animals were relatively normal, they presented a decrease in the number of Purkinje cells in the cerebellum and abnormal motor control (Zhou et al., 2003).

One of the first evidences of TGF-β1 induced Smad nuclear translocation in astrocytes was demonstrated by Burton et al. (2002). These authors reported that TGF-β1 induced the expression of the Alzheimer β amyloid precursor protein (APP) gene in human astrocytes. Treatment of astrocytes with TGF-β1 induced nuclear translocation of Smads 3 and 4 in contrast to Smad 2, which was kept in the cytoplasm (Burton et al., 2002). Further, they showed a significant increased expression and strong nuclear staining of Smad 4 in Alzheimer cases. More recently, astrocyte APP upregulation by TGF-β1 was described to be the result of cooperation between Smad 3 and 4 and the transcription factor Sp1 (Lesné et al., 2003; Docagne et al., 2004). These data suggest that TGF-β1/Smad 3–4 pathway might play a key role in Alzheimer disease progression.

As we previously discussed, secretion of TGF-β1 by astrocytes has a primary neuroprotective role in nervous system. However, the signaling pathway involved in this event is not completely elucidated. Works of Vivien's group provided additional comprehension on this subject. They demonstrated that transfection of astrocytes with a plasmid construct encoding Smad 3 leads to an overexpression of type-1 plasminogen activator inhibitor (PAI-1) mRNA and further a neuroprotective activity against *N*-methyl-D-aspartate (NMDA)-induced neuronal death (Docagne et al., 2002). These results suggest that the direct modulation of intracellular effectors such as Smad proteins could represent a neuroprotective strategy in cerebral pathologies.

Although the molecular mechanisms of TGF-β1 directed transcription have been extensively studied in other systems,

this is still a growing field for CNS. Recent studies underlying the intracellular TGF-β/Smad pathway in astrocytes demonstrated that the ultimate intensity of TGF-β signaling is tightly modulated by nuclear Smad binding partners, which interact with Smads controlling their availability (Shi and Massagué, 2003; Docagne et al., 2004; Law et al., 2004). Understanding these molecular events will certainly help to understand the role of TGF-β1 in CNS development and injury.

5. Role of TGF-β1 as mediator of astrocyte and radial glia development

Over the past decade, a considerable effort has been made to elucidate the mechanisms that underlie glia function in central nervous system (CNS) development and disease. Although glial cells have been regarded so far as elements of structural and trophic support, today they might represent a key element in neural development. Astrocytes are macroglial cells, which contribute to several processes of brain development, such as neurogenesis (Lim and Alvarez-Buylla, 1999; Song et al., 2002), neuronal migration (as radial glial cells, which are neural progenitors; Rakic, 1972; Nadarajah and Parnavelas, 2002), proliferation and differentiation (Gomes et al., 1999b; Martinez and Gomes, 2002), neural signaling (Fróes et al., 1999), synapse formation (Pfrieger and Barres, 1997; Nägler et al., 2001; Ullian et al., 2001), blood–brain barrier organization (Abbott, 2002; Garcia et al., 2004) and axonal guidance (Garcia-Abreu et al., 1995; Goodman and Tessier-Lavigne, 1997). More recently, astrocytes have been pointed as putative neural stem cells (Doetsch et al., 1999).

In the injured CNS, astrocytes appear as a key component of reactive gliosis, a process that characterizes the response of nervous system to many kinds of lesions such as ischemia, excitotoxic lesion and several neurodegenerative diseases (Hatten et al., 1991; Ridet et al., 1997). Following CNS injury, there is a dramatic increase of TGF-β1 by astrocytes, an event clear implicated in glial scar formation (da Cunha et al., 1993; Pratt and McPherson, 1997; Flanders et al., 1998).

Despite the widespread effects reported for TGF-β1 in CNS injury, where it has been implicated in the organization of the glial scar (Moon and Fawcett, 2001), relatively little has been reported in physiological situations.

TGF-β1 affects motility and morphology of astrocytes. When administrated in vitro, in the presence of fibroblast growth factor (FGF), TGF-β1 causes profound change in cellular morphology including the development of branched processes characteristic of hypertrophic astrocytes and colony formation (Labourdette et al., 1990; Toru-Delbauffe et al., 1990; Flanders et al., 1993). In vitro, it has been reported to reduce cell–cell-contacts and increase focal contacts, thus inducing astrocyte motility (Gagelin et al., 1995). TGF-β1 stimulates the production of laminin and fibronectin and their incorporation into the ECM of primary cultures of cerebellar astrocytes (Baghdassarian et al., 1993). It promotes the appearance of actin stress fibers and increases the cell actin content. In vivo administration of TGF-β1 has been reported to induce several neuronal and astrocytic cytoskeleton genes such as GFAP and tubulin (Laping et al., 1994). Overproduction of TGF-β1 in astrocytes from transgenic mice results in increased production of laminin and fibronectin (Wyss-Coray et al., 1995). More recently, the role of TGF-β1 in ECM production was highlighted by the demonstration that TGF-β1 knockout mice present reduced expression of laminin (Brionne et al., 2003).

TGF-β1 interferes with the metabolism of the extracellular matrix through up-regulation of type-1 plasminogen activator inhibitor (Plow et al., 1995), an inhibitory peptide for the serine–protease tissue-type plasminogen activator (t-PA). t-PA activity is implicated in a variety of processes during NS development as well as in the physiology of adult brain such as neurite outgrowth (Gurwitz and Cunningham, 1988), cell migration (Pittman and DiBenedetto, 1995), synaptic plasticity (Qian et al., 1993; Seeds et al., 1995) and in cerebral ischemia (Tsirka et al., 1997). Works from Vivien's group demonstrated that neuroprotective activity of TGF-β1 against NMDA neurotoxicity is associated with the modulation of tPA/PAI-1 axis. PAI-1, produced by astrocytes, is up-regulated by TGF-β1 treatment (Buisson et al., 1998; Docagne et al., 2002). This event leads to modulation of the NMDA-evoked calcium influx in neurons and is responsible for the neuroprotective effects of TGF-β1 against NMDA injury. Overexpression of PAI is not induced by other members of TGF-β family, such as bone morphogenetic proteins and glial cell line-derived neutrophic factors (Docagne et al., 1999). These results underline the potential neuroprotective effect that would be obtained by a modulation of the t-PA/PAI-1 axis and highlights the role of TGF-β1 modulation of astrocytic extracellular matrix components.

ECM has been implicated in several steps of CNS development, such as neuronal migration, survival and axonal growth. Changes promoted by TGF-β1 in astrocyte shape, cytoskeleton and ECM production may have relevance for understanding the mechanisms of action of this growth factor during brain development.

TGF-βs are well known inhibitors of astrocyte proliferation (Toru-Delbauffe et al., 1990; Lindholm et al., 1992; Morganti-Kossmann et al., 1992; Baghdassarian et al., 1993). It can inhibit the proliferation directly or it can either enhance or antagonize the action of other growth factors (Vergeli et al., 1995; Krieglstein et al., 1998a,b,c). Hunter et al. (1993) demonstrated that the three isoforms of TGF-β(1, 2, 3) inhibited the proliferation of cerebral hemisphere cultured astrocytes induced by FGF, epidermal growth factor (EGF), platelet-derived growth factor (PDGF), interleucin alpha (ILα) and interleucin 2 (IL2). They reported a TGF-β1 isoform specificity of anti-proliferation actions; TGF-β1 is

more effective in suppressing proliferation induced by PDGF, whereas TGF-β3 is more effective in suppressing FGF-mediated proliferation. In the majority of tissue types studied, TGF-β induces growth suppression associated with the increased expression or activities of several cyclin-dependent kinase inhibitors, including p15, p21 and p27 (Hannon and Beach, 1994). A similar mechanism might occur in TGF-β inhibition of astrocyte growth. Rich et al. (1999) demonstrated that induction of the cyclin dependent kinase inhibitor (Cdk1) p15 appears to be an important component of the TGF-β-mediated proliferative response in primary astrocytes. Further, the loss of this protein in gliomas is associated with the loss of the growth inhibitory response to TGF-β in these tumors.

Region-specific responses of astrocytes to TGF-β1 have also been reported (Johns et al., 1992); whereas TGF-β1 stimulates proliferation of brainstem astrocytes, it has no effect on forebrain astrocytes. Apparently, astrocytes derived from different encephalic regions, distinctly respond to TGF-β1. Recently, our group demonstrated that cortical neurons activate GFAP gene and differentiation from cortex astrocytes by secretion of TGF-β1. This event presents a neuroanatomical regional specificity since GFAP gene from cerebellum and midbrain is not activated in response to TGF-β1/neurons (Box 1). Together these data highlight (1) the great complexity of neuron–glia interactions and suggest a distinct mechanism underlying modulation of GFAP gene in heterogeneous population of astrocytes and (2) the great context-dependent action of TGF-β1 through out CNS.

Emerging data have been accumulating recently suggesting a role for TGF-β1 in radial glia development. Although radial glia cells are recognized today as neural stem cells they were originally described as specialized non-neuronal cells, which served solely as migratory scaffolding and disappeared early in development by transforming into astrocytes (Rakic, 1972; Voigt, 1989; Nadarajah and Parnavelas, 2002). The distribution of TGF-β ligands and receptors in the developing cortex in vivo are consistent with the notion that TGF-β might be involved in neuronal migration and thus radial glia–neuronal interaction: TGF-β among others. In order to gain insight into astrocyte differentiation induced by neurons, we have focused on GFAP expression, which is the major component of the astrocytic intermediate filaments (Eng et al., 1971; Bignami et al., 1972). In the rodent CNS, astrocyte maturation is followed by a replacement in the expression of vimentin by GFAP (Dahl, 1981; Pixley and De Vellis, 1984). By using transgenic mice bearing 2 kbp of the 5′ flanking region of the GFAP gene linked to the β-galactosidase (β-Gal) reporter gene, we have demonstrated that cortical neurons activate the GFAP gene promoter followed by transgenic astrocyte differentiation in vitro. This event was dependent on the brain origin of the neurons and was followed by an arrest of astrocyte proliferation and induction of glial differentiation. Addition of conditioned medium derived from cortical neurons had a similar effect, suggesting that a soluble factor derived from neurons might be responsible for the induction of the GFAP gene promoter (Gomes et al., 1999a). Recently, we identified TGF-β1 as the major mediator of this event (de Sampaio e Spohr et al., 2002; Fig. 1). We demonstrated that both cell types, neurons and astrocytes, synthesize and secrete this factor. However, addition of neurons to astrocyte monolayers greatly increased TGF-β1 synthesis and secretion by astrocytes (de Sampaio e Spohr et al., 2002). This event presents a neuroanaotomical regional specificity: cortical neurons or their conditioned medium do not activate the GFAP gene promoter of transgenic astrocytes derived from midbrain and cerebellum (Sousa et al., 2004). Surprisingly, they 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 (Sousa et al., 2004; Fig. 2). Thus, indicating that unresponsiveness of cerebellar and midbrain GFAP gene to TGF-β1 is not due to a defect in TGF-β1 signaling. An interesting observation is that neurons derived from cerebellum or midbrain, although secreting TGF-β1, do not induce GFAP expression with high efficacy. This observation leads to the suggestion that other factor might act in synergism with TGF-β1 in order to yield full activation of the GFAP gene in our coculture model. A good candidate for this function is glutamate, the major excitatory neurotransmitter of the mammalian CNS. Works by the Nicoletti's group demonstrated that activation of group-II mGlu receptors in astrocytes in vitro leads to increased synthesis of TGF-β1 and TGF-β2 by these cells which in turn act as neuroprotective factors against NMDA toxicity (Bruno et al., 1997, 1998; D'Onofrio et al., 2001). Our work was pioneering in revealing a physiological function of TGF-β1 on astrocyte development and GFAP expression. They support the concept that neuronal signals might provide a source responsible for astrocyte development and strongly implicate TGF-β1 in this process.

Box 1. *A novel role for TGF-β1 in neuron-astrocyte interactions:* Neuron–glia interactions are of fundamental importance in several processes of brain development (Lim and Alvarez-Buylla, 1999; Gomes et al., 2001; Ullian et al., 2001; Martinez and Gomes, 2002; Song et al., 2002). Several evidences have been recently accumulating pointing TGF-β1 as a novel mediator of neuron–glia interactions, specially during astrocyte differentiation. A way to study astrocyte differentiation is by evaluating levels of proteins which expression patterns vary during development such as the cytoskeleton molecule glial fibrillary acidic protein (GFAP) (Eng et al., 1971), the enzyme glutamine synthetase (Kvamme et al., 1982) and glutamate transporters (Würdig and Kugler, 1990),

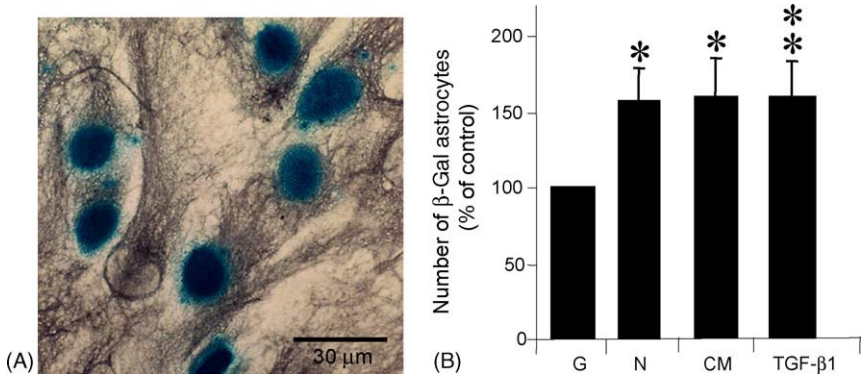

Fig. 1. Neurons induce *GFAP* gene promoter and astrocyte differentiation by secreting TGF-β1. Cerebral cortex astrocytes derived from newborn transgenic (bearing part of the GFAP gene promoter linked to the β-galactosidase (β-Gal) reporter gene) were kept for 12 days in culture (10 days in the presence of serum and 2 additional days in serum-free medium). After this period astrocytes monolayers were maintained for 24 h in the presence of: serum-free medium, control (G); 200,000 neurons derived from cerebral cortex of 14 days (E14) embryonic mice (N); conditioned medium derived from these neurons (CM) or 10 ng/ml of TGF-β1 (R&D Systems, Buckinghamshire, UK). GFAP promoter-directed expression of *lacZ* was revealed by X-Gal (gray nuclei) prior to anti-GFAP immunocytochemistry (cytoplasmic staining) (A). After β-Gal activity detection, β-Gal positive astrocytes were quantified. Each point represents the average of three independent experiments done in triplicate. Statistical significance: $^{*}p < 0.001$; $^{**}p < 0.00001$ (mean + S.D.). Addition of embryonic neurons, CM or TGF-β1 increased β-Gal astrocyte number.

ligands and receptors are expressed by neurons and radial glia (see itens 2 and 3). Supporting this idea, recently, Siegenthaler and Miller (2004) demonstrated that TGF-β1 alters cell migration in the developing cortex. By using cortical organotypical cultures, the authors showed that administration of TGF-β1 altered cell migration in a concentration-dependent manner: at low concentrations, cell migration was promoted whereas at high concentrations TGF-β1 impaired migration. This event was followed by increase in the levels of several adhesion proteins, such as

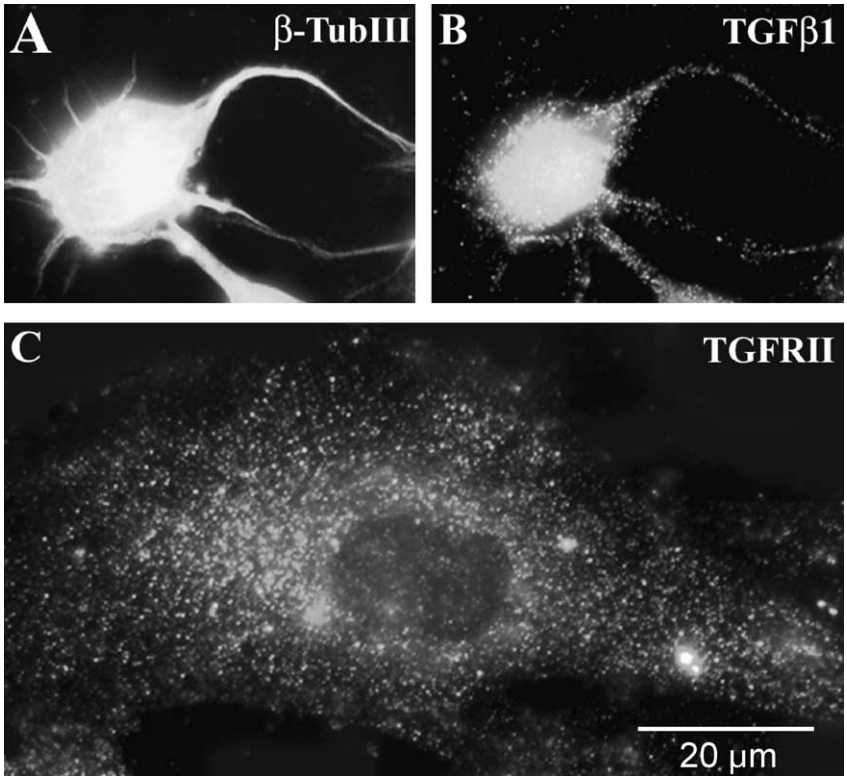

Fig. 2. Expression of TGF-β1 and TGFRII by neurons and astrocytes of the cerebral cortex in vitro, respectively. (A and B) Neurons derived from 14-days embryonic cerebral cortex were cultured for 24 h and immunostained for the neuronal marker, β-tubulin III (β-Tub III; mouse anti-human β-tubulin III antibody; Promega Corporation) (A) and TGF-β1 (TGF-β1; rabbit anti-transforming growth factor-beta; Sigma Chemical Co.) (B). Note the punctate arrangement of TGF-β1 staining in the processes of cortical neurons. (C) Astrocytes derived from newborn cerebral cortex were cultured for 10 days and immunostained for TGFRII (TGFRII; rabbit anti-transforming growth factor-beta receptor type II; Santa Cruz Biotechnology, Inc.). Astrocytes present a punctate pattern of TGFRII expression spread all over the cellular membrane.

nCAM and integrins (α3, α5 and β1). These data reported evidences of a novel function for TGF-β1 in the developing nervous system; by modulating the levels of cell adhesion proteins present in radial glia and migrating neurons, TGF-β1 controls cortical lamination.

Although there are several compelling data on TGF-β1 effects on astrocyte development in vitro, data from in vivo studies do not strongly support this hypothesis: (1) first, TGF-β1−/− knockout mice do not present alteration in GFAP labeling in the cerebral cortex (Brionne et al., 2003); (2) Smad 4 knockout mice do not present detectable alteration in the distribution and morphology of the Bergmann glia of cerebellum, revealed by GFAP staining (Zhou et al., 2003); (3) injection of neutralizing antibodies against TGF-β1 did not impair radial glia development in the chicken midbrain (Farkas et al., 2003). There are several possible explanations for this apparent discrepancy between in vitro and in vivo results: (1) one is that TGFβ/Smad signaling is not involved in radial glia and astrocyte development in vivo and thus results from culture studies may not completely reflect the in vivo situation; (2) alternatively, TGF-β/Smad signaling indeed plays a role in radial glia and astrocyte development, however a great biological redundancy with other molecules (specially BMPs) might compensate their effects in vivo in TGF-β−/− and Smad 4−/− mice; (3) in the case of Smad 4 mutant mice we cannot completely rule out a TGF-β1 Smad 4-independent signaling; (4) finally, in the case of Smad 4 mutant mice, as we have already commented above, the authors claim that Smad signaling might not be completely inactivated, thus it is possible that low levels of Smad 4 may be sufficient for certain TGF-β effects. Additional studies with double or triple knockout mice will certainly contribute in the future to elucidate these questions.

6. Concluding remarks

The molecular mechanisms that underlie TGF-β effects are relatively known for several organs and systems; however, only recently elucidative data on nervous system have emerged. Most of them were achieved from studies in invertebrate model organisms where TGF-β signaling has been implicated in several processes such as synaptic growth, neurotransmitter release, neuron survival and differentiation (Sanyal et al., 2004). In mammals, role of TGF-β in the healthy brain is only now being dissected with generation of several models of TGF-β/signaling mutants. However, several questions still remain unanswered. For example, the fact that although target perturbations of TGF-β signaling in different model systems have demonstrated its essential role in diverse developmental processes, there are only few examples of genetically defined human pathologic correlates (Cohen, 2003). This observation either reflects a survival dependency on or extensive functional redundancy in TGF-β-regulated programs. The second hypotheses might explain discrepancy between in vivo and in vitro data.

Although we have learned much about the physiology of glial cells over the last 10 years, our knowledge of the function and development of glia is still rudimentary. Several growth factors involved in gliogenesis have been identified and this will certainly be crucial for a better understanding of glial cell functions and interactions with neurons. The possibility that TGF-β might be a novel mediator of astrocyte–neuron interaction either in pathological conditions such as ischemia or glutamate toxity or recent data during astrocyte differentiation in vitro will certainly contribute to better understanding cellular interactions in the brain. Generation of in vivo models to access the role of TGF-β1 in astrocyte differentiation will be decisive to validate in vitro results.

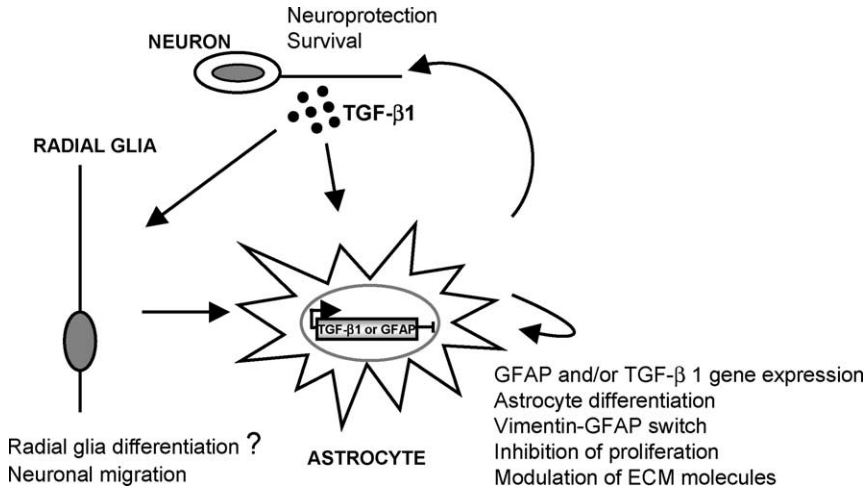

Fig. 3. TGF-β1 network as mediator of astrocyte interactions. Neurons increase production and release of TGF-β1 by astrocytes, which in turn protects neighboring neurons against excitotoxic death. Additionally, TGF-β1 secreted by neurons modulates neuronal migration along radial glia fibers and might be involved in radial glia differentiation. In addition to promoting neuronal survival, TGF-β1 modulates several events in astrocytes such as: induction of the GFAP gene expression and differentiation; inhibition of proliferation and modulation of secretion of extracellular matrix proteins (ECM). Such network summarizes the role of TGF-β1 in astrocyte biology and suggests that this cytokine must play key function as mediator of neuron–glia interactions during CNS development.

Another point, which remains opened is the signaling pathway activated by TGF-β1 in the healthy brain. Study of Smad-dependent and independent pathways will be helpful to unravel TGF-β actions. It is worthy to remember that signaling pathways make part of the overall signaling network of the cells. Thus, many more signals are likely to influence the cellular response to TGF-β (by either acting in synergism or antagonism) affecting, for example, the composition of Smad partners. This is certainly an emerging and promising field in the area of growth factors.

Interesting for glial cells (astrocytes and radial glia) has grew during the last few years since these cells have been pointed as putative neural stem cells (Doetsch et al., 1999; Gotz et al., 2002; Noctor et al., 2002). Within this context, it will be very useful in the future to elucidate the effect and signaling mechanisms involved in TGF-β1 induced radial glia–astrocyte differentiation.

Today, the list of TGF-β1 role in nervous system has been extensively enlarged. As illustrated in Fig. 3, besides its role in injury and its well known role as a neuroprotective factor, the endogeneous TGF-β1 system has been clearly implicated in several processes of brain development, such as GFAP gene expression and astrocyte differentiation, neuronal migration and cortex lamination and neuronal survival. As we have outlined in this review, studying the diversity of astrocytes responses to TGF-β1 and the underlying mechanisms may not only provide insights into the basic processes of CNS homeostasis but also a better understanding of the role of this cytokine in neurodegenerative disorders.

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ANEXO 2

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Quantum dots as fluorescent bio-labels in cancer diagnostic

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In this work we present and discuss some results concerning the application of colloidal semiconductor quantum dots for cancer diagnostic. We have prepared and applied different core-shell semiconductor quantum dots such as Cadmium Teluride-Cadmium Sulfide (CdTe-CdS) and Cadmium Sulfide – Cadmium Hydroxide ($CdS/Cd(OH)_2$). For the purpose of diagnostic with living cells, the $CdS/Cd(OH)_2$ presented best results, maintaining high levels of luminescence as well high stability in biological media. The quantum dots were obtained in aqueous medium, by reacting Cd^{2+} and S^{2-} in the presence of sodium polyphosphate as the stabilizing agent. Subsequent surface passivation with $Cd(OH)_2$ was carried out to improve luminescence. At a pH of 7,2 the quantum dots were functionalized with a 0.01% glutaraldehyde solution and then, incubated with living healthy and neoplastic cells (glial, glioblastoms and cervical) and tissues (breast) in culture medium. Tissue and cell staining were evaluated by the laser scanning confocal microscopy. Fluorescence Microscopy was used as a primary tool in order to explore the labeling of the samples. The procedure presented in this work, shown to be very efficient as a potential tool for fast and precise cancer diagnostic.

1 Introduction

Fluorescence provides an important tool for the investigation of basic physical properties of biological structures. The high sensitivity of fluorescence, combined with the advances in measurement techniques, permits detection of ultra small quantities of specific species present in the biological system. There is a great number of compounds used to generate fluorescence, such as organic molecules, fluorescent proteins, metal chelators, chemi- and bioluminescent agents. All of these fluorophores present one or more of the following disadvantages: lack of brightness, broad emission bands and high photobleaching rate (see Fig. 1). In the last decade a new class of fluorescent materials known as quantum dots (QDs) is

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being tested as biolabels. Quantum dots are nanometric inorganic crystals, which present special characteristics due to the fact that they are in quantum confinement regimen [1–3]. In the case of semiconductor quantum dots, one of these special characteristics is the capability of tuning their optical properties, particularly their emission spectra [4] by controlling the size of the particles. The first biological applications of quantum dots were reported in 1998 [5, 6]. Bruchez et al. [5] and Chan et al. [6] used CdSe QDs coated with silica and mercaptoacetic acid layers, respectively, and both groups showed specific labeling by covalent coupling of ligands to these surfaces. Subsequently, several authors have reported labeling of whole cells and tissue sections using several different surface modifications of QDs [7–11].

By attaching biomolecules to nanometer-sized bits of semiconductors, a sensitive and potentially widely applicable method for detecting biomolecules and for scrutinizing biomolecular processes was developed [12, 13]. The quantum dot-labeled molecules remain active for biochemical reactions and the tagged species produce brightly colored products [5, 6]. This methodology takes advantage of the efficient fluorescence and high photostability of the semiconductor dots, representing a new class of biological stains.

Quantum dots application in the investigation of neoplastic processes, which may give rise to a wide variety of cancer, constitutes a topic of crescent interest and with many questions that still wait for precise answers. In the pursuit of sensitive and quantitative methods to detect and diagnose cancer, nanotechnology has been identified as a field of great promise. Hydrophilic quantum dots in water medium and at physiological pH conditions, have the potential to expand conventional protocols used for cancer diagnostic, which needs previous tissue/cell fixation, and extend it to investigate living cellular and tissular neoplastic mechanisms in real time. In this work we show and discuss the results obtained by conjugating $CdS/Cd(OH)_2$ highly fluorescent quantum dots with living healthy and neoplastic breast, glial and cervical tissues and cells.

2 Methodology

The core-shell luminescent $CdS/Cd(OH)_2$ QDs were obtained reacting Cd^{2+} and S^{2-} in aqueous solution in the presence of sodium polyphosphate as the stabilizing agent. Subsequent surface passivation with $Cd(OH)_2$ was carried out to improve luminescence. At a pH of 7,2 the QDs were functionalized with a 0.01% glutaraldehyde solution (QD-glut), as described in a previous work [14]. The glutaraldehyde is an organic functionalizing agent that intermediates the interaction of the QDs with the living healthy and neoplastic cells. All samples were in culture medium and the incubation with the QDs was performed at room temperature (25 ^{o}C). Tissue and cell staining were evaluated by the laser scanning confocal microscopy Leica TCS SP2 AOBS (breast and cervical cells/tissues) and LSM 510 Carl Zeiss (for glial and glioblastoms cells) confocal microscopes, using the same acquisition parameters in order to be compared. The images were further processed using the software Leica Lite and LSM 510 (Carl Zeiss Inc). Laser Scanning Confocal Microscopy and Fluorescence Microscopy (Carl Zeiss, Jena, Germany) measurements were also performed at room temperature and different time intervals in order to monitor the time evolution of the interaction between the QDs and the cells. The Fluorescence Microscopy was used as a primary tool in order to explore the labeling of the samples.

Here there will be presented the results obtained by Laser Scanning Confocal Microscopy, using an apochromatic water immersion, 63x with numerical aperture of 1.2, objective lens. Two wavelengths were used to promote excitation of the marked samples: 488 and 543 nm. The recorded image was taken using dual-channel scanning and consisted of 1024x1024 pixels. For each cell type the images were reproduced at least three times, and to establish a comparative analysis of the luminescence intensity maps the parameters related to the acquisition of confocal images, such as pinhole, filters, beam splitters and photomultiplier gain and off-set were maintained constant.

3 Results and discussion

Structural characterization of the QDs was perfomed by X-Ray Diffraction and by Electronic Transmission Microscopy experiments. A representative Transmission Electronic Microscopy image of the core-shell $CdS/Cd(OH)_2$ quantum dots functionalized with glutaraldehyde is shown in Fig. 1, in which the

scale bar corresponds to 40 nm. The averaged size of the QDs is about 9 nm. Optical characterization of the QDs was done by spectroscopic techniques. Figure 2 illustrates the excitation and emission spectra for as prepared core-shell $CdS/Cd(OH)_2$ quantum dots, in which may be observed a broad excitation band and a narrow Gaussian emission band of about 50 nm (FWHM).

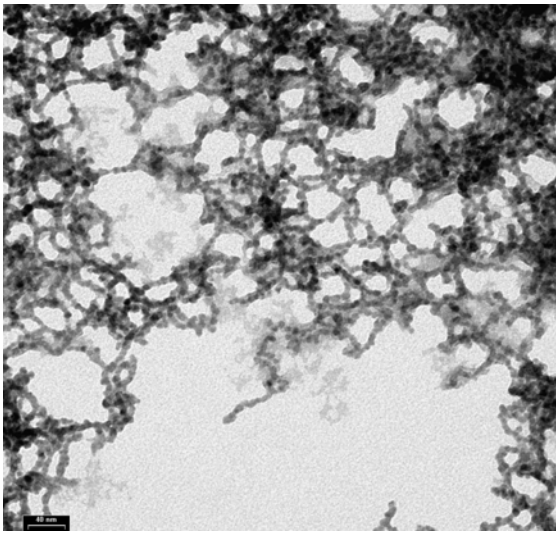

Fig. 1 Transmission Electron Microscopy image of the $CdS/Cd(OH)_2$ core shell quantum dots. Scale bar: 40 nm.

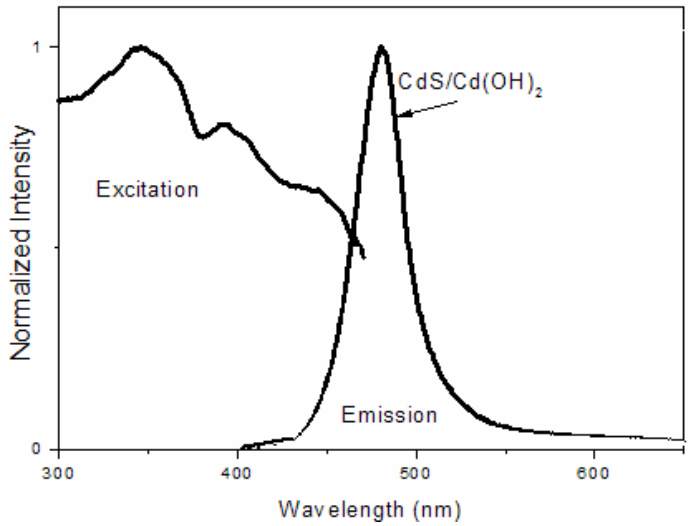

Fig. 2 Excitation and emission spectra of as prepared $CdS/Cd(OH)_2$ quantum dots.

Figures 3 and 4 show confocal microscopy images (left) and corresponding fluorescence intensity maps (right) for the healthy and neoplastic glial cells and the corresponding incubation time of the cells with the functionalized QDs-glut. At the fluorescence intensity maps, the blue (dark grey at printed version) regions corresponds to the absence of fluorescence, while the green, yellow and red regions (lighter but more intense grey at printed version) corresponds to regions of highest fluorescence intensities respectively.

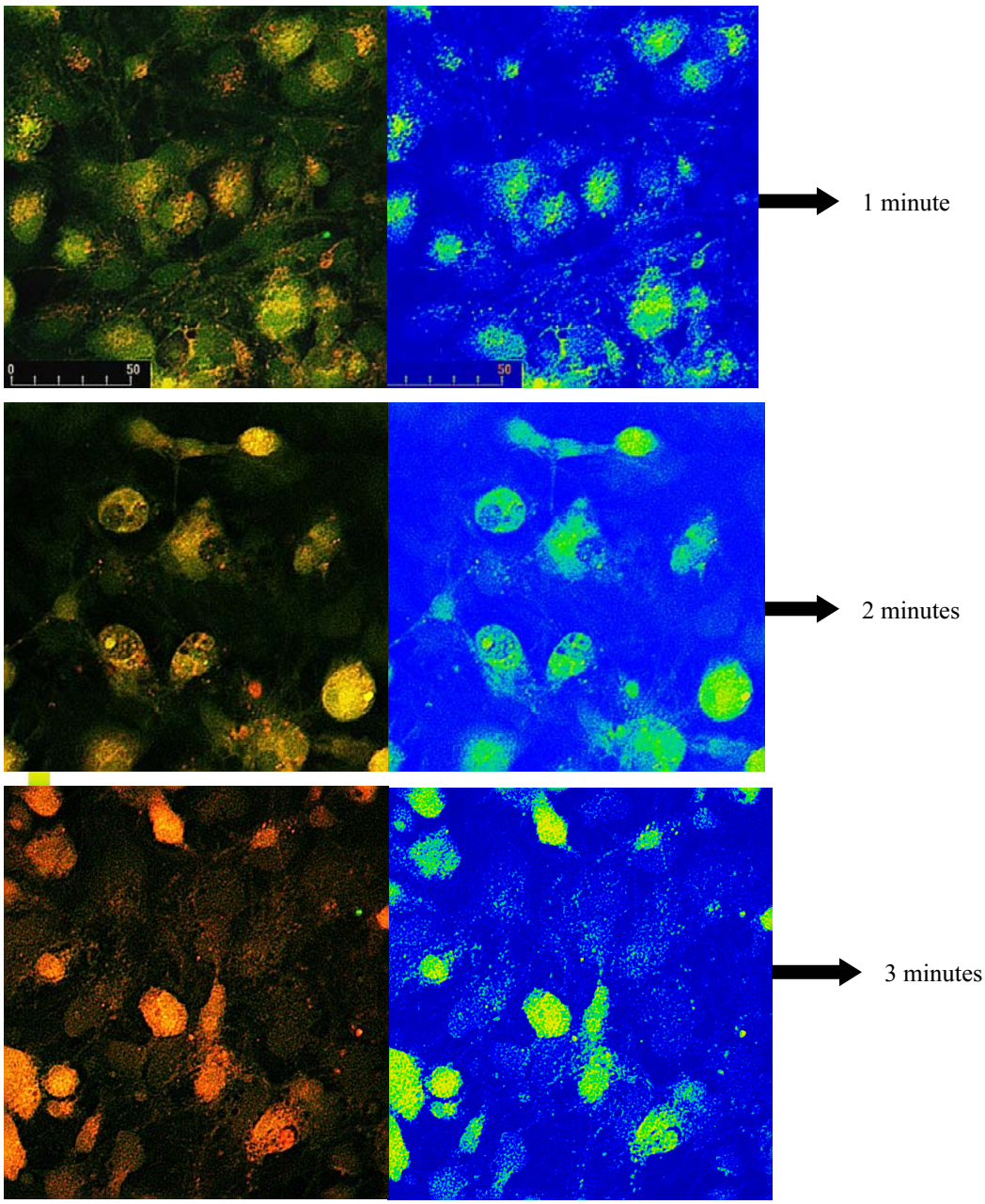

Fig. 3 Healthy glial cells incubated with QDs-Glut. Left: Confocal Microscopy image. Right: corresponding fluorescence intensity maps.

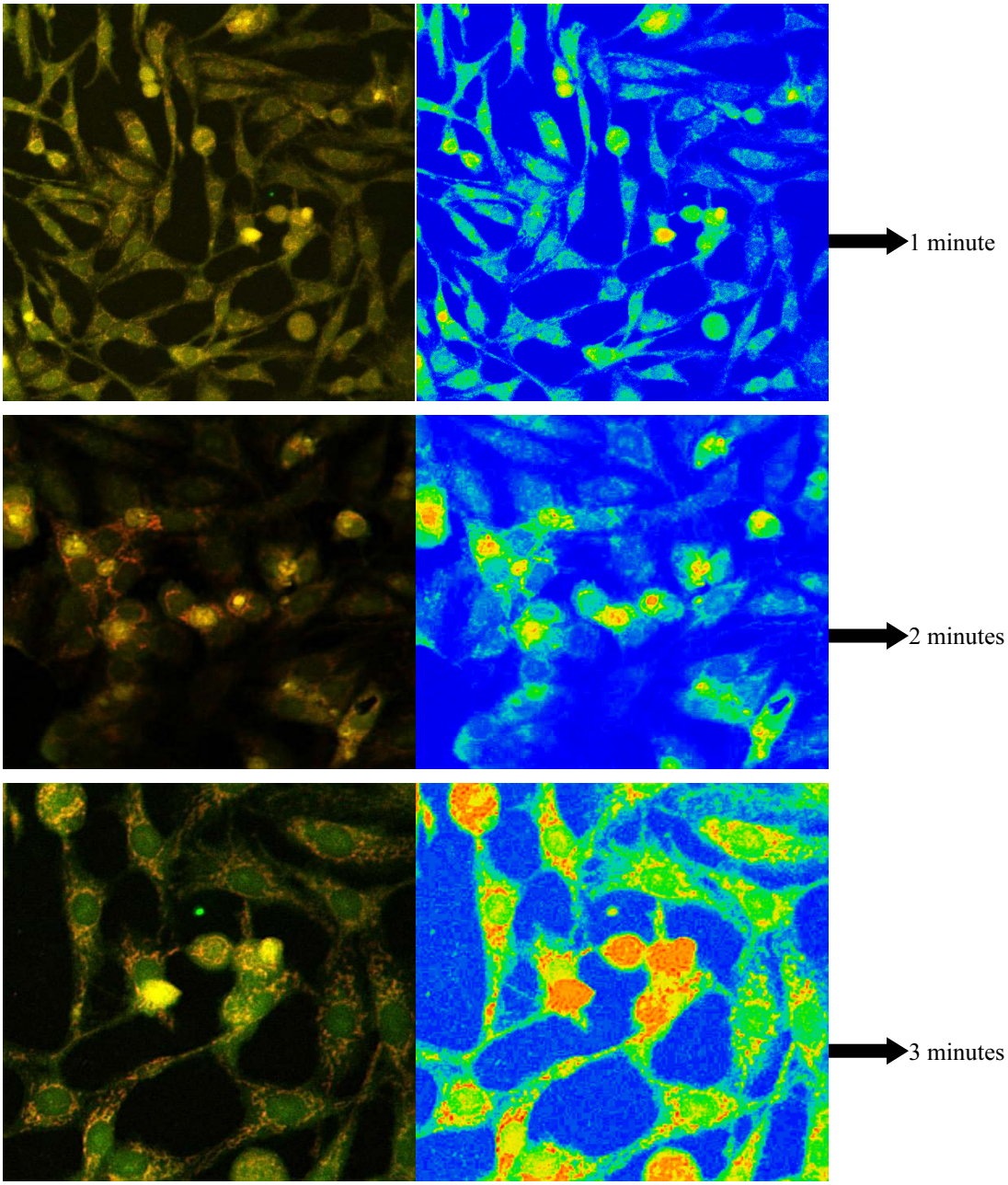

Fig. 4 Neoplastic glial (glioblastoma) cells incubated with QDs-Glut. Left: Confocal Microscopy image. Right: corresponding fluorescence intensity maps.

Figure 5 shows Transmission Electronic Microscopy image of glioblastoma labeled cells, in which the highest QDs concentration is nearby the nuclear envoltorium.

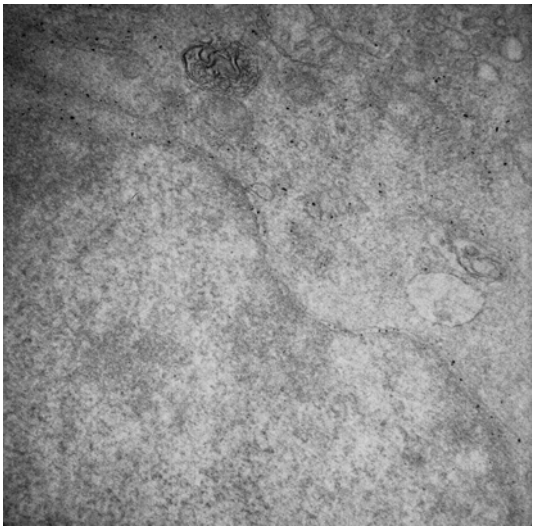

Fig. 5 Transmission Electron Micrography image of Glioblastoma labeled cells: highest QDs concentration at the nuclear envoltorium.

Breast cancer tissue samples were incubated with the functionalized QDs-glut, and as can be noticed from Fig. 6, the neoplastic cells filling up the mammary duct clearly show highest QD concentrations compared with the normal cells of the same sample.

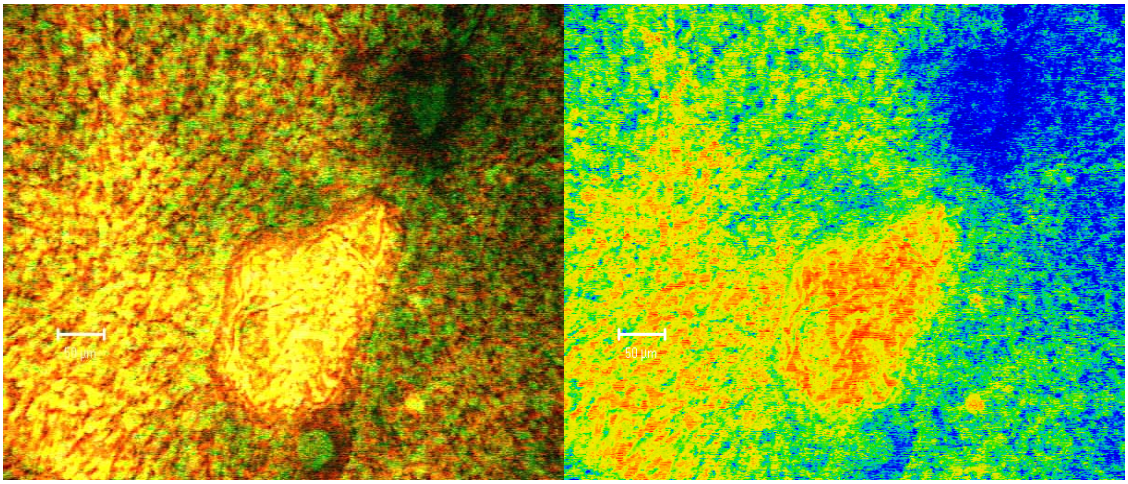

Fig. 6 Left: Confocal Microscopy image of filling ductal carcinoma. Right: Corresponding fluorescence intensity map.

Figures 7 and 8, show respectively confocal microscopy images and fluorescence intensity maps for QDs labeled normal cervical cells and cervical intra-epithelial neoplastic cells 3 (INC3), while Fig. 9 shows confocal microscopy image, fluorescence intensity map and transmission microscopy overlapped with fluorescence image of cervical cells presenting severe dyskaryosis, which is the last stage prior to cervical cancer. The images shown in Figs. 8 and 9, represent processes which result from the infection of cervical cell by Human Papillomaviruses (HPV).

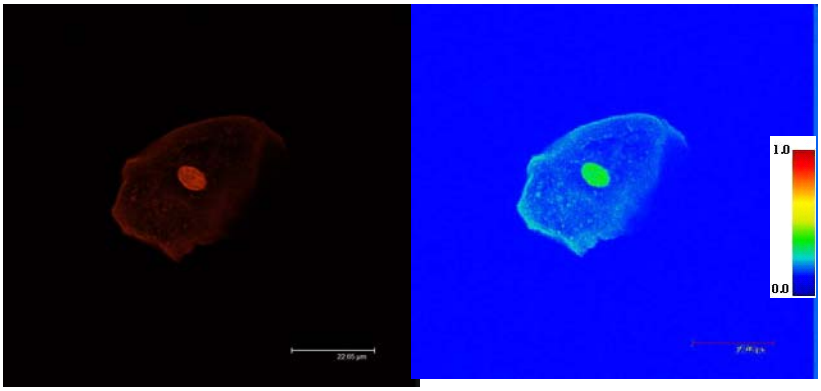

Fig. 7 Left: Confocal microscopy image of normal squamous cervical cell. Right: Corresponding fluorescence intensity map.

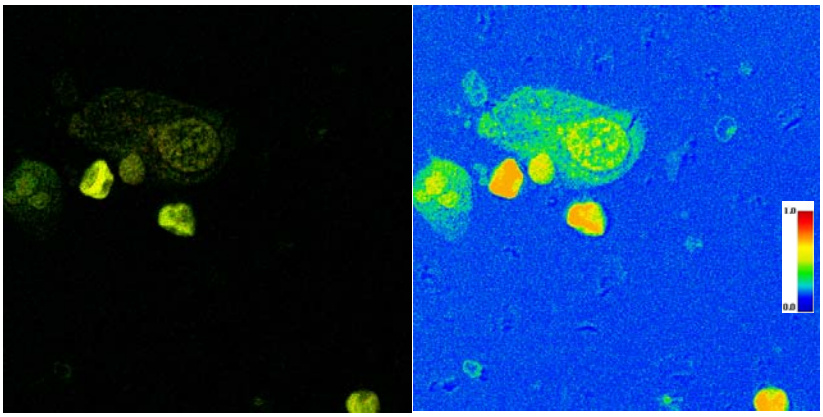

Fig. 8 Cervical intra-epithelial neoplastic cells 3 (INC3). Left: Confocal microscopy image. Right: Fluorescence intensity map.

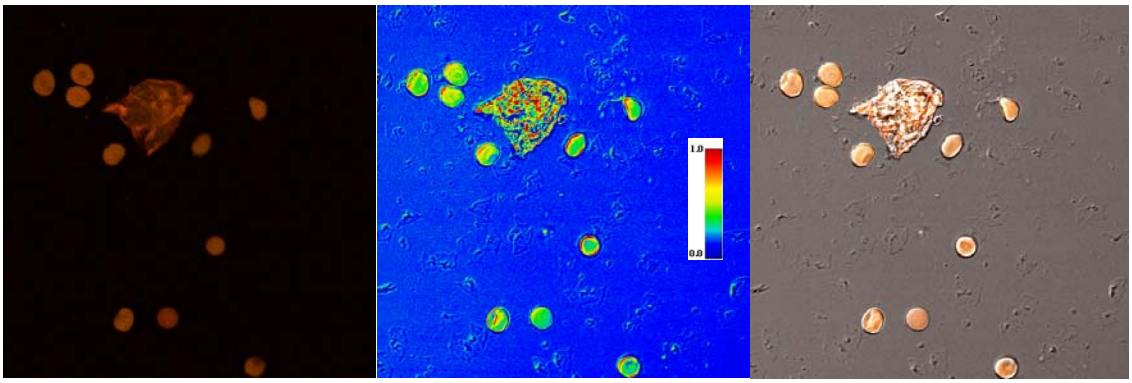

Fig. 9 Confocal microscopy image (left), fluorescence intensity map (center) and transmission microscopy overlapped with fluorescence image (right) of cervical cells presenting severe dyskaryosis.

The living cells showed no sign of damage after the conjugation procedure with the QDs and maintained their integrity even after five days of incubation time, demonstrating the low toxicity of the QDs for *in vitro* studies. The time evolution of the interaction cells-QDs shown in Figs. 3 and 4 above clearly reveals different labeling patterns as well as different fluorescence intensities. It also can be noticed that the CdS-$Cd(OH)_2$-Glut QDs easily interacts with both healthy and neoplastic. As the glutaraldehyde is a homofunctional bidentade ligand, it establishes hemi-acetal interaction with the QDs outer shell at the same time that binds to cell proteins by Schiff's base interactions [13, 14]. Due to the differences that rise in neoplastic processes, such as: metabolic rates and regimen, cell membrane permeability and fluidity, the fluorescence intensities and patterns are quite different for healthy and neoplastic cells. The mechanisms Neoplastic mechanisms almost always result in cancer. They present some singular aspects such as: fast endocytic rates and lack, selectivity of cell membrane, which allows the presence of large quantities of QDs at the citoplasmatic region, in special at the lisossomes. These results show that a simple procedure of synthesis, functionalization and incubation of aqueous quantum dots at physiological conditions, with healthy and neoplastic cells may represent a potential tool for fast and precise diagnostic of different kinds of cancer.

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ANEXO 3

Dopamine Affects the Stability, Hydration, and Packing of Protofibrils and Fibrils of the Wild Type and Variants of α-Synuclein†

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ABSTRACT: Parkinson's disease (PD) is characterized by the presence of cytoplasmic inclusions composed of α-synuclein (α-syn) in dopaminergic neurons. This suggests a pivotal role of dopamine (DA) on PD development. Here, we show that DA modulates differently the stability of protofibrils (PF) and fibrils (F) composed of wild type or variants of α-syn (A30P and A53T) as probed by high hydrostatic pressure (HHP). While in the absence of DA, all α-syn PF exhibited identical stability, in its presence, the variant-composed PF acquired a greater stability ($DAPF_{wt} < DAPF_{A30P} = DAPF_{A53T}$), implying that they would last longer, which could shed light onto why these mutations are so aggressive. When α-syn was incubated for long times (18 days) in the presence of DA, we observed the formation of F by electronic microscopy, suggesting that the PF trapped in the presence of DA in short times can evolve into F. The stability of F was also altered by DA. DAF_{wt} was more labile than F_{wt}, indicating that the former would be more susceptible to breakage. PF_{A30P} and $DAPF_{A30P}$, when added to mesencephalic and cortical neurons in culture, decreased the number and length of neurites and increased the number of apoptotic cells. Surprisingly, these toxic effects of PF_{A30P} and $DAPF_{A30P}$ were practically abolished with HHP treatment, which was able to break the PF into smaller aggregates, as seen by atomic force microscopy. These results suggest that strategies aimed at breaking and/or clearing these aggregates is promising in alleviating the symptoms of PD.

Parkinson's disease (PD)[1] is the second most common neurodegenerative brain disorder, after Alzheimer's disease, affecting ∼1% of the population over 65 years of age (*1*). The symptoms of PD include difficulty in initiating movements, resting tremor, muscular rigidity, impaired postural reflexes, and bradykinesia, resulting from the loss of dopaminergic neurons and depletion of dopamine (DA) in the regions of the brain involved in motor functions [the *substantia nigra* (SN)] (*2*). The remaining neurons of the SN are characterized by the presence of an intracellular inclusion, known as Lewy bodies (LB), composed primarily of fibrillar α-synuclein (α-syn) (*3*−*6*).

α-syn is a 140 amino acid, "natively unfolded" protein with unknown function, although recent studies suggest that it may play a role in regulating the reserve pool of synaptic vesicles in the brain (*7*, *8*). Genetic findings support a strong link between α-syn and PD. Three mutations, A30P, E46K, and A53T, are linked to rare, early onset forms of PD (*9*−*11*). Duplication and triplication in the gene encoding α-syn have also been described in familial PD (*12*), suggesting that increased expression is sufficient to cause PD. Although fewer than 10% of Parkinson's cases are linked to the presence of these mutations in the α-syn gene (*13*), detailed characterization of these variants may provide critical insight

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[1] Abbreviations: PD, Parkinson's disease; α-syn, α-synuclein; HHP, high hydrostatic pressure; Tris, tris(hydroxymethyl-aminomethane); PF, protofibrils; F, fibrils; DAPF, dopamine−protofibrils; DAF, dopamine−fibrils; AFM, atomic force microscopy; EM, eletronic microscopy; Thio-T, thioflavin-T; bis-ANS, 1,1′-bis(4-aniline-5-naphthalene sulfonate).

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into the mechanism by which these mutations alter the properties and/or function of α-syn and thus may help to unravel the mechanism(s) of α-syn-induced neurodegeneration in PD.

Neuropathologic findings based on postmortem examination of normal and diseased human brains as well as studies of transgenic mouse and flies overexpressing α-syn (wild type and PD variants) support the hypothesis that prefibrillar α-syn aggregates (PF) might be the pathogenic species in PD and suggest that fibril formation by α-syn may be protective in PD (*14*, *15*). First, a transgenic mouse model expressing human or mouse wild-type α-syn shows PD-like symptoms, including degeneration of dopaminergic nerve terminals and formation of cytoplasmic, nonfibrillar α-syn inclusions (*16*). Second, all mutations (A30P, A53T, and E46K) associated with familial PD accelerate α-syn protofibril (A53T and A30P) and/or fibril (A53T and E46K) formation in vitro when compared to the wild-type protein (*11*, *17*−*19*). Furthermore, protofibrillar forms of α-syn but not monomers or F were shown to permeabilize synthetic vesicles in vitro; the permeabilization activity of α-syn was enhanced in the case of the disease variants A30P and A53T (*20*).

DA has been shown to interact with α-syn to form α-syn−DA adduct(s) which are thought to inhibit the conversion of α-syn PF into F. These findings support the hypothesis that protofibrillar α-syn is the pathogenic species and offer a possible explanation for the selective degeneration of dopaminergic neurons in PD (*15*, *21*, *22*). The inhibitory activity of DA depends upon its oxidative breakdown and seems to be selective for the protofibril−fibril conversion (*15*, *21*). In addition, DA, L-dopa, and other catecholamines were able to prevent and/or disaggregate amyloid F of α-syn produced in vitro. These findings suggest that the α-syn interaction with DA promotes the accumulation of potentially toxic protofibrillar intermediates both during amyloid formation and dissociation and support the hypothesis that α-syn fibrillization reflects a detoxification strategy by which dopaminergic neurons sequester toxic intermediates.

Recently, we demonstrated that amyloid F composed of transthyretin or α-syn were destabilized by high hydrostatic pressure (HHP) (*23*). In the case of α-syn, the F produced from the disease-associated variants were more sensitive than wild-type F to HHP. This result suggests that, although all amyloid F share a common architecture (the cross β sheet) (*24*, *25*), regardless of the primary sequence or structure of the precursor amyloidogenic protein, the side-chain contacts play a significant role in the fine adjustment of fibril architecture, packing, and stability (*23*).

The aim of the present study was to evaluate the effects of DA on the stability of the in vitro grown PF and F composed of the wild type or variant (A53T and A30P) α-syn. Any change in PF and F stabilities modulated by DA could represent an enhancement or a decrease in their longevity in dopaminergic neurons and consequently in their toxicity. Although other neuronal groups are also affected in PD, mainly in the patients bearing mutations (*8*), any change in the stability of these aggregates promoted by DA could shed light onto why the dopaminergic neurons are so vulnerable in PD. It has been shown that DA inhibits protofibril−fibril conversion (*15*, *21*, *26*); however, in longer incubation times, as used here (18 days), we observe the appearance of F in the presence of DA. Thus, we grew PF and F from wild-type and variant α-syn in the absence or presence of DA (here called DAPF and DAF) and subjected all of them to HHP. Our results show that, in the absence of DA, the conversion of PF into F is accompanied by a gain in stability, especially in the case of the wild type; this would render the nontoxic fibril a very stable species, freeing the nondopaminergic regions of the brain from the toxic effects of the PF. In the presence of DA (mimicking the conditions in dopaminergic neurons), the DAPF composed of the variants were more resistant to pressure denaturation than $DAPF_{wt}$, suggesting that the former would last longer in dopaminergic regions of the brain, thus explaining the faster progression of the disease in patients with α-syn mutations with an increased susceptibility of the dopaminergic neurons in PD. We also investigated the toxicity of PF_{A30P} and $DAPF_{A30P}$ added to cortical and mesencephalic embryonic neurons in culture. Both aggregates of A30P were highly toxic to these neurons. Interestingly, the HHP treatment significantly reduced the toxicity of PF_{A30P} and $DAPF_{A30P}$, an observation that reinforces their toxic role in PD.

EXPERIMENTAL PROCEDURES

Production of F and PF of α-syn. Recombinant α-syn was expressed and purified as described (*27*). Solutions with 50 μM wild type, A30P, or A53T were incubated in buffer A [5 mM tris(hydroxymethyl-aminomethane) (Tris)-HCl and 100 mM NaCl at pH 7.0] at 37 °C at atmospheric pressure under gentle stirring. Fibril formation was monitored by thioflavin T (Thio-T) binding until the steady state was reached. α-syn PF were generated by incubating the protein solutions as described for 2 days only. Then, the solutions were centrifuged at 5000*g* for 5 min, and the supernatant was applied to the Superdex 200 (HR 10/30) column to remove the remaining monomers. The peak corresponding to the oligomeric form was collected and immediately subjected to HHP treatment. DAPF and DAF were produced as described (*21*, *22*). Briefly, 50 μM of the wild type or variants of α-syn were incubated with equimolar concentrations of DA and aggregated for 2−3 days at 37 °C to produce DAPF or for 18 days to produce DAF. The influence of oxygen on DAF formation was investigated by incubating the samples under the same conditions but in an atmosphere enriched in nitrogen gas.

Thio-T Binding. Aliquots of 10 μL of the aggregating solutions were withdrawn and mixed with 50 μL of 500 μM Thio-T solution (Sigma Co.) prepared in 50 mM glycine at pH 8.5, in a total volume of 1 mL. Thio-T binding was evaluated by monitoring the changes in the emission spectra of Thio-T from 460 to 570 nm upon exciting the samples at 446 nm. The presence of DA in the aggregating solutions did not interfere with the fluorescence emission of Thio-T or 1,1′-bis(4-aniline-5-naphthalene sulfonate) (bis-ANS).

Bis-ANS Binding. Aliquots of 5 μL of the aggregating solutions were mixed with 5 μL of an aqueous solution of 1.5 mM bis-ANS (Molecular Probes) in a total volume of 1 mL, and the binding of bis-ANS to the aggregates was determined by measuring the spectral area of the emission spectrum (400−600 nm) upon exciting the samples at 360 nm.

Dissociation of α-syn Aggregates by HHP. The aggregated protein samples were diluted to a final concentration of ∼16

μM in buffer A and then subjected to HHP treatment. The high-pressure cell equipped with optical windows has already been described (*28*) and was purchased from ISS (Champaign, IL). To evaluate the average size distribution of α-syn aggregates in solution, light scattering (LS) was measured by exciting the samples at 320 nm and collecting the light at 90° from 315 to 325 nm in an ISS K2 spectrofluorometer. To evaluate the time dependence of the pressure-induced dissociation of α-syn aggregates, kinetic experiments were performed by keeping the pressure fixed at 2170 bar and recording the LS at intervals of 5 min until the steady state was reached (60 min). In the experiments where the pressure dependence was measured, the pressure was increased from 1 to 3000 bar in steps of 270 bar, waiting 15−20 min before each measurement. All measurements were performed at 37 °C or as stated in the figure captions.

Transmission Electron Microscopy (TEM). The ultrastructure of the different aggregates of α-syn was characterized using negative-staining TEM. The α-syn F and α-syn−DAF solutions (50 μM) were diluted 12-fold, and 10 μL of this solution was deposited on a Formvar-coated copper grid. The samples were stained with 10 μL of uranyl acetate (2% aqueous solution) for 1 min, dried, and then examined in a JEOL 1200EX, operated at 80 kV.

Atomic Force Microscopy (AFM). A total of 10 μL of each sample was adsorbed onto the mica surface, dried at room temperature for 1−2 h, and then rinsed twice gently with 50 μL of MilliQ water before drying with nitrogen. AFM images were obtained at room temperature using a MFP-3D (Asylum Research, Santa Barbara, CA) in intermittent contact mode at 0.5 Hz and Si cantilevers (AC240TS, Olympus; with a spring constant of 1.32 N/m and nominal tip radius lower than 10 nm). Spring constants were measured by the thermal noise method. Image analysis was performed with the Igor Pro 5.0 image processing package (WaveMetrics, Inc., Portland, OR).

Neuron Primary Cultures. Neurons from the midbrain and cerebral cortex of 14-day-old Swiss mice (E14) were prepared as previously described (*29*) and modified by Garcia-Abreu and co-workers (*30*). All animals were kept under standard laboratory conditions according to National Institutes of Health guidelines. Mice anaesthetized by hypothermia were decapitated; the brain structures were removed; and the meninges were carefully stripped off. Dissociated cells were plated on coverslips previously coated with polyornithine (1.5 μg/mL, molecular weight = 41 000; Sigma Chem. Co., St Louis, MO), in serum-free neurobasal medium supplemented with B27 (Invitrogen, Carlsbad, CA). For immunocytochemistry assays, cells were plated on polyornithine-treated glass coverslips. The cultures were incubated at 37 °C in a humidified 5% CO_2 and 95% air chamber for 24 h.

Immunocytochemistry. Immunocytochemistry was performed as previously described (*31*). Briefly, cultured cells were fixed with 4% paraformaldehyde for 20 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) in phosphate-buffered saline (PBS) (blocking solution) for 1 h and incubated overnight at room temperature with the specified primary antibodies diluted in blocking solution. For peroxidase staining, prior to the primary antibody incubation, endogenous peroxidase activity was abolished with 3% H_2O_2 for 10 min, followed by extensive washing with PBS. The primary antibody was mouse antihuman β-tubulin III antibody (Promega Corporation; 1:500). 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 antimouse; Sigma Chemical Co.; 1:100) 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.). Nuclear DNA was counterstained with 0.1 μg/mL 4,6-diamidino-2-phenylindole (DAPI; Sigma) for 5 min at room temperature, washed with PBS, and mounted in fluorescence medium. Negative controls were performed by omitting the primary antibody during staining. In all cases, no reactivity was observed when the primary antibody was absent.

Toxicity of α-syn PF and DAPF Composed of A30P to Neuronal Cell Cultures. After 24 h of incubation, neuron cultures were treated with either PF_{A30P} or $DAPF_{A30P}$ generated by incubation of 50 μM A30P for 2 days at 37 °C in the absence or presence of DA (50 μM), respectively, as explained above. The toxic effects of these aggregates (at a concentration of 10 μM) before and after HHP treatment (60 min under 2.7 kbar at 37 °C) were evaluated after 24 h of incubation in neuronal cell cultures. Toxicity was evaluated by counting the number of neurites per neuron, their length, and also analyzing nuclear fragmentation. Neurons were maintained in serum-free medium supplemented with B27. Buffer A was used as a control.

Morphometry and Statistical Analysis. Neurons stained with β-tubulin III antibody were photographed using a Nikon microscope (Nikon Eclipse TE300). Photos were used to analyze the number of neurites and the total neurite length by using the Sigma Scan Pro Software (Jandel Scientific). In each experiment (at least three independent experiments were done), about 100 neurons per well, encompassing five randomly chosen fields, were scored per condition. The data were stored and analyzed graphically and statistically using the Sigma Scanplot and Microsoft Excel version 7.0.

RESULTS

PF Are Lesser Packed than F: Effects of DA on Protofibril Stability. To examine the effect of DA on the stability of α-syn PF, we produced purified, 2-day-old PF in the absence (called PF) or presence (called DAPF) of DA using wild-type, A30P, or A53T proteins. Using electronic microscopy (EM) and AFM, we were able to confirm the absence of mature F in all of these preparations. By EM imaging (parts A and B of Figure 1), the PF_{A30P} and $DAPF_{A30P}$ solutions presented small spherical aggregates with very few short F. When these solutions were imaged by AFM (parts C and D of Figure 1, respectively), the spherical aggregates were shown to be heterogeneous in diameter (graphs below C and D). The diameter of the PF_{A30P} ranged in size from 90 to 240 nm (graph below C), while the diameter of the $DAPF_{A30P}$, although more homogeneous, ranged from 180 to 240 nm (graph below D). The images of the PF and DAPF composed of the wild-type and A53T proteins were very similar and are not presented.

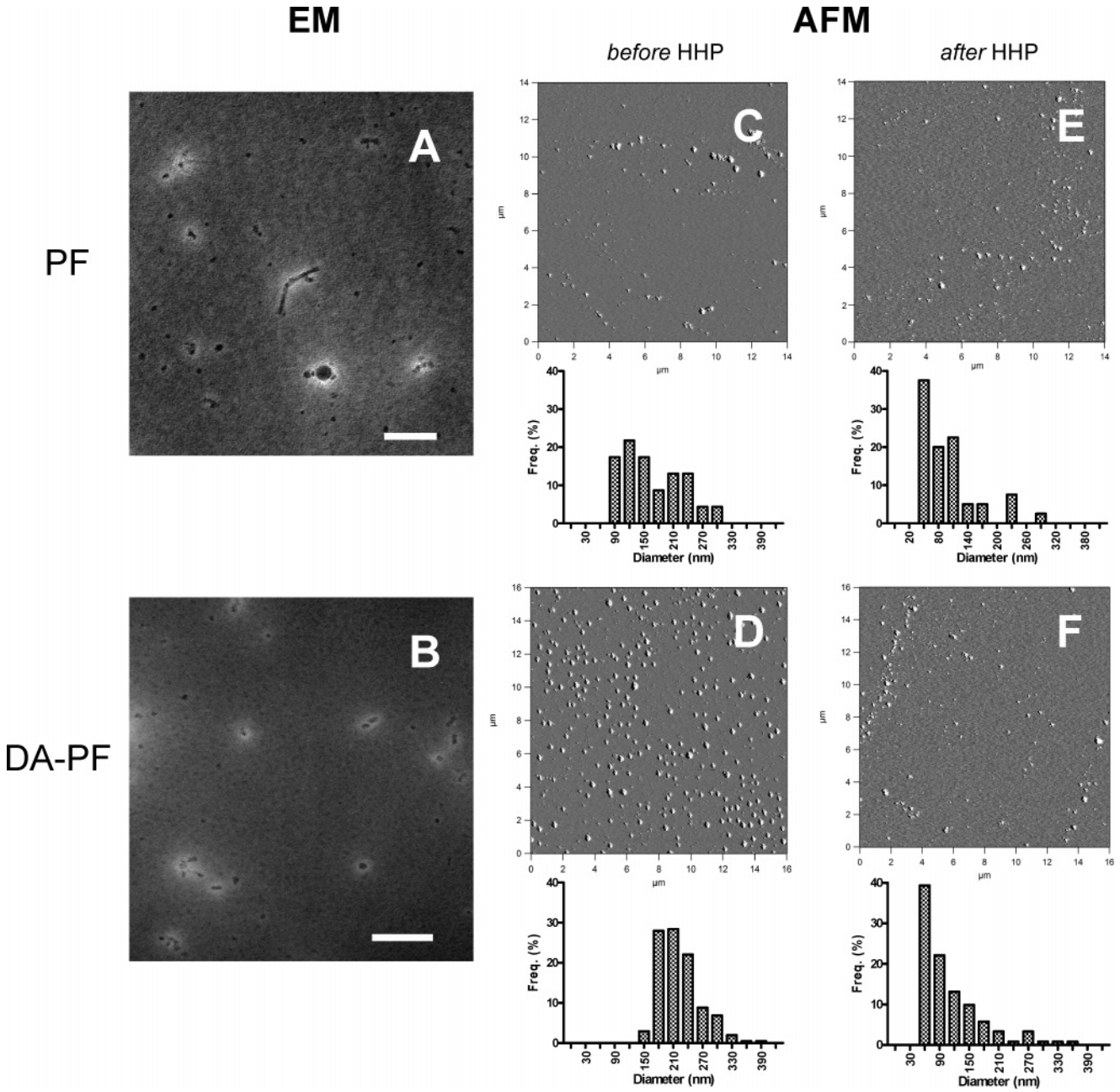

FIGURE 1: Analyzing the morphology of the PF_{A30P} and $DAPF_{A30P}$ by EM and AFM. A30P (50 μM) was incubated under aggregating conditions in the absence (PF) or presence of 50 μM DA (DAPF) and imaged by EM (PF = A, and DAPF = B) and AFM (PF = C and E, and DAPF = D and F). These samples were subjected to 2170 bar/h at 37 °C and then analyzed by AFM (E and F). In AFM images, the diameters of the aggregates were measured and are shown in the graphs below each image. Scale: EM = 150 nm.

The morphology of the α-syn PF has already been characterized by EM and AFM, where several different morphologies were described, including spherical, annular, pore-like, tube-like, and chain-like structures, and the extent of each species observed is hightly dependent upon the medium conditions (salts and pH) as well as on the time of incubation (*32*). It has been shown that α-syn forms spherical oligomers in the presence of DA (12 h of incubation at 37 °C), displaying a diameter of about 25 nm based on AFM data (*33*). Here, we have used a longer incubation time (2 days), and this could explain the larger diameter observed for the PF.

When all of these PF and DAPF were on hand, their stabilities were then probed by subjecting them to increasing pressures while monitoring the changes the LS to infer about their integrity (Figure 2). Figure 2A shows that the LS signal decreased by ∼90% for all three PF samples, compatible with their dissociation into smaller species. Interestingly, in the absence of DA, all three PF exhibited virtually identical stability against HHP. The $p_{1/2}$ values (pressure required to achieve 50% dissociation) were in the range of 1000−1300 bar (Table 1). After pressure removal, the LS values remained low (isolated symbols on the left), suggesting the irreversibility of the process. This irreversible behavior is expected because aggregation of α-syn depends upon sample agitation. In the case of the mature F, as we reported before (*23*), there were marked differences in stability among wild type and variants, where F_{wt} presented the highest stability followed by F_{A30P} and F_{A53T} (Table 1 and see also hollow symbols in the right of Figure 4). When these data are taken together,

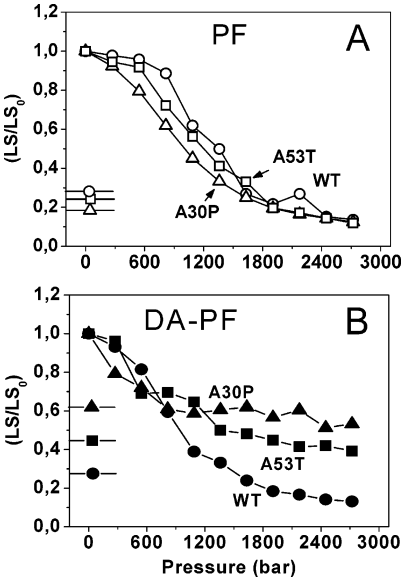

FIGURE 2: Pressure-induced dissociation of PF and DAPF composed of wild type or variants of α-syn. A total of 50 μM of each soluble α-syn was incubated for 2 days under aggregating conditions in the absence (PF) or presence of 50 μM DA (DAPF). Then, the solutions were centrifuged to remove large aggregates, and the supernatant was applied to the Superdex 200 (HR 10/30) column to remove the remaining monomers. The peak corresponding to the oligomerers was collected and immediately subjected to increasing pressures at 37 °C, and the LS was recorded and divided by the initial value (LS/LS_0). A and B show, respectively, the pressure−dissociation profiles of the PF and DAPF. Wild type (○ and ●), A30P (△ and ▲), and A53T (□ and ■). The isolated symbols on the left represent the LS values observed after decompression. Data are means of at least three independent experiments.

Table 1: $p_{1/2}$ Values for the Dissociation of All Aggregates of α-syn Calculated from Figures 2 and 3[a]

	wild type (bar)	A30P (bar)	A53T (bar)
PF	1350 ± 210	1000 ± 90	1200 ± 120
F (*23*)	2250	1650	950
DAPF	940 ± 130	ND[b]	ND
DAF	1100 ± 110	ND	1850 ± 190

[a] Data are means ± standard error of the mean of at least three independent experiments. [b] ND = values not accessible because of incomplete dissociation under those conditions.

they suggest that, at the protofibrillar stage, the contacts within these aggregates are still weak and are dominated by the main-chain contacts with little contribution from the side chains of the amino acids. However, during maturation, the conversion of PF into F seems to be accompanied by structural changes within the fibril involving mainly side-chain contacts, which contribute to fibril stability and individuality.

In the presence of DA (Figure 2B), DAPF of α-syn (wild type and variants) responded differently to HHP, with $DAPF_{wt}$ being less stable than the DAPF composed of variants (Table 1). Indeed, the latter dissociates by only 40−50%, even at the highest pressure attainable in our experimental setup (3000 bar). Again, after pressure removal, the LS values remained low (isolated symbols on the left of B), suggesting the irreversibility of the process. Thus, the interaction between α-syn PF and DA confers a great stability to the PF composed of the disease-associated α-syn (A30P and A53T) with a modest change in PF_{wt} stability (the $p_{1/2}$ for the dissociation of PF_{wt} and $DAPF_{wt}$ was almost the same, ∼1000 bar). In addition, the presence of DA seems to anticipate PF individuality. These observations have important physiological implications, given that in dopaminergic neurons the presence of DA would stabilize the toxic PF_{A30P} and PF_{A30T} species, which could explain the early onset of the disease in the patients bearing these mutations.

Production of F in the Presence of DA (DAF) at Long Incubation Times: Effects of DA on Fibril Stability. Under appropriate conditions, α-syn fibrillization takes ∼6−7 days (*20*, *26*). In this time range, previous studies demonstrated that DA inhibits α-syn fibrillization, resulting in the accumulation of oligomers and PF (*15*, *21*, *32*, *33*). It was also shown that an oxidized form of DA is responsible for this activity (*22*, *33*). Here, α-syn (wild type and variants) were incubated in the presence of DA for an extended period of time (18 days), resulting in the formation of amyloid F with a typical morphology (upper images in Figure 3A), suggesting that the PF formed in the presence of DA can evolve into amyloid F. These F are referred to here as DAF.

Initially, we compared the binding of bis-ANS and Thio-T to the wild-type, A53T, and A30P DAF with that displayed by the respective fibril (Figure 3B), whose binding was taken as 100%. As seen, the DAF composed of wild type or variants displayed only ∼10% of the total Thio-T binding capacity (hollow bars) of the fibril, while the bis-ANS binding to DAF (filled bars) was ∼40% of that of the fibril, except for A30P. It is possible that Thio-T and bis-ANS compete with DA for the same binding sites within the amyloid fibril, which could explain their diminished binding to these F. When the proteins were incubated in the presence of DA in an inert atmosphere, Thio-T binding increased to ∼40% (hollow bars with an upper N_2 in B), consistent with previously reported data demonstrating that an autoxidation product(s) of DA is the active species in binding and inhibiting α-syn fibrillization (*21*, *22*).

To evaluate the effects of DA on fibril stability, we subjected all DAF to HHP at 37 °C and the dissociation of the F was monitored by LS (Figure 3C). As seen, DAF_{wt} (●) was the most unstable species, followed by DAF_{A53T} (■), while DAF_{A30P} (▲) was only partially dissociated under these conditions. The $p_{1/2}$ values for the dissociation of DAF_{wt} and DAF_{A53T} were 1110 ± 110 and 1850 ± 190 bar, respectively (Table 1). Interestingly, in the case of the F formed in the absence of DA (*23*) (Table 1), the sequence of stability was exactly the opposite, with the variant-composed F being the most unstable species ($F_{A53T} < F_{A30P} < F_{wt}$). These results suggest an important role of DA in modulating F as well as PF (Figure 2B) stability.

Because DAF_{A30P} was poorly dissociated by HHP at 37 °C, a pressure−titration curve was performed at 1 °C (△ in Figure 3C). However, even at this low temperature, the dissociation of DAF_{A30P} was still incomplete, which suggests that hydrophobic interactions are not the main contributing factor in DAF stability.

Time-course experiments performed with all DAF confirmed the difference in stability among DAF (Figure 3D). While the complete dissociation of DAF_{wt} at 2170 bar required less than 20 min (●), the dissociation of DAF_{A53T} occurred in ∼40 min (■). Again, DAF_{A30P} did not dissociate completely at this pressure value, and the LS decreased only 50%, even after 60 min under high pressure (▲). Thus, the

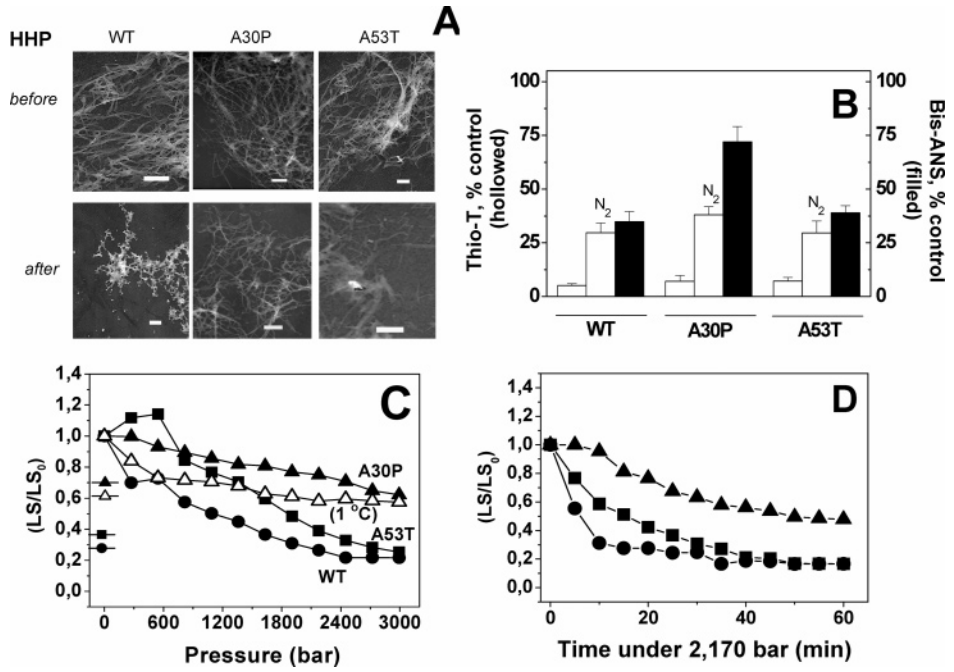

FIGURE 3: α-syn forms F in the presence of DA after long incubation times, comparing the effects of HHP on DAF composed of the wild type or variants. (A) EM images of the DAF formed after incubation of the wild type, A30P, and A53T (50 μM) under agitation at 37 °C for 18 days in the presence of 50 μM DA before (upper images) or after (2170 bar/h; lower images) HHP treatment (bar = 400 nm). (B) Binding of Thio-T (hollow bars) and bis-ANS (filled bars) to DAF as compared to the control F, without DA (taken as 100%). The influence of oxygen on the DA effect was evaluated by incubating the samples at 37 °C in an atmosphere enriched in nitrogen gas (bars with N_2). (C) Pressure-induced dissociation of wild type (●), A53T (■), and A30P (▲) DAF (obtained in the presence of oxygen) as followed by the LS decrease at 37 °C. The pressure was increased in steps and recorded after 15 min at each pressure value. (△) Dissociation profile of DAF_{A30P} at 1 °C. The isolated symbols on the left represent the LS values observed after decompression. (D) DAF were compressed at 2170 bar at 37 °C, and the time course of dissociation was evaluated by the decrease in the LS. Data are means of at least three independent experiments.

stability of DAF followed the same sequence as that of DAPF (wild type < A53T < A30P) and was the opposite of that observed for the F grown in the absence of DA (A53T < A30P < wild type) (Table 1) (*23*).

This partial decrease in the LS observed with DAF_{A30P} might represent either the dissociation of a subpopulation of pressure-sensitive DAF_{A30P} or the homogeneous fragmentation of all DAF_{A30P} into oligomers still large in size. To answer this question, EM images of the species formed after decompression were obtained (lower images in Figure 3A). As seen, after decompression (2170 bar/h), while the DAF_{wt} was drastically affected by the HHP treatment, in the DAF_{A30P}, there was a large population of intact F, suggesting that only a minor fraction of DAF_{A30P} may be sensitive to HHP. In the case of DAF_{A53T}, although several F were still present after pressure treatment, there was a considerable reduction in their concentration, because several fields of the EM grid presented little or no F to be imaged.

Differences in PF and F Stability Might Contribute to Dopaminergic Neuronal Loss in PD. In Figure 4, the dissociation profiles of all PF (left panels) and F (right panels) are grouped in pairs (grown in the absence (hollow symbols) or presence (filled symbols) of DA) to give an idea of what would be their expected behavior in nondopaminergic versus dopaminergic neurons. With the wild-type proteins (upper panels), the stabilities of the PF, DAPF, and DAF were almost identical ($p_{1/2} \sim 1000-1200$ bar, Table 1), whereas the F_{wt} was considerably more stable. This means that, in the dopaminergic region of the brain, the weak DAF_{wt} would easily brake into smaller toxic species, while in the nondopaminergic regions, the nontoxic F_{wt} ($p_{1/2} = 2250$ bar)

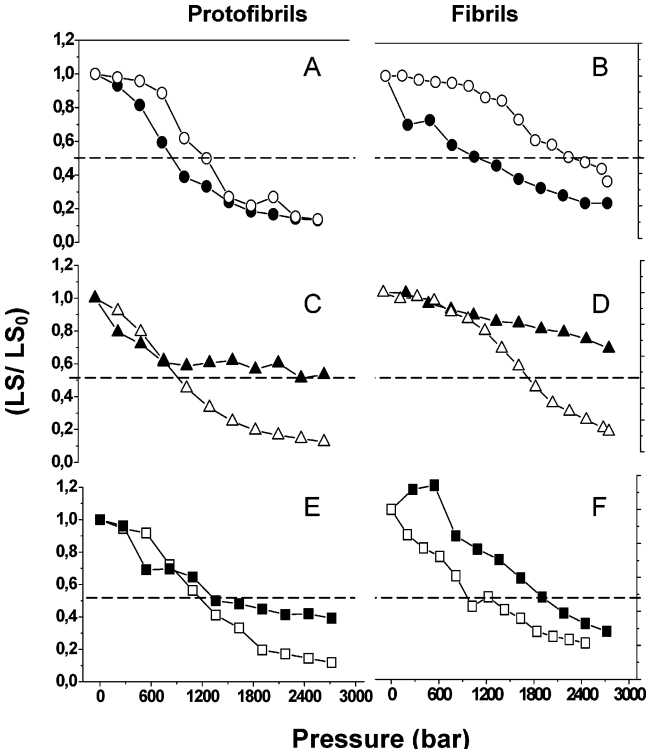

FIGURE 4: Comparing the stability of PF (left graphs) and F (right graph) grown in the absence (○, △, and □) or presence (●, ▲, and ■) of DA. A and B = wild type, C and D = A30P, and E and F = A53T. Other conditions are the same as in Figures 2 and 3.

would prevail. This would tend to free the nondopaminergic regions of the brain from the toxic effects of the smaller

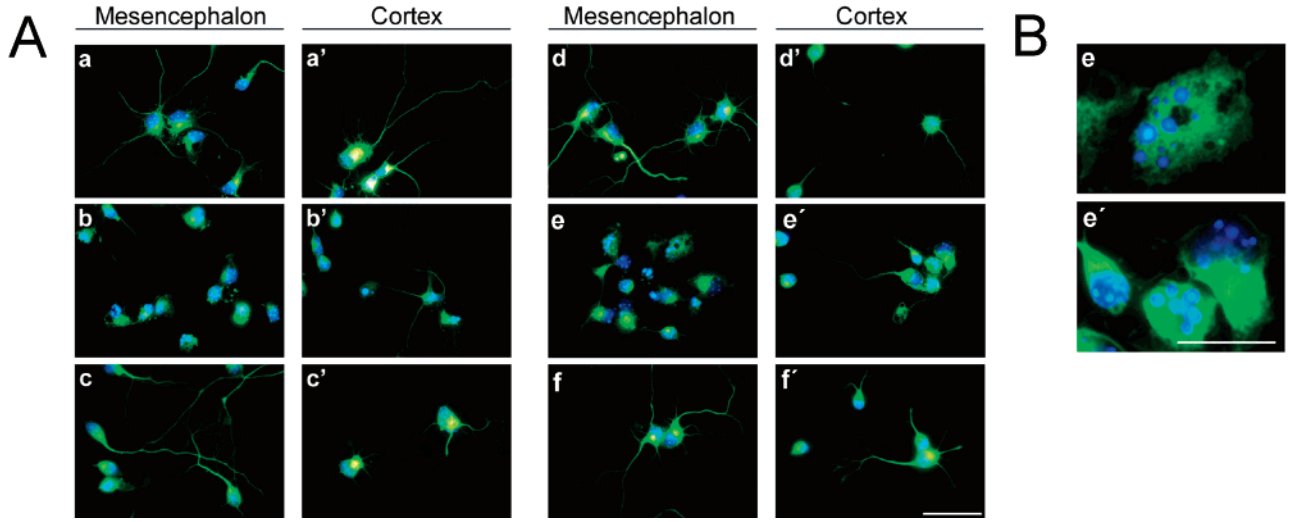

FIGURE 5: Evaluating the toxic effects of PF_{A30P} and $DAPF_{A30P}$ to mesencephalic and cortical neurons in culture. (A) Images a−f are from the mesencephalic, and images a′−f′ are from the cortical neurons from a 14-day-old embryonic (E14) mouse brain maintained for 24 h in culture before subsequent treatment. Each cell culture was incubated for 24 h in the presence of a and a′, buffer (5 mM Tris and 100 mM NaCl at pH 7.0); b and b′, PF_{A30P} (grown in the absence of DA for 2 days); c and c′, PF_{A30P} treated for 60 min at 2.7 kbar; d and d′, 50 μM DA solution; e and e′, $DAPF_{A30P}$ (grown for 2 days in the presence of 50 μM DA); and f and f′, $DAPF_{A30P}$ treated for 60 min at 2.7 kbar. Aggregate concentrations were 10 μM (monomer concentration). The neurons were stained with β-tubulin III antibody (green) and DAPI (blue). (B) Magnification of the images shown in e (mesencephalic neurons treated with $DAPF_{A30P}$) and e′ (cortical neurons treated with $DAPF_{A30P}$), where nuclear fragmentation as a sign of apoptosis is observed. Scale bar = 40 μm (a−f′) and 5 μm (lower images).

aggregates, and indeed, the sporadic form of the disease affects mostly the dopaminergic region (*9*).

In the case of the PF and F composed of the variants (parts C and D of Figure 4 = A30P, and parts E and F of Figure 4 = A53T), DA alters considerably their stabilities. It is clear that DA enhances stability at the level of the variant PF (filled symbols on parts A, C, and E of Figure 4), which might mean that, in dopaminergic neurons, when the toxic PF are formed, they are already stable enough to persist and damage these neurons. This effect is not observed with the wild-type protein, where PF and DAPF have approximately equal stabilities (Figure 4A and Table 1). Thus, although the DAF composed of variants are more stable than their respective F (parts D and F of Figure 4), the effects of DA in terms of increasing stability are already perceptible at the level of PF. Thus, we can postulate from these data that DA renders the PF composed of the variants very stable, enabling them to evade the cellular strategies that might dissolve these toxic aggregates. This could explain their enhanced toxicity in the dopaminergic regions of the brain. In the nondopaminergic regions, PF composed of the variants are either very unstable (as in the case of A30P) or as stable as the F (as in the case of A53T).

The Toxicity of PF_{A30P} and $DAPF_{A30P}$ to Neurons in Culture Is Eliminated by HHP Treatment. To further characterize the cytotoxic effect of the PF, DAPF, as well as the species recovered from the HHP treatment, we selected the A30P for this analysis. Initially, AFM imaging was employed to better characterize the morphology of the species present after HHP treatment (parts E and F of Figure 1 and graphs below them). As seen, after pressure treatment, the PF_{A30P} and $DAPF_{A30P}$ solutions presented only spherical aggregates homogeneous in diameter (75−120 and 86−103 nm, respectively), being smaller than the aggregates present before compression [diameter ranging from 130 to 182 nm (PF) and 213 to 224 nm (DAPF)]. Those values are 95% of the confidence interval of the geometrical mean, and it suggests that HHP induces the fragmentation of these spherical aggregates into smaller species.

To evaluate the toxic effects of PF_{A30P} and $DAPF_{A30P}$, these aggregates were added to mesencephalic and cortical neurons from 14-day-old embryonic (E14) mouse brains maintained for 24 h in culture before morphological analysis of the neurons. The length and number of neurites per neuron, as well as the morphology of the nuclei [DNA staining with 4,6-diamidino-2-phenylindole (DAPI)] were evaluated (Figures 5 and 6). These experiments were performed with PF (2−3 days old) because of the difficulty in keeping the solutions sterile for longer times, when F appear.

Figure 5A shows images of healthy mesencephalic (a) and cortical (a′) neurons in culture. They exhibited prolonged processes and no sign of nuclear fragmentation (not shown in detail). The addition of DA (50 μM) to these neurons did not change their healthy appearance (d−d′). However, when PF_{A30P} (b and b′) or $DAPF_{A30P}$ (e and e′) were present, neurite outgrowth and the number of neurites per neuron decreased dramatically, as further quantified in Figure 6. In addition, all of these toxic effects were accompanied by an evident nuclear fragmentation with an apparent preservation of the cytoplasm, suggesting an initial apoptotic process rather than necrosis induced by PF_{A30P} (not shown in magnification) and $DAPF_{A30P}$ either in the mesencephalon (e in Figure 5B) or in the cortex (e′ in Figure 5B).

Interestingly, the toxic effects of both PF_{A30P} and $DAPF_{A30P}$ were strongly reduced when the samples were previously subjected to HHP treatment (2700 bar/h) (c and c′ and f and f′ in Figure 5A). As seen, these neurons presented prolonged processes and no sign of nuclear fragmentation (not shown in magnification). When these data are taken together, they suggest that, being able to break the PF and DAPF composed of A30P into smaller aggregates (parts D and F in Figure

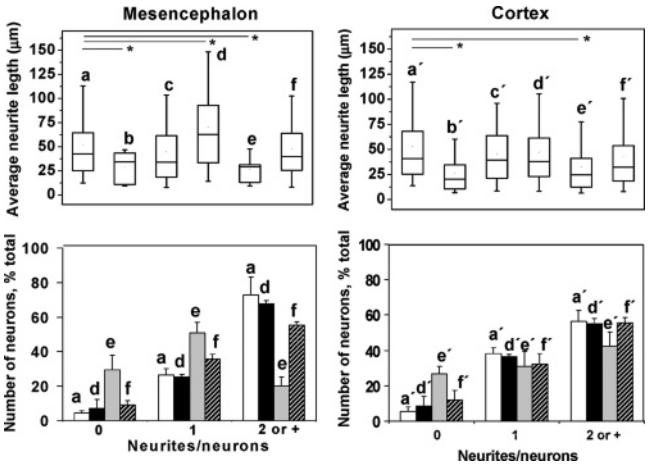

FIGURE 6: Evaluation of neuritogenesis in each cell culture treated with the aggregates of A30P shown in Figure 1. A total of 100 neurons chosen randomly were analyzed, and the results are expressed using the percentile plot, (*) $p < 0.01$. Upper panels show the length of neurites from mesencephalic (left) and cortical (right) neurons growing in the conditions described in the Experimental Procedures. The boxes enclose the 25th and 75th percentiles and are bisected by the median; whiskers indicate the 5th and 95th percentiles. Note that the bars related to the pressure-treated samples (c and f; c′ and f′) are similar to the controls (a and a′). Also note that treatment with DA alone stimulated neurite growth in the mesencephalon (bar d). An asterisk represents significant differences ($p < 0.01$). Lower panels show the number of neurites per neuron (0, 1, 2, or more) in mesencephalon (left) or in the cortex (right). Again, the mesencephalic neurons treated with $DAPF_{A30P}$ (bare) were more affected than the cortical neurons (e′). The distribution of neurites per neuron in the cells incubated with pressure-treated $DAPF_{A30P}$ (f and f′) was quite similar to the control.

Table 2: Average Neurite Length on Mesencephalic and Cortical Neurons from the Brains of E14 Mice Treated with Different Aggregates of A30P before and after HHP Treatment

condition	mesencephalic neurons (μm)	cortical neurons (μm)
control (buffer)	80−100	90−100
$+PF_{A30P}$	40	70
+after pressure PF_{A30P}	80−100	80−100
+DA (50 μM)	130−140	90−100
$+DAPF_{A30P}$	40	70−80
+after pressure $DAPF_{A30P}$	80−100	80−100

1), HHP treatment strongly reduces the associated toxicity of these aggregates to neurons in culture.

The number of neurites per neuron as well as neurite length were quantified as described in the Experimental Procedures and are presented in Figure 6 and Table 2. In mesencephalic cultures (left in Figure 6), the average neurite length of the neurons treated with PF (bar b) or DAPF (bar e) was much less (40 μm) than the average length in the control cultures (80−100 μm, bar a). The addition of DA to these neurons (bar d) increased neurite outgrowth (130−140 μm), suggesting that they might be dopaminergic neurons.

In the case of the cortical neurons (right in Figure 6 and Table 2), the average neurite length was 90−100 μm (bar a′) and the presence of DA (bar d′) did not change this value (90−100 μm). As observed with the mesencephalic neurons, the presence of PF_{A30P} (bar b′) or $DAPF_{A30P}$ (bar e′) decreased the average length of the neurites to 70 or 70−80 μm, respectively.

As mentioned before, the toxic effects of both PF_{A30P} and $DAPF_{A30P}$ were greatly reduced when the samples were previously subjected to HHP treatment. As seen, the cultures incubated in the presence of the "after pressure PF" (bars c and c′ and Table 2) displayed the same neurite length (80−100 μm in both neuron cultures) as the controls (bars a and a′), while the "after pressure DAPF" (bar f and f′) had the same neurite length as DA alone (d and d′) (80−100 μm in both neuron cultures).

In regard to the number of neurons with 0, 1, or ≥2 neurites (lower graphs in Figure 6), it is noteworthy that, in the control cultures, the majority of neurons have more than two neurites per neuron, whether in mesencephalon (bar a) or cortex (bar a′). DA alone did not affect the number of neurites/neuron either in the mesencephalon (bar d) or cortex (bar d′). The addition of DAPF to these cultures caused a significant reduction in the number of neurites/neuron (bars e and e′), where the majority of mesencephalic neurons present one neurite per neuron, while the majority of cortical neurons present one or more than two neurites. Interestingly, the pattern of neurite distribution was recovered in the cultures incubated in the presence of $DAPF_{A30P}$ previously subjected to HHP treatment (compare a−f and a′−f′).

DISCUSSION

Amyloid F, regardless of the precursor protein, display a common core structure that is characterized by the cross β structure (*24*, *25*). The structural properties, morphology, and kinetics of amyloid-fibril formation are strongly influenced by the amino acid sequence of the precursor protein as well as the changes in the solution conditions under which they were grown (*34*, *35*). Studies from our laboratory and others have shown that the primary sequence of the constituent protein also plays an important role in modulating the protein−protein contacts within the fibril, thus influencing fibril stability (*23*, *36*). For example, F composed of the wild-type α-syn proved to be more stable to pressure denaturation than the F composed of the disease-linked variants (A53T and A30P). Also, the V30M variant of transthyretin (TTR) was incompletely dissociated by HHP, while the wild-type protein was completely dissociated in less than 5 min at 2500 bar (*23*).

In the case of β2m F, pressure treatment in the absence of any added denaturant induced their conversion into a species able to bind more Thio-T, suggesting that even the amyloid F can assume different conformation, and HHP is useful in revealing these new conformational states (*37*). Recently, Bosharova and collaborators were able to show that aggregates of the prion protein (PrP), when subjected to heat treatment in the presence of Triton X-100, also undergo rearrangements within the amyloid core, resulting in the extension of the proteinase-K-resistant core. These data argue in favor of the existence of different fibrilar states, which might have important physiological implications in the ethiology of their respective diseases (*38*).

Recently, Dirix and co-workers also reported that early aggregates of a peptide from TTR were pressure-sensitive, while mature aggregates derived from the same peptide were pressure-resistant (*36*). These data are consistent with the results presented here, where we show that, in going from PF to F, there is a gain in stability (mainly observed with wild type and A30P) (Table 1). Further, while all PF exhibited an identical susceptibility to HHP regardless of

the primary sequence of the constituent protein, the F respond differently, with the F_{wt} being the most stable among them. These results suggest that during fibrillogenesis several structural changes take place within the amyloid structure, as summarized below:

monomer	→	PF	→	F
unpacked		not well packed		well packed
no cavities		cavities		fewer cavities
unstructured		main-chain contacts		side-chain contacts
highly hydrated		less hydrated		less hydrated

Differences in stability among all of these species are probably related to the presence of void volume and packing defects. In general, the species that are more susceptible to HHP are the ones with internal void volumes (water-excluded cavities), dry interfaces, and the presence of hydrophobic and electrostatic interactions (*39*−*41*).

Previous studies performed by Lansbury and others have shown that, with short incubation times, DA interacts with α-syn and inhibits the conversion of PF into F (*21*). However, as shown here, upon longer incubations, mature amyloid F of α-syn were observed to form in the presence of equimolar concentrations of DA, an observation that has not been reported before. It is possible that, with aging, a fraction of the α-syn-bound DA molecules detach from the PF, allowing the latter to evolve into F. However, because the F grown in the absence and presence of DA responded differently to HHP treatment, we postulate that at least some DA molecules remain bound to the protein in the F and modulate their stability, possibly through modulating structural changes involved in protein−protein interactions within the F.

The data presented here examine for the first time the idea that there is an effect of DA on the thermodynamic stability of the PF and F of α-syn, with important correlations with PD etiology. Our data show clearly that DA exerts a considerable effect in modulating PF and F stability, and this modulation depends upon the protein that constitutes the aggregates (Figure 4). Variant PF, when grown in the presence of DA, exhibited a notable gain in stability (Figures 2 and 4), and this could be associated with their early onset in PD patients. Although other regions of the brain are also affected in patients bearing these mutations (*8*), the dopaminergic region presents an increased susceptibility that has been attributed to the presence of the catecholamines. Thus, here, we propose an additional role of DA that is its differential modulation of the stability of the aggregate species of α-syn. This stabilizing effect is not observed in the case of wild-type α-syn, where PF_{wt} and $DAPF_{wt}$ are equally stable under pressure (Figure 4). However, DAF_{wt} is much more unstable than F_{wt}. It is tempting to suggest that, in the dopaminergic neurons, the presence of DA would render the F composed of wild-type α-syn very labile, enhancing the possibility of their breaking into smaller, toxic species and causing damage specifically to this region of the brain. In nondopaminergic neurons, because of the absence of DA, F_{wt} as a stable entity would remain as an inert material, sparing this region from damage. Considering that the vast majority of PD cases are sporadic and involve the wild-type protein, these observations have important physiological implications.

It is noteworthy that α-syn aggregation and toxicity are also implicated in the pathogenesis of several neurodegenerative diseases, including Alzheimer's disease, multiple-system atrophy, dementia with LB, Down syndrome, and neurodegeneration with brain iron accumulation, collectively referred to as synucleinopathies (*42*, *43*). The presence of α-syn aggregates within LB and Lewy neurites in brain regions other than the substantia nigra and within different population of neurons (dopaminergic and nondopaminergic) suggests that toxicity of the α-syn aggregates does not depend exclusively upon the presence of DA. However, it is likely that the stability of α-syn aggregates within these inclusions is also modulated by the interactions between α-syn and other macromolecule, as well as by the post-translational modifications that take place with α-syn.

To understand the role of DA in modulating the toxic properties of α-syn aggregates, we evaluated the toxicity of PF_{A30P} and $DAPF_{A30P}$ before and after HHP treatment using mesencephalic and cortical neurons in cultures (Figures 5 and 6). Our data show that the mesencephalic neurons seem to be more sensitive than cortical neurons to the presence of either aggregate, although the percentage of dopaminergic neurons in these cultures is in the range of 17−21% (*44*). Strikingly, when the neuronal cultures are incubated in the presence of samples treated under pressure at 2700 bar for 1 h, these neurons present a healthy aspect, suggesting that the species rescued from high pressure loses its toxicity. AFM images (parts C−F of Figure 1) revealed that, after pressure treatment, the average diameter of the spherical aggregates present before compression decreased significantly (Dunn's multiple comparison test, $p < 0.01$), and this may explain the decrease in toxicity. Besides, nuclear fragmentation was observed in all cultures treated with PF_{A30P} and $DAPF_{A30P}$ (Figure 5), and again, these effects are reversed by HHP treatment. It has been reported that the overexpression of human α-syn in rat neuronal cells results in a significant reduction of neurite expansion (*45*). However, very few data in the literature describe the toxic effects of adding aggregates of α-syn to neurons in culture as we show here. This approach has an important implication because the presence of α-syn in plasma has recently been described (*46*). Besides, because the addition of aggregates is much easier than α-syn transfection, it could be used as a first approach in drug screening.

The observation that the cytotoxicity of the $DAPF_{A30P}$ is almost completely abolished after pressure treatment despite the fact that these PF are only partially dissociated by HHP (Figure 2) suggests that α-syn toxicity is mediated by specific protofibrillar structures that are susceptible to pressure denaturation. Recently, in an elegant study, Silveira and coworkers (*47*) were able to isolate a subpopulation responsible for infectivity and by the converting activity of the PrP(res) in transmissible spongiform encephalopathy. This subpopulation contains aggregates of a size compatible with only 14−28 molecules of the PrP.

The mechanism by which these aggregates of α-syn exert their toxic effects remains unclear now. An important question that arises from this observation is the precise site of action of these aggregates on the outside of the cells. Further studies are necessary to address this issue.

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ANEXO 4

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ORIGINAL ARTICLE

Transforming growth factor α promotes sequential conversion of mature astrocytes into neural progenitors and stem cells

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An instability of the mature cell phenotype is thought to participate to the formation of gliomas, primary brain tumors deriving from astrocytes and/or neural stem cells. Transforming growth factor α (TGFα) is an erbB1 ligand overexpressed in the earliest stages of gliomas, and exerts trophic effects on gliomal cells and astrocytes. Here, we questioned whether prolonged TGFα exposure affects the stability of the normal mature astrocyte phenotype. We first developed astrocyte cultures devoid of residual neural stem cells or progenitors. We demonstrate that days of TGFα treatment result in the functional conversion of a population of mature astrocytes into radial glial cells, a population of neural progenitors. TGFα-generated radial glial cells support embryonic neurons migration, and give birth to cells of the neuronal lineage, expressing neuronal markers and the electrophysiological properties of neuroblasts. Lengthening TGFα treatment to months results in the delayed appearance of cells with neural stem cells properties: they form floating cellular spheres that are self-renewing, can be clonally derived from a single cell and differentiated into cells of the neuronal lineage. This study uncovers a novel population of mature astrocytes capable, in response to a single epigenetic factor, to regress progressively into a neural stem-like cell stage via an intermediate progenitor stage.
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Keywords: erbB1; differentiation; radial glia; gliogenesis; oncogenesis; EGFR

Introduction

Gliomas are the most common primitive central nervous system (CNS) tumors thought to derive from macroglial cells (astrocytes and oligodendrocytes) and/or neural progenitors. Mechanisms underlying brain tumors formation and progression are largely unknown. Aside the rapid progression of glioblastomas with a poor prognosis, low-grade gliomas slowly progress over years before they change into more aggressive stages (Wechsler-Reya and Scott, 2001). Transforming growth factor α (TGFα) and its receptor erbB1 (or epidermal growth factor receptor) constitute one of the signalling modules most frequently deregulated in gliomas. TGFα overexpression is found in about 80% of them, and is observed from the initial steps of their development (Junier, 2000), whereas the overexpression of erbB1 appears in 20–40% of them in later phases (Wechsler-Reya and Scott, 2001). We previously showed that TGFα acts as a gliatrophin on mouse and human cortical astrocytes, promoting their growth as well as their survival (Sharif *et al*., 2006b), in agreement with the recent report of increased astrocyte apoptosis in erbB1 knockout mice (Wagner *et al*., 2006). These results suggest that TGFα might participate in the early stages of tumoral transformation through the deregulation of astrocyte proliferation and apoptosis. However, the recent discoveries of cancerous neural stem-like cells in human gliomas, and the demonstration that neural progenitors are more sensitive than differentiated astrocytes to the transforming effects of various oncogenic manipulations, have raised the possibility that a trouble in cell differentiation might also participate to the formation of these tumors (Dai and Holland, 2003; Harris, 2004; Singh *et al*., 2004). Such a possibility is supported by the consequences of the introduction of a mutated, constitutively active form of erbB1 in cultured astrocytes (Bachoo *et al*., 2002). The activation of erbB1, insufficient alone to ensure wild-type astrocyte transformation, results in the dedifferentiation of cultured mutant astrocytes lacking the INK4a/ARF tumor suppressor gene locus, and their transformation into tumoral cells capable to form high-grade gliomas when transplanted in the brain (Bachoo *et al*., 2002).

Interestingly, we and others noted that several days of TGFα exposure leads *in vitro* to morphological alterations of wild-type astrocytes, reminiscent of the bipolar shape of a population of neural progenitors, the radial glial cells (Miller *et al*., 1996; Zhou *et al*., 2001; Figiel *et al*., 2003; Liu and Neufeld, 2004; Sharif *et al*., 2006b).

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This led us to examine if exposing differentiated astrocytes to TGFα compromises the stability of their phenotype in the absence of any genetic alteration. TGFα acts as a mitogenic or differentiating factor at different steps of the pathway leading from a neural stem cell to a mature astrocyte according to the low or high levels of erbB1 expressed by the cell (Lillien, 1995; Burrows *et al*., 1997; Sun *et al*., 2005). Any analysis of TGFα effects thus requires a careful characterization of the cell population studied. Mature astrocytes are routinely identified by their expression of the intermediate filament glial fibrillary acidic protein (GFAP). However, a distinct, much less abundant population of GFAP-expressing cells behaving as neural stem cells coexists with astrocytes in the germinal zones of the post-natal and adult brain, and possibly in non-neurogenic regions (Doetsch, 2003). The presence of such cells has been detected at a low frequency in astrocyte cultures through their capability to form floating cellular spheres (Laywell *et al*., 2000; Imura *et al*., 2003). Consequently, we developed culture conditions depleted of this cell type. Using these thoroughly defined primary cultures of cortical astrocytes, devoid of any residual neural progenitors or stem cells, we demonstrate that prolonged exposure of differentiated astrocytes to TGFα results in a two-step progressive and functional conversion of the cells, first into neural progenitor-like cells, and second in cells exhibiting properties of neural stem cells.

Results

TGFα induces cortical astrocytes to acquire a bipolar shape through the activation of erbB1

Astrocyte cultures (4–6 days with serum followed by 3 days in totally defined minimal medium) were established from 1- to 10-day-old C57Bl6 mouse cortices (see Materials and methods). No morphological rearrangement was noted upon serum starvation. No induced death was observed following either of the treatments performed (i.e. serum starvation, TGFα, heregulinβ1 or basic fibroblast growth factor (bFGF) addition). In control conditions, astrocytes adopted a typical flat polygonal morphology with an actin network predominantly arranged as sub-cortical fibers just beneath the membrane (Figure 1a). TGFα (10 or 50 ng/ml) initiated profound morphological rearrangements at 2–3 days *in vitro* (DIV), including elongation of the nucleus, retraction of the cytoplasm, extension of two opposite processes and appearance of actin stress fibers (Figure 1b). As TGFα treatment was prolonged, the elongation of the nucleus and the retraction of the cytoplasm were accentuated. At 7 DIV, half of the cell population ($53 \pm 1.4\%$, mean $\pm$ s.e.m., $n = 6$) presented a complete bipolar morphology with a very thin layer of cytoplasm surrounding the fusiform nucleus and two long thin processes extending in opposite directions (Figure 1c). TGFα effect was fully reversed upon serum addition (not shown). To check for the specificity of TGFα effect, we evaluated the activation state of erbB1

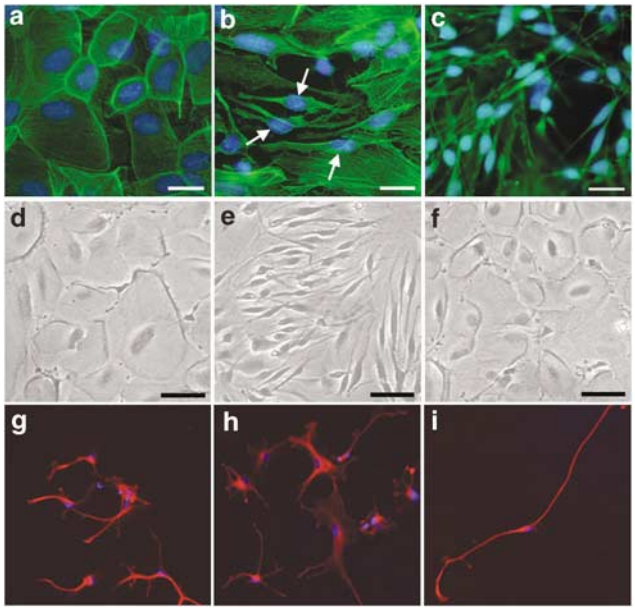

Figure 1 TGFα induces mouse and human astrocytes to acquire a bipolar shape. (**a**–**c**) Labelling of the mouse astrocyte actin cytoskeleton with Alexa 488-coupled phalloidin. In serum-free medium, mouse astrocytes have a flat, polygonal shape (**a**). After 3 days in the presence of TGFα, astrocytes start to exhibit an elongation of the nucleus accompanied by cytoplasmic retraction, and adopt an elongated shape (**b**, arrows). After 7 days, TGFα-induced morphological rearrangements are complete and astrocytes exhibit a bipolar shape with elongation of the nucleus and extension of two thin processes running in opposite directions (**c**). Scale bars = 20 μm. (**d**–**f**) TGFα-induced morphological changes require activation of erbB1. Morphological changes were monitored using phase-contrast microscopy. (**d**) Untreated astrocytes. (**e**) TGFα-treated astrocytes. (**f**) Inhibition of erbB1 activation by the specific inhibitor AG1478 abrogated TGFα-induced morphological changes. Scale bars = 50 μm. (**g**–**i**) Human astrocytes adopted a bipolar elongated morphology when exposed to TGFα. Actin cytoskeleton was labelled with rhodamine-coupled phalloidin. In serum-free medium, human astrocytes had a ramified stellate shape (**g**), which remained unchanged for 3 days in the presence of heregulinβ1, a ligand of the erbB3 receptor (**h**). In contrast, human astrocytes responded to TGFα as mouse astrocytes by adopting a bipolar, highly elongated shape after 3 days (**i**). Scale bars = 60 μm.

that we previously evidenced to be activated up to 3 days of TGFα treatment (Sharif *et al*., 2006b). We observed that the activated, phosphorylated form of erbB1 was still detected after 7 DIV in the presence of TGFα, despite downregulation of the protein levels of the receptor (Supplementary Figure 1). In addition, TGFα-induced morphological changes were abolished upon inhibition of erbB1 tyrosine kinase activity with AG1478, as observed at 7 DIV (Figure 1d–f). The specificity of TGFα-induced morphological changes was further demonstrated by the lack of effect of bFGF when substituted to TGFα (data not shown). We also analysed primary cultures of astrocytes derived from human embryonic cortices. In addition to erbB1 and erbB2, these astrocytes express the erbB3 receptor, activated by the heregulinβ1 ligand (Sharif *et al*., 2006a), another member of the epidermal growth factor (EGF) super-family. Heregulinβ1 treatment did not

alter the overall stellate astrocyte morphology, although a thickening of the processes was noted (Figure 1g and h), whereas TGFα induced human astrocytes to acquire a bipolar shape as their mouse counterparts (Figure 1i).

These results pointed to a specific effect of TGFα on astrocyte morphology, dependent on the activation of erbB1.

TGFα-induced bipolar cells express the molecular markers of radial glial cells

The bipolar morphology induced by TGFα exposure strikingly resembled that of radial glial cells, normally restricted to the developing nervous system, where they act as neural progenitors (Kriegstein and Gotz, 2003).

We therefore examined the expression of three proteins commonly used for their *in situ* identification (Kriegstein and Gotz, 2003). GLAST, a glutamate glial-specific transporter, and brain lipid-binding protein (BLBP) are expressed *in vivo* by radial glial cells and astrocytes in the developing brain and *in vitro* by astrocytes for GLAST (Feng *et al*., 1994; Sutherland *et al*., 1996). RC2, a post-translationally modified isoform of nestin, is considered as the most specific molecular marker of radial glial cells in the mouse (Chanas-Sacre *et al*., 2000), whereas nestin is expressed in all progenitors, reactive astrocytes and astrocytes grown in culture (Pekny *et al*., 1998). As expected, untreated astrocytes expressed GLAST in the form of a punctuated labelling enriched at the plasma membrane (Figure 2a), and TGFα-treated astrocytes retained a robust GLAST-immunoreactive signal (Figure 2b). A striking upregulation of BLBP expression was observed in TGFα-induced bipolar cells (Figure 2c and d). No RC2-immunoreactive polygonal astrocytes were observed in control conditions (Figure 2e). In contrast, at 7 DIV $36.3 \pm 2.9\%$ (mean $\pm$ s.e.m., $n = 3$) of the bipolar cells expressed RC2 in TGFα-treated cultures (Figure 2f). TGFα-induced RC2-immunoreactive bipolar astrocytes co-expressed GLAST (Figure 2i–k) and BLBP (Figure 2l–n). GFAP expression was observed in half of the RC2-immunoreactive bipolar astrocytes (Figure 2o–q and r–t). We also examined the expression of the transcription factor Olig2. Olig2 is a marker of neural progenitors, radial glial cells included (Kriegstein

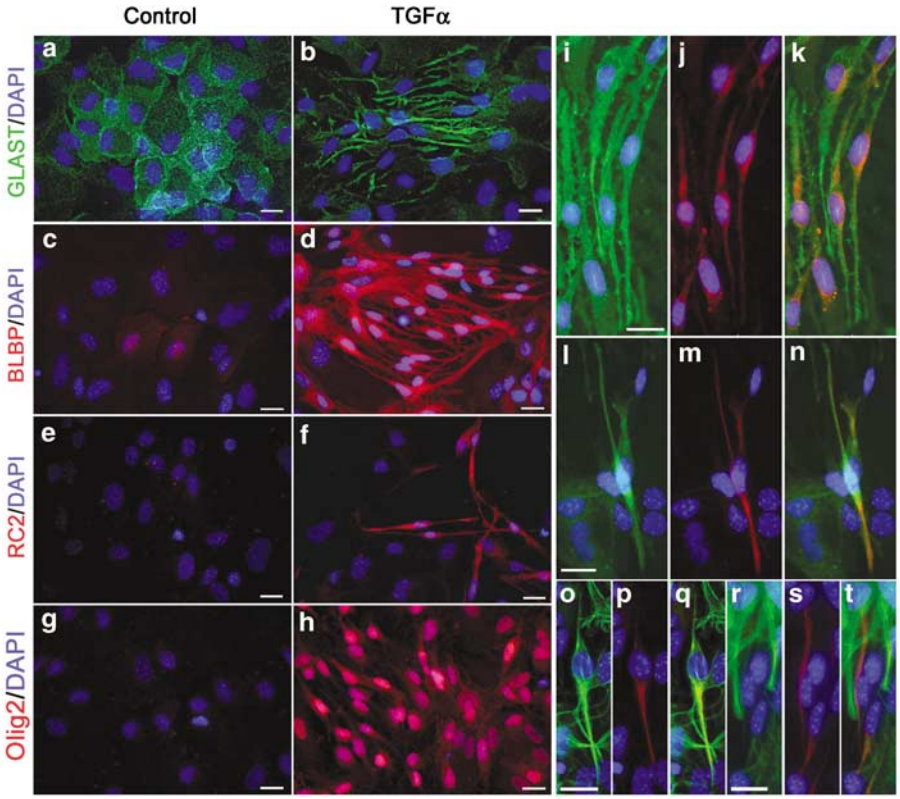

Figure 2 TGFα-induced bipolar astrocytes present the antigenic profile of radial glial cells. (**a**–**f**) Immunofluorescent labelling of astrocytes treated for 7 days in the absence (control) or presence of TGFα with antibodies directed against GLAST (**a**, **b**), BLBP (**c**, **d**) and RC2 (**e**, **f**). Nuclei were counter-stained with DAPI (blue). GLAST expression was observed in control astrocytes as punctuate staining (**a**), and was maintained in TGFα-induced bipolar astrocytes (**b**). A low BLBP-immunoreactive signal observed in control astrocytes (**c**) increased sharply upon TGFα action (**d**). Astrocytes did not express RC2 in control cultures (**e**), whereas RC2-immunoreactive bipolar cells were observed in TGFα-treated astrocyte cultures (**f**). (**g**, **h**) Immunodetection of Olig2, a marker of neural progenitors also expressed in immature astrocytes, yielded a barely detectable signal in control astrocytes (**g**), whereas TGFα-induced bipolar astrocytes exhibited a strong nuclear-immunoreactive signal after 7 DIV (**h**). Double immunofluorescent labelling showed that the RC2-immunoreactive bipolar cells (red) present in TGFα-treated cultures at 7 DIV co-expressed GLAST (**i**–**k**) and BLBP (**l**–**n**). RC2-immunoreactive cells (red) either co-expressed GFAP (green) (**o**–**q**) or were devoid of GFAP-immunoreactivity (**r**–**t**). Nuclei were counter-stained with DAPI (blue). Images were obtained on a fluorescent microscope. Scale bars = 20 μm.

and Gotz, 2003; Hack *et al*., 2004). Olig2 inhibits the differentiation of neural progenitors into astrocytes (Fukuda *et al*., 2004; Muroyama *et al*., 2005) and its expression is downregulated *in vitro* and *in vivo* in mature astrocytes (Fukuda *et al*., 2004; Marshall *et al*., 2005). Accordingly, a hardly detectable Olig2-immunoreactive signal was observed in control astrocytes (Figure 2g), whereas TGFα-treated cells exhibited a strong nuclear Olig2-immunoreactive signal (Figure 2h).

Taken together, these results showed that TGFα induces astrocytes to acquire the morphological and molecular hallmarks of radial glial cells while retaining for part of them the expression of GFAP, which is normally absent from radial glial cells in rodents (Bignami and Dahl, 1974).

Absence of residual neural progenitors or stem cells in the control astrocyte cultures

Our *in vitro* differentiated astrocyte cultures consisted of an adherent monolayer of 91–96% GFAP-positive cells, the complement cells (9–4%) corresponding to CD11b-positive microglial cells. The size of the population affected by TGFα and the progressiveness of the morphological alterations allowed a clear identification of GFAP-immunoreactive polygonal astrocytes as the target cells. However, previous reports, using growth factors cocktails including EGF, the functional analog of TGFα, have detected the presence of neural stem cells at a low frequency (0.1–10%) in astrocyte cultures (Laywell *et al*., 2000; Imura *et al*., 2006). We thus verified whether residual radial glia and/or neural stem cells could account for part of our observations.

Our dissection procedure did not encompass the germinal ventricular zone where the cellular bodies of the radial glial cells are located (Kriegstein and Gotz, 2003). Accordingly, freshly dissociated cells of post-natal day 1 cortices contained numerous GFAP-immunoreactive cells, but no RC2-immunoreactive signals (not shown). This was further confirmed by observing the same TGFα-induced morphological changes and RC2 expression on astrocyte cultures derived from the superficial layers of the cortex (compare Figure 3a and b to Figure 2e and f). We then examined the effects of TGFα on cultured astrocytes from 10-day-old cortices, as RC2-immunoreactive radial glial cells are detectable in the cortex of mice up to 5 days of age (Figure 3c), but not at 10 days of age (Figure 3d). Furthermore, the numbers of neural progenitors that can be derived from the cortex at that age is known to decrease sharply as compared to 1-day-old cortex (Seaberg *et al*., 2005). Again, response of astrocytes derived from 10-day-old mouse cortices to TGFα was indistinguishable from that of astrocytes derived from 1-day-old mouse cortex (compare Figure 3e and f to Figure 2e and f).

We next sought for the development of neurospheres that would testify to the presence of residual neural stem cells, reported to differentiate into radial glial cells in the presence of TGFα (Gregg and Weiss, 2003). Astrocyte cultures, treated or not with TGFα, were trypsinized, re-suspended in serum-free medium containing TGFα and bFGF (20 ng/ml each) (Laywell *et al*., 2000) and further cultured for 10 days. In these conditions, we did not observe the development of cellular spheres, and trypan blue exclusion assays showed that the few floating elements detected were debris. Likewise, cellular suspensions obtained from 1-day-old post-natal cortices directly cultured in serum-free medium supplemented with TGFα and bFGF did not yield cellular spheres up to 26 DIV. On the opposite, use of neurosphere medium allowed the development of cellular spheres after 21 DIV, at a rate $<1\%$ per viable cell initially seeded, as expected for non-neurogenic brain regions (Seaberg *et al*., 2005). We additionally evaluated whether the medium used for astrocyte cultures could sustain the development of already established neural stem cells. Neurosphere cultures derived from E12 mouse cortices were transferred into defined medium supplemented with TGFα. Neurospheres attached within 24 h before dying within the following 3 days (data not shown). These results demonstrate that the method we used to prepare astrocyte cultures did not allow the isolation of viable neural progenitors or stem cells.

Altogether, these results evidenced that RC2-immunoreactive bipolar cells observed in TGFα-treated cultures did not stem from the amplification by the

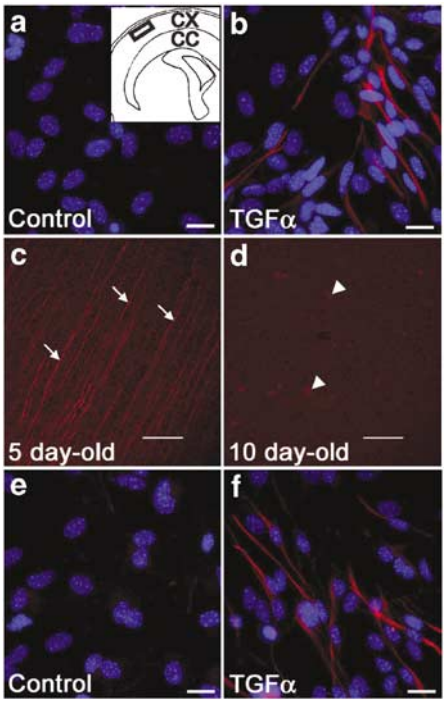

Figure 3 (**a**, **b**) TGFα-induced acquisition of bipolar morphology and RC2 expression in astrocyte cultures derived from post-natal day 1 tissue pieces dissected out from the superficial layers of the cortex away from the germinal zone (**a**, inset), and from cortices of 10-day-old mice (**e**, **f**), when RC2-immunoreactive cells are no longer detected *in vivo* in the cortex (**d**), as compared to 5-day-old mouse cortex (**c**). In (**c**) and (**d**), microphotographs are centered on the upper half of the cortex. Arrowheads in (**d**) point to nonspecific staining of blood vessel-like structures, which is also observed with the secondary antibody alone. Images in (**a**), (**b**), (**e**) and (**f**) were obtained on a fluorescent microscope; images in (**c**) and (**d**) were obtained on a confocal microscope. Scale bars = 50 μm (**c** and **d**), and 20 μm (**a**, **b**, **e** and **f**).

growth factor of radial glial cells, or the amplification and subsequent differentiation in radial glial cells of other neural progenitors or stem cells present in the initial cellular suspension.

TGFα induces the conversion of astrocytes into functional radial glial cells

Acquisition of the morphology of radial glial cells as well as of the synthesis of RC2 and Olig2 are not sufficient to give proof of a functional status.

The initial function assigned to radial glial cells is the support of neuronal migration from the germinal zones of the CNS towards their final locations (Rakic, 2003). We evaluated this function using co-cultures of astrocytes derived from cortices of mice bearing an actin-green fluorescent protein (GFP) transgene, and cortical neurons derived from 16-day-old wild-type mouse embryos. Embryonic brain cellular suspension was added upon actin-GFP astrocytes seeded at low density and treated for 7 days with TGFα, and the migration of immature neurons (doublecortin positive (Francis *et al*., 1999); Figure 4b) along the glial bipolar processes was monitored 24 h later using time-lapse microphotography as described (Gregg and Weiss, 2003). The bipolar processes extended by TGFα-treated astrocytes supported neuronal migration (Figure 4e–i). Of the 39 neurons monitored, 15 moved in a directional way along the bipolar glial processes at speeds ranging from 3 to 51 μm/h (mean $\pm$ s.e.m.: 17.95 $\pm$ 3.16 μm/h, $n = 15$). In contrast, neurons apposed to polygonal astrocytes did not move (double arrows, Figure 4e and i).

Radial glial cells are also recognized as an essential neural progenitor population in the developing CNS (Kriegstein and Gotz, 2003). Cultured astrocytes initially exposed for 7 DIV to TGFα were further cultured for 18 days in neurobasal medium containing the B27 supplement and TGFα. This additional differentiating period resulted in the appearance of cells expressing the neuronal markers βIII-tubulin (Figure 5b) and microtubule associated protein 2 (MAP2) (Figure 5d), absent from the control cultures (Figure 5a and c). Neuronal markers were also absent in astrocyte cultures maintained for 7 plus 18 DIV in defined medium supplemented with TGFα (not shown). We further evaluated the functional properties of the cells transferred in neurobasal medium containing B27 and TGFα, as compared to cells never exposed to TGFα and transferred to neurobasal medium containing only B27, by characterizing their electrophysiological properties using standard whole-cell patch-clamp recordings (Figure 5e–h). All cells recorded from TGFα-treated cultures displayed voltage-activated currents ($n = 25$, Figure 5f1 and f2). These cells had an input resistance (R_i) ranging from 40 MΩ to 1.3 GΩ (244 $\pm$ 50.6 MΩ, mean $\pm$ s.e.m., $n = 25$), and an input capacitance of 14.8 $\pm$ 0.8 pF (mean $\pm$ s.e.m., $n = 25$). All cells tested showed an outward delayed current (Figure 5f1 and f2). This current, activated mostly at −20 mV, was decreased in the presence of 10 mM tetraethylammonium (TEA) (Figure 5g), identifying voltage-gated potassium channels. Twelve out of 25 cells also expressed voltage-gated sodium channels (holding potential $V_h = -80$ mV), characterized by a voltage-dependent inward current (Figure 5f1 and f2), partly blocked by the application of tetrodotoxin (TTX) ($\approx$80%) (Figure 5h). This sodium current was activated at −40 mV and reached a maximum between −10 and 0 mV ($V_h = -80$ mV). Its averaged maximal amplitude was 168.5 $\pm$ 22.3 pA (mean $\pm$ s.e.m., $n = 12$). Such a combination of voltage-gated delayed outward rectifier K^+ currents and voltage-gated inward Na^+ currents has been reported in differentiating neural progenitors (see

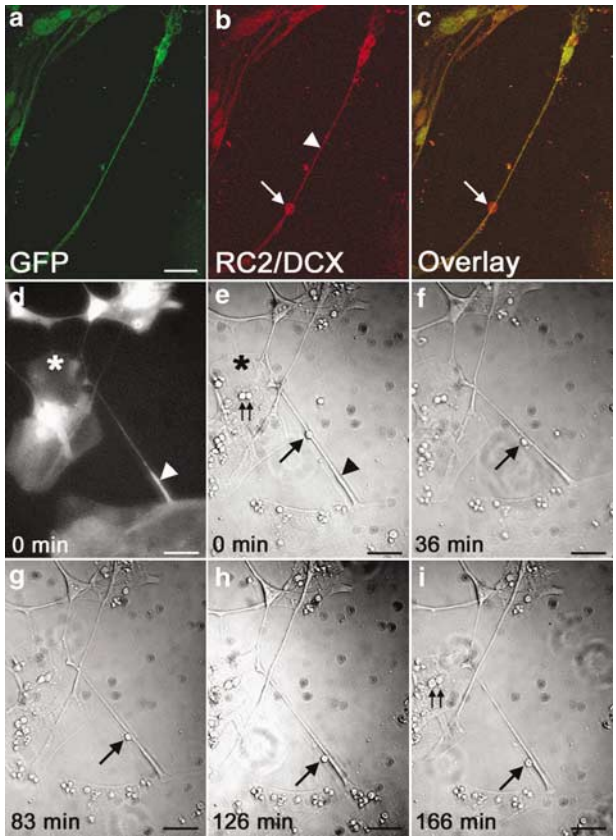

Figure 4 TGFα-induced bipolar astrocytes support the migration of immature neurons along their processes. Astrocyte cultures were derived from cortices of actin-GFP transgenic mice, allowing visualization of astrocytes by their green fluorescence (**a** and **d**). After treatment of astrocytes with TGFα, immature neurons derived from cortices of wild-type C57Bl6 16-day-old embryos were seeded onto the astrocytes. (**a**–**c**) Astrocyte bipolar processes express GFP (**a**) and RC2 (red, **b**, arrowhead). Immature migrating neurons were immunoreactive for doublecortin (DCX) (red, **b** and **c**, arrow). (**c**) Overlay of (**a**) and (**b**). (Owing to technical constraints, RC2 and doublecortin were both detected with red fluorochromes. Lack of RC2 immunolabelling in neurons apposed onto the glial bipolar processes was therefore verified before performing doublecortin labelling.) Images were acquired on a confocal microscope. Scale bar = 20 μm. (**e**–**i**) Example of a neuron migrating along a process of a bipolar astrocyte. Displacements were monitored using phase-contrast time-lapse microphotography. The arrow points to a neuron attached to a bipolar astrocyte (arrowhead in (**d**) and (**e**)) and that migrates along the process. Polygonal astrocytes (asterisk in (**d**) and (**e**)) could not support directional migration of immature neurons (double arrowhead in (**e**) and (**i**)). Scale bars = 50 μm.

Discussion). In contrast, none of the cells cultivated in the absence of TGFα displayed voltage-activated currents (Figure 5e1 and e2, $n=15$). Cells in control cultures had R_i values ranging from 20 to 329 MΩ (79.4±20.5 MΩ, mean±s.e.m., $n=15$), which were significantly lower than values recorded for cells in the TGFα-treated cultures (unpaired *t*-test, $P<0.01$). Similar electrical patterns were observed whether astrocytes were in contact between each other or isolated.

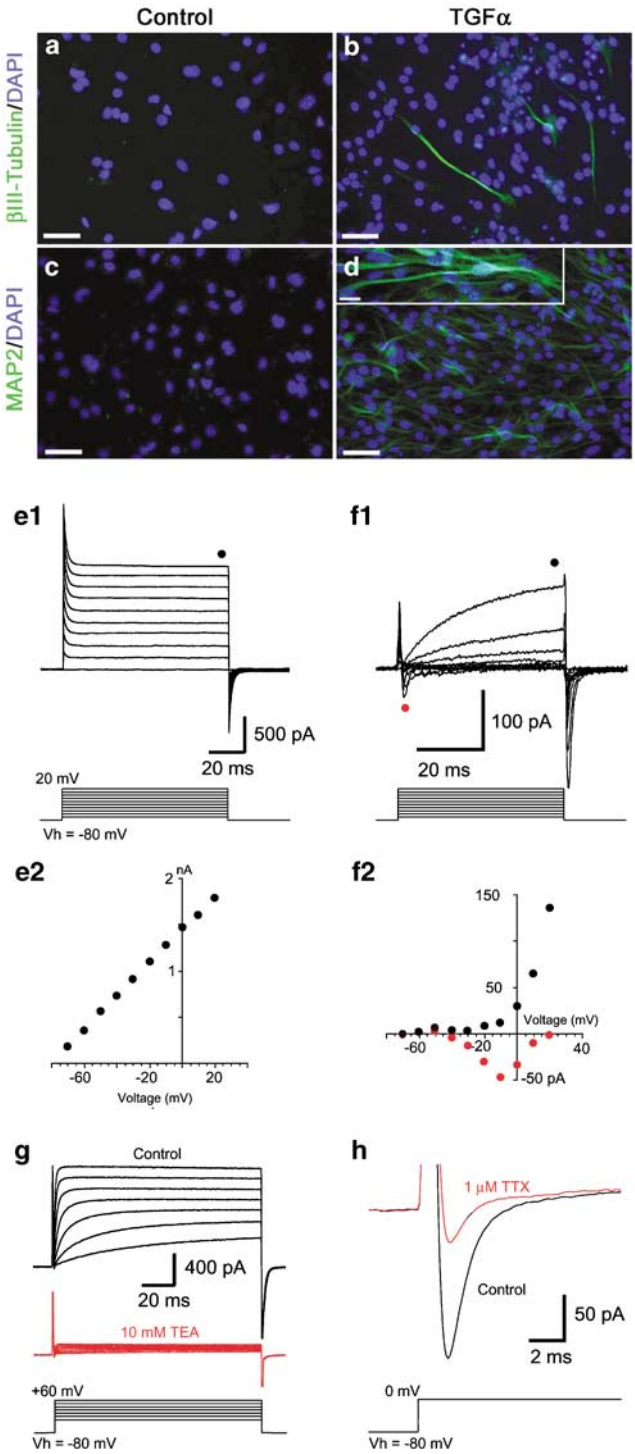

Figure 5 TGFα-induced bipolar astrocytes can behave as progenitors. Astrocytes cultured for 7 days in defined medium supplemented with TGFα, and subsequently transferred to neurobasal medium for 18 days, expressed the neuronal markers βIII-tubulin (**b**) and MAP2 (**d**). Nuclei were counter-stained with DAPI (blue). Control cultures, submitted to similar conditions but never exposed to TGFα, were devoid of βIII-tubulin (**a**) and MAP2 (**c**) expression. Images were obtained on a fluorescent microscope. Scale bars = 50 μm in (**a**)–(**d**), 20 μm in inset. (**e–h**) Electrophysiological properties of cells maintained in neurobasal medium in the absence (control) or presence of TGFα were recorded using the whole-cell patch-clamp technique. (**e1–e2**) None of the recorded cells cultivated in the absence of TGFα displayed voltage-activated currents. (**e1**) Superimposed traces showing responses to depolarizing voltage step pulses. The command voltage is shown at the bottom of the traces. Pipette capacitance and series resistance (R_s) were not compensated ($R_s=6$ MΩ). (**e2**) Current–voltage (*I–V*) relationship of the responses shown in **e1**. (**f–h**) Recording from TGFα-induced bipolar cells. All recorded cells displayed voltage-activated currents. (**f1**) Superimposed traces showing responses to depolarizing voltage step pulses ($R_s=5$ MΩ). Note the occurrence of a rapidly activating and rapidly inactivating inward current (red dot). This inward current is followed by a slow activating outward current (black dot). (**f2**) Current–voltage (*I–V*) relationship of the inward current (red dots) and the outward current (black dots). (**g**) The voltage-dependent delayed outward current was blocked by the addition of 10 mM TEA. (**h**) The fast-activating and fast-inactivating inward current was 79% blocked by the addition of 1 μM TTX. Traces in (**f**), (**g**) and (**h**) were obtained from different cells.

Altogether, these results showed that TGFα induced the conversion of astrocytes into functional radial glial cells, able to support neuronal migration and to behave as neuroprogenitors.

TGFα-induced radial glial cells convert into neural stem-like cells upon lengthened TGFα action

Development of 0.5–1 mm cellular blocks at the top of the cellular layer was observed in astrocyte cultures maintained for 10 months in TGFα-supplemented defined medium (untreated cultures died after 2 months). Blocks were collected from three different wells of two independent cultures, dissociated and the cells further cultured in six-well plates in neurosphere medium. The cells kept growing free-floating, forming cellular spheres (Figure 6a) that multiplied and have been maintained in culture for more than 5 months. The presence of TGFα or EGF in the neurosphere medium was required for long-term maintenance of these cellular spheres. Dissociating the cellular spheres and expanding them during 12–22 successive passages further ascertained their self-renewal capabilities. Their clonal potential was determined after seeding dissociated cellular spheres at a density of 20 cells/250 μl/well. Visual observations confirmed that each well contained 5–20 cells at the time of seeding. After 1 week *in vitro*, 12.6±0.7 cellular spheres per well (mean±s.e.m., $n=24$) were observed, indicating that these spheres can be clonally derived. The cellular spheres were then seeded onto polyornithine-coated glass slides, and cultured for 3–7 days in 1 or 5% fetal calf serum-containing medium (Figure 6b–g). In these conditions, the spheres attached, and individual cells appeared

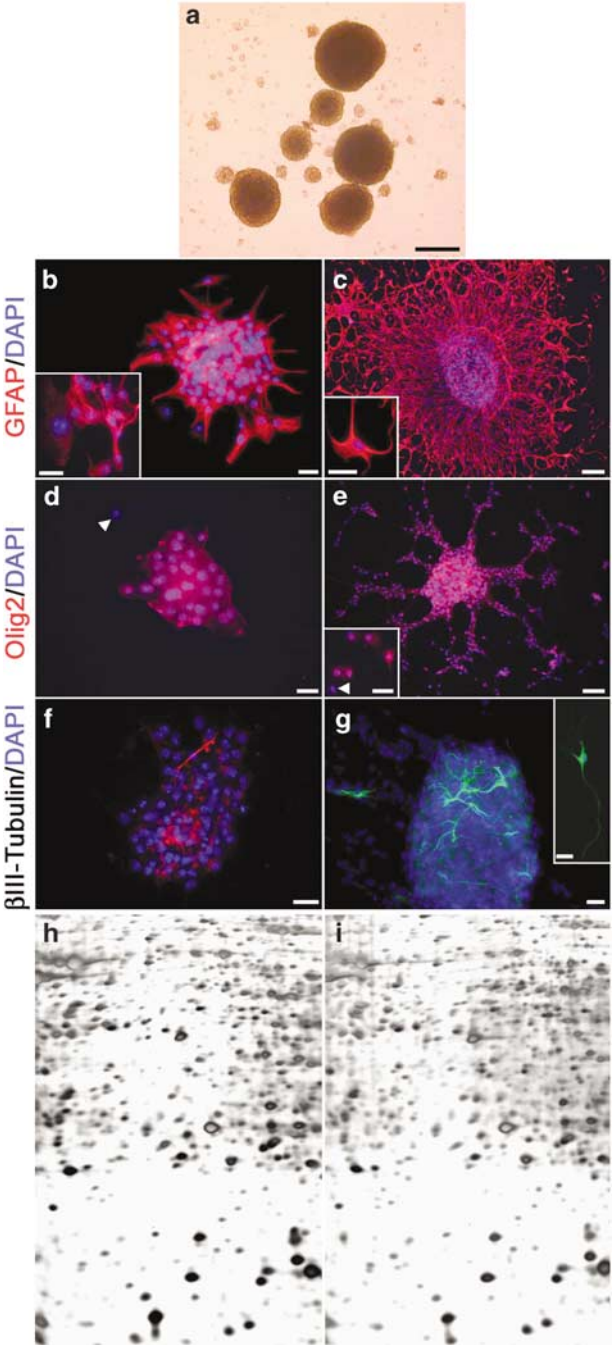

Figure 6 Prolonged TGFα treatment induces astrocytes to regress towards a stem-like cell phenotype. Blocks of cells detached from long-term TGFα-treated cultures formed free-floating cellular spheres in neurosphere medium (**a**). When transferred to DMEM:F12 supplemented with 1% fetal calf serum (**b**, **d** and **f**) or to neurosphere medium supplemented with 5% fetal calf serum (**c**, **e** and **g**), spheres attached to the bottom of the vial. Cells expressed GFAP (red, **b** and **c**), and Olig2 (red, **d** and **e**, arrowheads point to Olig-2-immunonegative cells) and a subset expressed βIII-tubulin (red in **f** and green in **g**). Images were acquired on a phase-contrast microscope in (**a**), and on a fluorescent microscope in (**b**)–(**g**). Scale bars = 200 μm (**a**), 100 μm (**c**), 50 μm (**e**), 20 μm (**b**, **d**, **f** and **g**, insets in **b**, **c**, **e** and **g**). Neural stem cells derived from 12-day-old mouse embryo cortices (**h**) and cellular spheres derived from 10-month-old TGFα-treated astrocyte cultures (**i**) exhibited highly similar protein patterns following 2D electrophoresis analysis (pI range: 3–10, MW range: 16–70 kDa).

either at their immediate limit or at further distances (Figure 6b–g). Numerous GFAP (Figure 6b and c), Olig2 (Figure 6d and e) and nestin-immunoreactive cells (not shown) were then observed, as were lower numbers of βIII-tubulin-immunoreactive cells (Figure 6f and g). Taken altogether, these capacities make TGFα-generated cellular spheres very similar to neural stem cells. This led us to use two-dimensional (2D) sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis to compare the pattern of expression of several hundreds of proteins from control astrocytes, 7-day TGFα-treated astrocytes, cellular spheres derived from long-term TGFα-treated astrocytes and neural stem cells derived from the cortex of 12-day-old mouse embryos. The 2D maps obtained from control astrocytes and 7-day TGFα-treated astrocytes were different, although numerous overlaps were observed as expected, as only half of the cells had responded to TGFα at that time (Supplementary Figure 2a and b). Comparison of the protein maps using computer-assisted analysis showed a 0.44 coefficient of correlation. On the contrary, maps obtained from the cellular spheres derived from long-term treated cells did not overlap even in part with the protein patterns of control or 7-day TGFα-treated astrocytes (coefficients of correlation = 0). In contrast, cellular spheres derived from long-term TGFα-treated astrocyte cultures exhibited a global pattern of protein migration very similar to neurospheres derived from embryonic cortices (coefficient of correlation = 0.74), within the ranges of molecular weight (MW) and pI examined (Figure 6h and i and Supplementary Figure 2c and d). These results together with the fact that sphere-forming cells in astrocytic cultures exposed to TGFα for several months have self-renewal properties, the ability to be clonally derived and the potential to differentiate into cells of the neuronal lineage, demonstrated that those cells acquired properties of neural stem cells.

Discussion

The existence of a population of GFAP-expressing neural stem cells in adult germinal regions of the mammal CNS, distinct from astrocytes, is now recognized (Doetsch, 2003). The demonstration that neurospheres, considered as signing for the presence of neural stem cells (Reynolds *et al*., 1992), can be derived from astrocytes sub-cultured for a week in EGF- or EGF- and FGF-containing medium (Laywell *et al*., 2000; Imura *et al*., 2003), has additionally pointed to the possibility that mature astrocytes themselves might behave as stem cells at least *in vitro*. But the brevity of the time course required to obtain neurospheres from astrocyte cultures (7 DIV) and its similarity with that required to obtain neurospheres from brain germinal zones (Reynolds *et al*., 1992) suggest that they correspond to GFAP-expressing neural stem cells preserved in the cortical astrocyte cultures used (Imura *et al*., 2006). In contrast, in neural stem cell and progenitor-deprived cultures, we observed a slow and sequential conversion of a

population of astrocytes into functional neural progenitors. In addition, we did not observe floating cellular spheres before several months of treatment. Furthermore, we obtained the same and constant proportion of converted cells, whether the cultures derived from 1- to 2- or 10-day-old mouse cortices. Indeed, a tremendous reduction of the numbers of neural stem cells that can be derived from the mouse cortex occurs between postnatal days 1 and 10, the numbers falling then almost to zero (Seaberg *et al*., 2005). Lack of Olig2 expression in control cultures further supports astrocyte maturity and initial lack of progenitors. Indeed, whereas mature astrocytes lack Olig2 expression, this transcription factor is present in radial glial cells, glial-restricted progenitors and immature astrocytes (Marshall *et al*., 2005), and its expression has been shown to repress astrocytes differentiation (Fukuda *et al*., 2004; Muroyama *et al*., 2005). Serum starvation of the cultures prior growth factors addition, and possibly the repeated washes with ice-cold phosphate saline buffer initially designed to remove the microglial and oligodendroglial cells, which reside at the top of the cellular layer, might account for the lack of neural progenitors and stem cells in our astrocyte cultures.

After a week exposure, TGFα-treated astrocytes share molecular markers and functional properties of radial glial cells, that is, they support the migration of neurons and give birth to neuroblasts. Indeed, TGFα-generated radial glia transferred to neuronal growth medium expressed a combination of voltage-gated delayed outward rectifier K^+ currents and voltage-gated inward Na^+ currents repeatedly reported in differentiating neural progenitors (Feldman *et al*., 1996; Jung *et al*., 1998; Bahrey and Moody, 2003; Fukuda *et al*., 2003). In contrast, astrocytes maintained in control conditions showed the passive membrane conductances with no voltage-dependent Na^+ currents as described previously for mature astrocytes (Kressin *et al*., 1995, reviewed by Verkhratsky and Steinhauser, 2000). Taken as a whole, these results demonstrate that astrocytes can indeed be converted into a *bona fide* novel and functional cell type, that of the radial glial cells.

The actions of TGFα and erbB1 on glial cells appear complex. TGFα was first found to promote neural stem cell/progenitor proliferation both *in vitro* and *in vivo*. Further studies extended its intervention to the adoption of an astrocyte fate by the neural progenitors (reviewed by Junier, 2000). We now demonstrate that the same molecular system is active in a bidirectional manner, as the conversion of astrocytes into progenitors also requires erbB1 activation. It is likely that the cellular context plays a major role in the biological outcome of the mobilization of the TGFα-erbB1 pathway. The distinct actions exerted by TGFα on cortical neural progenitors, mitogenic or promoting an astrocytic fate according to the developmental stage, depend on the low or high expression levels of its receptor, respectively. During the late stage of embryonic development, when gliogenesis takes over neurogenesis, it favours the preferential differentiation of neural progenitors into astrocytes over neurons, an effect mediated by enhanced expression of erbB1, and segregation of the receptors into the daughter cell that will ultimately acquire an astrocyte phenotype (Lillien, 1995; Burrows *et al*., 1997; Sun *et al*., 2005). Interestingly, we observed that conversion of mature astrocytes into progenitor-like cells correlated with the downregulation of the whole-cell erbB1 content, suggesting that modulation of the receptor levels might be part of the mechanisms by which TGFα alters the astrocyte phenotype. This possibility remains however to be demonstrated.

The very lengthened TGFα treatment required for the genesis of neural stem-like cells from astrocytes appears puzzling. One may envisage that such prolonged treatment of glial cells with a growth factor well known to participate in oncogenesis in numerous organs (Lee *et al*., 1995) may result in transforming events necessary for the genesis of these cellular spheres. The striking similarities between the patterns of protein expression of cellular spheres derived from the astrocyte cultures and neural stem cells derived from the 12-day-old embryonic cortex does not, however, support such a possibility.

This study uncovers a remarkable capability of astroglial cells to undergo *in vitro* a functional de-differentiation in response to a single change in their extracellular environment. This property of astrocytes may be of significance for both nervous tissue repair and glial oncogenesis. *In vivo*, the only known example of conversion of a differentiated cell into a less mature state in the mammal nervous system is provided by Schwann cells (Raff, 2003). Upon nerve injury, these glial cells of the peripheral nervous system de-differentiate into their immediate precursors before proliferating and differentiating into myelinating mature cells (Scherer, 1997). Although the upregulation of the intermediate filament nestin and its isoform RC2, normally present in progenitors, and enhanced TGFα synthesis, have been reported in astrocytes following CNS injury (Frisen *et al*., 1995; Lin *et al*., 1995; Leavitt *et al*., 1999; Junier, 2000), their capability to behave *in situ* as regenerative cells remains to be uncovered. However, contrary to Schwann cells, astrocytes do not respond to injury by entering *en masse* into the cell cycle, and this tight control of their proliferation (Norenberg, 1994; Dihne *et al*., 2001) might limit their *in vivo* plasticity. Modelling of gliomas in transgenic mice showed that neural progenitors are more sensitive than astrocytes to the oncogenic effects of genetic mutations identified in human gliomas (Dai and Holland, 2003). Our results suggest that a deregulation of TGFα metabolism may result in an increase of neural progenitors representing new potential targets for an additional transformation event.

Materials and methods

C57Bl6/J mice (Janvier, France) were bred and housed in an air-conditioned room with free access to water and food. All experiments carried out on cells derived from human fetuses were performed in compliance with French bioethics laws. Recombinant human TGFα was obtained from AbCys and

RnDSystems (Lille, France). Recombinant human bFGF was from AbCys. Recombinant human heregulinβ1 was from Neomarkers (Montluçon, France). AG1478 was from Calbiochem (La Jolla, CA, USA).

Cell culture preparations
Cultures of mouse astrocytes were prepared from cortices of 1- to 2-, 5- and 10-day-old mice following previously described procedures (Prevot *et al*., 2005) in defined medium containing 10% fetal calf serum (Biowest, Nuaillé, France). Medium was changed every 3 days following washes with ice-cold phosphate-buffered saline (PBS). When confluency was reached (8–10 DIV), cultures were shaken overnight (250 r.p.m.), trypsinized and seeded on poly-L-ornithine-coated glass slides (80 000 cells/cm^2). The cells were further cultured for 4–6 days, with washes with ice-cold PBS preceding each medium renewal, until an 80–90% confluent cell layer had formed. The cultures were then transferred to serum-free defined medium for 3 days, before be fed with fresh defined medium containing the appropriate growth factor and drugs or the corresponding vehicles. Enrichment of the cultures in astrocytes was determined using immunocytochemical detection of GFAP (monoclonal mouse anti-GFAP antibody, 1:400, ICN, Strasbourg, France) and microglia were identified using anti-CD11b receptor immunocytochemical detection (monoclonal rat Mac1 antibody, 1:200, PharMingen, Le Pont de Claix, France). Human astrocyte cultures were prepared from fetal cortices (9–12 gestational weeks), and grown on poly-D-lysine-coated plastic dishes and glass slides. The cortices were crushed on an 80 μm nylon cloth (Buisin, Clermont, France), filtered through a 20 μm Nitex cloth, and the dissociated cells were cultured in Dulbecco's modified Eagle's medium (DMEM)-F12 supplemented with 10% fetal calf serum (Invitrogen, Cergy Pontoise, France) for 1 month in 75 cm^2 culture flasks, before seeding at 50 000 cells/well in 12-well plates on coverslips for experimentation. GFAP-immunoreactive astrocytes accounted for 91–96% of the cells in all culture types. In mouse cultures, the remaining 4–9% of cells were CD11b receptor-immunoreactive microglia.

Blocks of cells detaching from the cell monolayer in astrocyte cultures exposed to TGFα for 10–12 months were taken, dissociated and further cultured in neurosphere medium (see below). The spheres were dissociated at each medium renewal (once a week). For differentiation assays, spheres were seeded onto poly-L-ornithine-coated slides and cultured for 4–8 days in the presence of 1 or 5% fetal calf serum.

Mouse 12.5-day-old embryos were used to prepare cortical neural stem cell cultures. Embryos were collected from time-mated C57/Bl6 mice. The cortex was dissected and mechanically dissociated as described (Tropepe *et al*., 1999). The cells were spun down and cultured in a 75 cm^2 flask at 10 cells/μl in neurosphere medium composed of a 1:1 mixture of DMEM and F12 nutrient (Invitrogen) containing 0.6% glucose, 2 mM glutamine, 13 mM sodium bicarbonate, 5 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer, 5 IU/ml penicillin and 5 μg/ml streptomycin, and the B27, N-2 and G5 supplements (10 μl/ml each, Invitrogen). Cells were passaged once a week by centrifugation at 172 *g* for 10 min, followed by mechanical re-dissociation and re-plating in the neurosphere medium described above.

Characterization of the morphological effect of TGFα
Control and TGFα-treated (50 ng/ml) cultures were monitored daily for morphological changes under phase-contrast microscopy. Ten nanograms per milliliter TGFα also proved to be efficient, but the highest concentration was used to minimize the frequency of the medium renewals. After fixation in 4% paraformaldehyde, actin was labelled with phalloidin coupled to Alexa 488 or rhodamine (1/1500, Molecular Probes, Cergy Pontoise, France). Nuclei were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI) or Hoechst. Cultures were treated for 7 days with AG1478 (1 μM) in the presence or absence of TGFα.

Immunolabelling
Immunocytochemical and immunohistochemical procedures were performed as described (Sharif *et al*., 2004) using the following primary antibodies: polyclonal guinea-pig anti-GLAST (1:1000, Chemicon, Paris, France), polyclonal rabbit or monoclonal anti-GFAP (1:500, Dako or ICN), polyclonal rabbit anti-BLBP antibody (1:4000), monoclonal anti-RC2 (1:200), monoclonal anti-nestin (1:200, Chemicon), monoclonal anti-βIII-tubulin (1:1000, Tuj1, Sigma, Lyon, France), monoclonal anti-MAP-2 (1:1000, Sigma), polyclonal rabbit anti-doublecortin (1:2000, Tebu, Le Perray en Yvelines, France).

Image acquisition
Immunofluorescence was observed with a fluorescent microscope (Eclipse E800, Nikon, USA). Images were acquired on a digital still camera (DXM 1200, Nikon, Champigny-Sur-Marne, France) using Lucia software (Laboratory Imaging Ltd., Nikon, Champigny-Sur-Marne, France). We used custom-made filters (Chroma, Rockingham, VT, USA): the excitation wavelengths were 440–480 nm for Alexa 488, 530–545 nm for CY3 and Texas red, and 340–380 for DAPI. The emission wavelengths were 535–550 nm for Alexa 488, 620–660 nm for CY3 and Texas red, and 435–485 nm for DAPI. Texas red-coupled phalloidin was acquired using a fluorescent system (DMRB microscope, DC300FX camera, FW4000 software, Leica, Bensheim, Germany). For confocal immunofluorescence, samples were observed using a Leica TCS SP2 confocal microscope. Two lasers were used depending on the fluorochrome. The excitation wavelengths were 543 nm for Texas red and rhodamine and 488 nm for GFP, and the emission wavelengths were 580–660 nm for Texas red and rhodamine and 500–550 nm for GFP. Samples were observed with the 40 and 63 Å objectives, with the 1.25 and 1.32 numerical aperture lenses, respectively (Leica, Germany). The pinhole was set on the airy spot and the images were acquired in a sequential scan mode for double immunofluorescent labelling. The images were prepared using Adobe Photoshop software (Adobe Systems, San Jose, CA, USA).

Neuronal migration assay
Astrocytes derived from cortices of 1- to 2-day-old transgenic mice expressing GFP under the control of the actin promoter (Hadjantonakis *et al*., 1998) were seeded at a density of 26 000 cells/cm^2 and treated for a week in defined medium supplemented or not supplemented with TGFα. Neurons were prepared from cortices of C57Bl6 embryos at E16 following previously described procedures (Francis *et al*., 1999). They were seeded onto the astrocyte monolayer at a density of 260 000 cells/cm^2 and further maintained for up to 4 days in defined medium containing or not containing TGFα. Astrocytes were visualized by their green fluorescence, and neuronal migration was followed using phase-contrast time-lapse microphotography using an inverted microscope (Leica). Images were acquired on a Photometrics CoolSNAP camera (Roper Scientific Inc., Evry, France) using MetaVue software (Molecular Devices Corporation, Sunnyvale, CA, USA). Cells

were then gently fixed in paraformaldehyde and subjected to immunocytochemical labelling.

Neuronal differentiation assay
Astrocytes were treated for 7 days in the absence or presence of TGFα, before transfer for 18 days to neurobasal medium supplemented with glutamax (200 mM), B27 (20 μl/ml), 5 IU/ml penicillin and 5 μg/ml streptomycin (Invitrogen), in the presence or absence of TGFα. Cells were then subjected to electrophysiological recordings, and to βIII-tubulin and MAP2 immunolabelling.

Electrophysiological recordings
Standard whole-cell recordings (Hamill *et al*., 1981) from glial cells were made under direct visualization (Nikon Optiphot microscope). The cell culture was continuously perfused at room temperature (20°C) in a recording chamber (0.5 ml) with an oxygenated bathing solution (2 ml/min) containing (in mM): NaCl 145; KCl 1.5; $CaCl_2$ 2; $MgCl_2$ 1; glucose 10, HEPES 10 (pH 7.3) with the osmolarity adjusted to 300 mosM. Patch-clamp electrodes were pulled from thick-wall borosilicate glass (GC150F-10: CEI, Harvard Apparatus Ltd., Kent, UK), fire-polished and filled with (in mM): CsCl 135 or KCl 135; $MgCl_2$ 2; Na_3ATP 4; ethyleneglycol tetracetate 10; HEPES 10 (pH 7.2) (290 mosM). Electrodes had resistances of 2–4 MΩ. Currents were recorded using an Axopatch 200B amplifier (Axon Instruments, Sunnyvale, CA, USA), filtered at 5 kHz and stored using a digital tape recorder (DAT DTR 1201, Sony, Tokyo, Japan). Series resistances (6–20 MΩ) were 60–80% compensated. Recordings with a series resistance >20 MΩ were rejected. Voltage command pulses were generated using pClamp9 software (Axon Instruments). Currents were recorded at a sampling frequency of 40 kHz and transferred onto a G4 Macintosh computer. The quality of the seal was verified for each cell having a low R_i. Analysis was performed using Axograph 4.9 software (Axon Instruments) after digital filtering of the traces at 1–4 kHz. Leakage and capacitive currents were digitally subtracted. TEA (10 mM) and 1 μM TTX (Sigma, Lyon, France) were applied to the bath using a single barrel connected to a manifold with reservoirs filled with different solutions.

Immunoprecipitation and Western blot analyses of erbB1 and erbB2
erbB1 and erbB2 expression and activation upon TGFα application were analysed following previously described procedures (Prevot *et al*., 2005). Activated forms of erbB1 and erbB2 were detected using immunoprecipitation of the proteins with their respective antibodies, followed by immunoblotting with anti-phosphotyrosine antibodies. Detection of the whole protein levels of the receptors was achieved by direct Western blot analysis of the protein lysates.

2D proteomic analysis
Cells were washed three times with PBS for 10 min with gentle shaking, before lysis in buffer containing 8 M urea, 4% 3-[(3-cholamidopropyl)-dimethylammonio]-2-hydroxy-1-propanesulfonic acid and 40 mM Tris. The samples were shaken for 1 h and centrifuged for 20 min at 16 000 *g*. The protein pellets were dissolved in isoelectric focusing buffer and quantified using the Micro BCA protein assay kit (Pierce, Brebieres, France). First-dimension isoelectric focusing was performed with the Ettan IPGphor system (Amersham Biosciences, Saclay, France) at 20°C with a maximum current setting of 50 mA/strip. Immobiline DryStrip gels (IPG stripes, Amersham Biosciences) with a pH gradient of 3–10 or 4–7 and a length of 13 cm were rehydrated in 250 μl sample solution, containing 80 μg proteins. Series of voltage steps were applied until 16 kVh was reached (step-and-hold at 100 V for 30 min, then 1000 V gradient for 30 min, followed by a 5000 V gradient for 60 min, and terminated with step-and-hold at 8000 V). Before SDS–PAGE analysis, IPG stripes were equilibrated in second-dimension equilibration buffer (6 M urea, 2% SDS, 50 mM Tris–HCl, pH 8.8, 30% glycerol) containing 1% dithiothreitol (Sigma) to reduce the disulfide bonds of the proteins. After 15 min, the solution was replaced by equilibration solution containing 4% iodoacetamide (Sigma) and incubated for another 15 min in the dark at room temperature. The second dimension was carried out using 12.5% gradient polyacrylamide gels at constant 20 mA current per gel. Comparison of the protein maps was achieved using the PD-Quest software according to the manufacturer instructions (BioRad, Marnes-la-Coquette, France).

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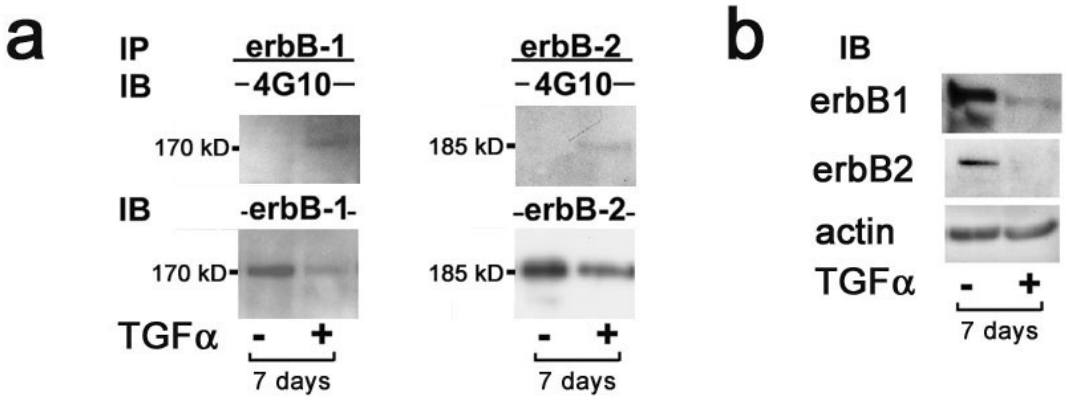
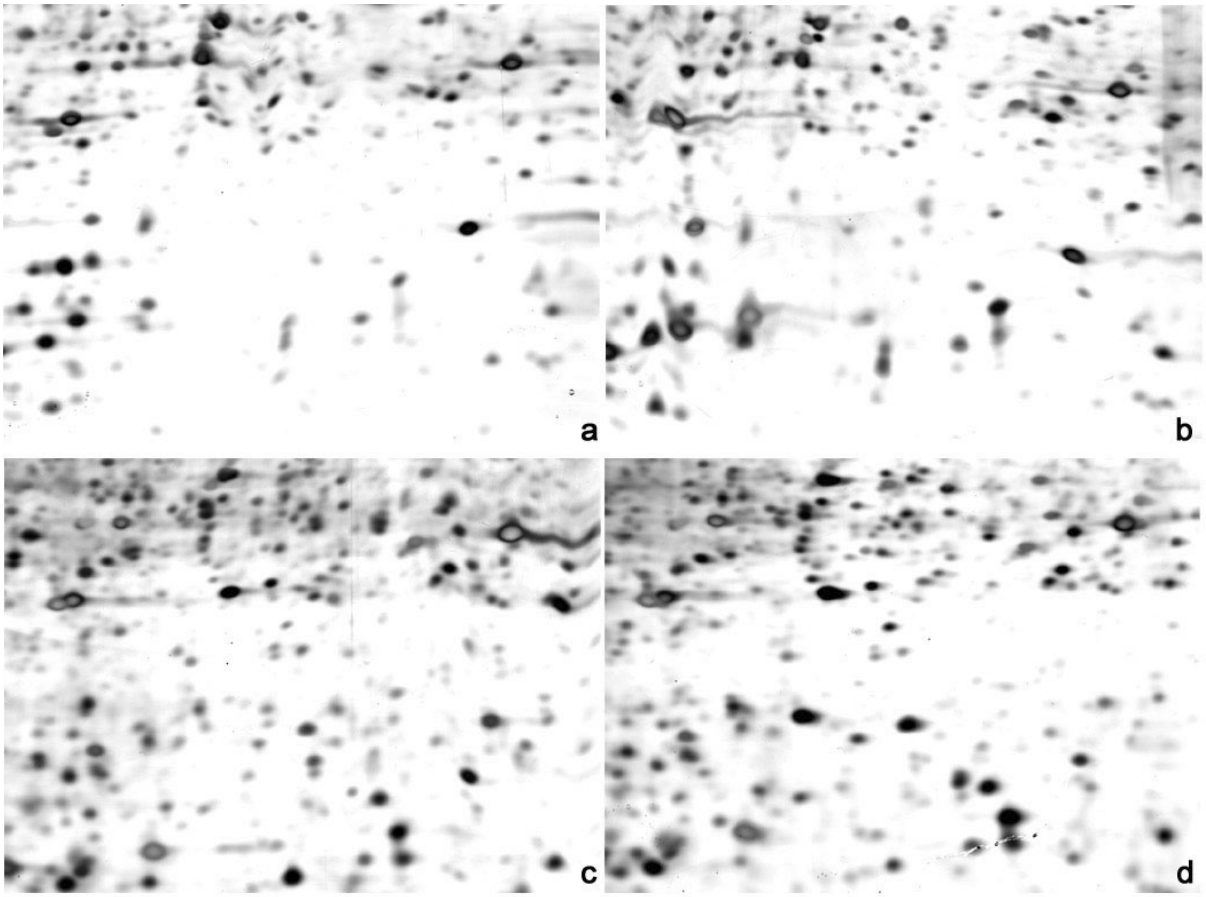


Supplementary Figure 1



Supplementary Figure 2

ANEXO 5

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Guanine Derivatives Modulate Extracellular Matrix Proteins Organization and Improve Neuron-Astrocyte Co-Culture

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Guanine derivatives (GD) have been shown to exert relevant extracellular effects as intercellular messengers, neuromodulators in the central nervous system, and trophic effects on astrocytes and neurons. Astrocytes have been pointed out as the major source of trophic factors in the nervous system, however, several trophic effects of astrocytic-released soluble factors are mediated through modulation of extracellular matrix (ECM) proteins. In this study, we investigated the effects of guanosine-5′-monophosphate (GMP) and guanosine (GUO) on the expression and organization of ECM proteins in cerebellar astrocytes. Moreover, to evaluate the effects of astrocytes pre-treated with GMP or GUO on cerebellar neurons we used a neuron-astrocyte coculture model. GMP or GUO alters laminin and fibronectin organization from a punctate to a fibrillar pattern, however, the expression levels of the ECM proteins were not altered. Guanine derivatives-induced alteration of ECM proteins organization is mediated by activation of mitogen activated protein kinases (MAPK), CA^{2+}-calmodulin-dependent protein kinase II (CaMK-II), protein kinase C (PKC), and protein kinase A (PKA) pathways. Furthermore, astrocytes treated with GMP or GUO promoted an increased number of cerebellar neurons in coculture, without altering the neuritogenesis pattern. No proliferation of neurons or astrocytes was observed due to GMP or GUO treatment. Our results show that guanine derivatives promote a reorganization of the ECM proteins produced by astrocytes, which might be responsible for a better interaction with neurons in cocultures.

Key words: GMP; guanosine; laminin; fibronectin; cerebellar astrocytes

Guanine derivatives (GD) have well known multiple intracellular roles mainly in the modulation of signal transduction processes. In higher vertebrates, important extracellular effects of GD have been described, such as intercellular messengers and neuromodulators in the central nervous system (CNS) (Neary et al., 1996; Rathbone et al., 1999; Ciccarelli et al., 2001). Astrocytes have been considered the main source of extracellular adenine and guanine derivatives (Ciccarelli et al., 1999), although it has been shown that GTP, like ATP, is taken up and stored into neuronal synaptic vesicles (Santos et al., 2006), suggesting a GD vesicular released. Specific purinergic receptors for adenine derivatives belong to both adenosine P1 and ATP P2 receptors (Stone, 1991). The GD, guanosine (GUO), or GTP, may bind to adenine derivatives receptors with low affinity, suggesting GD may have distinct cellular targets than already known purinergic receptors (Neary et al., 1996). Evidences for putative receptors or specific binding sites for GD have been shown in cultured astrocytes (Chen et al., 1993), in PC12 cells (Gysbers et al., 2000; Bau et al., 2005), in chick cerebellar membranes (Tasca et al., 1999a), and in rat brain membranes (Traversa et al., 2002). A major role for GD as glutamate receptors antagonists (Tasca et al., 1999b) and as neuroprotective agents has been shown in vitro (Oliveira et al., 2002;

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Molz et al., 2005) and in vivo models of glutamate toxicity (Schmidt et al., 2000; Tavares et al., 2005).

GUO and GTP in the extracellular medium display trophic effects on glial cells, hippocampal neurons and PC12 cells. They induce cellular proliferation, differentiation, neurite arborization and outgrowth (Rathbone et al., 1999; Ciccarelli et al., 2001). GD-induced trophic effects can occur indirectly by increasing the synthesis and release of neurotrophin and pleiotrophins (Ciccarelli et al., 2001). Mitogenic effects of GD on astrocytes have been shown to be dependent on the micro-environment and increase proportionally with the number of microglial cells present in culture (Ciccarelli et al., 2000).

Astrocytes have been pointed out as the major source of trophic factors in the CNS (Garcia-Segura et al., 1999; Gomes et al., 1999; Dong and Benveniste, 2001), however, several of the trophic effects of the soluble factors are mediated through modulation of extracellular matrix (ECM) proteins (Trentin et al., 2003). ECM is organized as a supramolecular architecture composed mainly of laminin (LN), fibronectin (FN), collagen IV, and proteoglycans. The ECM provides positional and environmental information that is essential for tissue function. ECM, through their specific receptors, determines the cytoskeleton organization, as well as the localization and activation of signaling molecules (Hay and De Boni, 1991; Streuli, 1999; Pesheva and Prosbtmeier, 2000). FN participates in cellular adhesion, spreading, and migration of diverse cell types (Trentin et al., 1995, 2003). Beyond displaying a role as an adhesive substrate to cells, LN improves conditions for neurite outgrowth (Letourneau et al., 1988; Liesi et al., 1995; Luckenbill-Edds, 1997).

We investigated the effects of guanosine-5′-monophosphate (GMP) and GUO on the organization of ECM proteins in cerebellar astrocytes in vitro. Moreover, by using a neuron-astrocyte coculture model, we investigated the effects of GMP- and GUO-treated astrocytes on cerebellar neuronal differentiation in culture. We show that GMP and GUO modulate ECM astrocyte organization but not the expression levels of the ECM proteins, LN and FN. We also provide evidences showing ECM proteins modulation by GD is mediated by activation of proteins kinases pathways. Furthermore, astrocytes treated with GMP or GUO contributed to an increased number of cerebellar neurons, but did not alter the neuritogenesis pattern, neither neuronal nor glial proliferation.

MATERIALS AND METHODS

Astrocyte Primary Cultures

Astrocyte cultures were prepared from cerebella derived from newborn Wistar rats from our own breeding colony, following the procedure described previously by Trentin et al. (1995). Rats were killed by decapitation, cerebella were removed, and the meninges were stripped off carefully. Tissues were washed in PBS, 0.6% glucose (Merck, Darmstadt, Germany) and dissociated into single cells in a medium consisting of Dulbecco's modified Eagle's medium (DMEM) and nutrient mixture F-12 (Gibco, Carlsbad, CA). Cells were plated onto plastic culture flasks (Sigma, St. Louis, MO) or glass cover slips (24-well plates; Sigma), coated previously with polyornithine (10 μg/ml, Sigma) in DMEM-F12 medium supplemented with 10% FBS (Gibco). The cell cultures were incubated at 37°C in a humidified 5% CO_2, 95% air atmosphere. Cell culture medium was changed 24 hr after plating and subsequently every third day until reaching confluence, which usually occurred after 7–10 days in vitro.

Guanine Derivatives Treatment

After reaching confluence, astroglial monolayers were carefully and extensively washed with serum-free DMEM-F12 medium and incubated with 1 μM, 100 μM, or 1 mM guanosine or guanosine-5′-monophosphate (GUO or GMP, Sigma) for 24 hr in serum-free medium. Control cell cultures were maintained in serum-free DMEM-F12 medium.

Signaling Pathways Evaluation

Astrocytes monolayers were treated with specific intracellular signaling pathways inhibitors for 30 min in serum-free DMEM-F12 medium and then incubated concomitantly with GUO 100 μM for additional 24 hr. The inhibitors used were: PD98059 (50 μM) a mitogen-activated protein kinase (MAPK) extracellular signal regulated kinase kinase (MEK)-specific inhibitor; KN-62 (10 μM) a CA^{2+}-calmodulin dependent protein kinase II (CaMK-II) specific inhibitor; chelerythrine (10 μM) a protein kinase C (PKC) specific inhibitor; Wortmannin (1 μM) a phosphatidylinositol-3 kinase (PI3-K)-specific inhibitor and H-89 (5 μM) a protein kinase A (PKA) specific inhibitor.

Cocultures

Neuronal culture was prepared from cerebella derived from 7-day-old Wistar rats (P7) as previously described by Faria et al. (2006). Briefly, neurons were freshly dissociated from cerebellum, and 5×10^4 cells were plated over an astrocytic monolayer in serum-free DMEM-F12 medium. In the coculture assays, astrocytes monolayer was pretreated with GMP or GUO for 24 hr in serum-free DMEM-F12 medium. Cerebellar granule cells were plated onto nontreated or astroglial monolayer carpets treated previously. Cocultures were kept for 24 hr at 37°C in a humidified 5% CO_2, 95% air atmosphere.

Immunocytochemistry

Immunostaining of cultured cells was carried out as described previously by Faria et al. (2006). Briefly, cells were fixed with 4% paraformaldehyde for 5 min (for extracellular matrix protein labeling) or 30 min (for cytoskeleton protein labeling assay), carefully washed with PBS, and in the case of cytoskeleton protein labeling, permeabilized with 0.2% Triton X-100. For peroxidase assays, endogenous peroxidase activity was inactivated by incubation with 3% H_2O_2 for 15 min followed by extensive washing with PBS. Cells were incubated with 5% BSA (Sigma) in PBS (blocking solution) for 1 hr and subsequently with specific antibodies, diluted in blocking solu-

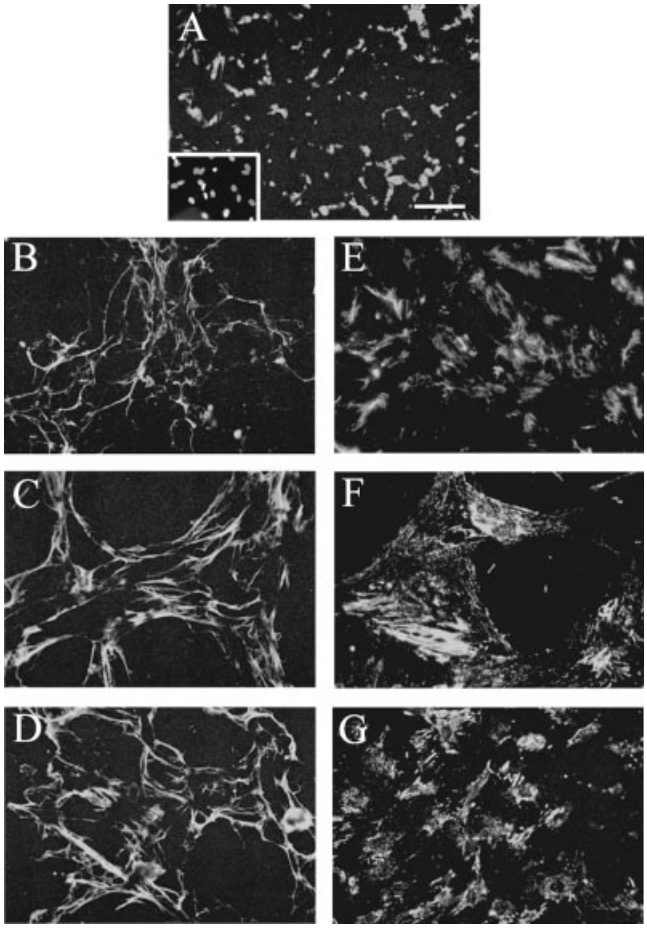

Fig. 1. Guanine derivatives modulate astrocyte organization of fibronectin. Cerebellar astrocytes cultures treated with GMP (**B–D**) or GUO (**E–G**) (1 μM, 100 μM, and 1 mM) were immunostained for the ECM protein fibronectin. Inset in (A) shows a nuclear immunolabeling of control astrocyte culture, which is equivalent for all conditions. The experiments were done three times and triplicate to each one. Scale bar = 20 μm.

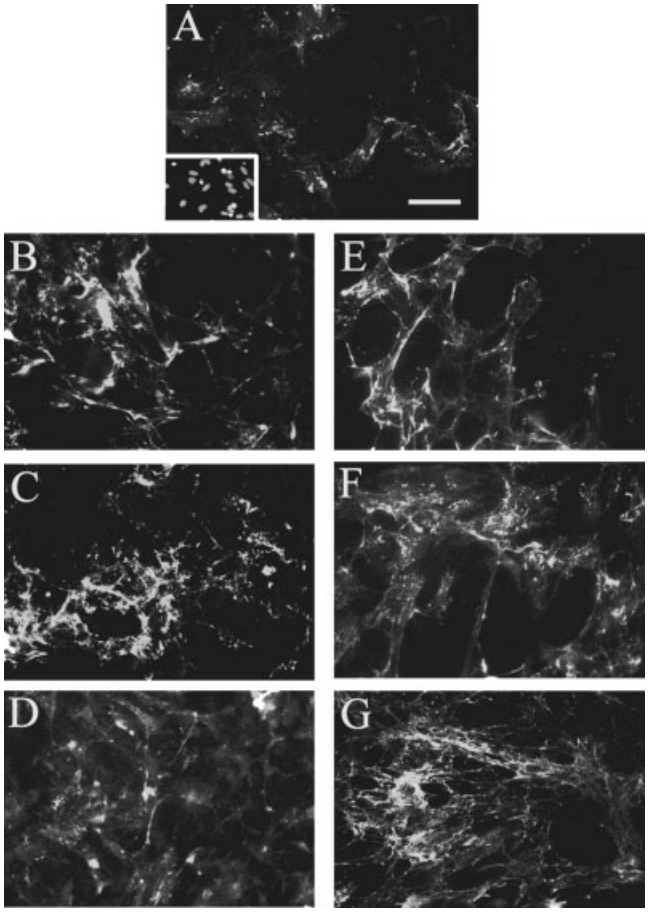

Fig. 2. Guanine derivatives modulate astrocyte organization of laminin. Cerebellar astrocytes cultures treated with GMP (**B–D**) or GUO (**E–G**) (1 μM, 100 μM, and 1 mM) were immunostained for the ECM protein laminin. Inset in (**A**) shows a nuclear immunolabeling of control astrocyte culture, which is equivalent for all conditions. The experiments were done three times and triplicate to each one. Scale bar = 20 μm.

tion, overnight at 4°C. Primary antibodies used were: mouse anti-human β-tubulin III (1:400 dilution; Sigma), rabbit anti-mouse laminin (1:30 dilution; Sigma), rabbit anti-human fibronectin (1:400 dilution; Dako, Carpinteria, CA), and rabbit anti-mouse glial fibrillar astrocytic protein (GFAP, 1:500 dilution; Dako). Secondary antibodies were conjugated with Cy3 (sheep anti rabbit, 1:5,000 dilution; Sigma) or horseradish peroxidase (goat anti-mouse, 1:200; Invitrogen, Carlsbad, CA). Peroxidase activity was shown with VIP kit (Vector, Burlingame, CA). Negative controls were carried out omitting primary antibodies during staining. When necessary, cells were incubated with 5 mg/ml Hoechst 33342 for 1 hr to nuclei staining. Cell preparations were mounted directly on N-propyl gallate glycerol.

Immunoblot Analysis

The expression of extracellular matrix proteins, fibronectin, and laminin, was determined by Western blot analysis as described by Cordova et al. (2004) with some modifications. Cultured astrocytes were solubilized with SDS-stopping solution (4% SDS, 2 mM EDTA, 8% β-mercaptoethanol, and 50 mM Tris, pH 6.8). Samples (100 μg of total protein/track) were separated by 6.5% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% skim milk (1 hr), followed by second blockage (1 hr) with 2.5% gelatin, both solutions in TBS (Tris 10 mM, NaCl 150 mM, pH 7.5). All steps were followed by washing three times with TBS-T (Tris 10 mM, NaCl 150 mM, Tween-20 0.05%, pH 7.5). Membranes were incubated with a specific primary antibody overnight at 4°C and were then exposed to a secondary antibody for 1 hr at room temperature. The antibodies used were polyclonal antibody for fibronectin (1:5,000 dilution; Dako), polyclonal antibody for laminin (1:2,000 dilution; Dako) and anti-rabbit IgG (1:2,000 dilution; Sigma). Immunocomplexes were visualized using the enhancing chemiluminescence detection system (ECL, GE Healthcare, Waukesha, WI). Densitometric analyses were carried out for the quantification of the immunoblots using the Sion Image Software

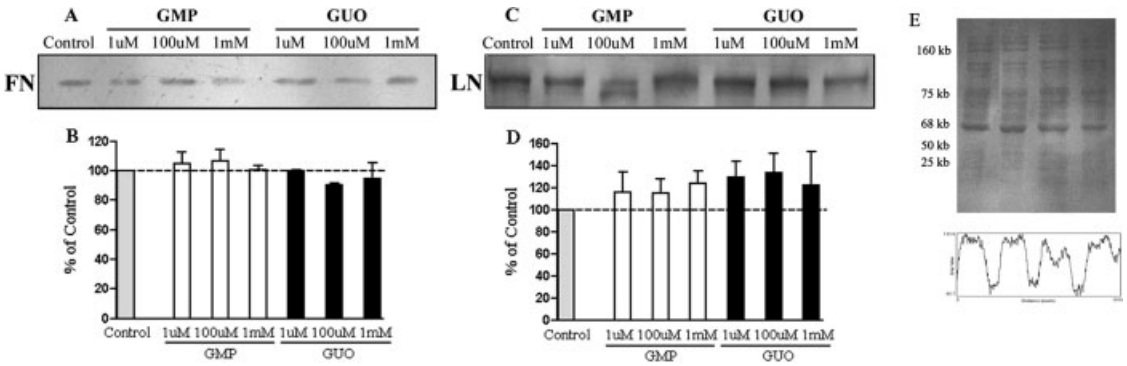

Fig. 3. Guanine derivatives did not alter the astrocytic expression levels of fibronectin or laminin. Cerebellar astrocytes cultures treated with GMP or GUO (1 μM, 100 μM, and 1 mM) were solubilized, proteins were separated by 6.5% SDS-PAGE and transferred to nitrocellulose membranes according to Materials and Methods. Representative immunoblotting anti-fibronectin (**A**) or anti-laminin (**C**) shows that GMP or GUO did not alter the expression levels of fibronectin or laminin. (**B**) and (**D**) show the quantitative analysis expressed as percentage of the control (considered as 100%). The experiments were done three times and triplicate to each one. To confirm the proteins extracts loading in the slab-gel we have chosen samples with 1-GMP 1 μM comparatively to 2-GMP 1 mM, 3-GUO 1 μM, 4-GUO 100 μM to densitometric analysis of serum albumin (68 kDa) present in each total treatment of cultures (**E**).

(Scion Corporation, Frederick, MD). To confirm the proteins extracts loading in the slab-gel we have chosen samples of lane 1 (GMP 1 μM) comparatively to lane 2 (GMP 1 mM), 3 (GUO 1 μM) and, 4 (GUO 100 μM) to densitometric analysis of soroalbumin (68 kDa) present in each total treatment of cultures. The gel was running, proteins transferred to nitrocellulose sheet, stained by Ponceau Red (0.5% ponceau red/1% acetic acid) and these 68 kDa proteins from each lane submitted to Sion Image Software (Scion Corporation).

Analysis of Cell Proliferation

Proliferation of both neuronal and astrocytic cells was evaluated in co-culture. Astrocytes were treated with GMP or GUO for 24 hr in the presence of 0.1 mg/ml BrdU. To evaluate the proliferation of neuronal cells, 0.1 mg/ml BrdU was also added when the neurons were plated on the pre-treated astrocytes monolayer. After 24 hr, cells were fixed with 4% paraformaldehyde for 20 min, washed three times with PBS and twice with distilled H2O. Cells were incubated with 2 N HCl and 0.1 M Borate buffer (pH 8.5) at 50°C. Cells were incubated with PBS/10% BSA/0.01% Triton X-100 (blocking solution) for 30 min and subsequently in the presence of specific antibody, diluted in blocking solution, overnight at 4°C. Primary antibody was anti-BrdU (1:500) and secondary antibody was conjugated with Cy3 (sheep anti-rabbit, 1:5,000 dilution; Sigma). After carefully washing with PBS, cells were incubated with 5 mg/ml Hoechst 33342 for 1 hr to nuclei staining. Cell preparations were mounted directly on N-propyl gallate glycerol.

Morphometry and Statistical Analysis

Images of neurons stained with anti-β-tubulin III antibody were captured by a system coupled to a Nikon fluorescent microscope (Nikon Eclipse TE300). The number of neurons, neurites per neuron, and the neurite length were analyzed by using the Sigma Scan Pro Software (Jandel Scientific, San Rafael, CA). Three independent experiments assayed in triplicates, and at least 100 neurons/well, encompassing five randomly chosen wells, were analyzed. The data were stored and morphometric analyses were carried out using the Microsoft Excel version 2000.

Statistical Analysis

For all experiments, data are expressed as mean ± SEM. Statistical analyses were carried out by using one-way ANOVA followed by Duncan's test, when necessary.

RESULTS

GMP and GUO Modulate the Organization But Not Expression of the Extracellular Matrix Proteins in Astrocytes

The organization of ECM proteins in astrocytes treated with GMP or GUO was evaluated by immunolabeling for LN and FN. As shown in Figures 1 and 2, both proteins had their pattern of organization altered after GMP or GUO treatment for 24 hr in serum-free conditions. In the control cultures, LN and FN were restrict to certain groups of cells and predominantly organized in a punctual form (Fig. 1A, 2A). After GMP or GUO treatment, FN and LN were distributed with punctate and fibrous arrangements. Both patterns of organization were found in different fields of the cultures (Fig. 1B–G, 2B–G). Astrocytes treated with GMP or GUO did not alter GFAP expression or astrocytic morphology, which suggest GD-protein modulation to be specific to ECM proteins (data not shown).

To evaluate if the GMP- or GUO-induced alteration of FN and LN organization was an extracellular action, an inhibitor of nucleosides transporter, dipyridamole, was used. Incubation of astrocytes with GMP or GUO in the presence of dipyridamole did not prevent the GD-modulation of ECM proteins, showing that the GMP or GUO effects were extracellular (data not shown).

The expression of ECM proteins was determined by immunoblotting analysis. We verify that regardless of the spatial organization distribution, LN and FN were quantitatively expressed with similar levels after GD treatment (Fig. 3). The loading of the proteins was controlled by previous determination of protein concentration sample by sample and confirmed by densitometric analysis of some lanes to measure the soroalbumin (68 kDa) pixels. These pixels were equivalent at least in four lanes examined (Fig. 3E).

These observations suggest that the GUO effect is to reorganize the extracellular proteins architecture more than to change the expression of these two ECM proteins.

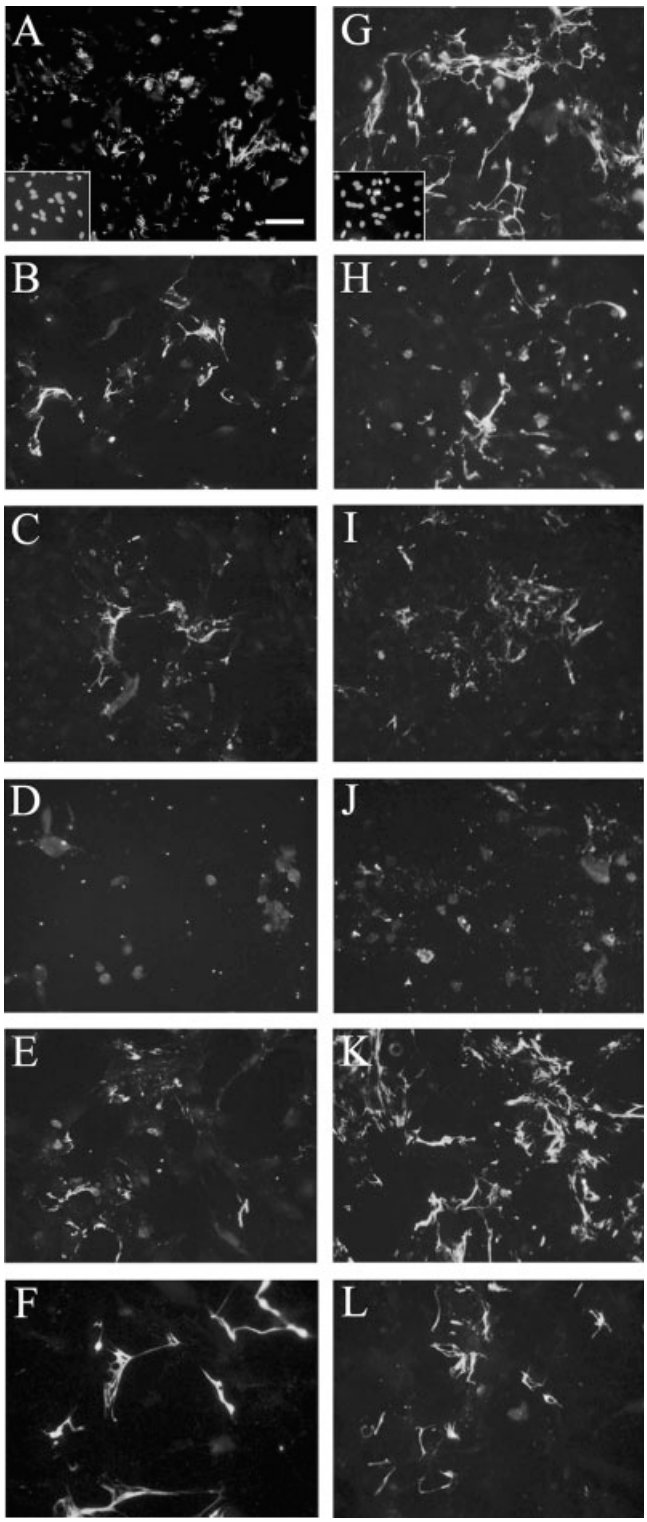

Fig. 4. Evaluation of signaling pathways involved on GUO-induced fibronectin reorganization in astrocytes. Cerebellar astrocytes cultures were treated with GUO (100 μM) in the presence of specific protein kinases inhibitors according to Experimental Procedures. After treatments, cultures were immunostained for the ECM protein fibronectin. Note that GUO greatly modified fibronectin organization in astrocytes (**G**), when compared to the control. (**A**) Control; (**B**) PD 98059, 50 μM; (**C**) KN-62, 10 μM; (**D**) chelerythrine, 10 μM; (**E**) Wortmannin, 1 μM; (**F**) H-89, 5 μM; (**G**) GUO, 100 μM; (**H**) GUO + PD 98059; (**I**) GUO + KN-62; (**J**) GUO + chelerythrine; (**K**) GUO + Wortmannin; (**L**) GUO + H-89. Insets show nuclear immunolabeling of control astrocyte cultures and GUO-treated cultures. The experiments were done four times and triplicate to each one. Scale bar = 20 μm.

Evaluation of Signaling Pathways Involved on GUO-Induced Modulation of Extracellular Matrix Proteins Organization in Astrocytes

To evaluate the signaling pathways involved on GD-induced ECM protein reorganization, cerebellar astrocytes cultures were treated with GUO (100 μM) and several specific protein kinases inhibitors (Fig. 4). After treatment, cultured cells were immunostained for FN. As shown in Figure 1, GD modulates astrocyte organization of FN, when compared to the control group. The addition of PD 98059 (50 μM), a selective inhibitor of MEK, greatly prevents this phenomenon (Fig. 4H). Similar results were obtained by incubation with KN-62 (10 μM), a specific inhibitor of CaMKII (Fig. 4I), Chelerythrine (10 μM), a specific inhibitor of PKC (Fig. 4J), and H-89 (5 μM), a specific inhibitor of PKA (Fig. 4L). Wortmannin (1 μM), a specific PI3-K inhibitor (Fig. 4K), had no effect on preventing GD-modulation of ECM proteins spatial distribution.

Astrocytes Treated With GMP or GUO Increase the Number of Cerebellar Neurons in Coculture

After treatment of cerebellar astrocytes with GMP or GUO for 24 hr, cerebellar neurons were plated onto astrocytic monolayer. After 24 hr, cells were fixed, immunostained for the neuronal marker β-tubulin III, and the total number of neurons was calculated. This analysis showed an evident increase between the numbers of neurons plated onto treated astrocytic monolayers when compared to controls (Fig. 5). An increase of 116%, 154%, and 139% was observed in the number of neurons plated onto 1 μM, 100 μM, and 1 mM GMP-treated astrocyte monolayers, respectively. In addition, an

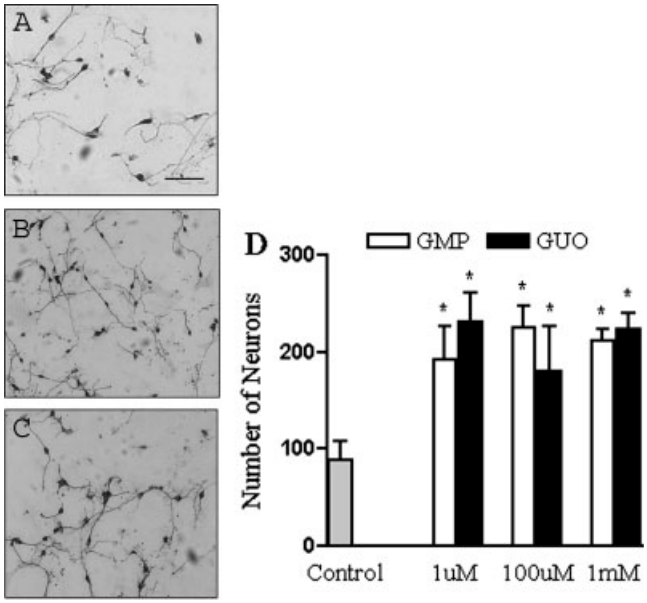

Fig. 5. Astrocytes treated with GMP or GUO increase the number of cerebellar neurons in coculture. Cerebellar neurons obtained from P7 rats were cultivated for 24 hr onto control (**A**) and astrocytes monolayers treated with 1 μM GMP (**B**) or GUO (**C**). Total number of neurons (**D**) was obtained by counting the total cellular bodies positive to anti-β-tubulin III stain. In all cases, at least five fields chosen randomly were observed from triplicate experiments. A higher density of neurons can be observed on guanine derivatives-treated astrocyte carpets (B,C). Statistical significance was observed for all groups ($\star P < 0.05$), using ANOVA statistical test. Scale bar = 20 μm.

increase of 160%, 103%, and 151% was noticed when neurons were plated onto 1 μM, 100 μM, and 1 mM GUO treated astrocyte monolayers, respectively. These values correspond to means of 136 ± 19% and 138 ± 30% when astrocytes were treated with GMP or GUO, respectively (Fig. 5D).

Treatment of Astrocytes With GMP or GUO Did Not Alter the Neuritogenesis in Coculture

To investigate the involvement of the ECM proteins organization in neurite outgrowth, the number of neurites per neuron and the length of neurites were measured. After treatment of cerebellar astrocytes with GMP or GUO, cerebellar neurons were plated onto astrocytic monolayer, and after 24 hr the cells were fixed and immunostained for β-tubulin III for neurite outgrowth analysis. The treatment of astrocytes with 1 μM, 100 μM, and 1 mM GMP or GUO did not induce any alteration in the number of neurites per neuron, in the distribution of neurite length, or in the total neurite length. Control and treated co-cultures presented a similar pattern of neuritogenesis. The total neurite length was 210.4 ± 22.7 μm in the control cultures and remained statistically unaltered after GMP or GUO addition to the co-culture. Similarly, the percentage of neurite length distribution and, the number of neurites per neurons was not different from control when co-cultures were treated with GD (Table I).

Treatment of Astrocytes With GMP or GUO Did Not Alter the Proliferation of Neurons or Astrocytes

To determine if the increase in the number of neurons observed after treatment of astrocytes with GMP or GUO was promoted by increased neuronal proliferation, BrdU incorporation was evaluated in a double labeling protocol with GFAP, to discriminate between neurons or astrocytes incorporating BrdU. As shown in Figure 6A, treatment of astrocytes with GMP or GUO did not alter the number of BrdU positive neurons, showing that the increase in the number of neurons was not due to neuronal proliferation. Also, neither GMP nor GUO treatment altered cerebellar astrocytes proliferation, when compared to control cells culture (Fig. 6B) that was evaluated using one-way ANOVA, followed by Duncan's test, when necessary. Treatment of astrocytes with GMP (1 mM) showed an increased variability, which is possible to reflect the heterogeneity of astrocytes in culture and a bigger concentration claims to a different sensibility of the cells to GMP. Statistical analysis confirms no difference on the proliferative rates.

DISCUSSION

In the present study, guanine derivatives (GD) modulated the extracellular matrix (ECM) proteins organization in astrocytic cells. The addition of GMP or GUO to astrocytes in culture alters the organization, but not the expression levels of laminin (LN) and fibronectin (FN). Alterations of LN or FN organization and expression in cultured cerebellar astrocytes have been observed due to thyroid hormone treatment (Martinez and Gomes, 2002; Trentin et al., 2003). Alterations on the organization pattern of ECM proteins induced by GD have not been reported previously. The transition of ECM proteins organization from punctate to fibrillar has been associated to a higher complexity of protein interaction (Garcia-Abreu et al., 1995a,b). In most cases, differences in the supramolecular organization of ECM proteins were either attributed to interactions with other macromolecules, such as proteoglycans, or cellular receptors, or due to specific properties of the various ECM proteins isoforms (Freire et al., 2002). In the nervous system, FN participates in the cellular adhesion, spreading, and migration of diverse cell types (Trentin et al., 1995, 2003), whereas LN improves the conditions to neurite outgrowth displaying a role as an adhesive substrate (Letourneau et al., 1988; Liesi et al., 1995; Luckenbill-Edds, 1997). Therefore, these biologic events are controlled by the ECM proteins organization (Freire and Coelho-Sampaio, 2000).

We have used three different concentrations of GD: 1 μM, 100 μM, and 1 mM. The precise cerebral concentration of GD is not well determined. In human

TABLE I. Decker et al.

		GMP			GUO		
	Control	1 μM	100 μM	1 mM	1 μM	100 μM	1 mM
Neurite length distribution (μm)			% neurons				
0–50	44 ± 1.0	43 ± 7.0	45 ± 2.0	46 + 6.0	40 + 2.0	47 + 8.0	39 + 5.0
50–100	31 ± 4.0	33 ± 1.0	33 ± 7.0	30 + 3.0	31 + 4.0	30 + 6.0	36 + 4.0
100–200	18 ± 10.0	19 ± 1.0	19 ± 5.0	19 + 1.0	21 + 2.0	22 + 6.0	19 + 3.0
> 200	07 ± 10.0	02 ± 7.0	03 + 6.0	05 + 1.0	07 + 0.5	10 + 6.0	05 + 3.0
Total neurite length (μm)	210.4 + 22.7	210.6 + 3.7	195.1 + 23.2	194.8 + 5.2	219.3 + 6.4	213.7 + 26.8	202.6 + 6.4
		GMP			GUO		
	Control	1 μM	100 μM	1 mM	1 μM	100 μM	1 mM
Neurites/Neuron			% neurons				
0	16 ± 5.7	17 ± 8.3	33 ± 11.7	30 ± 4.5	19 ± 8.0	21 ± 12.2	20 ± 8.1
1	19 ± 9.3	16 ± 8.5	14 ± 7.1	15 ± 4.1	20 ± 8.6	17 ± 7.2	18 ± 5.3
2	17 ± 8.6	18 ± 3.1	15 ± 2.0	16 ± 4.2	22 ± 5.3	16 ± 4.5	20 ± 4.7
3 and +	47 ± 11.5	45 ± 12.5	37 ± 4.0	40 ± 2.1	40 ± 3.0	48 ± 6.1	45 ± 4.9

cerebral fluid, 200 μM were detected to GMP (Regner et al., 1997). The mechanisms of cellular release of guanine nucleotides are poorly defined, but can detected in concentrations exceeding 100 μM in the extracellular compartment (Morciano et al., 2004). Moreover, Gysbers and Rathbone (1992) showed that 30–300 μM of GUO elicited the extension of neurites from PC12 cells and Molz et al. (2005) showed that GMP 1 mM acted as a neuroprotective agent against glutamatergic toxicity. To glial cells, concentrations of 0.1–100 μM of GUO induced an increase in astrocytic CA^{2+} intracellular levels (Chen et al., 2001). In our experiments, the three concentrations showed similar effects and were cytotoxic.

The interaction of ECM proteins with cytoplasmic molecules and actin cytoskeleton through integrins is critical in initiating a variety of intracellular signaling pathways, like MAPK, PKC, and PKA pathways (Wierzbicka-Patynowski and Schwarzbauer, 2003). The MAPK cascade is a key element of signal transduction involved on cellular proliferation, differentiation and stress response (Davis, 1993), whereas PKC and PKA pathways display important regulation on FN organization (Lin et al., 2002; Yang et al., 2002). Furthermore, the activation of PI3-K pathway has been shown to be required for some alterations on LN and FN organization (Martinez and Gomes, 2002). The signaling pathways involved on the reorganization of ECM proteins induced by GMP or GUO were evaluated. In this sense, GUO was chosen as a representative compound, because GMP may be hydrolyzed in neural cells to GUO in minutes (Tasca et al., 1999b; Frizzo et al., 2003), suggesting that the observed effect on ECM proteins organization was induced mainly by GUO. To evaluate whether GMP or GUO was acting via an extracellular interaction with membrane proteins, a non-specific inhibitor of nucleoside transport, dipyridamole, was used. The blockage of GUO entrance into astrocytes did not alter the ECM proteins modulation induced by GD (data not shown), suggesting that the effect of guanine derivatives (GD) was extracellular. In astrocytes treated with GUO, the specific inhibitors of MAPK/ERK, PKC, CaMKII, and PKA signaling pathways blocked the modulatory effect of GUO on FN organization (Fig. 4). A specific inhibitor of PI3-K had no effect on GUO-induced FN organization changes when compared to GUO-treated astrocytes (Fig. 4K), however. Traversa et al. (2002) have shown that GUO activates MAPK cascade in cultured astrocytes, an event blocked by pretreatment of the cells with pertussis toxin, but not with purinergic P1 or P2 receptors antagonists. This implies that GUO acts through a G-protein coupled receptor distinct from those activated by adenine derivatives. Regarding our data on Figure 4, GUO may active a receptor mobilizing intracellular calcium, and promoting PKC and CaMKII activation accompanied by the activation of PKA, which may contribute to the rise of intracellular calcium levels (Koehler et al., 2006). The activation of MAPK/ERK cascade could be result of the binding of ECM proteins to integrins, which stimulate intracellular responses to affect cytoskeleton proteins, alteration in cell adhesion or protein expression (Rocha et al., 2001).

The trophic effects induced by GD have been shown in astrocytic cultures and in PC12 cells (Kim et al., 1991; Rathbone et al., 1999; Ciccarelli et al., 2000, 2001; Gysbers et al., 2000), however, no previous studies have evaluated the effects of GD on neuron-astrocyte interaction. Thus, the trophic effects of GD were evaluated in neurons cocultured with astrocytes pre-treated with GMP or GUO. Guanine derivatives promote a significant increase in the number of neurons in coculture (Fig. 5), but did not alter the pattern of neuritogenesis in those neurons when compared to the control situation (Table I). These results seem to oppose previous reports using PC12 cells, which GUO- or GTP-promoted neurite outgrowth (Berridge, 1993; Gysbers et al., 2000). It is worth mentioning that PC12 cells differ from primary neuronal culture in many aspects, such as growth, division and differentiation

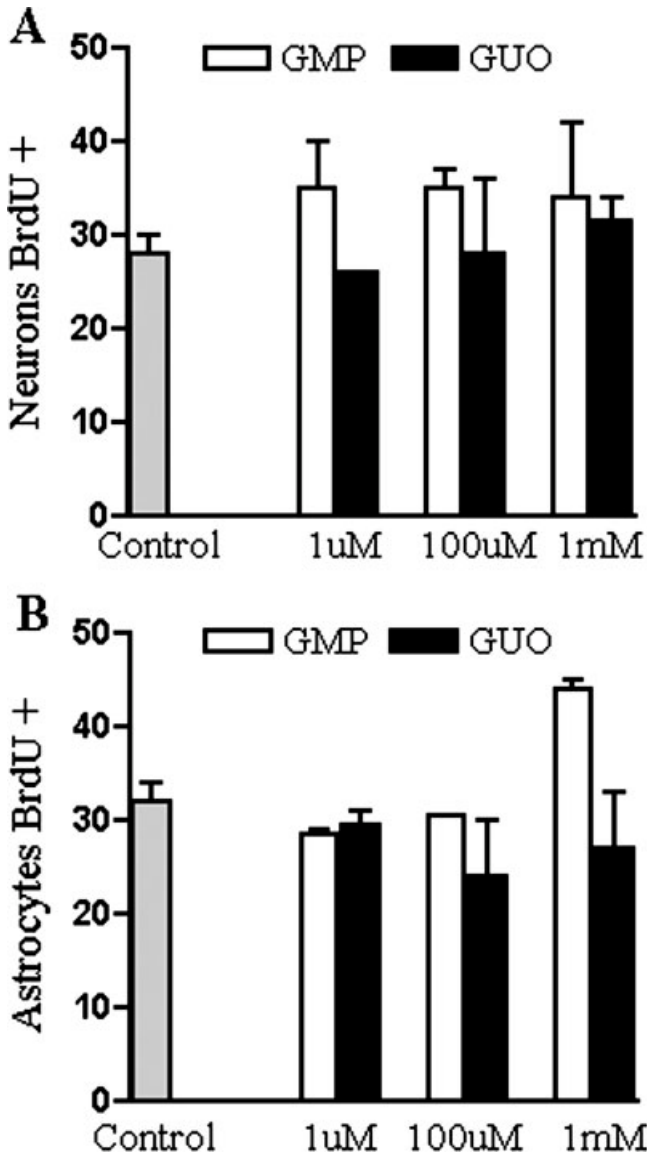

Fig. 6. Treatment of astrocytes with GMP or GUO did not alter the proliferation of neurons or astrocytes. Astrocytes were treated with guanine derivatives (GD) for 24 hr. Cerebellar neurons were cultivated for 24 hr onto pre-treated or control astrocytic monolayers. After 24 hr of treatment of astrocytes or neuron/astrocyte coculture, cells were fixed and submitted to an immunocytochemistry for BrdU. Graphics express the percentage of neurons (**A**) or astrocytes (**B**) that incorporated BrdU due to cell proliferation (BrdU +). In all cases, at least five fields chosen randomly were observed from triplicate experiments. Statistical significance was not observed in any group.

mechanisms. GD promoted proliferation and cellular differentiation in cortical astrocytes in culture (Kim et al., 1991; Rathbone et al., 1999; Ciccarelli et al., 2000). The treatment of cerebellar astrocytes with GMP or GUO did not induce neuronal or astrocytic proliferation, showing that the increased number of neurons in coculture was not due to neuronal proliferation (Fig. 6). This increased number of neurons might indicate an enhanced neuronal attachment to the astrocytic monolayer.

Our data show a special role of guanine derivatives on ECM proteins. These guanine derivatives modulate laminin and fibronectin reorganization, increasing adhesion of neurons in culture on astrocytic substrate. This effect of GD on attachment of cell–cell interaction could play a role on neurogenesis or neuronal migration, as neuronal migration through radial glia during brain development.

Considering no neuronal proliferation was observed, we are evaluating currently whether the increase in neuronal cells induced by GD could be due to improvement of neuronal viability or increase on the adhesion molecules through ECM proteins reorganization. It is well known that ECM laminin constitute a better substrate for the neurons outgrowth (Mangoura et al., 1988; Chamak and Prochiantz, 1989; Garcia-Abreu et al., 1995b; Faria et al., 2006).

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ANEXO 6

Luciana Ferreira Romão

Curriculum Vitae

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Luciana Ferreira Romão
Curriculum Vitae

Dados Pessoais

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2003 - 2007 Doutorado em Ciências Morfológicas.
Universidade Federal do Rio de Janeiro, UFRJ, Rio De Janeiro, Brasil
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Título: Interação neurônio-glia: efeito de TGF-beta1 e CTGF na biologia glial normal e tumoral, Ano de obtenção: 2007
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1998 - 2003 Graduação em Ciências Biológicas Modalidade Médica.
Universidade Federal do Rio de Janeiro, UFRJ, Rio De Janeiro, Brasil
Título: Efeitos morfológicos da interação astrócito-sinaptossomas em animais eutireóideos e hipotireóideos
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Formação complementar

2000 - 2000 Curso de curta duração em Biology of embrionic and adult stem cells.

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Estágio:
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Laboratório de Biologia Celular - Profa. Andrea Thompson Da Poian - Diferenças na fosforilação e atividade da caseína cinase II (CKII) durante a infecção pelo vírus mayaro

02/1999 - 12/2002 Estágio, Instituto de Ciências Biomédicas, Departamento de Anatomia
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Laboratório de Morfogênese Celular - Prof. Vivaldo Moura Neto - Interação astrócito-sinaptossoma in vitro: efeitos morfológicos

Áreas de atuação

1. Citologia e Biologia Celular
2. Desenvolvimento Neural

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2004 Membro da Sociedade Americana de Biologia Celular, Sociedade Americana de Biologia Celular, Sociedade Americana de Biologia Celular

2004 Menção Honrosa - XII Congresso da Sociedade Brasileira de Biologia Celular e IX Congresso da Sociedade Iberoamericana de Biologi, Sociedade Brasileira de Biologia Celular

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Artigos completos publicados em periódicos

1. Cristian Follmer, ROMÃO, L., Tasca, C.I., Carla Einsiedler, Thaís C.R. Porto, Flávio Alves Lara, Moncores M, Weissmuller G, Lashuel HA, Peter Lansbury, MOURA NETO, V., Jerson L. Silva, Debora Foguel
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2. Sharif Ariane, Prévot Vincent, Allet Cécile, ROMÃO, L., Studler Jeanne-Marie, Chneiweiss Hervé, Junier Marie-Pierre
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Palavras-chave: Transforming Growth Factor alpha, Astrocytes, Stem cells

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laminin organization.. Differentiation (London). , v.9-10, p.562 - 572, 2006.
Palavras-chave: Glioblastoma, Laminin, neuron-glioblastoma interactions

5. FARIAS, P., SANTOS, B., MENEZES, F., FERREIRA, R., FONTES, A., Carvalho H, ROMÃO, L., MOURA NETO, V., Amaral J, LENZ-CEZAR, C., Figueiredo R, Lorenzato F
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6. Gomes FC, De Oliveira Sousa V, ROMÃO, L.
Emerging roles for TGFb1 in nervous system development. International Journal of Developmental Neuroscience. , v.5, p.413 - 424, 2005.
Palavras-chave: Glial fibrillary acidic protein (GFAP), Astrocytes, transforming growth factor-beta 1, neuron-astrocyte interactions
Áreas do conhecimento : Diferenciação astrocitária,Morfologia,Neurobiologia celular
Setores de atividade : Neurociências

7. De Oliveira Sousa V. *, ROMÃO, L., MOURA NETO, V., Gomes FC, *V.d.O.S. and L.R. contributed equally to this work.
Glial fibrillary acidic protein gene promoter is differently modulated by transforming growth factor-beta 1 in astrocytes from distinct brain regions. European Journal of Neuroscience. , v.7, p.1721 - 1730, 2004.

Comunicações e Resumos Publicados em Anais de Congressos ou Periódicos (resumo)

1. Tasca, C.I., Francisco, Sheila Regina Schmidt, ROMÃO, L., Decher, Helena, MOURA NETO, V.
Trophic effects of GMP and guanosine on cerebellar neuron-astrocyte coculture. In:
Journal of Neurochemistry. , 2004. v.90. p.75 - 75
Palavras-chave: guanine derivatives, neuron-astrocyte interactions, trophic effects, extracellular matrix
Áreas do conhecimento : Neuroquímica,Citologia e Biologia Celular
Setores de atividade : Neurociências
Referências adicionais : Estados Unidos/Inglês. Meio de divulgação: Impresso

Eventos
Participação em eventos

1. Apresentação de Poster / Painel no(a) **XXI Reunião Anual da Federação de Sociedades de Biologia Experimental-FESBE**, 2006. (Congresso)
EVALUATION OF THE MECHANISMS INVOLVED ON THE INCREASE OF GRANULAR CEREBELLAR NEURONS IN CULTURE INDUCED BY GUO OR GMP.

2. Apresentação de Poster / Painel no(a) **XXI Reunião Anual da Federação de Sociedades de Biologia Experimental-FESBE**, 2006. (Congresso)
INTERAÇÃO NEURÔNIO X GLIOBLASTOMAS: A PROLIFERAÇÃO TUMORAL.

3. Apresentação de Poster / Painel no(a) **XXI Reunião Anual da Federação de Sociedades de Biologia Experimental-FESBE**, 2006. (Congresso)
INTERACTIVE PROPERTIES OF HUMAN GLIOBLASTOMA CELLS WITH BRAIN NEURONS IN CULTURE AND NEURONAL MODULATION OF GLIAL LAMININ ORGANIZATION.

4. Conferencista no(a) **XXI Reunião Anual da Federação de Sociedades de Biologia Experimental-FESBE**, 2006. (Congresso)
Interactive properties of human glioblastoma cells with brain neurons in culture and neuronal modulation of GLIAL laminin organization.

5. Apresentação de Poster / Painel no(a) **XII Congress of the Brazilian Society for Cell Biology**, 2004. (Congresso)
Application of CDS Nanoparticules in Neural Cells as New High Efficient Fluorescent Labels.
Palavras-chave: CDS, Neurons, Glioma
Áreas do conhecimento : Citologia e Biologia Celular,Química

6. Apresentação de Poster / Painel no(a) **Federação de Sociedades de Biologia Experimental**, 2004. (Congresso)

Diferenciação astrocitária induzida através da interação astrócito-sinaptossoma e o efeito do hipotireoidismo.
Palavras-chave: Astrócito, Sinaptossoma, Hipotireoidismo
Áreas do conhecimento : Morfologia,Desenvolvimento Neural

7. Apresentação de Poster / Painel no(a) **XII Congress of the Brasilian Society of Cell Biology**, 2004. (Congresso)
Glutamate Indution of GFAP Gene Promoter is Mediated by TGF-beta1 and Involves MAPK/PI-3-K Pathways.
Palavras-chave: Astrocytes, Glial fibrillary acidic protein (GFAP), transforming growth factor-beta 1, Neurons
Áreas do conhecimento : Citologia e Biologia Celular

8. Apresentação de Poster / Painel no(a) **XII Congress of the Brasilian Society for Cell Biology**, 2004. (Congresso)
Influence of Normal Neurons on Laminin Organization on the Glioblastoma Cells Stablished in Culture.
Palavras-chave: Glioma, Neurons, Laminin
Áreas do conhecimento : Citologia e Biologia Celular

9. Apresentação de Poster / Painel no(a) **Federação de Sociedades de Biologia Experimental-FeSBE**, 2004. (Congresso)
INTERAÇÃO NEURÔNIO-GLIA: PRODUTO SINÁPTICO NA DIFERENCIAÇÃO ASTROCITÁRIA.
Palavras-chave: Glutamato, Astrócito, TGF-beta1
Áreas do conhecimento : Morfologia,Desenvolvimento Neural,Diferenciação astrocitária

10. Apresentação Oral no(a) **Federação de Sociedades de Biologia Experimental-FeSBE**, 2004. (Congresso)
INTERAÇÕES CELULARES NO SISTEMA NERVOSO: O GLIOBLASTOMA HUMANO.
Palavras-chave: Neurônios, Glioblastoma, Laminina
Áreas do conhecimento : Citologia e Biologia Celular

11. Apresentação de Poster / Painel no(a) **XII Congresss of the Brasilian Society for Cell Biology**, 2004. (Congresso)
Neo-Natal Neurons modulates CTGF Expression in Glioblastoma Cells.
Palavras-chave: Neurons, Glioma, CTGF
Áreas do conhecimento : Citologia e Biologia Celular

12. Apresentação de Poster / Painel no(a) **Federação de Sociedades de Biologia Experimental-FeSBE**, 2004. (Congresso)
O PROMOTOR DO GENE DE GFAP É DIFERENTEMENTE MODULADO POR TGF-β1 EM ASTRÓCITOS DE REGIÕES DISTINTAS DO CÉREBRO.
Palavras-chave: Astrócito, GFAP, TGF-beta1
Áreas do conhecimento : Morfologia,Diferenciação astrocitária,Citologia e Biologia Celular

13. Apresentação de Poster / Painel no(a) **Federação de Sociedades de Biologia Experimental**, 2004. (Congresso)
Papel do TGF-ß1 e do glutamato na ativação do promotor de GFAP in vitro.
Áreas do conhecimento : Diferenciação Astrocitária

14. Apresentação de Poster / Painel no(a) **SBBq**, 2004. (Congresso)
Trophic effects of GMP and GUO on cerebellar neuron-astrocyte coculture..
Palavras-chave: guanine derivatives, neuron-astrocyte interactions, Astrocytes, Laminin, Fibronectin
Áreas do conhecimento : Neuroquímica,Diferenciação astrocitária,Desenvolvimento Neural
Setores de atividade : Neurociências

15. Apresentação de Poster / Painel no(a) **XII Congress of the Brazilian Society for Cell Biology**, 2004. (Congresso)
T3 Effect on Myosin 5a expression in the Central Nervous System.
Palavras-chave: Astrocytes, T3, Miosin 5a
Áreas do conhecimento : Citologia e Biologia Celular

16. Apresentação de Poster / Painel no(a) **Federação de Sociedades de Biologia Experimental**, 2003. (Congresso)
Modulação da expressão de miosina 5a por T3 no sistema nervoso central.
Palavras-chave: Astrócito
Áreas do conhecimento : Citologia e Biologia Celular,Desenvolvimento Neural

17. Apresentação de Poster / Painel no(a) **XVI Reunião Anual da Federação de Sociedades de Biologia**

Experimental (FeSBE), 2002. (Congresso)
Efeito do Hipotireoidismo na Interação Astrócito Sinaptossoma.
Palavras-chave: Astrócito, Sinaptossoma
Áreas do conhecimento : Morfologia,Citologia e Biologia Celular,Desenvolvimento Neural

18. Apresentação Oral no(a) **XVI Reunião Anual da Federação de Sociedades de Biologia Experimental (FeSBE)**, 2002. (Congresso)
Efeito do Hormônio T3 na Expressão da Miosina 5a no Sistema Nervoso Central.
Palavras-chave: Astrócito, Hormônio T3
Áreas do conhecimento : Morfologia,Citologia e Biologia Celular

19. Apresentação de Poster / Painel no(a) **International Symposium on Extracellular Matrix (SIMEC)**, 2002. (Simpósio)
Effects of Hypothyreoidism on Astrocyte-Synaptosome Interactions.
Palavras-chave: Astrócito, Sinaptossoma
Áreas do conhecimento : Desenvolvimento Neural,Morfologia

20. Apresentação de Poster / Painel no(a) **XXIV Congresso Brasileiro de Neurocirurgia**, 2002. (Congresso)
Invasividade do Tumor Cerebral e Matriz Extracelular: Modelo Experimental com Glioma Humano em Murino.
Palavras-chave: Glioma
Áreas do conhecimento : Morfologia,Citologia e Biologia Celular

21. Apresentação de Poster / Painel no(a) **International Symposium on Extracelullar Matrix (SIMEC)**, 2002. (Simpósio)
T Cells from Trypanosoma Cruzi - Infected Mice Interect with Neurons in vitro: Role of Extracellular Matrix.
Palavras-chave: Tripanossoma Cruzi, Neurônios
Áreas do conhecimento : Desenvolvimento Neural

22. Apresentação de Poster / Painel no(a) **Molecular Biology of the Cell**, 2002. (Congresso)
The Effect of T3 on Myosin 5a Expression in the Central Nervous System.
Palavras-chave: Astrocytes, T3, Miosin 5a
Áreas do conhecimento : Citologia e Biologia Celular

23. Apresentação de Poster / Painel no(a) **I Simpósio Nacional sobre Pesquisa em Oncologia Experimental**, 2001. (Simpósio)
I Simpósio Nacional sobre Pesquisa em Oncologia Experimental.

24. Apresentação de Poster / Painel no(a) **XVI Reunião Anual da Federação de Sociedades de Biologia Experimental (FeSBE)**, 2001. (Congresso)
Interação Astrócito-Sinaptossoma in vitro: Efeitos de Mudança Morfológica.
Palavras-chave: Astrócito, Sinaptossoma
Áreas do conhecimento : Morfologia,Citologia e Biologia Celular,Desenvolvimento Neural

25. Apresentação de Poster / Painel no(a) **XXII Jornada de Iniciação Científica da Universidade Federal do Rio de Janeiro**, 2000. (Congresso)
Interação Astrócito Sinaptossoma in vitro: Efeito da Mudança Morfológica.
Palavras-chave: Astrócito, Sinaptossoma
Áreas do conhecimento : Morfologia,Citologia e Biologia Celular,Desenvolvimento Neural

26. Apresentação de Poster / Painel no(a) **International Symposium on Extracelullar Matrix (SIMEC)**, 2000. (Simpósio)
Synaptosome-astrocyte in vitro: morphogenetic effects.
Palavras-chave: Astrócito, Sinaptossoma
Áreas do conhecimento : Morfologia,Desenvolvimento Neural,Citologia e Biologia Celular

Bancas
Participação em banca de trabalhos de conclusão

Graduação

Participação em banca de Marissol dos Santos F. T. Pereira. **Papel da Netrina-1 na formação de circuitos corticais**, 2006
(Ciências Biológicas Modalidade Médica) Universidade Federal do Rio de Janeiro

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Comunicações em anais de congressos e periódicos (proceedings e suplementos)..... 1

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