Extracellular Signal-Regulated Protein Kinase Signaling Is Uncoupled From Initial Differentiation of Central Nervous System Stem Cells to Neurons

Maria Enarsson,[1,3] Anna Erlandsson,[1,3] Helena Larsson,[1] and Karin Forsberg-Nilsson[1,2,3]

[1]Department of Genetics and Pathology, Rudbeck Laboratory, Uppsala University, Uppsala, Sweden; and [2]Biovitrum AB, Stockholm, Sweden

Abstract

Knowledge about signaling pathways in response to external signals is needed to understand the regulation of stem cell proliferation and differentiation toward particular cell fates. The Ras/extracellular signal-regulated kinase (ERK) pathway has been suggested to play an essential role in neuronal differentiation. We have examined ERK signaling in the transition from multipotent stem cell to post-mitotic progeny using primary stem cells from the rat embryonic cortex. Fibroblast growth factor-2 (FGF-2) is a stem cell mitogen, whereas platelet-derived growth factor AA (PDGF-AA) expands a pool of committed neuronal precursors from stem cells. When comparing ERK activation by these growth factors, we found that FGF-2 stimulates high and PDGF-AA lower levels of ERK phosphorylation in stem cells. Differentiation was monitored as down-regulation of the bHLH transcription factor mammalian achaete-scute homologue-1 (MASH1). Even in the absence of active ERK, MASH1 became down-regulated and microtubule-associated protein 2-positive cells could form. Thus, ERK activation seems dispensable for the earliest steps of CNS stem cell differentiation.

Introduction

Fibroblast growth factor-2 (FGF-2) is a major mitogen for central nervous system (CNS) stem cells in culture. It represses differentiation, maintains the cells in a proliferative mode, and recruits quiescent cells into the cell cycle (1). By withdrawal of the mitogen, differentiation is induced by default within 24 h (2). Several epigenetic factors have been shown to regulate stem cell differentiation, but the pathways that govern fate choice of stem cells are only partly delineated. Signaling via the ciliary neurotrophic factor (CNTF) receptor leads to astrocytic differentiation (2–4). Addition of bone morphogenetic proteins (BMPs) encourages either glial fate choice (5) or smooth muscle differentiation (6, 7), displaying that CNS stem cells could give rise also to peripheral nervous system progenitors. Platelet-derived growth factor (PDGF) is a family of polypeptide chains (A, B, C, and D) that form homo- and heterodimers in different combinations. The dimers bind to and signal through two receptor tyrosine kinases, PDGF-α and -β receptors (reviewed in Refs. (8, 9)). In this study, we have used PDGF-AA that increases the number of neurons from stem cell cultures (2, 10) by acting as a mitogen and survival factor for immature neurons during their transition from a multipotent stem cell to nerve cells (11).

FGF and PDGF elicit their effect on target cells by binding to two structurally related protein tyrosine kinase receptors, leading to tyrosine phosphorylation of various intracellular proteins as well as receptor autophosphorylation (12–14). The most important pathway for mitogenic signaling by tyrosine kinase receptors is the Ras/extracellular signal-regulated kinase (ERK) pathway. Activated Ras associates with Raf that phosphorylates and activates the mitogen-activated protein (MAP) kinase kinases MEK1 and MEK2, which in turn phosphorylate the MAP kinases ERK1 and ERK2. The ERKs then activate other cytoplasmatic proteins or translocate into the nucleus, where they regulate transcription factors (15–17).

CNS stem cell differentiation can be easily monitored by immunostaining with antibodies to cell lineage specific markers because these antigens appear after a few days. However, to study the role for ERK when stem cells initiate their differentiation, an earlier marker was required. The mammalian achaete-scute homologue-1 (MASH1) encodes a basic helix-loop-helix transcription factor, which controls the correct timing of differentiation during neuronal development (18–21). Neuronal differentiation coincides with down-regulation of MASH1. Consequently, decreased levels of MASH1 were used as a marker of initial stem cell differentiation in the presence or absence of active ERK. We found that even in the absence of ERK1/2 signaling, the cells ceased to express MASH1 protein. Furthermore, we show that microtubule-associated protein 2 (MAP2)-positive cells with a neuronal morphology can form without ERK1/2 activity. Thus, ERK signaling is dispensable for the earliest steps of CNS stem cell differentiation.

Received 02/26/02; revised 09/17/02; accepted 10/04/02.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Grant support: Swedish Cancer Foundation, the Children Cancer Foundation of Sweden, Magnus Bergwall's Foundation, Åke Wiberg's Foundation, Selander's Foundation, and Biovitrum AB.
[3]Present address: Department of Medical Biochemistry and Microbiology, Box 582, Biomedical Center, Uppsala University, 751 23 Uppsala, Sweden.
Requests for reprints: Karin Forsberg-Nilsson, Department of Medical Biochemistry and Microbiology, Box 582, Biomedical Center, Uppsala University, 75123 Uppsala, Sweden. Phone: 46-18-471-4158; Fax: 46-18-471-4244.
E-mail: karin.nilsson@imbim.uu.se

Results

FGF-2 and PDGF-AA Mediate ERK1/2 Phosphorylation in CNS Stem Cells

This study was conducted in primary cultures of cortical stem cells from the embryonic rat brain (E15) grown in serum-free medium using FGF-2 as a mitogen. A continuous supply of FGF-2 has been shown to repress differentiation and to maintain multipotential stem cells in a proliferative state (2). In contrast to FGF-2, which causes rapid proliferation of CNS stem cells from both embryonic and adult sources, PDGF treatment leads to expansion of a committed neuronal precursor, thereby increasing the amounts of neurons from stem cells. FGF-2 and PDGF tyrosine kinase receptors signal through many common pathways although the net cellular effects by the two polypeptide growth factors are different. We therefore aimed to compare the pattern of tyrosine phosphorylation on activation of FGF and PDGF receptors, respectively.

Stem cell cultures were stimulated with FGF-2 or PDGF-AA and subjected to Western blot analysis with anti-phosphotyrosine antibodies. The phosphorylation pattern shown in Fig. 1A revealed few distinct differences between the stimuli. As expected, a protein product corresponding to the PDGF-α receptor (M_r 180,000) was tyrosine phosphorylated in PDGF-AA-stimulated cells, but not elsewhere. In the cells stimulated with FGF-2, a distinct band with $M_r \sim 40{,}000$ was detected. The corresponding protein product from cells treated with PDGF-AA was weaker. This protein species was not detected in control cultures.

We used specific antibodies and verified the tyrosine-phosphorylated protein as the MAP kinase ERK2. Fig. 1B shows an immunoblot of stem cells treated with FGF-2 or PDGF-AA for 10 and 30 min, incubated with an antibody against phosphorylated ERK1/ERK2 (M_r 44,000 and 42,000, respectively). The distinct band seen in the phosphotyrosine blot after FGF-2 or PDGF-AA stimulation was found to correspond to ERK2. Phosphorylation of ERK1 could also be detected, albeit weaker than for ERK2. Less phosphorylation of ERK1 and 2 was seen after PDGF-AA treatment. The Ras/ERK pathway is among the most important signaling pathways for receptor tyrosine kinases, and our results show that both FGF-2 and PDGF-AA stimulated ERK1/2 phosphorylation in neural stem cells.

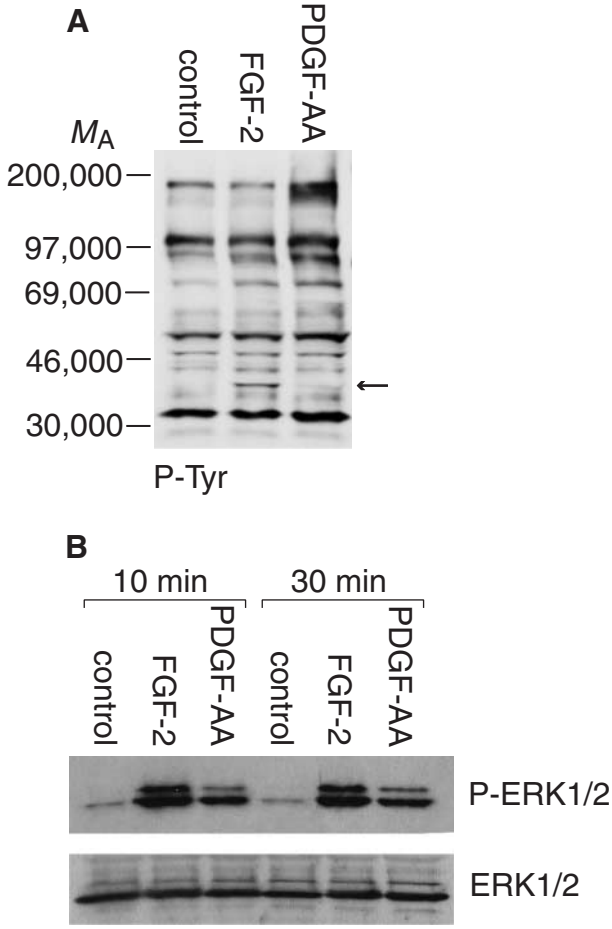

FIGURE 1. FGF-2 and PDGF-AA activate the Ras/ERK pathway in CNS stem cells. **A.** FGF-2 and PDGF-AA mediated intracellular signaling in neural stem cells. Subconfluent neural stem cell cultures in their first passage were either untreated (*control*) or treated with 100 ng/ml FGF-2 or PDGF-AA for 10 min. Total cell lysate was subjected to Western blot analysis. For immunoblotting, an antibody against phosphorylated tyrosine residues (PY20) was used. **B.** Neural stem cells were either untreated (*control*) or treated with 100 ng/ml FGF-2 or PDGF-AA for 10 and 30 min. Total cell lysate was immunoblotted with antibodies against phosphorylated ERK1 (M_r 44,000) and ERK2 (M_r 42,000) (*upper panel*) and subsequently with anti-ERK1/2 antibodies (*lower panel*).

Sustained Activation of ERK1/2 on Stimulation with FGF-2 or PDGF-AA

ERKs are implicated in the promotion of both cell proliferation and differentiation. The duration of ERK phosphorylation on growth factor stimulation has been suggested to impact the outcome of the cellular response. To determine if the ERK activation is transient or sustained, we treated cortical stem cells with FGF-2 or PDGF-AA for time points ranging from 30 min to 12 h. ERK activation was monitored using immunoblotting with an antibody to the phosphorylated ERK 1 and 2 isoforms (Fig. 2A). Fig. 2B shows the same immunoblot as in Fig. 2A quantified by densitometric scanning. Maximum stimulation of ERK phosphorylation after FGF-2 stimulation was reached between 30 min and 2 h. The phosphorylation remained over the 12 h of the experiment, but at a slightly lower level compared to peak values. Similarly, PDGF-AA-stimulated ERK phosphorylation remained well above control levels for the duration of the experiment. In accordance with the immunoblot shown in Fig. 1B, the signal was weaker than in FGF-2-treated cultures.

ERK1/2 Signaling Is Dispensable for Initial Differentiation of CNS Stem Cells

That the ERK pathway is critically needed for proliferation is well documented. Its role in differentiation and lineage commitment is less clear (22–28). We aimed to investigate the importance of ERK1/2 phosphorylation in the early steps of conversion from multipotential stem cells to post-mitotic progeny. To this end, we used the small molecule inhibitor PD098059, which blocks the phosphorylation of MEK, the upstream activator of ERK. The effect of PD098059 on FGF-2-

induced ERK1/2 phosphorylation in neural stem cells was verified by stimulating cells with FGF-2 for 10 min in the absence or presence of PD098059. This treatment almost fully reduced the ERK1/2 phosphorylation (Fig. 3A).

The cell culture system used throughout this study is critically dependent on a continuous supply of FGF-2 to promote stem cell proliferation. If FGF-2 is discontinued, the differentiation program is activated by default and neurons, astrocytes and oligodendrocytes will form in proportions previously described (2). The commitment of undifferentiated cells to specific cell lineages is classically monitored by expression of cell type-specific markers. Until neuron-specific markers, such as MAP2, have accumulated to allow detection, differentiation has proceeded for days rather than hours. To be able to couple rapid signal transduction events to initiation of differentiation, we took advantage of the expression pattern of MASH1, a basic helix-loop-helix transcription factor the expression of which is rapidly lost on differentiation of neural progenitors (18–21, 29). By immunofluorescence staining (Fig. 3B) and immunoblotting (Fig. 3C) using antibodies against MASH1, we show that MASH1 could readily be detected in FGF-2-supported neural stem cell cultures. The withdrawal of FGF-2 for 24 h sufficed to down-regulate MASH1 protein levels and at 2 days post-FGF-2, no MASH1 was detected with Western blot. We have therefore used the down-regulation of MASH1 expression as an indicator of early differentiation of neural stem cells.

To study the role of active ERK in the early differentiation process, FGF-2 was washed out and the cultures replenished with medium with or without the MEK inhibitor PD098059. FGF-2-induced ERK2 activation in neural stem cells follows a sustained kinetics (Fig. 2) and remained active for at least 12 h after FGF-2 addition. By 24 h post-FGF-2, however, ERK1/2 phosphorylation was not detected (Fig. 3C) neither in the absence or presence of PD098059. As can be seen from Fig. 3, B and C, MASH1 expression was discontinued also in cells treated with PD098059 for 1 and 2 days. This suggests that ERK1/2 signaling is dispensable for initiation of neural differentiation.

PDGF has a limited mitogenic effect when neural stem cells are in transition to the neuronal lineage (11), but cannot support continuous stem cell proliferation like FGF-2. In accordance, contrary to cell cultures that have been deprived of FGF-2 for 24 h, PDGF-AA-treated cultures maintained detectable levels of phosphorylated ERK2 (Fig. 3C) and expressed MASH1 protein (Fig. 3B). Still after 2 days in the presence of PDGF-AA, MASH1 was expressed, indicative of a delayed differentiation program (Fig. 3C). Both ERK1/2 phosphorylation and MASH1 expression were abrogated on combined treatment with the MEK inhibitor and PDGF-AA. This suggests that the PDGF-AA-stimulated cell division was blocked as a result of ERK pathway inhibition, shifting cell fate more toward differentiation.

MAP2-Positive Neurons Are Formed in the Absence of ERK1/2 Activation

Next we aimed to monitor if differentiation, as measured by morphological criteria and immunostaining of the neuronal marker MAP2, could take place in the absence of phosphorylated ERK1/2. In Fig. 4A, stem cells proliferating in response to FGF-2, representative of the starting cell population, are shown. As previously reported by numerous investigators (reviewed in Refs. (30, 31)), these cells incorporated 5′ -bromo-2′ -deoxyuridine (BrdU) and had no or limited MAP2 expression.

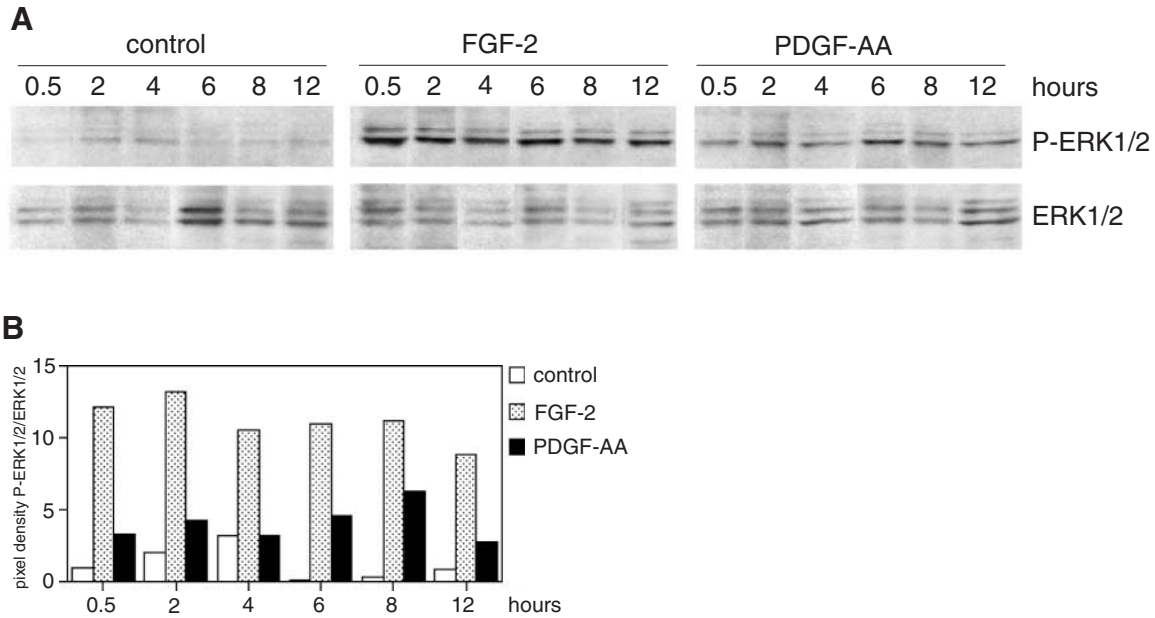

FIGURE 2. FGF-2 and PDGF-AA induce sustained phosphorylation of ERK1 and ERK2 in stem cells. **A.** Cultures were stimulated with FGF-2 or PDGF-AA (100 ng/ml) for different lengths of time, ranging from 30 min to 12 h, and total cell lysate was subjected to Western blot analysis. Control cultures received no growth factor. Immunoblotting was performed using anti-phospho-ERK1/2 antibodies (*upper panel*) and subsequently with antibodies against ERK1/2 (*lower panel*). **B.** Quantification by densitometric scanning of the Western blot shown in **A**. The levels of phosphorylated protein are given as the ratio of P-ERK1/2 to ERK1/2 pixels.

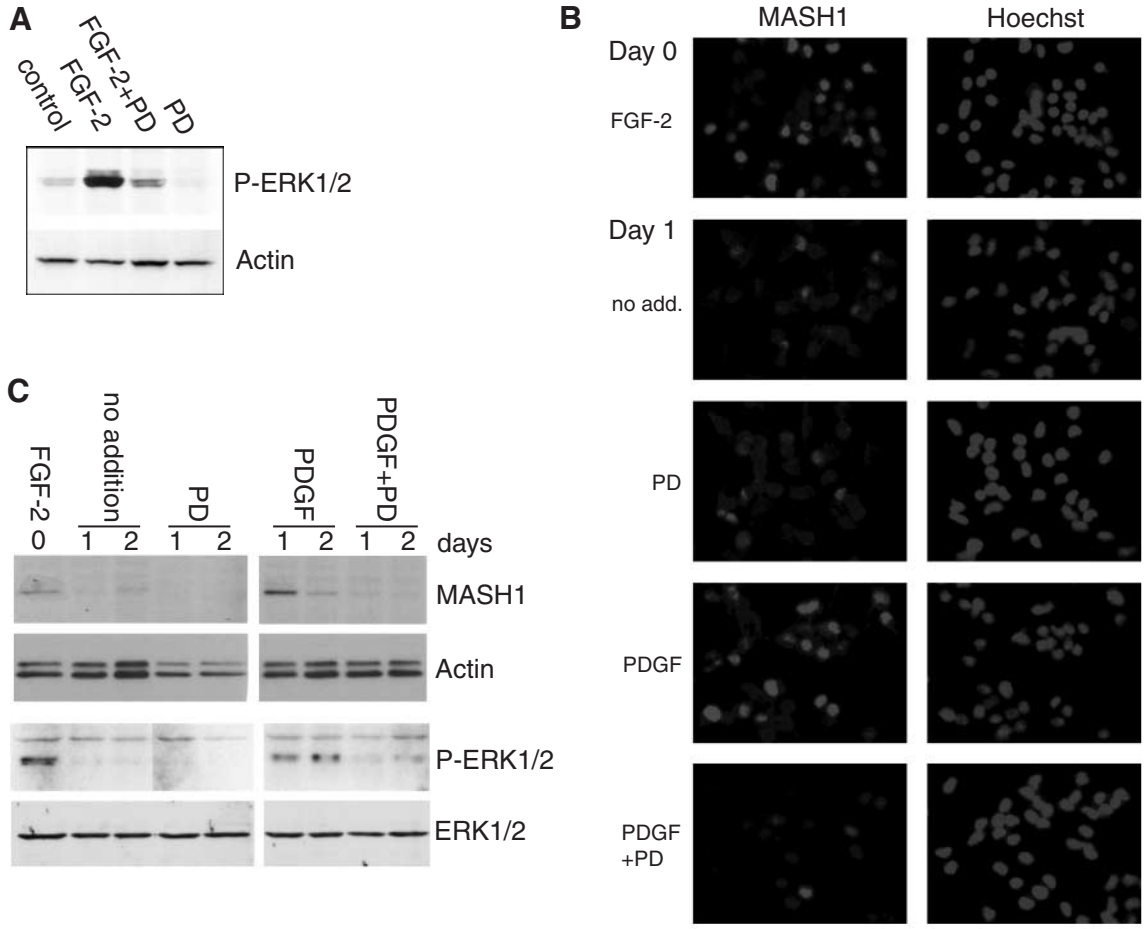

FIGURE 3. Neural stem cells can initiate differentiation without active ERK1/2. **A.** Neural stem cell cultures were either treated or untreated with PD098059 (*PD*, 50 μM) for 30 min, followed by stimulation with FGF-2 (100 ng/ml) for 10 min. Untreated cultures served as control. Total cell lysate was immunoblotted with phospho-ERK1/2 antibodies. To control for equal loading of proteins, antibodies against actin were used. **B.** Neural stem cell cultures were either untreated or treated with PD098059 (*PD*, 25 μM) and/or PDGF-AA (10 ng/ml) for 24 h. Cells proliferating in response to FGF-2 served as a positive control. On fixation, duplicate cultures were stained with anti-MASH1 antibodies, incubated with Hoechst 33342 solution to visualize nuclei, and photographed at ×40 magnification. Data are representative fields from three independent experiments. **C.** Cell lysates from cultures treated as in **B** were subjected to Western blot analysis using antibodies against phosphorylated ERK1/2 and MASH1 protein, respectively. Anti-ERK1/2 and anti-actin antibodies were used to control for equal loading of proteins.

In cultures that were induced to differentiate by withdrawal of FGF-2, MAP2 expression was detectable on the second day as neurite outgrowth begins (Fig. 4B, *open arrowheads*). At 4 days, the processes were longer and more abundant, often with a branched and more elaborate morphology. This morphology was seen also when the MEK inhibitor PD098059 was added at the time of FGF-2 withdrawal (*filled arrowheads*). PDGF-AA-treated cultures expressed MAP2, but did not extend processes even at 4 days (*arrows*) as previously reported (11). The immature appearance of these round MAP2-positive cells was the same in cultures that were simultaneously treated with PD098059 and PDGF-AA.

Fig. 4C shows the quantification of MAP2-positive cells under the same conditions as described for Fig. 4B. The amount of neurons staining positive for MAP2 increased to comprise between 50% and 55% of the cells 2 days after FGF-2 withdrawal. This ratio was kept at 4 days of differentiation. The concomitant addition of PD098059 to differentiating cells did not affect neuronal cell number either at 2 or 4 days post-FGF-2 withdrawal. This supports our initial finding that ERK phosphorylation is not critical for the early differentiation process. An attempt to continue culture in the presence of the MEK inhibitor beyond 4 days to monitor morphological maturation of neurons failed because this severely impaired cell viability. In PDGF-AA-treated cultures, which were grown in the presence or absence of the MEK inhibitor, more cells were MAP2-positive (around 65% after 4 days) than in cultures not receiving PDGF-AA. Whereas the percentage of MAP2-positive cells was equal comparing cultures grown in PDGF-AA with or without PD098059 after 4 days, the total cell number was higher in cultures treated with PDGF-AA alone (98 cells/field) than PDGF-AA and MEK inhibitor (74 cells/field) (Fig. 4D).

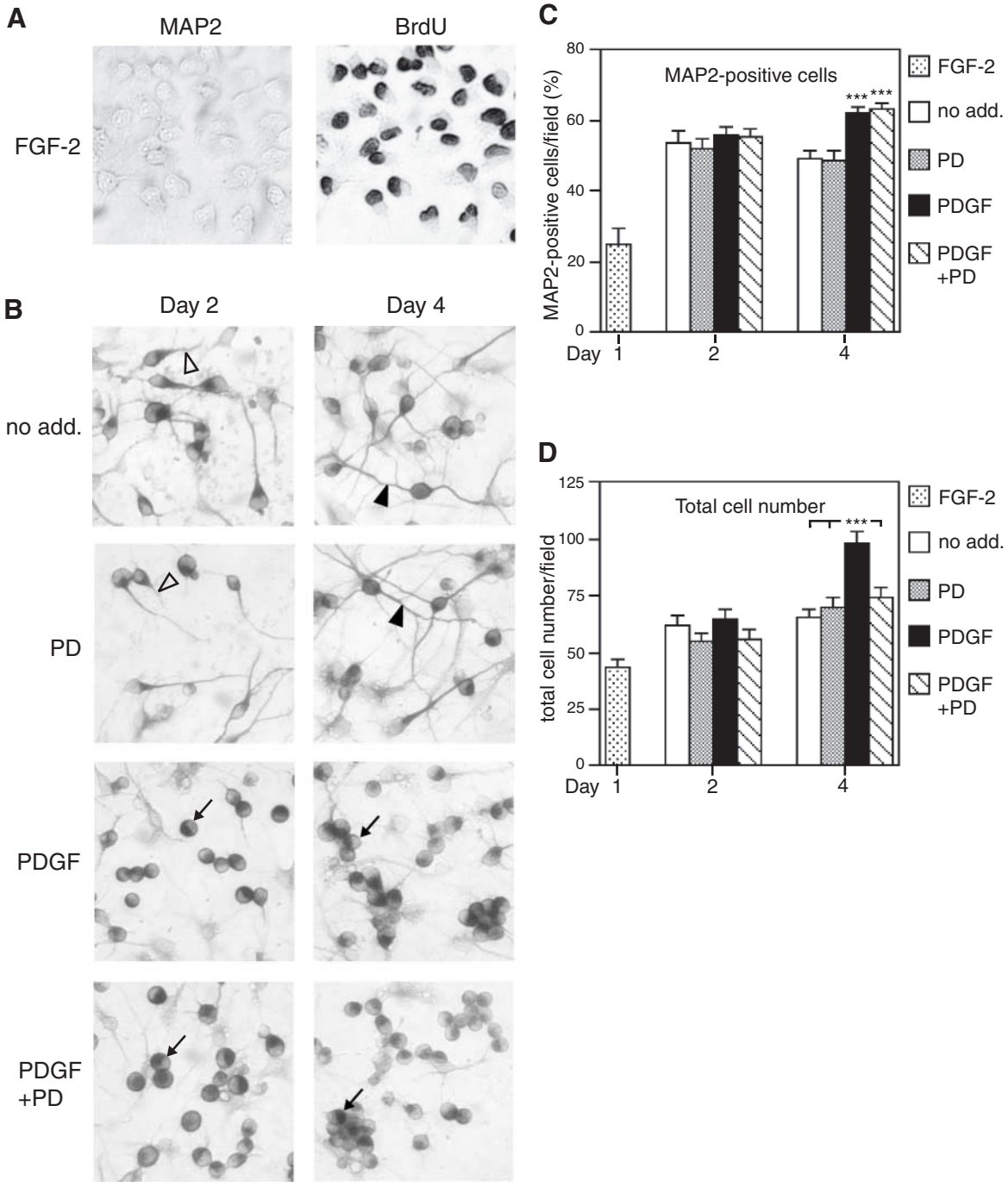

FIGURE 4. MAP2-positive neurons are formed in the absence of active ERK1/2. **A.** Neural stem cells grown in the presence of FGF-2 are BrdU-positive/MAP2-negative. Stem cell cultures were kept in FGF-2 and before fixation, the cultures were exposed to BrdU for 14 h. Duplicate cultures were stained for MAP2 and photographed. After staining with anti-BrdU antibodies, the same fields were photographed again using a computerized microscope. **B.** MAP2 staining of stem cell cultures during differentiation. Cultures were induced to differentiate by withdrawal of FGF-2 in the presence or absence of either the MEK inhibitor PD098059 (*PD*, 25 μM) and/or PDGF-AA (10 ng/ml) for 2 and 4 days. Duplicate cultures were fixed and stained with an antibody to MAP2 and photographed at ×40 magnification. *Open arrowheads,* newly formed processes; *filled arrowheads,* longer branched processes; *arrows,* immature round-shaped MAP2-positive cells. **C.** Quantification of MAP2-positive cells after treatment with PD098059 (*PD*) and/or PDGF-AA during differentiation. The experimental setup was as in **B**. The percentage of MAP2-positive cells/field was counted at ×40 magnification in 8–10 fields and the mean ± SE was calculated. The figures are one out of four independent experiments. ***, $P < 0.001$, comparing PDGF-AA cultures (±PD098059) to control cultures untreated or treated with PD098059. **D.** Total cell number of the experiment presented in **C**. Cells were counted at ×40 magnification in 8–10 fields and the mean ± SE was counted. ***, $P < 0.001$.

Discussion

A critical issue in understanding neuronal differentiation is the response of stem cells to extracellular signals. Primary cells are likely to better reflect the *in vivo* setting than transformed cell lines. Several protocols have been adapted for the study of primary stem cells *in vitro*, in which proliferation and differentiation can be efficiently controlled (reviewed in Refs. (31, 32)). In the presence of the mitogen FGF-2, it is possible to maintain and expand neural stem cells in a multipotent state *in vitro*, and on withdrawal of the mitogen, the cells start to differentiate (32). In previous reports (2, 10), PDGF has been implied to enhance neuronal differentiation and a recent study from our laboratory (11) has defined the role of PDGF-AA as a mitogen for stem cells in their transition from multipotency to committed neuron.

In this study, we aimed to examine the activated signaling cascades on stimulation with FGF-2 or PDGF-AA, because these growth factors affect stem cells differently while their structurally related receptors couple to the same basic signal transduction machinery. The major tyrosine-phosphorylated protein product was identified as ERK2. Our results show that both FGF-2 and PDGF-AA activated ERK1 and ERK2 although PDGF-AA-induced ERK1/2 phosphorylation levels were lower than those induced by FGF-2. Furthermore, we show that ERK1/2 signaling was dispensable for early neuronal differentiation under the conditions used in this study. It should be kept in mind that the total phosphotyrosine pattern is a crude measure of the intracellular signal transduction at a given time point, and can only be used to detect major differences between treatment groups. Therefore, subtle changes undetectable by this method in the level of activation of other signaling molecules may also occur, which affect the cellular outcome.

The ERK signaling pathway universally governs proliferation, differentiation, and cell survival, and activation of different MAP kinases is one of the most rapid cellular responses to growth and differentiation factors, as well as to various other external stimuli (33–35). It has been shown that the genes for ERK1/2 are highly expressed in the CNS (36) and at the protein level, both ERK2 and ERK1 are widely but differently expressed in various regions of the rat brain (37). Previous studies showed that FGF-2 activates ERK2 phosphorylation to a higher extent in embryonic cortical cells than in neurons from neonatal cells (38). This possibly reflects the rapid stem cell expansion in the embryonic cortex *versus* a later relative focus on differentiation.

It has been suggested that a rapid initial *versus* a sustained activation of the Raf/MEK/ERK cascade is distinctly regulated (39). From our studies of MAP kinase phosphorylation, it was evident that both the FGF-2- and PDGF-AA-stimulated ERK1/2 activation follow the sustained kinetics. The possible link between this type of ERK activation and proliferation *versus* differentiation is not clear. Several studies have shown that a sustained activation of MEK and ERK1/2 is sufficient for PC12 phaeochromocytoma cell differentiation (22–25, 40), whereas activation of ERK for a brief time period leads to proliferation (26, 40). However, in the hippocampal cell line, H19-7, prolonged MEK or ERK1/2 activation is not necessary for differentiation (27, 28). Also, neurite outgrowth of chicken dorsal root ganglia can occur in the absence of active ERK (41). Based on the results in this study, we propose that a sustained ERK activation is not necessary for neuronal differentiation.

A previous study of the ERK signaling pathway in neural stem cells shows that CNTF stimulates ERK1/2 phosphorylation with a more rapid kinetics than FGF-2 (4), and that ERK1/2 signaling is necessary for early astrocytic differentiation following CNTF stimulation. To determine precisely the effect on cell lineage commitment as measured by immunostaining of the neuronal marker MAP2, a prolonged exposure to the MEK inhibitor up to 6 days would be desirable. This, however, severely impaired cell survival and we could culture cortical stem cells exposed to PD098059 for no longer than 4 days. The fact that PD098059 had deleterious effects on cell survival may either reflect nonselectivity of the small molecule compound or a role for ERK1/2 to block apoptosis. An early differentiation marker would preferentially monitor the effect of exogenous factors on stem cells, before the expression of classical neuronal antigens appears in high-enough amounts to be detected, and makes experiments with the MEK inhibitor more feasible.

Members of the proneural genes forming the achaete-scute complex in *Drosophila* orchestrate neuronal differentiation and their homologues in mammalian have been characterized (20, 29). MASH1 is expressed in the developing CNS in a transient manner, and its expression is extinguished in postmitotic cells (19). The use of MASH1 antibodies enabled a determination of early effects on neuronal commitment after interfering with ERK1/2 signaling. After withdrawal of the mitogen FGF-2, down-regulation of MASH1 was seen within 24 h. Even in the continuous presence of the MEK inhibitor, PD098059, differentiation proceeded over 2 days as measured by MASH1 down-regulation. This suggests that although ERK1/2 phosphorylation was necessary for stem cell proliferation, it was not critical to the early differentiation process.

By extending the MEK inhibitor treatment for 4 days, we aimed to determine an effect of blocked ERK1/2 activation on formation of MAP2-positive cells. We found no difference in MAP2 cell number either after 2 or 4 days post-FGF-2 withdrawal in the absence or presence of the MEK inhibitor, supporting our data from MASH1 expression experiments. In cortical stem cell cultures that were treated with both PDGF-AA and PD098059, more MAP2-positive cells were found than in untreated cultures or cells being treated with PD098059 alone. Thus, it would seem that ERK1/2 activation is dispensable for early CNS stem cell differentiation.

In a previous work from our laboratory, we have shown that PDGF is a mitogen and survival factor for immature neurons (11). In line with those data, we observed that the total cell number in cultures treated with a combination of PDGF-AA and the MEK inhibitor was lower than in cells treated with PDGF-AA alone. This indicates that the PDGF-AA-induced proliferation and/or survival of MAP2-positive cells were abolished by PD098059 addition. Together with the prolonged expression of MASH1 protein in PDGF-AA-treated cultures, this may indicate that the ERK1/2 signaling pathway plays an important role for the mitogenic/survival effect on CNS stem cells by PDGF-AA, but not for the initial steps of neuronal differentiation.

Materials and Methods

Stem Cell Culture From Embryonic Rat Brain

The cell culture system used in this study has been described in detail (2). Briefly, E15 rat embryonic cortices were dissected from timed-pregnant Sprague-Dawley rats (B&K, Sollentuna, Sweden) in HBSS. The tissue was gently triturated, followed by sedimentation of meninges and larger cell clumps. The cell suspension was pelleted and resuspended in N2 medium (42). Cells were plated onto poly-L-ornithine- and fibronectin-coated tissue culture dishes in N2 medium with 10 ng/ml FGF-2. When subconfluent (6–7 days postdissection), cells were passaged using a cell lifter (Costar Corp., Cambridge, MA) and reseeded.

Cells in their first passage were used for experiments when subconfluent. Before stimulation with FGF-2 or PDGF-AA, the medium was aspired, and the plates washed and returned to the incubator for 3 h without FGF-2 to allow recycling of FGF receptors to the cell surface.

Preparation of Cell Lysates

Cell lysates were performed using either of the following two protocols, depending on the intracellular protein to be extracted.

Triton X-100 Lysis (Phosphotyrosine, Phospho-ERK, and ERK). Cells were harvested by scraping in ice-cold PBS, pelleted, and lysed for 5 min on ice in Triton-X-100 lysis buffer [1% Triton X-100, 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1% aprotinin (Trasylol, Bayer, Leverkusen, Germany), 2 mM phenylmethylsulfonyl fluoride (PMSF), 250 μM Na_3VO_4 (Sigma, St. Louis, MO)]. Lysates were clarified at 10,000 × *g* for 12 min at 4°C and supernatants were stored at −70°C.

2× Laemmli Sample Buffer Lysis (MASH1 and Actin). Cells were washed with ice-cold PBS containing 100 μM Na_3VO_4, harvested by scraping in 100 μl boiling 2× Laemmli sample buffer (43), and sonicated. Cell debris was then removed by centrifugation.

Western Blot Analysis

Total cell lysate (25–35 μg total protein) was boiled with 5% β-mercaptoethanol for 5 min and separated by SDS-PAGE using an 8%- to 16%-gradient Tris-Glycine gel (pre-cast mini-gel, Novex/Invitrogen, Groningen, the Netherlands). For immunoblotting, the samples were electrically transferred onto a nitrocellulose membrane [Hybond enhanced chemiluminescence (ECL) Nitrocellulose membrane, Amersham-Pharmacia Biotech, Uppsala, Sweden]. Non-specific protein binding to the filter was blocked by incubation in PBS containing 5% BSA and 0.05% Tween-20 for 1 h, RT, followed by incubation with primary antibody (overnight, 4°C). The filter was washed in Tris-buffered saline/Tween [TBS-T: 20 mM Tris base (pH 7.6), 137 mM NaCl, 0.2% Tween 20] and incubated with peroxidase-conjugated secondary antibody for 1 h, RT. After washing the filter in TBS-T, the filter was subjected to ECL detection (Amersham-Pharmacia Biotech).

Before incubation with other antibodies, the membrane was washed [62.5 mM Tris-HCl (pH 6.8), 2% SDS, 100 mM β-mercaptoethanol] at 50°C for 30 min.

Immunocytochemistry

For immunofluorescence staining with anti-MASH1 antibodies, duplicate cell cultures, which were grown on poly-L-ornithine- and fibronectin-coated glass coverslips, were fixed in 3% paraformaldehyde. The cultures were permeabilized (0.2% Triton X-100 in PBS, 10 min) and blocked [10% normal goat serum (NGS, DAKO A/S, Copenhagen, Denmark) and 0.1% BSA in PBS-T (0.1% Tween 20), 1 h] followed by overnight incubation with primary antibody (diluted in PBS-T containing 5% NGS/0.1% BSA/0.1% Triton X-100) in a humidified chamber, 4°C. The coverslips were washed in PBS and incubated with secondary antibody for 1 h. To visualize the cell nucleus, the coverslips were incubated with Hoechst 33342 (Molecular Probes, Eugene, OR) for 10 min after which they were washed with PBS-T and mounted with Vectashield Mounting Medium (Vector Laboratories, Inc., Burlingame, CA). Cells were photographed at ×40 magnification.

For MAP2 immunostaining, duplicate cell cultures were fixed in ice-cold acid ethanol (90% ethanol, 5% HAc, 5% H_2O). Endogenous peroxidase was quenched by 0.3% H_2O_2 in methanol. After washing with PBS, the cultures were permeabilized and blocked [0.1% Triton X-100 and 5% NGS (DAKO A/S) in PBS] for 30 min. Primary antibody was added for 1 h, RT, after which the culture dishes were washed in PBS. Secondary antibody was incubated for 30 min to 1 h. The reaction was developed using a streptavidin peroxidase kit (ABComplex, DAKO A/S) followed by diaminobenzidine (DAB SigmaFast, Sigma). Cells were counted at ×40 magnification and the ratio of MAP2-positive cells to total cell number was calculated on 8–10 parallel fields per plate. For detection of BrdU incorporation, a cell proliferation kit (Amersham-Pharmacia Biotech) was used.

For statistical analysis, we used an ANOVA software (StatView).

Materials

The materials used in this study were from the following sources: HBSS and fibronectin from Life Technologies Ltd. (Paisley, Scotland), poly-L-ornithine and the MEK inhibitor PD098059 (dissolved in DMSO) from Sigma. Recombinant FGF-2 and PDGF-AA were from Pepro Technologies (London, England).

For Western blot analysis, Rainbow RPN 800 and ^{14}C-Rainbow RPN 756 (Amersham-Pharmacia Biotech) were used as molecular weight markers. Antibodies used were PY20 (monoclonal anti-mouse, dilution 1:1000; Transduction Laboratories, San Diego, CA), phospho-specific MAPK antibody (polyclonal anti-rabbit, dilution 1:1000; New England Biolabs, Beverly, MA), EET (polyclonal anti-rabbit, against ERK1/2, dilution 1:300; a kind gift from the Ludwig Institute for Cancer Research, Uppsala, Sweden), ERK1 (polyclonal anti-rabbit, against ERK1/2, dilution 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), MASH1 antibody (monoclonal anti-mouse, dilution 1:250; PharMingen, San Diego, CA), and actin antibody (polyclonal anti-goat, dilution 1:200; Santa Cruz Biotechnology). The secondary antibodies used were peroxidase-conjugated sheep anti-mouse immunoglobulin,

peroxidase-conjugated donkey anti-rabbit immunoglobulin (dilution 1:5000; Amersham-Pharmacia Biotech), and peroxidase-conjugated rabbit anti-goat immunoglobulin (dilution 1:5000; DAKO A/S).

For immunocytochemistry, we used anti-MASH1 antibodies (monoclonal, dilution 1:5, a kind gift from Dr. F. Guillemot, IGBMC, CNRS/INSERM, Communauté Urbaine de Strasbourg, France) and anti-MAP2 antibodies (monoclonal, clone HM2, dilution 1:200; Sigma) as primary antibodies and Alexa488 goat anti-mouse highly cross-adsorbed immunoglobulin (Molecular Probes) and biotinylated goat anti-mouse immunoglobulin (Kirkegaard and Perry Laboratories, Gaithersburg, MD) as secondary antibodies.

Acknowledgments

The authors honor the memory of the late Meta Lindström and acknowledge her excellent technical assistance. We also thank Dr. L. Claesson-Welsh for valuable advice on experimental outline.

References

1. Cavanagh, J. F., Mione, M. C., Pappas, I. S., and Parnavelas, J. G. Basic fibroblast growth factor prolongs the proliferation of rat cortical progenitor cells *in vitro* without altering their cell cycle parameters. Cereb. Cortex, *7:* 293–302, 1997.

2. Johe, K. K., Hazel, T. G., Muller, T., Dugich Djordjevic, M. M., and McKay, R. D. Single factors direct the differentiation of stem cells from the fetal and adult central nervous system. Genes Dev., *10:* 3129–3140, 1996.

3. Bonni, A., Sun, Y., Nadal-Vicens, M., Bhatt, A., Frank, D., Rozovsky, I., Stahl, N., Yancopoulos, G., and Greenberg, M. Regulation of gliogenesis in the central nervous system by the JAK-STAT signaling pathway. Science, *278:* 477–483, 1997.

4. Rajan, P. and McKay, R. D. Multiple routes to astrocytic differentiation in the CNS. J. Neurosci., *18:* 3620–3629, 1998.

5. Gross, R. E., Mehler, M. F., Mabie, P. C., Zang, Z., Santschi, L., and Kessler, J. A. Bone morphogenetic proteins promote astroglial lineage commitment by mammalian subventricular zone progenitor cells. Neuron, *17:* 595–606, 1996.

6. Mujtaba, T., Piper, D. R., Kalyani, A., Groves, A. K., Lucero, M. T., and Rao, M. S. Lineage-restricted neural precursors can be isolated from both the mouse neural tube and cultured ES cells. Dev. Biol., *214:* 113–127, 1999.

7. Tsai, R. Y. and McKay, R. D. Cell contact regulates fate choice by cortical stem cells. J. Neurosci., *20:* 3725–3735, 2000.

8. Heldin, C. H. and Westermark, B. Mechanism of action and *in vivo* role of platelet-derived growth factor. Physiol. Rev., *79:* 1283–1316, 1999.

9. Betsholtz, C., Karlsson, L., and Lindahl, P. Developmental roles of platelet-derived growth factors. BioEssays, *23:* 494–507, 2001.

10. Williams, B. P., Park, J. K., Alberta, J. A., Muhlebach, S. G., Hwang, G. Y., Roberts, T. M., and Stiles, C. D. A PDGF-regulated immediate early gene response initiates neuronal differentiation in ventricular zone progenitor cells. Neuron, *18:* 553–562, 1997.

11. Erlandsson, A., Enarsson, M., and Forsberg-Nilsson, K. Immature neurons from CNS stem cells proliferate in response to PDGF. J. Neurosci., *21:* 3483–3491, 2001.

12. Heldin, C-H. and Purton, M. Signal Transduction. London: Chapman & Hall, 1996.

13. Jaye, M., Schlessinger, J., and Dionne, C. A. Fibroblast growth factor receptor tyrosine kinases: molecular analysis and signal transduction. Biochim. Biophys. Acta, *1135:* 185–199, 1992.

14. Schlessinger, J. How receptor tyrosine kinases activate Ras. Trends Biochem. Sci., *18:* 273–275, 1993.

15. Hill, C. S. and Treisman, R. Transcriptional regulation by extracellular signals: mechanisms and specificity. Cell, *80:* 199–211, 1995.

16. Janknecht, R., Ernst, W. H., Pingoud, V., and Nordheim, A. Activation of ternary complex factor Elk-1 by MAP kinases. EMBO J., *12:* 5097–5104, 1993.

17. Kortenjann, M., Thomae, O., and Shaw, P. E. Inhibition of v-*raf*-dependent c-*fos* expression and transformation by a kinase-defective mutant of the mitogen-activated protein kinase Erk2. Mol. Cell. Biol., *14:* 4815–4824, 1994.

18. Guillemot, F., Lo, L. C., Johnson, J. E., Auerbach, A., Anderson, D. J., and Joyner, A. L. Mammalian achaete-scute homolog 1 is required for the early development of olfactory and autonomic neurons. Cell, *75:* 463–476, 1993.

19. Horton, S., Meredith, A., Richardson, J. A., and Johnson, J. E. Correct coordination of neuronal differentiation events in ventral forebrain requires the bHLH factor MASH1. Mol. Cell. Neurosci., *14:* 355–369, 1999.

20. Porteus, M. H., Bulfone, A., Liu, J. K., Puelles, L., Lo, L. C., and Rubenstein, J. L. DLX-2, MASH-1, and MAP-2 expression and bromodeoxyuridine incorporation define molecularly distinct cell populations in the embryonic mouse forebrain. J. Neurosci., *14:* 6370–6383, 1994.

21. Torii, M., Matsuzaki, F., Osumi, N., Kaibuchi, K., Nakamura, S., Casarosa, S., Guillemot, F., and Nakafuku, M. Transcription factors Mash-1 and Prox-1 delineate early steps in differentiation of neural stem cells in the developing central nervous system. Development, *126:* 443–456, 1999.

22. Cowley, S., Paterson, H., Kemp, P., and Marshall, C. J. Activation of MAP kinase kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH 3T3 cells. Cell, *77:* 841–852, 1994.

23. Fukuda, M., Gotoh, Y., Tachibana, T., Dell, K., Hattori, S., Yoneda, Y., and Nishida, E. Induction of neurite outgrowth by MAP kinase in PC12 cells. Oncogene, *11:* 239–244, 1995.

24. Klesse, L. J., Meyers, K. A., Marshall, C. J., and Parada, L. F. Nerve growth factor induces survival and differentiation through two distinct signaling cascades in PC12 cells. Oncogene, *18:* 2055–2068, 1999.

25. York, R. D., Yao, H., Dillon, T., Ellig, C. L., Eckert, S. P., McCleskey, E. W., and Stork, P. J. Rap1 mediates sustained MAP kinase activation induced by nerve growth factor [see comments]. Nature, *392:* 622–626, 1998.

26. Traverse, S., Seedorf, K., Paterson, H., Marshall, C. J., Cohen, P., and Ullrich, A. EGF triggers neuronal differentiation of PC12 cells that overexpress the EGF receptor. Curr. Biol., *4:* 694–701, 1994.

27. Kuo, W. L., Abe, M., Rhee, J., Eves, E. M., McCarthy, S. A., Yan, M., Templeton, D. J., McMahon, M., and Rosner, M. R. Raf, but not MEK or ERK, is sufficient for differentiation of hippocampal neuronal cells. Mol. Cell. Biol., *16:* 1458–1470, 1996.

28. Kuo, W. L., Chung, K. C., and Rosner, M. R. Differentiation of central nervous system neuronal cells by fibroblast-derived growth factor requires at least two signaling pathways: roles for Ras and Src. Mol. Cell. Biol., *17:* 4633–4643, 1997.

29. Lo, L. C., Johnson, J. E., Wuenschell, C. W., Saito, T., and Anderson, D. J. Mammalian achaete-scute homolog 1 is transiently expressed by spatially restricted subsets of early neuroepithelial and neural crest cells. Genes Dev., *5:* 1524–1537, 1991.

30. Gage, F. H. Stem cells of the central nervous system. Curr. Opin. Neurobiol., *8:* 671–676, 1998.

31. McKay, R. Stem cells in the central nervous system. Science, *276:* 66–71, 1997.

32. Gage, F. H. Mammalian neural stem cells. Science, *287:* 1433–1438, 2000.

33. Blenis, J. Signal transduction via the MAP kinases: proceed at your own RSK. Proc. Natl. Acad. Sci. USA, *90:* 5889–5892, 1993.

34. Cobb, M. H., Boulton, T. G., and Robbins, D. J. Extracellular signal-regulated kinases: ERKs in progress. Cell Regul., *2:* 965–978, 1991.

35. Cobb, M. H., Robbins, D. J., and Boulton, T. G. ERKs, extracellular signal-regulated MAP-2 kinases. Curr. Opin. Cell Biol., *3:* 1025–1032, 1991.

36. Fukunaga, K. and Miyamoto, E. Role of MAP kinase in neurons. Mol. Neurobiol., *16:* 79–95, 1998.

37. Ortiz, J., Harris, H. W., Guitart, X., Terwilliger, R. Z., Haycock, J. W., and Nestler, E. J. Extracellular signal-regulated protein kinases (ERKs) and ERK kinase (MEK) in brain: regional distribution and regulation by chronic morphine. J. Neurosci., *15:* 1285–1297, 1995.

38. Ghosh, A. and Greenberg, M. E. Distinct roles for bFGF and NT-3 in the regulation of cortical neurogenesis [see comments]. Neuron, *15:* 89–103, 1995.

39. Howe, A. K. and Juliano, R. L. Distinct mechanisms mediate the initial and sustained phases of integrin-mediated activation of the Raf/MEK/mitogen-activated protein kinase cascade. J. Biol. Chem., *273:* 27268–27274, 1998.

40. Qui, M. S. and Green, S. H. PC12 cell neuronal differentiation is associated with prolonged p21*ras* activity and consequent prolonged ERK activity. Neuron, *9:* 705–717, 1992.

41. Klinz, F. J., Wolff, P., and Heumann, R. Nerve growth factor-stimulated mitogen-activated protein kinase activity is not necessary for neurite outgrowth of chick dorsal root ganglion sensory and sympathetic neurons. J. Neurosci. Res., *46:* 720–726, 1996.

42. Bottenstein, J. E. and Sato, G. H. Growth of a rat neuroblastoma cell line in serum-free supplemented medium. Proc. Natl. Acad. Sci. USA, *76:* 514–517, 1979.

43. Sambrook, J., Fritsch, E. F., and Maniatis, T. Molecular Cloning: A Laboratory Manual, 2nd Edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1989.