SMAD pathway mediation of BDNF and TGFβ2 regulation of proliferation and differentiation of hippocampal granule neurons

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Summary

Hippocampal granule cells self-renew throughout life, whereas their cerebellar counterparts become post-mitotic during early postnatal development, suggesting that locally acting, tissue-specific factors may regulate the proliferative potential of each cell type. Confirming this, we show that conditioned medium from hippocampal cells ($CM_{Hippocampus}$) stimulates proliferation in cerebellar cultures and, vice versa, that mitosis in hippocampal cells is inhibited by $CM_{Cerebellum}$. The anti-proliferative effects of $CM_{Cerebellum}$ were accompanied by increased expression of the cyclin-dependent kinase inhibitors p21 and p27, as well as markers of neuronal maturity/differentiation. $CM_{Cerebellum}$ was found to contain peptide-like factors with distinct anti-proliferative/differentiating and neuroprotective activities with differing chromatographic properties. Preadsorption of $CM_{Cerebellum}$ with antisera against candidate cytokines showed that TGFβ2 and BDNF could account for the major part of the anti-proliferative and pro-differentiating activities, an interpretation strengthened by studies involving treatment with purified TGFβ2 and BDNF. Interference with signaling pathways downstream of TGFβ and BDNF using dominant-negative forms of their respective receptors (TGFβ2-RII and TRKB) or of dominant-negative forms of SMAD3 and co-SMAD4 negated the anti-proliferative/differentiating actions of $CM_{Cerebellum}$. Treatment with $CM_{Cerebellum}$ caused nuclear translocation of SMAD2 and SMAD4, and also transactivated a TGFβ2-responsive gene. BDNF actions were shown to depend on activation of ERK1/2 and to converge on the SMAD signaling cascade, possibly after stimulation of TGFβ2 synthesis/secretion. In conclusion, our results show that the regulation of hippocampal cell fate in vitro is regulated through an interplay between the actions of BDNF and TGFβ.

Key words: Neurogenesis, Apoptosis, Neuronal differentiation, Hippocampus, Cerebellum, TGFβ2, BDNF, SMAD, Rat

Introduction

Control of cell fate, including the mechanisms that govern cell proliferation, differentiation and death, is a central theme in developmental neurobiology. Previous studies have indicated the importance of neurotrophic factors in neurogenesis (Yoshimura et al., 2001; Tao et al., 1997; Borghesani et al., 2002) and of cellular dialogue in cell fate decisions (Renfranz et al., 1991; Vicario-Abejón et al., 1995; Suhonen et al., 1996; Alder et al., 1999). Besides contributing to a better understanding of these biological processes, identification of the instructive and permissive factors and their signaling pathways will provide leads for prevention and cell-replacement strategies in neurodegenerative and other diseases of the brain.

Granule cells of the cerebellum and hippocampal dentate gyrus share several morphological commonalities (Ramon y Cajal, 1911); in addition, they both display dependencies on, or expression of, a common set of growth factors and signaling pathways (Dreyfus, 1998). However, cerebellar and hippocampal granule cells show distinct differences in their repertoire of glutamate receptor subtypes (Monyer et al., 1994) and gene expression profiles (Saito et al., 2002), features that most probably reflect their different physiological roles. These similar, but also divergent, properties make these two types of granule neuron interesting models for analyzing the intrinsic factors responsible for the regulation of their proliferation and maturation. Both dentate and cerebellar granule cells first appear late in embryogenesis, with peak numbers appearing during the first postnatal week (Altman, 1972; Altman and Bayer, 1990; Schlessinger et al., 1975). Hippocampal granule cells continue to proliferate throughout life, although the rate of proliferation wanes with age (Altman and Bayer, 1990; Kuhn et al., 1996; Cameron and McKay, 2001). By contrast, the genesis of cerebellar granule cells terminates within the first two weeks of life (Altman, 1972). Interestingly, gene profiling studies revealed that genes involved in oncogenesis and ribosomal protein synthesis are most strongly expressed at the peak of cerebellar granule cell production (Saito et al., 2002); of the five gene clusters analyzed in that study, none showed any particular temporal pattern of expression in the dentate gyrus.

To examine the hypothesis that tissue-specific factors may serve as 'start' and 'stop' controls of proliferation in different brain areas, we here measured neurogenesis by

bromodeoxyuridine (BrdU) incorporation in immunochemically characterized cells after exchanging conditioned medium (CM) between rat postnatal day 4 (P4) hippocampal and P7 cerebellar granule cell cultures; medium exchanges were performed over a period covering the appearance of the subgranular (secondary germinative) layer of the hippocampus (Altman and Bayer, 1990) and the start of the disappearance of the external granule layer of the cerebellum (Altman, 1972). Our studies reveal that TGFβ2 and BDNF of cerebellar origin have strong anti-proliferative and neuronal differentiating properties when applied to mitotic hippocampal granule cells.

Materials and methods

Primary cell cultures and conditioned medium

Hippocampal, cerebellar and cortical primary cell cultures were prepared as previously described (Crochemore et al., 2005). Briefly, hippocampal (P4) and cerebellar (P7) cells obtained from P4 Wistar rats were dissociated using the Papain Dissociation System (Worthington Biochemicals) and plated on poly-d-lysine-coated glass coverslips (400 cells/mm^2). Cultures were maintained (37°C, 5% CO_2/95% air, 90% relative humidity) in Neurobasal A medium/B27 Supplement and 1 mM GlutamaxI and 0.1 mg/ml kanamycin (all from Invitrogen), half of which was renewed every 3 days. Experiments were started 8-14 days after plating. Immunocytochemical analysis revealed that the cultures comprised ~40% neurons (NeuN-, TuJ1- or doublecortin-immunopositive), ~10% astroglial cells (glial fibrillary acidic protein-positive) and ~50% progenitor (nestin-positive) cells. Twenty-four hours before experiments, the culture medium was completely replaced with conditioned medium (CM) from either cerebellar ($CM_{Cerebellum}$) or hippocampal ($CM_{Hippocampus}$) cultures containing BrdU (20 μM). Treated cultures were fixed with 4% paraformaldehyde (PFA) 24 hours later and processed for the immunocytochemical detection of BrdU.

Slice culture

Cerebellar and hippocampal 'interface' slice cultures were prepared from P7 Wistar rats based on a protocol published by Noraberg et al. (Noraberg et al., 1999). Briefly, hippocampal and cerebellar slices (400 μm) were placed on Millicell semiporous membranes in six-well plates (Millipore). Slices from each brain area were placed adjacent to each other in a single well and bathed in 50% OPTI-MEM/Dulbecco's modified Eagle's Medium (DMEM), including 10% fetal bovine serum, 15% horse serum, 1 mM Glutamax and 0.1 mg/ml kanamycin in Hank's buffered saline solution (all from Invitrogen). Co-cultures were maintained at 37°C (90% humidity) for 16 days, with medium changes every 3 days. Cultures were treated with BrdU (20 μM, 24 hours) before fixation (4% PFA).

HiB5 hippocampal cell line

Neural precursor SV40 T large antigen-immortalized HiB5 cells (Renfranz et al., 1991) (kindly provided by Dr Nina Rosenqvist, Lund, Sweden) were maintained in DMEM containing 10% fetal calf serum and 1% kanamycin at the permissive temperature (32°C) and a 5% CO_2 environment.

Immunocytochemistry

Slice and dispersed cell cultures were fixed in 4% paraformaldehyde, permeabilized (0.3% Triton-X100/PBS) and incubated in 3% donkey serum/0.3% Triton (30 minutes) before incubation (1 hour; room temperature) with primary antibodies diluted 1:500 in 3% donkey serum/0.3% Triton X-100 in PBS: anti-BrdU (DAKO), anti-Nestin (Chemicon), anti-TuJ1 (Babco), anti-MAP2 (Sigma), anti-doublecortin (Santa Cruz Biotechnology), anti-GFAP (Sigma), anti-αMash1 and anti-αMath1 (kind gifts from Dr Jane Johnson, Dallas, TX). After washing in PBS, cells and slices were incubated (30 minutes, room temperature) with biotinylated anti-mouse or anti-rabbit secondary antibody (1:500; Sigma), washed and incubated (30 minutes) with FITC- or horseradish peroxidase-conjugated Avidin (1:500; Sigma). HRP was developed with diaminobenzidine. In some instances, nuclear staining was achieved using Hoechst 33342 (1:1000 in PBS; 15 minutes; Roche). Cells staining positive for BrdU or one of the various neural markers were counted with respect to the total number of cells in five randomly chosen microscopic fields (0.072 mm^2; 400× magnification) across the long axis of each object; an average of 1000 cells were sampled on each coverslip and the results shown represent values from 6-10 coverslips per treatment.

Cell death assay

Cell death was examined in 4% PFA-fixed cells by TUNEL histochemistry (Almeida et al., 2000) or Hoechst 33342 staining. Apoptotic cells were identified as dark-brown nuclear staining showing DNA fragmentation without plasma membrane damage. The relative number of apoptotic versus total number of cells was measured in at least five randomly chosen microscopic fields (400× magnification).

Western blotting

Cells were harvested in lysis buffer, briefly sonicated (on ice). Lysates were cleared by centrifugation, and proteins were electrophoretically resolved on 10 or 8% SDS polyacrylamide gels before transfer onto nitrocellulose membranes. Membranes were blocked (5% non-fat milk and 0.2% Tween-20 in PBS), and incubated with specific primary antibodies (anti-MAP2a/b, Sigma, 1:5000; anti-synapsin, Chemicon, 1:400; anti-p21, Pharmingen, 1:500; anti-p27, Santa Cruz, 1:200). Antigens were revealed by enhanced chemoluminescence (Amersham Biosciences) after incubation with appropriate horseradish peroxidase-IgG conjugates (Amersham).

Concentration and purification of conditioned medium from cerebellar cultures ($CM_{Cerebellum}$)

A total of 1 L of $CM_{Cerebellum}$ was collected from cultures between 8 and 14 days in vitro (d.i.v.). A 100-fold concentrate, containing peptides with a M_r greater than 6 kDa, was prepared using Vivaspin columns (Vivascience) before running through Q-ion exchange columns (Vivapure 20; Vivascience) and elution with a sequential salt gradient buffer. Bio-active fractions were further separated on Affigel blue columns (BioRad) and analyzed for their proliferative, differentiating and apoptotic properties (see above).

Heat lability test

Concentrated (100×) $CM_{Cerebellum}$ was boiled for 15 minutes before addition to hippocampal cultures and measurement of bioactivity (cell proliferation and neuronal markers).

Immunoneutralization

Antibodies against brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) were purchased from Santa Cruz Biotechnology; antibodies against TGFβ2 were from R&D Systems. All antisera were purified IgG, and species-matched purified IgG preparations (Chemicon) were used in controls. $CM_{Cerebellum}$ was adsorbed with these antisera for 1 hour (room temperature) before being added to cell cultures at dilutions ranging from 1:10 to 1:10,000, after which the biological activity of the $CM_{Cerebellum}$ was assessed (see above).

BDNF studies

Results from the BDNF immunoneutralization experiments were confirmed by adding BDNF (hBDNF, 10-100 ng/ml; Alomone Laboratories) to hippocampal cultures at 7 d.i.v., and monitoring for BrdU incorporation and MAP2a/b expression after 24 hours.

Further verification of BDNF effects was obtained by transiently transfecting primary hippocampal cultures with a BDNF expression vector (pcDNA3-BDNF-Citron, kindly provided by Dr Oliver Griesbeck, Martinsried, Germany) or pEGFP (as control). Transfection was carried out using 1 μg DNA/well and Lipofectamine 2000 (Invitrogen) under serum-free conditions. Twenty-four hours after transfection, media were exchanged between BDNF- and EGFP-transfected cells, and BrdU (20 μM) was added to the cultures. BrdU incorporation was assessed after a further 24 hours.

MAP kinase mediation of the pro-mitotic actions of BDNF was examined by assaying BrdU retention and MAP2a/b expression after treating cultures with the MEK inhibitor PD98059 (0.1 μM; Calbiochem). The ability of BDNF (100 ng/ml) to stimulate cytoplasm-to-nucleus translocation of key TGFβ signaling partners was analyzed by transiently transfecting hippocampal cultures with fluorescence-tagged *Smad2* and *Smad4* (see below) and microscopic examination.

Cellular contents of TGFβ2 after BDNF or $CM_{Cerebellum}$ treatment were measured by semi-quantitative immunocytochemistry, using recombinant TGFβ2 standards and ABTS [2,2'-azino-di-(3 ethylbenzthia-zoline sulfonic acid)] as chromogen. Attempts were made to measure secreted TGFβ using either a commercial ELISA kit (Promega) or the plasminogen activator inhibitor 1 (PA1)-luciferase assay described by Abe et al. (Abe et al., 1994). For the latter assay, mink lung epithelial cells (MLEC, clone 32) stably transfected with PA1-luciferase, were kindly provided by Dr D. Rifkin (New York, NY). Neither assay proved to be sufficiently sensitive to measure secreted TGFβ2.

Nuclear translocation of SMADs

To study nuclear translocation of SMAD, primary hippocampal neurons (7 d.i.v.), were transfected with pEGFP-SMAD2 or pEGFP-SMAD4 (kindly provided by Dr Kelly Mayo, Evanston, IL). Transfection was carried out using 1 μg DNA/well and Lipofectamine 2000. Transfection efficiency, judged in control transfections with pEGFP, was ~10%. Following transfection, cells were returned (3 hours) to standard growing medium or $CM_{Cerebellum}$ or exposed to TGFβ2 (1 ng/ml) or BDNF (100 ng/ml), stained with Hoechst 33342 and examined.

Receptor signaling

Analysis of BDNF (TRKB) and TGFβ receptor (TGFβ-RII) signaling was studied in primary hippocampal cells after transfection (see above) with 1 μg/well of the following plasmids: dominant-negative TGFβRII (pRK5-TβRII-DN-F; generously provided by Dr R. Derynck, San Francisco, CA, USA) and TRKB dominant-negative (pEF/BOS-TRKB.T1-Flag; kind gift from Dr Eero Castren, Helsinki, Finland); pEGFP was used as an internal control. Twenty-four hours after transfection and exposure to either control or $CM_{Cerebellum}$, the number of BrdU-positive or MAP2a/b-positive cells was counted as a proportion of all cells (stained with Hoechst 33342). Transfection efficiency was approximately 10%.

SMAD signaling

Analysis of SMAD signaling was studied in primary hippocampal cells after transfection (see above) with 1 μg/well of either dominant-negative SMAD3 [pCS2-FLAG-SMAD3(3S-A)] or dominant-negative SMAD4 [pCMV5-FLAG-DPC4(1-514)] (provided by Dr Joan Massague, New York, NY); pEGFP was used as an internal control. Transfection efficiency was ~10%. Twenty-four hours after transfection and exposure to either control or $CM_{Cerebellum}$, the number of BrdU-positive or MAP2a/b-positive cells were assessed with respect to the number of Hoechst 33342-stained cells.

3TP-Lux reporter assay

The p3TP-Lux reporter gene, containing a known TGFβ-inducible plasminogen activator inhibitor promoter (Wrana et al., 1994) (provided by Dr J. Massague), was transfected into HiB5 cells seeded in 24-well plates (4×10^4 cells per well), together with dominant-negative SMADs, dominant-negative TRKB and TGFβRII, or wild-type BDNF. Plasmid (625 ng of total DNA) was introduced using Lipofectamine 2000 into cells maintained in Neurobasal A medium/B27 supplement. Twenty-four hours after transfection (efficiency ~10%), cells were treated with $CM_{Cerebellum}$ (10 μl/ml). Cells were lysed after 24 hours in 100 μl of 1× lysis buffer (Promega), cleared by centrifugation and assayed for β-gal and luciferase activity. For β-gal detection, 10 μl of cellular extract was mixed with 100 μl of β-gal buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgCl_2$, 2 mM β-mercaptoethanol) and 20 μl of O-nitrophenyl-β-D-galactopyranoside (Sigma). The reaction was terminated with 50 μl of Na_2CO_3 (1 M) and luciferase activity was measured by mixing 30 μl of cellular extract with 50 μl of a buffer containing 75 mM Tris-HCl and 1 mM $MgCl_2$ (pH 8). Substrate D-(–) Luciferin (1 mM) was automatically injected and light emission (410 nm) was measured over 20 seconds in a luminometer.

Statistical analysis

All data are depicted as mean±s.d. and represent the observations from three to five independent experiments, with three or four replicates for each data point. Data were analyzed for statistical significance using ANOVA and appropriate post-hoc tests (Student-Newman-Keuls or Kruskal-Wallis multiple comparison procedures) in which $P<0.05$ was set as the minimum level of significance.

Results

Spontaneous expression of markers of neuronal proliferation, differentiation and apoptosis

Hippocampal and cerebellar cultures (8-19 d.i.v.) displayed a dense network of neurites specifically labeled with TuJ1 (anti-tubulin-βIII) (Fig. 1A). Postmitotic cerebellar cells were evidenced by positive labeling with anti-MATH1, a basic helix-loop-helix (bHLH) transcription factor known to be essential for cerebellar granule cell development (Ben-Arie et al., 1997), and with anti-MASH1, a neuronal commitment gene (Ross et al., 2003). Hippocampal cultures were MATH1 negative and MASH1 positive, indicating the presence of immature neurons (Pleasure et al., 2000). Cerebellar and hippocampal cultures displayed mitosis (BrdU incorporation; blue-black nuclei) and apoptosis (brown, TUNEL-stained cells with nuclear fragmentation) concomitantly.

Cerebellar 'stop' signals *vs.* hippocampal 'go' signals

BrdU incorporation by hippocampal cultures maintained in conditioned medium derived from cerebellar cultures ($CM_{Cerebellum}$) was used to test the hypothesis that cerebellar cells secrete factors that can serve as an 'instructive' micro-environment. As shown in Fig. 1B, exposure of hippocampal cultures to $CM_{Cerebellum}$ resulted in a significant reduction in BrdU incorporation ($P<0.001$); the converse experiment (cerebellar cultures treated with $CM_{Hippocampus}$) resulted in a significant increase of BrdU-positive cerebellar cells ($P<0.01$) (Fig. 1C). Similar results were obtained when hippocampal and cerebellar slices (donor age: 7 days) were co-cultured for 7 days. Monitoring of BrdU retention (20 μM, final 24 hours; Fig. 1D) showed that cell proliferation in hippocampal slices was significantly reduced in the presence of cerebellar slices when compared with when hippocampal slices were grown alone ($P<0.01$); by contrast, cell proliferation in cerebellar

slices was slightly, but not significantly, increased in the presence of hippocampal tissue.

The above results indicate that soluble factors with mitotic and anti-proliferative properties are secreted into $CM_{Hippocampus}$ and $CM_{Cerebellum}$, respectively; the remainder of our investigations focused on the anti-proliferative activity of $CM_{Cerebellum}$ on hippocampal cells. Incubation of hippocampal cell cultures with varying volumes of 100-fold concentrated $CM_{Cerebellum}$ established that its putative anti-proliferative activity dose-dependently reduces BrdU incorporation in primary hippocampal cells (Fig. 1E) and in a hippocampus-derived cell line, HiB5 (Fig. 1F). An insight into the physicochemical nature of the putative anti-proliferative factor(s) was gained by assaying the effects of boiled 100-fold concentrated $CM_{Cerebellum}$ on BrdU incorporation by recipient hippocampal cells. As Fig. 1E (last column) shows, the anti-mitotic activity of $CM_{Cerebellum}$ was abolished by boiling for 15 minutes, pointing to the peptidergic/proteinaceous nature of the anti-proliferative moieties.

Expression of negative regulators of the cell cycle and markers of neuronal differentiation

A series of experiments showed that factors present in $CM_{Cerebellum}$ can induce cell cycle arrest within that subpopulation of cells destined to become neurons while simultaneously accelerating neuronal maturation. $CM_{Cerebellum}$-induced arrest of progression through the cell cycle was demonstrated (western blot analysis) by increased expression of the cyclin-dependent kinase (CDK) inhibitors, p21 and p27 (Fig. 2A). These changes were accompanied by enhanced

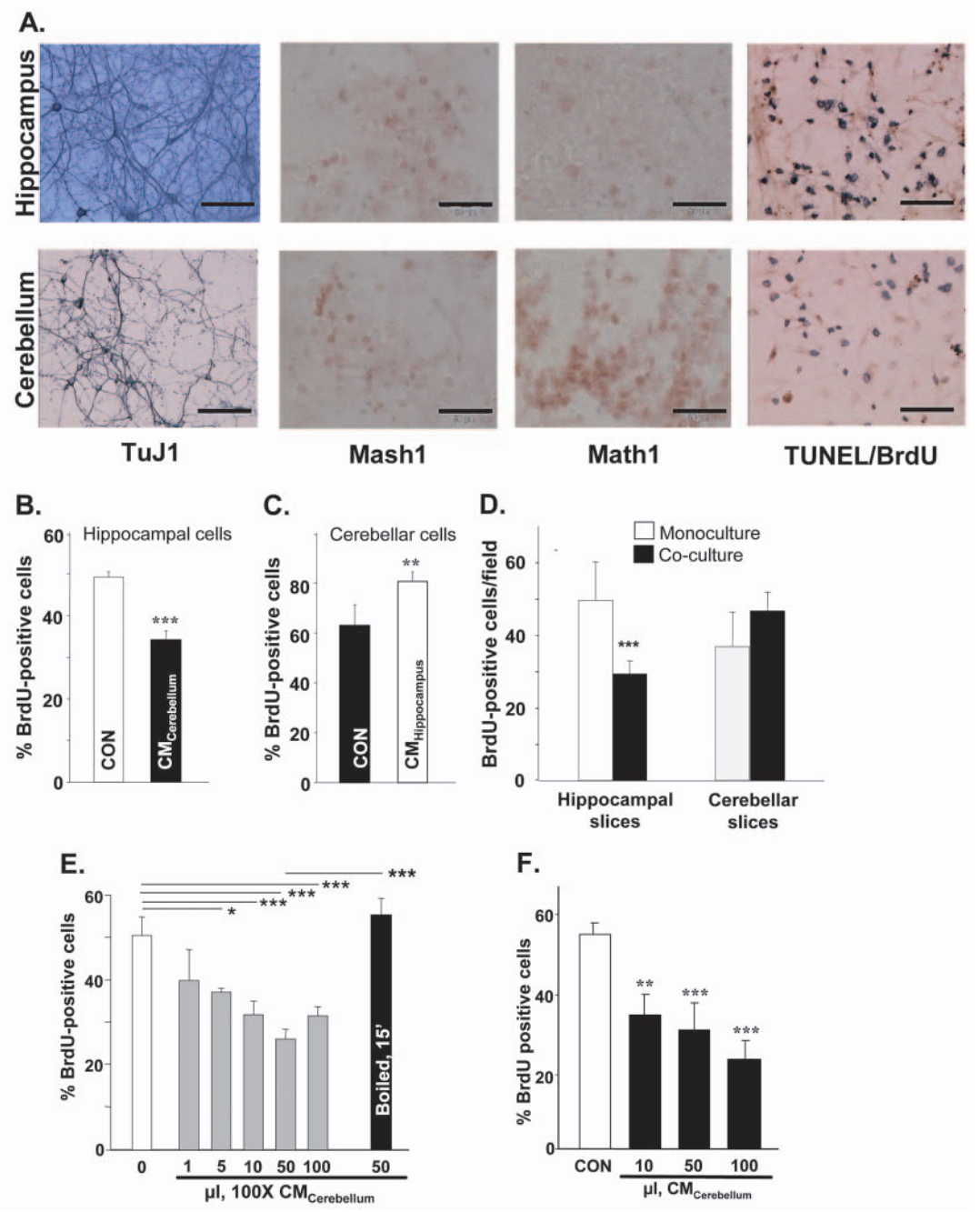

Fig. 1. Cerebellar 'stop' versus hippocampal 'go' signals. (A) Characteristics of hippocampal and cerebellar neuronal cultures under basal conditions, showing expression of the neuronal markers TuJ1, MASH1 and MATH1, and levels of apoptosis (TUNEL, dark brown) and proliferation (BrdU incorporation, blue-black). (B,C) Exposure of hippocampal cells to $CM_{Cerebellum}$ reduces BrdU incorporation (B); exposure of cerebellar cultures to $CM_{Hippocampus}$ stimulates BrdU uptake (C). (D) In hippocampal-cerebellar slice co-cultures, hippocampal cell proliferation is reduced in the presence of cerebellar slices, whereas cerebellar cell proliferation is slightly increased in the presence of hippocampal tissue. Dose dependency of $CM_{Cerebellum}$ anti-mitotic effects in hippocampal cultures are shown in E; the peptidergic nature of the anti-proliferative activity present in $CM_{Cerebellum}$ is indicated by the fact that anti-proliferative activity is lost after boiling $CM_{Cerebellum}$. $CM_{Cerebellum}$ also exerts anti-proliferative effects on hippocampus-derived HiB5 cells (F). Scale bar: 50 µm. Numerical data refer to mean±s.d. (n=4-6) *P<0.05, **P<0.01, ***P<0.001 (versus appropriate controls).

numbers of cells displaying signs of neuronal maturity (immunofluorescence and/or western blot analysis); specifically, exposure to $CM_{Cerebellum}$ resulted in increased synapsin (not shown) and MAP2a/b (Fig. 2B,C) expression and, concomitantly, in a small but significant (P<0.05) decrease in the number of cells immunoreactive for the marker of early postmitotic neuroblasts, doublecortin (Fig. 2D). Interestingly, $CM_{Cerebellum}$ had no significant effect on the relative number of glial (GFAP-immunopositive) cells in the hippocampal cultures (data not shown); in fact, the majority of BrdU-positive cells in hippocampal cultures grown in $CM_{Cerebellum}$ had differentiated into neurons after 3 days, as shown by double-labeling for BrdU and MAP2a/b (Fig. 2E).

Presence of multiple factors with differing anti-proliferative and pro-differentiation or pro-apoptotic properties in $CM_{Cerebellum}$

On the premise that more than one factor may account for the anti-mitotic actions of $CM_{Cerebellum}$, concentrated $CM_{Cerebellum}$ was fractionated according to ionic strength after minimizing albumin interference using Affigel Blue chromatography. The four fractions that were eluted with NaCl (0.1-1.5 M) showed differing potencies on the proliferative, differentiating and apoptotic potential of hippocampal cells (Table 1). Specifically, fractions eluting at 0.1 M NaCl had significant anti-mitotic (P<0.001) and anti-apoptotic (P<0.05) activities; fractions eluting at 1 M NaCl proved effective at promoting neuronal maturation (P<0.05) and strong anti-apoptotic activity was observed in eluates containing 1.5 M NaCl (P<0.001). Thus, $CM_{Cerebellum}$ contains a cocktail of factors that differentially direct hippocampal cell fate. Non-fractionated $CM_{Cerebellum}$ did not elicit any significant change in the incidence of apoptosis ($F_{11,24}$=1.373, P=0.28; Table 1).

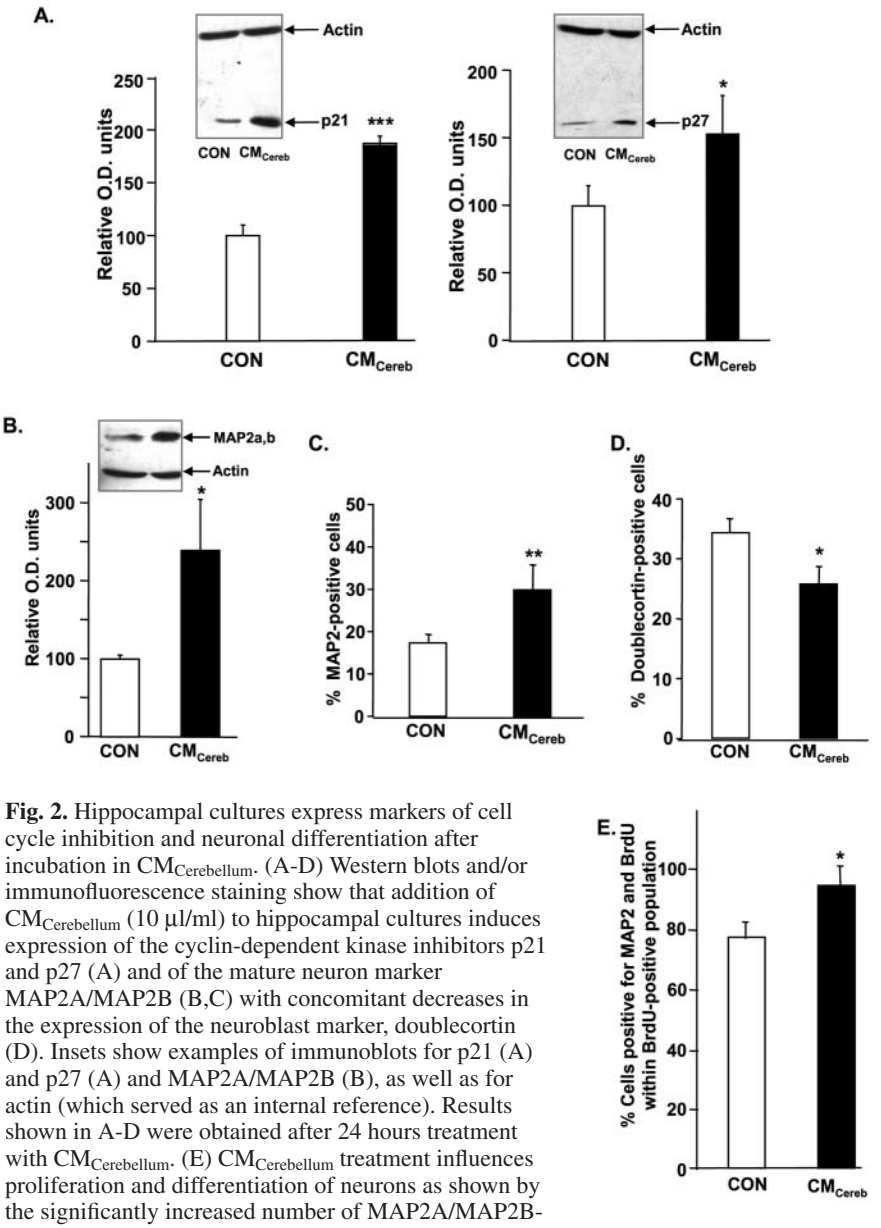

Fig. 2. Hippocampal cultures express markers of cell cycle inhibition and neuronal differentiation after incubation in $CM_{Cerebellum}$. (A-D) Western blots and/or immunofluorescence staining show that addition of $CM_{Cerebellum}$ (10 μl/ml) to hippocampal cultures induces expression of the cyclin-dependent kinase inhibitors p21 and p27 (A) and of the mature neuron marker MAP2A/MAP2B (B,C) with concomitant decreases in the expression of the neuroblast marker, doublecortin (D). Insets show examples of immunoblots for p21 (A) and p27 (A) and MAP2A/MAP2B (B), as well as for actin (which served as an internal reference). Results shown in A-D were obtained after 24 hours treatment with $CM_{Cerebellum}$. (E) $CM_{Cerebellum}$ treatment influences proliferation and differentiation of neurons as shown by the significantly increased number of MAP2A/MAP2B-positive/BrdU-positive double-stained cells relative to the total number of BrdU-positive cell population. For this analysis, cells growing in $CM_{Cerebellum}$ were exposed to BrdU for 8 hours, and washed and maintained in $CM_{Cerebellum}$ for 72 hours before fixing and processing for MAP2A/MAP2B and BrdU double immunocytochemistry. There was no statistical difference on the incidence of apoptosis in cultures grown in either control medium or $CM_{Cerebellum}$. Numerical data refer to mean±s.d. (n=4-6) *P<0.05, **P<0.01, ***P<0.001 (versus appropriate controls).

Identification of anti-proliferative and differentiating factors

Data reported above indicated that the active factor(s) of interest in $CM_{Cerebellum}$ were heat unstable and had a M_r greater than 6 kDa. Accordingly, we analyzed the effects of immunoneutralization of three candidate trophic factors, previously implicated in neuronal birth, differentiation and death, namely, NGF, BDNF and TGFβ2 (Minichilo and Klein, 1996; Massague et al., 2000; Borghesani et al., 2002; Vaudry et al., 2003), on BrdU incorporation and differentiation (MAP2a/b expression). Immunocytochemistry showed the presence of all these peptides in cerebellar and hippocampal cultures (data not shown). BrdU incorporation by hippocampal cells was not affected after incubation in anti-NGF-preadsorbed $CM_{Cerebellum}$. Anti-BDNF (1:10) significantly blocked the anti-proliferative effects of crude $CM_{Cerebellum}$ (P<0.05), as did anti-TGFβ2 (1:1000 and 1:100; P<0.01) (Fig. 3A). Control cultures (treated with species-appropriate purified IgG preparations) did not differ from naive controls (data not shown).

In accordance with our earlier results that showed that $CM_{Cerebellum}$ concomitantly blocks proliferation of hippocampal cells while promoting their maturation (Fig. 2), immunoneutralization against TGFβ2 (antibody

Table 1. Proliferative, differentiating and apoptotic activities in different fractions of $CM_{Cerebellar}$ purified by ion-exchange chromatography

Medium	Biological activity		
	Proliferative (% $BrdU^+$ cells)	Differentiating (% $MAP2^+$ cells)	Apoptotic (% $TUNEL^+$ cells)
Control	45.1±3.9	15.2±1.6	22.7±1.3
$CM_{Cerebellar}$Crude	13.5±1.2	26.4±0.5	27.1±1.5
Q-ion exchange chromatography $CM_{Cerebellum}$			
Eluate, 0.1 M NaCl	18.5±1.4	16.5±0.8	16.3±1.2
Eluate, 0.5 M NaCl	38.1±3.5	13.4±0.5	21.8±2.2
Eluate, 1 M NaCl	46.9±0.8	22.2±1.2	22.6±4.1
Eluate, 1.5 M NaCl	46.5±1.9	19.2±2.8	8.7±1.3

Multiple factors in $CM_{Cerebellum}$ contribute to its anti-proliferative, anti-apoptotic and differentiation-inducing properties. Concentrated $CM_{Cerebellum}$ was fractionated according to ionic strength and dye affinity (Q and Affigel Blue chromatography). The four fractions that eluted with NaCl (0.1-1.5 M) differentially influenced proliferation, apoptosis and differentiation in primary hippocampal cells. Specifically, fractions with 0.1 M NaCl displayed significant anti-mitotic ($P<0.001$) and anti-apoptotic ($P<0.05$) effects; strong anti-apoptotic activity was observed in fractions eluting with 1.5 M NaCl ($P<0.001$), whereas those eluting with 1 M NaCl promoted neuronal maturation ($P<0.05$).

concentrations that proved efficient at reversing the anti-mitotic effects) significantly reduced MAP2a/b expression ($P<0.01$; Fig. 3B). By contrast, anti-NGF (which did not influence proliferation) and anti-BDNF did not alter the relative number of MAP2a/b-immunoreactive cells, when compared with $CM_{Cerebellum}$ that had not been pre-adsorbed with these antisera (Fig. 3B).

As immunoneutralization experiments provide only qualitative information and may be compromised by factors such as antibody affinity and purity, we next examined hippocampal cell proliferation and maturation after addition of either BDNF or TGFβ2 to normal medium; for comparison, hippocampal cultures were grown in $CM_{Cerebellum}$. Significant reductions in BrdU incorporation were observed after treatment with either BDNF at 50 ng/ml ($P<0.05$) and 100 ng/ml ($P<0.001$) (Fig. 3C) or TGFβ2 at 1 ng/ml ($P<0.01$) and 10 ng/ml ($P<0.01$) (Fig. 3D). The results obtained with exogenous BDNF peptide were confirmed when hippocampal cells were transfected with a BDNF expression plasmid ($P<0.001$, compared with cells transfected with pEGFP; inset, Fig. 3C) and when medium from pBDNF-transfected cells (24 hours) was added to pEGFP-transfected cells (third bar in inset, Fig. 3C; $P<0.01$).

In addition to inhibiting cell proliferation, both exogenous BDNF and TGFβ2 were found to promote neuronal maturation, evidenced by significant increases ($P<0.01$ and 0.05, respectively) in the number of MAP2a/b-immunopositive cells after addition of purified forms of each peptide (Fig. 3E). These findings indicate that BDNF and TGFβ2 account for a large part of the pro-neuronal properties of $CM_{Cerebellum}$. Using another indicator of pro-neuronal activity, namely neurite extension ('long neurites' defined as neurites with lengths that were more than twice the diameter of the cell body), we observed that both BDNF (at 10-100 ng/ml; $P<0.05$) and TGFβ2 (at 1-10 ng/ml; $P<0.05$) stimulated neurite growth in a manner comparable with that observed with $CM_{Cerebellum}$ (Fig. 3E).

The temporal patterns of BDNF and TGFβ2 expression in hippocampal and cerebellar cultures were examined using immunocytochemistry after varying numbers of days in vitro. Generally, the intensity of BDNF and TGFβ2 staining was stronger in cerebellar versus hippocampal cultures (data not shown). When compared with hippocampal cells, a significantly larger number of cerebellar cells expressed BDNF on 3 and 6 d.i.v. ($P<0.001$ and $P<0.05$; data not shown). At 9 d.i.v. (peak), more cerebellar cells than hippocampal cells were immunoreactive for TGFβ2 ($P<0.05$; data not shown).

Anti-proliferative and differentiating effects of $CM_{Cerebellum}$ are mediated by TRKB and TGFβ-RII receptors

$CM_{Cerebellum}$ failed to inhibit proliferation in primary hippocampal cells expressing dominant-negative forms of the BDNF receptor TRKB (pEF/BOS-TRKB.T1-flag) or the TGFβ receptor, TGFβ-RII (pRK5-TβRII-DN-F) (Fig. 4A). In addition, hippocampal neuronal maturation induced by $CM_{Cerebellum}$, as measured by the number of MAP2a/b-immunoreactive cells, was significantly reduced in cells expressing dominant-negative forms of TRKB and TGFβ-RII (Fig. 4B). These results add further support for the roles of BDNF and TGFβ-2 as the anti-proliferative and pro-differentiation moieties in $CM_{Cerebellum}$.

Mediation of $CM_{Cerebellum}$, BDNF and TGFβ2 actions by SMAD pathways

TGFβ2 exerts its biological actions through the mediation of SMAD proteins. SMAD2 and SMAD3 specifically transduce TGFβ signals after dimerizing with co-SMAD4, translocating the resulting complex to the nucleus and modulating the transcriptional machinery (Attisano and Wrana, 2002). The data depicted in Fig. 4C show that pEGFP-SMAD2 and pEGFP-SMAD4 are translocated to the nucleus after exposure of primary hippocampal neurons to $CM_{Cerebellum}$, as well as to purified TGFβ2 and BDNF.

We showed in Fig. 1F that, as in primary hippocampal cells, $CM_{Cerebellum}$ exerts anti-mitotic actions in the hippocampus-derived HiB5 cell line. $CM_{Cerebellum}$ failed to inhibit proliferation or to stimulate differentiation in hippocampal cells expressing dominant-negative forms of SMAD3 (pCS2-FLAG-SMAD3-3SA) or co-SMAD4 (pCMV-FLAG-DPC4(1-514)) (Fig. 4D,E).

Another set of experiments in HiB5 cells showed that $CM_{Cerebellum}$ dose-dependently transactivates the specific SMAD reporter gene 3TP-Lux (Fig. 4F). Stimulation of reporter gene activity was abolished in the presence of dominant-negative forms of SMAD3 (pCS2-FLAG-SMAD3-3SA) or co-SMAD4 (pCMV-FLAG-DPC4(1-514) (Fig. 4G), again pointing to the involvement of SMAD signaling in the mediation of $CM_{Cerebellum}$ actions. These and the other above-reported data do not, however, completely rule out the participation of other SMAD-linked factors, as co-SMAD4 complexes with other members of the SMAD family, independently of TGFβ; indeed, as functional inhibition of SMAD4 resulted in stronger ($P<0.01$) inhibition of the anti-proliferative and differentiating effects of $CM_{Cerebellum}$ (Fig. 4D,E), when compared with those observed after SMAD3 inhibition, activation of other SMAD pathways by

non-TGFβ ligands is highly plausible (see Attisano and Wrana, 2002).

Last, as shown in Fig. 4H, $CM_{Cerebellum}$ transactivation of the 3TP-Lux SMAD reporter gene was significantly attenuated ($P<0.01$) when HiB5 cells were transfected with plasmids expressing the dominant-negative forms of TGFβRII or TRKB. These data demonstrate the essential role of TGFβRII or TRKB receptors in coupling $CM_{Cerebellum}$-initiated signals (putatively, through TGFβ2 and BDNF, respectively) with the SMAD signaling cascade.

BDNF actions involve convergence of MAPK and SMAD signaling pathways

Adding to the evidence that TGFβRII are involved in mediating the biological actions of BDNF, we observed that BDNF failed to exert anti-proliferative effects when it was applied to primary hippocampal cells that were transiently transfected with a dominant-negative form of TGFβRII (Fig. 5A).

As BDNF signaling pathways reportedly converge on those triggered by TGFβ2 following the activation of ERK1/2 by the neurotrophin (Segal and Greenberg, 1996; Pera et al., 2003), we pharmacologically tested the involvement of these kinases using the MEK inhibitor PD98059. This drug significantly abrogated the inhibitory effects of $CM_{Cerebellum}$ on BrdU incorporation ($P<0.001$; Fig. 5B). PD98059 also abolished the pro-differentiating potential of $CM_{Cerebellum}$ ($P<0.001$; Fig. 5C). Together, these observations indicate that $CM_{Cerebellum}$-induced effects on proliferation and differentiation are mediated through MAP kinases and, in accordance with earlier reports (Marshall, 1995; Du et al., 2003), they hint at the involvement of BDNF in these processes.

We showed earlier that $CM_{Cerebellum}$-stimulated 3TP-Lux reporter activity was attenuated when HiB cells were transiently transfected with a dominant-negative form of the BDNF receptor TRKB. In another set of experiments, we showed that expression of pBDNF also significantly increased 3TP-Lux reporter activity (Fig. 5D). However, transactivation of the reporter was not seen after co-transfection of cells with pBDNF and (1) dominant-negative TRKB, (2) dominant-negative SMAD4 or (3) PD98059 (Fig. 5D). These results

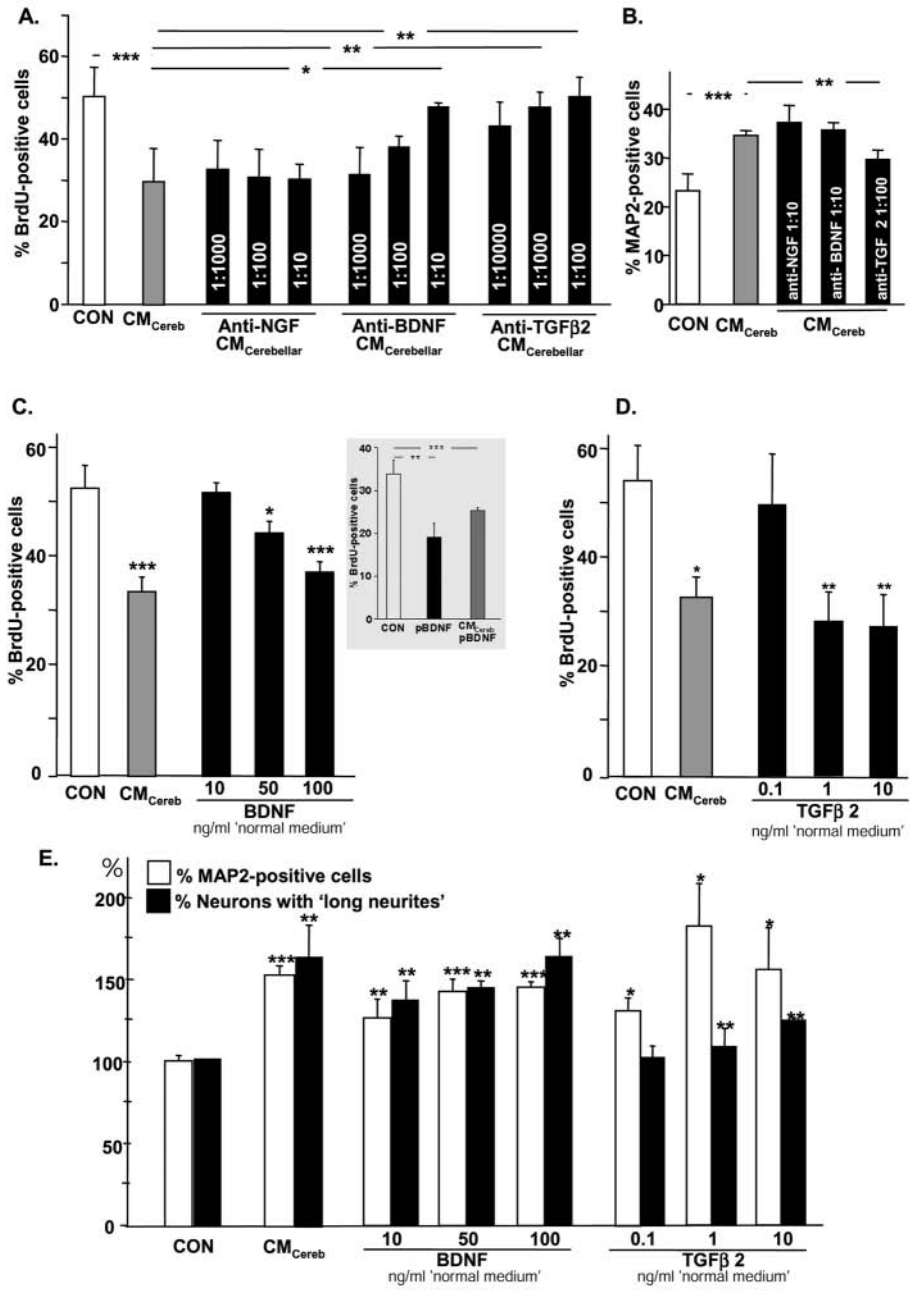

Fig. 3. Immunoneutralization of candidate anti-proliferative and differentiating factors in $CM_{Cerebellum}$ and replication of effects by exogenous BDNF and TGFβ2. (A) $CM_{Cerebellum}$ was preadsorbed with the indicated dilutions of anti-NGF, BDNF or TGFβ2. (B) Immunoneutralization of BDNF and TGFβ2 significantly attenuates the anti-proliferative actions of $CM_{Cerebellum}$; anti-TGFβ2 significantly attenuates the pro-differentiating effects of $CM_{Cerebellum}$, assessed by MAP2A/MAP2B expression. (C,D) Exogenous BDNF (C) and TGFβ2 (D) dose-dependently inhibit BrdU retention in hippocampal cells; the inset (C), shows that transient expression of pBDNF also reduces BrdU incorporation (pEGFP used as transfection control). (E) Exogenous BDNF and TGFβ2 promote neuronal maturation in hippocampal cultures (increased expression of MAP2A/MAP2B and neurons with neurite lengths more than twice the diameter of the soma). The control data in E, shown as 100%, represent 126/865 MAP2-positive cells (14.5%) with 'long neurites' in the BDNF studies, and 94/518 MAP2-positive cells (18.2%) with 'long neurites' in the TGFβ2 experiments. Numerical data refer to mean±s.d. (n=4-6) $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ (versus appropriate controls).

demonstrate that MAP kinases mediate the actions of BDNF on SMAD signaling. Interestingly, co-transfection of pBDNF with a dominant-negative form of the TGFβRII also abrogated BDNF-stimulated activity of the 3TP-Lux reporter (Fig. 5D), indicating that the TGFβRII is involved in the mediation of BDNF actions. This finding led us to examine the possibility that BDNF can stimulate TGFβ2 synthesis/secretion. Using a semi-quantitative immunocytochemical assay, we observed that exposure of primary hippocampal cells to BDNF (10-100 ng/ml) can dose-dependently increase the cellular content of TGFβ2 (Fig. 5E). Two different assay systems used to detect secreted TGFβ2 proved to be insufficiently sensitive (cf. Lutz et al., 2004).

Discussion

The pluripotency of neural cell progenitors (McConnell and Kaznowski, 1991; Coskun and Luskin, 2002) implies that their ultimate phenotype can be influenced by environmental factors. Phenotypic re-specification has been demonstrated, for

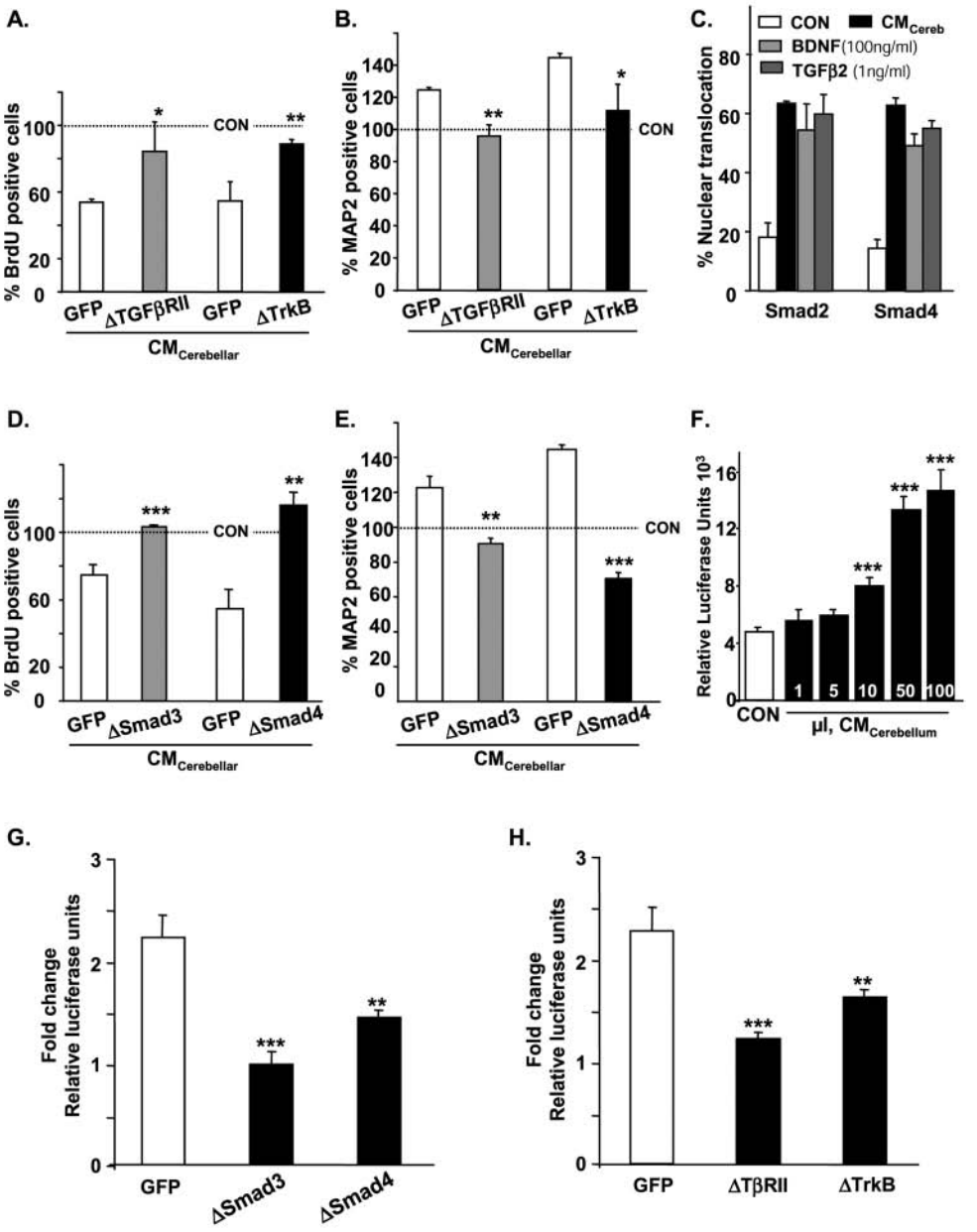

Fig. 4. Involvement of TRKB/TGFβ-RII and TGFβ2 signaling pathways in the anti-mitotic and differentiating effects of $CM_{Cerebellum}$. (A) Transfection of cells with a dominant-negative form (Δ) of TRKB (ΔTRKB) or TGFβ receptor II (ΔTGFβ-RII) before treatment with $CM_{Cerebellum}$ resulted in a significant increase in proliferation (versus controls transfected with GFP) (100% refers to BrdU incorporation in absence of $CM_{Cerebellum}$). (B) Results from cells treated as described above show that inhibition of expression of TGFβ-RII or TRKB prevents $CM_{Cerebellum}$-induced neuronal differentiation, as measured by number of MAP2A/MAP2B-positive cells (cells not exposed to $CM_{Cerebellum}$ provide the reference value of 100%). (C) Exposure of hippocampal cells to $CM_{Cerebellum}$, as well as to either TGFβ2 (1 ng/ml) or BDNF (100 ng/ml) induces nuclear translocation of the TGFβ2-specific partner SMAD2 and of co-SMAD4 within 3 hours, as shown by transient transfection experiments. (D,E) Introduction of dominant-negative forms of either SMAD3 (which specifically couples with TGFβ2) or of Co-SMAD4 abrogates the anti-mitotic (D) and differentiating (E) effects of $CM_{Cerebellum}$ on primary hippocampal cells; these changes do not reflect apoptosis as the number of TUNEL-stained and activated caspase 3 cells were not altered by the treatment (data not shown). (F-H) $CM_{Cerebellum}$ stimulates generation of luciferase from the TGFβ reporter gene 3TP-Lux in HiB5 cells (F), an effect abrogated when expression of either SMAD3 or Co-SMAD4 is blocked by transfection with their respective dominant-negative (Δ) forms (G); similarly, transactivation of 3TP-Lux is blocked in the presence of ΔTGFβ-RII and ΔTRKB (H). Each half set of data in A,B,D,E were obtained in independent experiments in which GFP was used to control for between-culture variability in transfection efficiency. Data in G,H represent ratios of luciferase expression (fold change in treatments versus non-treated cells). In all cases, transfections were performed 24 hours before addition of $CM_{Cerebellum}$, TGFβ2 or BDNF for 24 hours, after which the analysis was performed. Numerical data refer to mean±s.d. (n=4-6) *P<0.05, **P<0.01, ***P<0.001 (versus appropriate controls).

example, in studies involving hippocampal granule cell transplants into the cerebellum (Renfranz et al., 1991) and vice versa (Vicario-Abejón et al., 1995). This adaptive capacity, resulting from the interplay between lineage-specific and extrinsic factors, is gradually lost over time as the host environment becomes increasingly differentiated (Suhonen et al., 1996; Alder et al., 1999); thus, these processes appear to be spatiotemporally organized. Another intriguing aspect of neuronal development concerns the determination of optimal neuronal population sizes. It is recognized that rates of apoptosis and neurogenesis from embryonic development through to adulthood occur in a balanced manner. Earlier studies have suggested that autocrine or paracrine secretions may play a role in the proliferation, survival and differentiation of developing granule neurons (Gao et al., 1991; Mumm et al., 1996; Ueki et al., 2003; Wu et al., 2003). Although various experimental paradigms have indicated regulatory roles for the neurotrophin BDNF in these events (Lin et al., 1998; Borghesani et al., 2002) and other cytokines (Tao et al., 1997; Unsicker and Strelau, 2000; Pratt and McPherson, 1997; Alder et al., 1999; Angley et al., 2003), little is known about the identity of the intrinsic and environmental signals that maintain the equilibrium between neuronal birth, maturation and death.

The appearance and differentiation of cerebellar and hippocampal granule neurons overlap only transiently: cerebellar granule cells enter a post-mitotic state at P7-14, i.e. when hippocampal granule cell neurogenesis peaks before gradually declining with increasing age (Schlessinger et al., 1975; Altman and Bayer, 1990; Cameron and McKay, 2001). A recent DNA microarray analysis revealed that cerebellar and hippocampal granule cells display distinct gene expression profiles, even at times when both cell types are undergoing rapid mitosis (Saito et al., 2002).

In an analogous approach to those used previously in animals (Renfranz et al., 1991; Vicario-Abejón et al., 1995), the different developmental profiles in the hippocampus and cerebellum were exploited in the present study to identify factors contributing to the regulation of hippocampal granule cell proliferation and differentiation. By immunocytochemical monitoring of markers of neuronal maturity and proliferative potential (BrdU incorporation) in hippocampal and cerebellar cultures from animals of a given developmental age, we confirmed that, when compared with hippocampal cultures, cerebellar cultures were more mature and mainly postmitotic after 14 d.i.v. Next, cultures of one type were treated with conditioned medium from the other ($CM_{Cerebellum}$ and $CM_{Hippocampus}$). Analysis of BrdU uptake revealed that whereas $CM_{Hippocampus}$ stimulated proliferation in cerebellar cultures, $CM_{Cerebellum}$ treatment inhibited cell proliferation and accelerated neuronal maturation in hippocampal cultures in a dose-dependent manner. The anti-

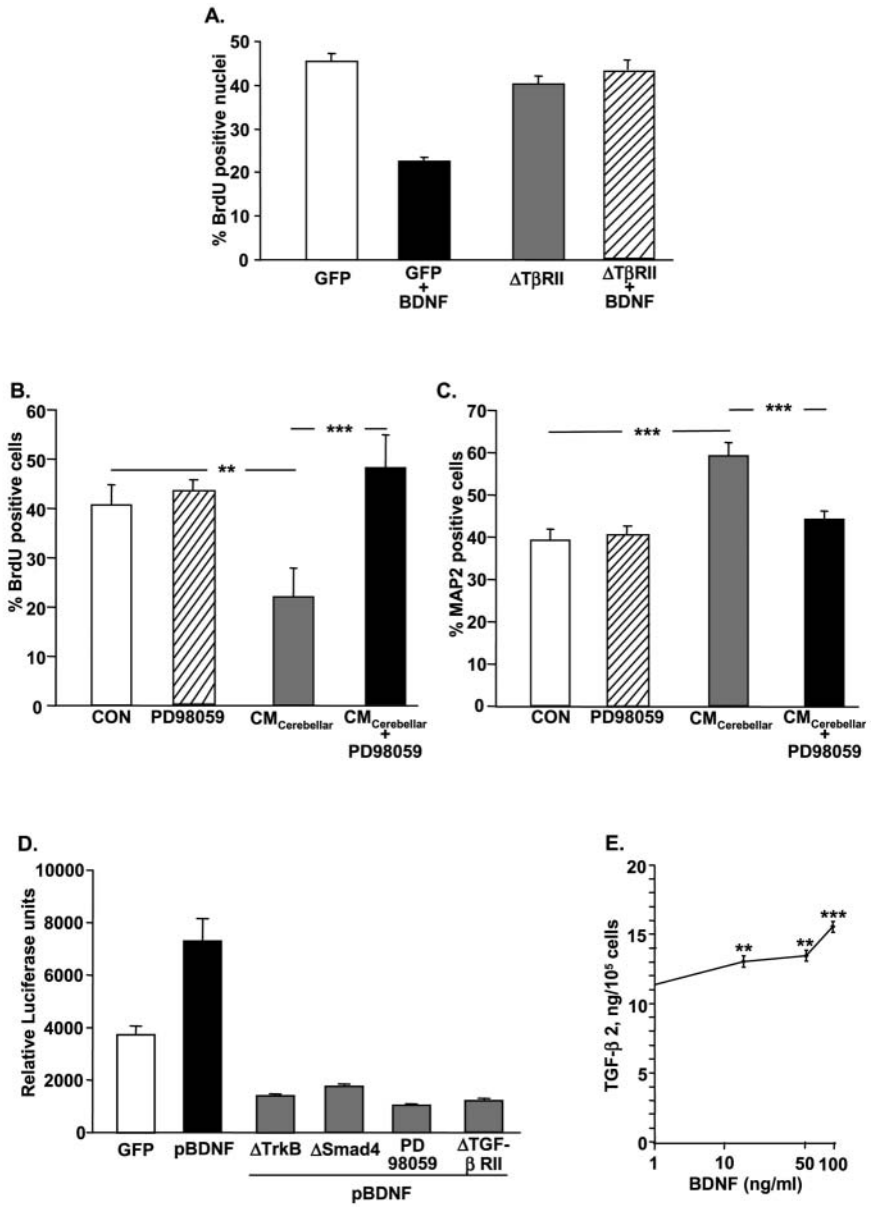

Fig. 5. Mechanisms underlying the anti-mitotic and differentiating effects of $CM_{Cerebellum}$ and BDNF – possible opportunities for crosstalk with TGFβ signaling pathways. The anti-proliferative effects of BDNF (measured by BrdU incorporation) are significantly attenuated in primary hippocampal cells expressing a dominant-negative form of TGFβ-RII. (B,C) The MEK inhibitor PD98059 (0.1 μM) counteracts the anti-proliferative (B) and pro-differentiating (C) effects of BDNF. (D) Transient transfection of HiB5 cells with pBDNF results in increased TGFβ2 reporter gene (3TP-Lux) expression, an effect that is significantly attenuated by co-transfecting dominant-negative forms (Δ) of either TRKB, TGFβ-RII or SMAD4, or by pretreatment with PD98059. (E) BDNF dose-dependently increases TGFβ2 protein content in primary hippocampal cells; numerical data refer to mean±s.d. (n=4-6) *P<0.05, **P<0.01, ***P<0.001 (versus appropriate controls).

proliferative/pro-differentiating effects of $CM_{Cerebellum}$ coincided with increased expression of p21 and p27, two cell cycle arrest-related molecules. Similar results were obtained after treating hippocampus-cerebellum slice co-cultures or a hippocampus-derived cell line (HiB5) with $CM_{Cerebellum}$. Together, these results indicate that cerebellar and hippocampal cells secrete cell type-specific factors in a temporally coordinated manner, and that these factors exert distinct influences on neurogenesis and differentiation.

The loss of biological activity after boiling $CM_{Cerebellum}$ hinted at the polypeptide nature of its anti-proliferative/pro-differentiating activities. Importantly, biological potency was retained in $CM_{Cerebellum}$ that was subjected to ion exchange chromatography, but the anti-mitotic, apoptotic and differentiating activities eluted at different ionic strengths. Immunoneutralization was used as an approach to identify the active moieties in $CM_{Cerebellum}$; the selection of candidates was based on reports that TGFβ2, NGF and BDNF are differentially expressed in hippocampal and cerebellar tissues during development (Unsicker et al., 1991; Sakamoto et al., 1998; Dieni and Rees, 2002). We found that immunoneutralization of BDNF and TGFβ2, but not NGF, abrogated the proliferative actions of $CM_{Cerebellum}$. At the same time, pre-adsorption of $CM_{Cerebellum}$ with anti-TGFβ2 (but not anti-BDNF or anti-NGF) inhibited the pro-differentiating actions of $CM_{Cerebellum}$.

Further studies focused on verifying the roles of TGFβ2 and BDNF in the observed $CM_{Cerebellum}$-induced effects on hippocampal cell development. Both TGFβ2 and BDNF were found to be more strongly expressed in age-matched cerebellar versus hippocampal cultures (data not shown), and treatment of hippocampal cultures with either cytokine resulted in reduced BrdU incorporation and increased signs of neuronal differentiation.

Three separate genes encode three isoforms of TGFβ: TGFβ1 (normally restricted to the choroid plexus); and the neuron- and glia-expressed isoforms TGFβ2 and TGFβ3 (Unsicker et al., 1991; Pratt and McPherson, 1997). TGFβ1 and TGFβ3 have been implicated in neuroprotection, while neurotrophic functions have been ascribed to TGFβ2 and TGFβ3 (Finch et al., 1993; Böttner et al., 2000; Pratt and McPherson, 1997). The latter include stimulation (Mahanthappa and Schwarting, 1993) or inhibition (Constam et al., 1994) of neurogenesis, or both (Kane et al., 1996), as well as the regulation of neuronal differentiation (Ishihara et al., 1994; Abe et al., 1996; Cameron et al., 1998). TGFβ2, the isoform focused on in this work, is expressed in the external granular (neurogenic) layer and in Purkinje and radial glia of the cerebellum according to a strict temporal pattern and, interestingly, appreciable levels of TGFβ2 are not seen in other brain sites of neuronal proliferation (Flanders et al., 1991; Constam et al., 1994; Unsicker and Strelau, 2000).

Members of the TGFβ superfamily signal by sequentially binding to two TGFβ receptors (TGFβ-R) that are transmembrane protein serine/threonine kinases; binding of TGFβ ligand to TGFβ-RII activates TGFβ-RI (expressed in the developing and adult rat hippocampus) (Böttner et al., 1996) and its substrates, the receptor-regulated SMAD proteins (R-SMADs). Upon phosphorylation, the latter bind co-SMAD4 and translocate to the nucleus where they form a transcriptionally active complex after association with DNA-binding partner(s). This complex binds to promoter elements of target genes whose functions include regulation of the cell cycle and differentiation (Moustakas et al., 2001; Chang et al., 2002; Shi and Massagué, 2003). For example, cell cycle arrest by TGFβ involves suppression of the oncogene Myc, a repressor of the CDK inhibitors p21 and p27 (Seoane et al., 2002; Gartel and Shchors, 2003). Supporting the view that TGFβ2 may be responsible for at least some of the anti-mitogenic activity of $CM_{Cerebellum}$ we here observed an upregulation of p21 and p27 after $CM_{Cerebellum}$ treatment of proliferating hippocampal neurons.

Additional evidence for a key role of TGFβ2 in the hippocampal cell fate-determining actions of $CM_{Cerebellum}$ was obtained by studying TGFβ signal-propagating SMAD proteins. Of the various members of the SMAD system, SMAD2 and SMAD3 mediate TGFβ signals. SMAD4 is a requisite partner for transcriptional activity of all SMADs, including SMAD2 and SMAD3; the generation of specific downstream responses is presumed to depend on the formation of specific R-SMAD-SMAD4 complexes that then recruit different sequence-specific DNA-binding factors (Massagué and Wotton, 2000). We demonstrated that $CM_{Cerebellum}$ treatment can induce nuclear translocation of EGFP-SMAD2 and EGFP-SMAD4. Essential roles for SMAD3 and SMAD4 were demonstrated insofar that transient expression of the dominant-negative forms of either of these molecules in hippocampal cells prevented $CM_{Cerebellum}$-induced transactivation of the TGFβ reporter gene (3TP-Lux) and abrogated the anti-proliferative and pro-differentiating effects of $CM_{Cerebellum}$. Further support for the view that TGFβ2, at least partially, accounts for the anti-proliferative activity present in $CM_{Cerebellum}$ is provided by the observation that expression of a vector containing a dominant-negative form of TGFβRII in either primary hippocampal cells or a hippocampus-derived cell line (Hib5) abolishes $CM_{Cerebellum}$-induced effects on BrdU incorporation and 3TP-Lux reporter activity.

As already mentioned, hippocampal cells responded to exogenous BDNF (or a BDNF-expressing plasmid) with an inhibition of BrdU uptake, and an increase in the number of MAP2a/b neurons and neuritic lengths; the effects of this neurotrophin therefore closely resembled those of TGFβ2. BDNF effects on neuronal differentiation are mediated through TRKB receptors (Klein et al., 1991) and BDNF can either promote or inhibit neuronal proliferation by activating the TRK-MAPK-ERK pathway (Marshall, 1995; Du et al., 2003), thus raising the issue of whether ERK signaling is involved in the biological actions of $CM_{Cerebellum}$. We found that upstream (MEK) inhibition of this pathway using PD98059 negates the proliferative and differentiating actions of $CM_{Cerebellum}$. Furthermore, we observed that transient expression of a dominant-negative form of TRKB in hippocampal cells abolishes $CM_{Cerebellum}$-induced effects on proliferation and differentiation, while, at the same time, abolishing the ability of $CM_{Cerebellum}$ to induce nuclear translocation of SMAD2 and SMAD4, and TGFβ 3TP-Lux transactivation. These findings are consistent with previous reports that suggested crosstalk (Lutz et al., 2004) or interdependence/synergism (Unsicker and Strelau, 2000) between these trophic factors and their signal transduction pathways. Additional support for this interpretation is

provided by our observation that BDNF cannot stimulate 3TP-Lux reporter activity after functional blockade of SMAD4 and TGFβ–RII expression, and that expression of a dominant-negative form of TGFβRII attenuates the anti-proliferative activity of BDNF. In an initial analysis of the upstream mechanisms that might be responsible for crosstalk between the BDNF and TGFβ pathways, we found that BDNF can stimulate the cellular content of TGFβ2 in hippocampal cells; unfortunately, limited assay sensitivity precluded information on whether BDNF can also stimulate TGFβ2 secretion. Seoane et al. (Seoane et al., 2002) showed that TGFβ can induce cell cycle arrest by activating cdk inhibitors such as p21 and p27. The present study shows that both these latter proteins are upregulated in hippocampal cells after exposure to $CM_{Cerebellum}$, raising the following questions for future research. Do BDNF-activated TRKB receptors induce cell cycle arrest? If so, is the TGFβ/SMAD pathway involved?

In summary, we have demonstrated that BDNF and TGFβ2 and their respective signaling machineries, by acting in a dynamic, but strictly coordinated, spatiotemporal fashion, play a decisive role in determining hippocampal cell fate by inhibiting cell proliferation and promoting neuronal differentiation. We have also shown that BDNF, better known for TRK receptor-mediated promotion of neurogenesis and differentiation (Klein et al., 1991; Gao et al., 1995; Pencea et al., 2001), can exert anti-proliferative and pro-differentiating effects on hippocampal granule cells by activating MAPK and, subsequently, TGFβ signaling pathways; the latter is a novel observation and provides a mechanism through which diverse cytokine signals can converge on a common signaling 'hub' to direct neuronal development.

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