PITUITARY ADENYLATE CYCLASE-ACTIVATING POLYPEPTIDE
INDUCES ASTROCYTE DIFFERENTIATION OF PRECURSOR CELLS
FROM DEVELOPING CEREBRAL CORTEX

Inmaculada Vallejo* and Mario Vallejo

Endocrine Unit, Massachusetts General Hospital, Harvard Medical School, Boston, and
Instituto de Investigaciones Biomédicas “Alberto Sols”, Consejo Superior de
Investigaciones Científicas/Universidad Autónoma de Madrid, Calle Arturo Duperier 4,
28029 Madrid, Spain. *Present address: Laboratorio de Microbiología, Facultad de
Ciencias del Mar, Universidad de Cádiz, 11510 Puerto Real (Cádiz), Spain.

Running Title: Astrocyte differentiation promoted by PACAP

Correspondence:
Mario Vallejo, M.D., Ph.D.
Instituto de Investigaciones Biomédicas “Alberto Sols”
Calle Arturo Duperier, 4
28029 Madrid, Spain
Tel: 34-91-585 4890
Fax: 34-91-585 4587
e-mail: mvallejo@iib.uam.es
ABSTRACT

Ciliary neurotrophic factor and bone morphogenetic proteins induce astrocytogenesis in the developing rat brain by stimulating STAT- and Smad-dependent signaling, respectively. We previously found that stimulation of the cAMP-dependent signaling pathway also triggers differentiation of cerebral cortical precursor cells into astrocytes, providing an additional mechanism to promote astrocyte differentiation. In this study, we show that pituitary adenylate cyclase-activating polypeptide (PACAP), but not the related vasoactive intestinal peptide, induces astrocyte differentiation of cortical precursor cells, even after a transient exposure. Cortical precursors were found to express predominantly the short isoform of the PACAP-specific PAC1 receptor, which couples to adenylate cyclase. Consistent with this notion, we determined that exposure of cortical precursors to PACAP resulted in a dose-dependent increase in cAMP production. Pretreatment of cells with the cAMP antagonist Rp-cAMPS prevented astrocyte differentiation. Thus, PACAP acts as an extracellular signal to trigger cortical precursor cell differentiation into astrocytes via stimulation of intracellular cAMP production.
INTRODUCTION

During development, the central nervous system becomes populated by cells that arise from neuroepithelial precursors that proliferate in the ventricular zone of the neural tube. Those cells, generated through several rounds of cell division, differentiate to yield different types of neurons and glial cells, following a tightly regulated spatial and temporal arrangement. It has become widely accepted that neural progenitor cells that proliferate in the embryonic central nervous system are pluripotent. By virtue of this property, they have the capacity to generate different neuronal or glial phenotypes in response to specific extracellular signals that instruct them to follow specific pathways of differentiation. Thus, phenotypic diversity of cells in the central nervous system appears to be due to a large extent to the nature of the neurotrophic factors that act on precursor cells at a particular time during development.

In the developing rat brain, production of neurons takes place during a period of time spanning from embryonic day 13 (E13) to E19, approximately, whereas generation of astrocytes takes place towards the end of the gestational period and proceeds postnatally (Jacobson, 1993). Several neurotrophic factors have been discovered that limit the differentiation capacity of progenitor cells and trigger their phenotypic transition toward neuronal or glial lineages. These neurotrophic factors regulate neurogenesis, gliogenesis and proliferation of neural cells, and include, among others, basic fibroblast growth factor (bFGF), ciliary neurotrophic factor (CNTF), bone morphogenetic proteins (BMPs), platelet-derived growth factor (PDGF), neurotrophin 3, brain-derived neurotrophic factor (BDNF) and neuregulins (Bonni et al., 1997; Gross et al., 1996; Johe et al., 1996; Park et al., 1999; Buonanno and Fischbach, 2001).
Among neurotrophic factors discovered so far, at least two, CNTF (Bonni et al., 1997; Johe et al., 1996; Park et al., 1999; Shimazaki et al., 2001) and BMP-2 (Gross et al., 1996; Mabie et al., 1999; Nakashima et al., 2001) appear to be major signaling factors that act on neural precursors to induce astrocyte differentiation during brain development. Integrity of receptors for CNTF is required to ensure adequate intracellular signaling to generate proper number of astrocytes, as indicated by targeted gene disruption via homologous recombination in mice (Koblar et al., 1998; Nakashima et al., 1999; Ware et al., 1995). Intracellular signaling by CNTF relies on the activation by phosphorylation of the receptor-associated JAK and STAT proteins, which results in the stimulation of GFAP gene expression mediated by the transcriptional transactivation properties of STAT1 and STAT3 (Bonni et al., 1997; Nakashima et al., 1999; Park et al., 1999; Rajan and McKay, 1998; Sun et al., 2001). Absence of astrocyte differentiation in cells that lack receptors for CNTF can be overcome by treatment with BMP-2 (Koblar et al., 1998), indicating that neural precursors are able to respond to different astrocyte differentiation stimuli through the activation of independent intracellular signaling pathways. The signaling pathway activated by members of BMP family involves activation of Smad transcription factors (Ebendal et al., 1998; Zhang et al., 1998), that can act on the GFAP promoter independently or in synergy with CNTF-activated STAT proteins to induce astrocyte differentiation (Nakashima et al., 1999; Sun et al., 2001).

Previous studies carried out in our laboratory led to the identification of an additional or alternative mechanism for the generation of cortical astrocytes from undifferentiated precursor cells based on the activation of the cAMP-dependent signal transduction pathway (McManus et al., 1999). We found that treatment of cortical precursors maintained in vitro with the cAMP analog 8Br-cAMP or with forskolin
results in a potent antimitotic effect and an increase in GFAP gene expression, along with changes in the cellular morphology that accompany the differentiation response. Those studies suggested the possible existence of extracellular factors in the developing cortex that promote astrocytogenesis by acting on G protein-coupled receptors that activate cAMP-dependent signaling. In the present study, we report that treatment of cortical precursor cells with pituitary adenylate cyclase activating polypeptide (PACAP), a member of the vasoactive intestinal peptide (VIP)/secretin/glucagon family, results in elevated levels of cAMP and astrocyte differentiation.

**RESULTS**

**Exposure of cortical precursor cells to brain monoamines does not induce astrocyte differentiation**

Based on our previous observation that cAMP stimulation induces astrocyte differentiation of cortical precursor cells, we sought to identify extracellular factors, such as monoamines and peptides, that could exert this action during development by acting on G protein-coupled receptors in the embryonic central nervous system. The monoamines serotonin, noradrenaline and dopamine are present in axons that originate in the brainstem and midbrain, reaching the developing cerebral cortex before the onset of gliogenesis. Receptors for these monoamines are expressed in neural precursors in the developing brain, and there is evidence indicating that they have effects on cortical development (Lauder, 1993; Lavdas et al., 1997; Levitt et al., 1997; Naqui et al., 1999). For this reason, we tested whether primary precursor cells from embryonic rat cortex differentiate into astrocytes after exposure to these substances.

Primary cortical cells prepared from E17 rat brains cultured in defined medium in the presence of bFGF (20 ng/ml) are proliferating, nestin-positive neuroepithelial
precursors (McManus et al., 1999). We treated these cells with serotonin, noradrenaline or dopamine for two days after withdrawal of bFGF. As positive controls, we used cells treated in parallel with 8Br-cAMP (McManus et al., 1999). Processing of these cells for immunocytochemistry for GFAP revealed that astrocyte differentiation, evidenced by morphological changes and GFAP immunoreactivity, was detected only in cells treated with 8Br-cAMP, but not in cells treated with any of the monoamines (Fig. 1A). This lack of effect of monoamines was not likely to be due to absence of appropriate membrane receptors on cultured cortical precursors, because we observed phosphorylation of transcription factor CREB within minutes of treatment with 8Br-cAMP or monoamines, as evidenced by western immunoblot with a specific anti-phospho-CREB antiserum (Fig. 1B).

**PACAP induces astrocyte differentiation of cortical precursor cells**

We then turned our attention to neuropeptides, as a role for some of them in neural development has been suggested (Cameron et al., 1998). Among them, we considered PACAP and the closely related VIP (vasoactive intestinal peptide), because PACAP is known to increase intracellular levels of cAMP by activating adenylate cyclase (D'Agata et al., 1996; Grimaldi and Cavalaro, 1999; Lu and DiCicco-Bloom, 1997; Spengler et al., 1993; Vaudry et al., 1998).

As in previous experiments, primary cortical cells from E17 rat brains were cultured in defined medium in the presence of bFGF for at least 24 hours after plating, and bFGF was withdrawn prior to treatment with PACAP. Exposure of cells to PACAP resulted in a concentration-dependent increase in the number of GFAP-positive cells generated over a period of two days in culture (Fig. 2A). In contrast with the results obtained with PACAP, cells treated in parallel with the structurally related VIP did not
exhibit morphological changes and did not become immunoreactive for GFAP (Fig. 2A). The observed GFAP immunoreactivity was correlated with an increase in GFAP expression by Western immunoblot (Fig. 2B).

Immunocytochemical analyses confirmed that withdrawal of bFGF did not significantly alter nestin immunostaining, and that the number of cells that appeared positive for GFAP was less than 7% after two days, consistent with our own previous studies (McManus et al., 1999). In contrast, in dishes in which bFGF had been replaced with PACAP (100 nM), most cells had become GFAP-positive after 2 days, and had undergone significant morphological changes, acquiring a stellate morphology typical of astrocytes and extending cellular processes (Fig. 3). These processes developed with time, becoming morphologically more elaborate and reaching lengths of up to 50-100 µm after 5 days. In addition, treatment of cortical precursor cells with PACAP led to downregulation of the neuroepithelial precursor marker nestin (Fig. 3). Finally, PACAP-treated cells also expressed S100β but did not become immunoreactive for MAP-2, NCAM, calbindin, or galactocerebroside (not shown), suggesting that PACAP acts on cortical precursor cells to promote astrocyte differentiation without influencing neuronal or oligodendroglial lineages.

It has been reported that short exposure of neural precursor cells to specific neurotrophic signals is sufficient to induce their differentiation towards neuronal or glial phenotypes, a response that persists even after the inducing agent has been withdrawn (Park et al., 1999; Williams et al., 1997). To determine whether transient exposure to PACAP can induce astrocyte differentiation, cortical precursors were treated with PACAP for different periods of time after bFGF withdrawal. Processing of cells for GFAP immunocytochemistry revealed that PACAP can initiate astrocyte
differentiation even after a pulse of treatment lasting only 30 minutes, as assessed two
days after the onset of treatment (Fig. 4).

**PACAP decreases proliferation of cortical precursors**

To determine whether primary cortical cells prepared from E17 rat brains
decrease their proliferation rate in response to PACAP, as has been described for neural
precursors derived from embryos obtained at an earlier developmental stage, we
determined the relative number of cells that incorporate bromodeoxyuridine (BrdU) in
the presence or absence of PACAP. In these experiments, cells were treated with
PACAP (100 nM) for 16 hours in the presence of bFGF, and BrdU (10 μM) was added
during the last 5 hours. When cells were maintained in the presence of bFGF alone, the
percentage of them that incorporated BrdU was 67 ± 6.2 (mean ± s.e.m.). In contrast, in
the presence of PACAP, the percentage of cells labeled with BrdU was reduced to 36 ±
2.9. This effect was specific, because a similar treatment with VIP (100 nM) resulted in
a proportion of BrdU-labeled cells not significantly different from controls (60 ± 5.1%).
These results are consistent with previous findings showing that PACAP provides a
potent antimitotic signal for cortical precursor cells (Lu and DiCicco-Bloom, 1997;
Nicot and DiCicco-Bloom, 2001; Suh et al., 2001; Carey et al., 2002), and with our
previous observations of the antiproliferative effects of cAMP stimulation on neural
precursors (McManus et al., 1999).

**Cortical precursor cells express PACAP type I receptors**

The capacity of PACAP to induce astrocyte differentiation suggests the presence
of specific receptors whose activation is responsible for this effect. Thus, we initiated
experiments to characterize PACAP receptor isoforms expressed by these cells.
PACAP can bind specifically to G protein-coupled receptors encoded by at least three different genes. These receptors are known as PAC1, which is selective for PACAP, and VPAC1 and VPAC2, both of which exhibit similar affinities for VIP and PACAP (reviewed in Harmar et al., 1998). To determine whether one or more of these receptors are expressed in primary cortical precursor cells, we carried out RT-PCR/Southern blot hybridization experiments for the amplification of specific transcripts corresponding to each type of receptor. These experiments indicated that cortical precursor cells express predominantly the PACAP-selective PAC1 receptor (Fig. 5). Significantly lower levels of VPAC2 receptor transcripts were observed, whereas transcripts encoding the VPAC1 receptor were undetectable (Fig. 5).

In G protein-coupled receptors with seven transmembrane-spanning domains, the third intracellular loop regulates coupling to G proteins that link to second messenger pathways (Strader et al., 1995). The PAC1 receptor exhibits different isoforms depending on the presence or absence of one or two 28 amino acid domains, named Hip and Hop, inserted into the third intracellular loop (Spengler et al., 1993) (Fig. 6A). The Hip and Hop domains, are encoded by two different exons, and affect the signal transduction pathways activated upon peptide binding to the PAC1 receptor (Spengler et al., 1993). To determine which one of the PAC1 receptor isoforms are expressed in primary cortical precursor cells, we carried out RT-PCR/Southern blot hybridization experiments, using specific oligonucleotides as PCR primers or internal probes, as indicated in Fig. 6A.

These experiments revealed that the most abundant transcripts in these cells correspond to the short isoform that lacks Hip and Hop domains (Fig. 6B), although transcripts with one of these two domains, mostly the Hop domain, were also detected (Fig. 6B). Notably, we observed that treatment of cortical precursors with 1 mM 8Br-
cAMP, which induces astrocyte differentiation of these cells as described above, results in a disappearance of the band corresponding to the Hip or Hop isoforms, and in an apparent increase in the intensity of the band corresponding to the short receptor isoform (Fig. 6C). These results indicate that differentiation of astrocytes is accompanied by changes in the relative proportion of PAC1 receptor isoforms such that the expression of the short isoform, which couples to cAMP production, is favored over the expression of the Hip or Hop isoforms, which also activate the polyphosphoinositide-specific phospholipase C-dependent signaling pathway (Nicot and DiCicco-Bloom, 2001; Spengler et al., 1993). This circumstance could be important during neural development, as the short PAC1 receptor isoform has been associated to inhibition of proliferation of neural cells, and the PAC1Hop isoform with proliferative effects (Nicot and DiCicco-Bloom, 2001).

**Exposure of cortical precursor cells to PACAP results in elevated levels of intracellular cAMP**

As mentioned earlier, PACAP receptors use the cAMP-dependent pathway as a major intracellular signaling mechanism of peptide activation. Therefore, we determined intracellular levels of cAMP in primary cultures of cortical precursor cells following treatment with PACAP. For these experiments cells were treated with PACAP or VIP in the presence of 2 mM isobutylmethylxantine (IBMX). Control cells were treated with IBMX only. Cells were harvested 15 minutes after the onset of treatment, and cAMP levels were determined by radioimmunoassay, using a cAMP-specific antiserum provided by Dr. E. Schipani (Massachusetts General Hospital, Boston).
Levels of intracellular cAMP were undetectable in control cells but increased significantly after exposure of cells to PACAP (Fig. 7A). Treatment of cells with VIP also resulted in increased levels of cAMP in these cells, but this response was significantly smaller than the one observed with PACAP (Fig. 7A).

To further support the notion that the differentiating response of cortical precursors to PACAP is due to the observed increase in intracellular levels of cAMP, we investigated whether blockade of intracellular cAMP effectors with the cAMP-specific antagonist Rp-cAMPS would prevent the differentiation of astrocytes by PACAP. We observed that cells pretreated with Rp-cAMPS (100 µM) added 5 minutes before PACAP (100 nM), did not differentiate into astrocytes, as did those treated only with PACAP (Fig. 7B).

DISCUSSION

Our study shows that PACAP can induce astrocyte differentiation of cortical precursor cells. This effect is due to the stimulation of intracellular cAMP levels following activation of PAC1 receptors, a notion supported by the expression of this type of receptors on the target cells, by the observed elevation of intracellular levels of cAMP within minutes of the onset of PACAP treatment, and by inhibition of PACAP-induced astrocytogenesis by the cAMP antagonist Rp-cAMPS. Together with our previous studies indicating that cAMP-stimulation induces astrocyte differentiation of cortical precursor cells instructively (McManus et al., 1999), our data provide evidence in support of the notion that the PACAP-cAMP pathway provides an alternative signaling route to astrocyte differentiation in addition to those provided by the previously described CNTF-STAT and BMP-Smad signaling pathways.
The notion that PACAP can influence cortical astrocyte differentiation is also consistent with the observed expression of both PACAP and PACAP receptors in the developing cortex. Thus, studies carried out by Skoglosa et al. (1999), Jaworski and Proctor (2000); and Suh et al. (2001) using both in situ hybridization and immunohistochemistry show that in the developing cortex both PACAP and the PAC1 receptor are expressed at significantly high levels in the ventricular zone during late gestation and postnatally. Since the ventricular zone contains proliferating precursor cells that differentiate during cortical development, and astroglial cells are generated there at a time coincident with PACAP and PAC1 receptor expression, it is likely that in vivo PACAP influences cortical gliogenesis.

Differentiation to acquire specific cellular phenotypes in the developing brain coincides with the withdrawal of neuroepithelial precursors from the cell cycle. Therefore, it is possible that the same factors that instruct cells towards differentiation act also as signals to inhibit mitosis. This would occur during development even in the presence of endogenous mitogens, such as bFGF, that ensure the generation of appropriate numbers of cells. Although PACAP can stimulate proliferation of specific types of neural cells (Lee et al., 2001; Lu et al., 1998), it is well documented, and our data confirm, that this peptide has a potent antimitotic effect on cortical precursor cells of the developing central nervous system (Carey et al., 2002; Lu and DiCicco-Bloom, 1997; Lu et al., 1998; Nicot and DiCicco-Bloom, 2001; Suh et al., 2001).

Previous studies indicate that PACAP can exert different developmental actions in the central nervous system (Lee et al., 2001; Lu and DiCicco-Bloom, 1997; Zupan et al., 1998). Recently, in experiments involving transuterine intraventricular administration of PACAP into developing rat brains, Suh et al. (Suh et al., 2001) suggested that PACAP affects neuronal lineages without altering gliogenesis or
precursor cell fate in the cerebral cortex. This conclusion was based on the observation that in PACAP-treated animals, subsequent administration of BrdU resulted in the appearance of cortical plate cells in which BrdU immunoreactivity colocalized with neuronal markers, but not with glial markers. However, this observation appears to be consistent with an effect of PACAP on neural precursors that would differentiate into astrocytes. Indeed, if PACAP inhibits proliferation by preventing entry into S-phase (Suh et al., 2001) and induces astrocyte differentiation in vivo, one would expect to find that GFAP-positive cells are not immunopositive for BrdU.

Studies in which neuronal differentiation of cortical precursors was observed to be enhanced by PACAP were performed using cells derived from E13-E14.5 embryos (Carey et al., 2002; Lu and DiCicco-Bloom, 1997; Suh et al., 2001), a time when neurogenesis is actively taking place. In the present study, cells were taken from older (E17) embryos, at a time close to the onset of gliogenesis. Thus, it is possible that PACAP could have effects on neuronal as well as glial lineages, depending on the different cell populations affected or on the relative developmental stage of target cells. It is known that cortical precursors from different stages of development react differently to CNTF and BMP signaling, which act as inducers of astroglial differentiation during late gestation. In relatively early stage cortical precursors, CNTF does not induce astrocyte differentiation (Molné et al., 2000), and BMPs can cause either apoptosis (Mabie et al., 1999), neuronal differentiation (Li et al., 1998) or proliferation, depending on the type of receptor isoform expressed by target cells (Pachinsion et al., 2001). Interestingly, it has been shown that one reason why only older cortical precursor cells can respond to CNTF or BMP stimulation by differentiating into astrocytes is that these cells express low levels of neurogenin. Younger cortical precursors express higher levels of neurogenin, and this inhibits
astrocyte differentiation by interfering with CNTF/STAT- and BMP/Smad-dependent signaling (Sun et al., 2001). Thus, neurogenin expression at different times of development affects cell-fate specification in response to similar extracellular signals. Whether a similar type of mechanism applies in the case of PACAP-dependent signaling remains to be investigated.

The type of receptor isoform on target cells can also determine the specific biological actions of PACAP. Different PACAP receptor isoforms generated by alternative splicing have been described. Some splice variants generate changes in the N-terminal extracellular domain of the protein, thus influencing the selectivity of the receptor for the ligands PACAP-27 and PACAP-38 (Pantaloni et al., 1996). Another splice variant, named PACAPR (PAC1) TM4 (Chatterjee et al., 1996; Harmar et al., 1998), differs by the presence of small changes in the amino acid sequence of the fourth transmembrane-spanning domain. This receptor isoform does not work by stimulating adenylate cyclase or phospholipase C, but by stimulating calcium entry through an L-type calcium channel (Chatterjee et al., 1996). In the present study, we focussed our attention on splice variants generated by changes in the third intracellular loop, as they affect directly the coupling of the receptor to cAMP-dependent signaling mechanisms.

Expression of PACAP receptor isoforms has been detected in the developing central nervous system, including the rat cerebral cortex (D'Agata et al., 1996; Jaworski and Proctor, 2000; Matsuo et al., 1994; Sheward et al., 1998; Suh et al., 2001; Waschek et al., 1998; Zhou et al., 1999). PAC1Hop isoforms are associated with cellular proliferation, whereas activation of the short isoform of the PAC1 receptor lacking the Hip and Hop cassettes is associated to an antiproliferative effect (Lu et al., 1998; Nicot and DiCicco-Bloom, 2001). In this context, the observed disappearance of the Hip/Hop isoforms and relative enhancement of the short PAC1 receptor isoform may represent a
functional switch to more efficiently coupling PACAP signaling to cAMP production during the differentiation process.

Acting as a second messenger within the cell, cAMP can activate at least three different signal transduction pathways. One of them, triggers the activation of PKA, which phosphorylates different types of substrates including transcription factors such as CREB. Another pathway acts through the PKA-independent activation of the GTPase Rap-1, triggered by the direct stimulation by cAMP of a guanine nucleotide exchange protein called cAMP-GEF or Epac (De Rooij et al., 1998; Kawasaki et al., 1998). Finally, a third pathway has been described whose effector is another guanine nucleotide exchange protein called CNrasGEF, which activates Ras directly (Pham et al., 2000). To what extent PACAP stimulates any one of these pathways remains to be determined. It has been suggested that generation of different responses following specific stimuli acting via cAMP production may be due to intracellular compartmentalization allowing the coexistence of distinct cAMP signals, possibly generated by differential activation of these pathways (Mei et al., 2002; Rich et al., 2001). Thus, it is possible that these differences account for the lack of astrocyte differentiation found after treatment with monoamines, despite the observation of CREB phosphorylation in these cells. In any case, it seems that CREB phosphorylation per se is not sufficient to trigger the astrocyte differentiation response. Indeed, we have observed CREB phosphorylation in cortical precursor cells treated with agents such as glutamate that do not induce differentiation (not shown).

We observed that a brief exposure to PACAP induces differentiation of cortical precursors into astrocytes. Other investigators have found that a short exposure of neural precursors to CNTF or BMP triggers astrocyte differentiation (Gross et al., 1996; Park et al., 1999), whereas a short exposure to PDGF turns neural precursors into
neurons (Williams et al., 1997). These observations imply that once receptor activation is accomplished, intracellular mechanisms leading to changes in gene expression are activated in such a way that the differentiation response can proceed without further agonist action. Although at least some of the cells that respond in this manner have been shown to be bipotent at a minimum (Park et al., 1999), it has been shown that glial fates can be regionally restricted early in development even before neurogenesis starts (McCarthy et al., 2001). This notion would be in line with the observation that CNTF, BMP and possibly PACAP, do not induce astrocyte differentiation from precursor cells isolated at earlier developmental times.

In conclusion, we have demonstrated that PACAP induces cortical precursor cell differentiation into astrocytes via the cAMP-dependent signaling pathway. Thus, PACAP adds to the neurotrophic signals that can act during cortical development to regulate astrocytogenesis.

**EXPERIMENTAL METHODS**

**Materials.** Radioactive compounds were obtained from Du Pont-New England Nuclear (Boston, MA). Nucleotides were purchased from Pharmacia-LKB (Piscataway, NJ). Tissue culture medium and reagents were obtained from Gibco-BRL (Grand Island, NY). Basic FGF was from PeproTech Inc. (Rocky Hill, NJ). Rp-cAMPS (cAMP monophosphothioate, Rp-isomer) was purchased from Research Biochemicals International (Natik, MA). PACAP38 and all other reagents were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise specified.

**Primary cortical cell cultures.** The cerebral cortex from fetal brains of E17 Sprague-Dawley rats were dissected as described (McManus et al., 1999). Cell suspensions were
prepared by trituration of the dissected pieces in Hank's Balanced Salt Solution. Once dissociated, cells were pelleted and resuspended in serum-free DMEM containing N1 supplement (Sigma) and 1mM sodium pyruvate (defined medium) (Bottenstein and Sato, 1979), to which bFGF (20 ng/ml) was added. Cells were seeded into polyornithine-coated 10-cm dishes at a density of 2-4 x 10⁴ cells/cm², and incubated at 37°C. Medium was replaced every two days, and cells were passaged before reaching confluence (usually 5-6 days after plating) by treatment with 0.025% trypsin, which was inactivated by adding DMEM/10% FBS. Cells were then washed, resuspended in defined medium containing bFGF, and seeded either into 10-cm dishes for expansion (1:3 ratio) or into 35 mm dishes for differentiation experiments, in which case plating density was 3 x 10⁴ cells/cm². Cells were not trypsinized more than twice before an experiment was carried out.

For differentiation experiments, cells incubated at 37°C for at least 24 hr after plating were used. Basic FGF-containing medium was replaced with bFGF-free defined medium, and one of the following compounds were immediately added: 8Br-cAMP (1 mM), noradrenaline (50 µM), dopamine (50 µM), serotoinile (50 µM), PACAP (1-100 nM), or VIP (1-100 nM). After the addition of these compounds cells were incubated for 2 or 5 days, at the end of which they were processed for immunocytochemistry.

Assessment of proliferation rates. Proliferation studies were carried out by assessing BrdU incorporation using an RPN 20 cell proliferation kit (Amersham, Arlington Hights, IL) as described (McManus et al., 1999). Briefly, after treatment with BrdU (10 µM), cells were fixed in 90% ethanol/5% acetic acid and processed for immunocytochemistry with a monoclonal anti-BrdU antibody. Control cells that had not been exposed to BrdU were processed in parallel in an identical manner.
Immunodetection was carried out with a biotinylated horse anti-mouse antiserum (Bio-Rad, Hercules, CA) using nickel-ammonium-enhanced immunoperoxidase staining. BrdU incorporation was quantified by determining the percentage of BrdU-labeled cells relative to the total number of cells per field of vision as observed by phase-contrast microscopy.

**Immunocytochemistry.** Immunocytochemistry was carried out with cells plated into poly-ornithine-coated 35 mm tissue culture dishes after fixation in 4% paraformaldehyde for 5 minutes. Cells were washed in phosphate buffered saline (PBS) and permeabilized with methanol for 2 min at -20°C. Blocking was carried out with normal goat serum (for polyclonal antisera) or normal horse serum (for monoclonal antibodies) for 1 hr, and then cells were incubated overnight with the corresponding primary antisera at 4°C. Polyclonal antisera used were: nestin (1:10,000 dilution) (McManus et al., 1999), calbindin D-28K (1:1,000 dilution) (Moratalla et al., 1996), NCAM (1:500 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA) and galactocerebroside (1:1,000 dilution) (Sigma). Monoclonal antibodies used were: MAP-2 (1:300 dilution) (clone HM-2, Sigma), S100β (1:500 dilution) (clone SH-B1, Sigma) and GFAP (1:300 dilution) (clone G-A-5, Sigma). Immunodetection was carried out with secondary biotinylated goat anti-rabbit or horse anti-mouse antisera (Bio-Rad Laboratories, Hercules, CA) using immunoperoxidase staining with a Vectastain ABC kit (Vector Laboratories, Burlingame, CA).

**Western immunoblots.** Cells were lysed in buffer containing 125 mM Tris-HCl (pH 6.8), 4% SDS, 15% glycerol, 10% β-mercaptoethanol, and 10 mM dithiothreitol. Proteins were resolved by SDS-PAGE and blotted onto a nitrocellulose membrane.
GFAP immunoreactivity was detected with a monoclonal antibody (clone G-A-5; Sigma) used at a 1:10,000 dilution, followed by incubation with a horse anti-mouse peroxidase-conjugated secondary antibody (1:5,000 dilution) (BioRad, Hercules, CA). Phosphorylated CREB was detected with an anti-phospho-CREB monoclonal antibody (1:2000 dilution) (New England Biolabs, Beverly, MA). CREB immunoreactivity was detected with a rabbit polyclonal antiserum (1:500 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA), followed by incubation with a goat anti-rabbit peroxidase-conjugated secondary antibody (1:10,000 dilution) (BioRad, Hercules, CA). Immunoreactive bands were visualized using an enhanced chemiluminescence detection system (ECL, Amersham, Buckinghamshire, England).

**RT-PCR/Southern blot hybridization.** Total RNA (10 µg) was purified from primary cortical cells prepared from rat embryos using Triazol (Gibco), and then it was primed with poly-(dT)$_{15}$ and incubated with avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim Biochemicals, Indianapolis, IN) to synthesize cDNA. Three different aliquots of this cDNA were used as templates to detect the expression of the PACAP or VIP receptors PAC1, VPAC1 and VPAC2 by PCR. Specific pairs of primers used to amplify cDNAs coding for each receptor were identical to those described earlier to detect their expression in the rat retina (D'Agata and Cavallaro, 1998). These were as follows: PAC1 receptor forward primer, 5’-CATCCTTGTACAGAAGCTGC-3’; PAC1 receptor reverse primer, 5’-GGTGCTTTGAAGTCCATAGTG-3’; VPAC1 receptor forward primer, 5’-CACGAGTGTGAGTACCGTCA-3’; VPAC1 receptor reverse primer, 5’-CGGTCTTCACGGTATTGTAG-3’; VPAC2 receptor forward primer, 5’-TCTTCAGGAAGCTGCACTGC-3’; and VPAC2 receptor reverse primer, 5’-CAAACACCATGTAGTGAGC-3’.
PCR conditions for amplifications were 95 °C for 5 minutes, followed by 27 cycles of 94 °C for 30 seconds, 60 °C for 30 seconds, and 72 °C for 30 seconds, after which a 5 minute incubation at 72 °C followed. The expected sizes of the different PCR products are as follows: 303 bp for the unspliced form of PAC1; 387 bp for PAC1 isoforms containing either the Hip or the Hop cassettes; 471 bp for the PAC1 isoform containing both Hip and Hop cassettes; 333 bp for the VCAP1 receptor; and 583 bp for the VPAC2 receptor. After PCR, aliquots of each reaction were resolved in 1% agarose gels. Identical aliquots were distributed in three different segments of the gel, which were then blotted onto three separate nylon membranes. Each membrane was probed with a different 32P-labeled oligonucleotide specific for each type of receptor, designed to hybridize within the amplified segment. The sequences of these oligonucleotides are as follows: PAC1 receptor probe, 5’-ATGGAGGAGCTGGAAGGTGAAC-3’, corresponding to nucleotides 1660-1681 of the PAC1 receptor cDNA (Hashimoto et al., 1993); VPAC1 receptor probe, 5’-AGCTGCACTGAAGAGGGCTGGT-3’, corresponding to nucleotides 368-389 of the VPAC1 receptor cDNA (Ishihara et al., 1992); and VPAC2 receptor probe, 5’-TGTTTCCTGGCCTACCTTCTTA-3’, corresponding to nucleotides 783-804 of the VPAC2 receptor cDNA (Lutz et al., 1993). Membranes were subsequently washed and autoradiographed at -70°C.

To determine which one of the different splice variants of the PAC1 receptor are expressed in primary cortical cells from rat embryos, PCR reactions were carried out as described above. After PCR, aliquots of the reactions were resolved in 1% agarose gels, blotted onto nylon membranes, and probed with 32P-labeled oligonucleotides that hybridize specifically to either the Hip cassette (5’-AGACTGAGAGTCCCCCAAGAAAAAC-3’) or to the Hop cassette (5’-CAAGATGTCAGAACTATCCACCA-3’) (Spengler et al., 1993). The PAC1 receptor
probe described in the previous paragraph is common for both spliced and unspliced isoforms of the PAC1 receptor (see Fig. 6A).

**Determination of intracellular levels of cAMP.** Cells were treated with either VIP or PACAP in the presence of 2 mM isobutylmethylxantine (IBMX, Sigma). Control cells were treated with IBMX only. Fifteen minutes after the onset of treatment, the medium was aspirated and cAMP was extracted by adding 50 mM HCl. The concentration of cAMP was then determined by radioimmunoassay as described (Schipani et al., 1993).

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**REFERENCES**


FIGURE LEGENDS

**Figure 1.** A. Lack of astrocyte differentiation in response to exposure of cortical precursor cells to monoamines. Cortical cells from E17 rat brains were cultured in serum-free defined medium containing 20 ng/ml bFGF. After withdrawal of bFGF, cells were left untreated (-) or were treated for 2 days with 8Br-cAMP (1 mM), noradrenaline (NA, 50 µM), dopamine (DA, 50 µM), or serotonine (5-HT, 50 µM). Cells were then fixed and processed for immunocytochemistry with a monoclonal anti-GFAP antibody. The percentage of GFAP-positive cells relative to the total number of cells per field of vision (fov) is depicted. Values represent mean ± s.e.m. of data gathered from three experiments carried out in duplicate. At least 10-12 non-overlapping fov per dish were examined. B, Phosphorylation of CREB in response to treatment of primary cortical precursor cells with monoamines or 8Br-cAMP at concentrations indicated above. Cells were lysed 10 minutes after the onset of treatment and processed for western immunoblot with an anti-phospho-CREB antiserum (top panel) or with an antiserum that does not discriminate between CREB and phospho-CREB (bottom panel).

**Figure 2.** A, Generation of GFAP-expressing cells from primary cortical precursor cells induced by PACAP, but not by VIP. Cortical cells from E17 rat brains were cultured in serum-free defined medium containing 20 ng/ml bFGF. After withdrawal of bFGF, cells were left untreated (-) or were treated for 2 days with PACAP or VIP at the indicated concentrations. Cells were then fixed and processed for immunocytochemistry with a monoclonal anti-GFAP antibody. The percentage of GFAP-positive cells relative to the total number of cells per field of vision (fov) is depicted. Values represent mean ± s.e.m. of data gathered from three experiments carried out in duplicate. At least 10-12
non-overlapping fov per dish were examined. B, Western immunoblot showing the increase in GFAP induced by PACAP. After withdrawal of bFGF, cells were left untreated (-) or were treated with 100 nM PACAP for 2 days. CREB immunoreactivity was used as a control to monitor equal protein loading into each lane.

**Figure 3.** Astroglial differentiation of primary cortical precursor cells induced by PACAP. Cells were cultured in defined medium in the presence of bFGF (20 ng/ml), and processed for immunostaining of GFAP (left panel) or nestin (right panel). After withdrawal of bFGF, cells were treated with PACAP (100 nM) for either 2 days or 5 days, after which they were fixed and processed for immunocytochemistry for GFAP (left panels) or nestin (right panels). Note that cells with more elaborate morphology exhibit less intense nestin immunostaining (bottom right panel).

**Figure 4.** Transient exposure of cortical precursor cells to PACAP results in astrocyte differentiation. Cortical cells from E17 rat brains were cultured in serum-free defined medium containing 20 ng/ml bFGF. After withdrawal of bFGF, cells were left untreated (-) or were treated for the indicated periods of time with PACAP (100 nM). After PACAP treatment, defined medium without bFGF was replaced. Two days after bFGF withdrawal, cells were fixed and processed for immunocytochemistry with a monoclonal anti-GFAP antibody. The percentage of GFAP-positive cells relative to the total number of cells per field of vision (fov) is depicted. Values represent mean ± s.e.m. of data gathered from three experiments carried out in duplicate. At least 10-12 non-overlapping fov per dish were examined.
**Figure 5.** Cortical precursor cells express predominantly the PACAP-selective PAC1 receptor. Depicted is a representative RT-PCR/Southern blot hybridization experiment. Total RNA purified from primary cortical precursor cells cultured in serum-free defined medium in the presence of bFGF was primed with poly-(dT)$_{15}$ and incubated with avian myeloblastosis virus reverse transcriptase to synthesize cDNA. Three independent aliquots of this cDNA were used for PCR amplifications with primers specific for PAC1, VPAC1, or VPAC2 receptors. After PCR, aliquots of the reactions were resolved in 1% agarose gels. Three separate gels were used, and these were blotted onto nylon membranes, each of which was probed with a $^{32}$P-labeled internal primer specific for each type of receptor, designed to hybridize within the amplified fragment. Probes used to hybridize each membrane are indicated at the bottom.

**Figure 6. A,** Schematic depiction of the structure of the PAC1 receptor. Alternative splicing of exons encoding the Hip and Hop domains of the third intracellular loop can generate four different PAC1 receptor isoforms: 1) No cassette spliced in; 2) Hip; 3) Hop; and 4) Hip and Hop. Indicated by arrows and bars are regions of the receptor encoded by the PCR primers and oligonucleotide probes used in RT-PCR/Southern blot hybridization experiments carried out to determine which of these receptor isoforms are expressed in primary cortical precursor cells. **B,** Splice variants of PAC1 receptor isoforms expressed in primary cortical precursor cells. RT-PCR/Southern blot hybridization was carried out with total RNA (10 µg) purified from primary cortical precursor cells cultured in the presence of bFGF. PCR amplification was carried out with forward and reverse PAC1-specific primers as indicated in A. After PCR, aliquots of the reactions were resolved in 1% agarose gels, blotted onto nylon membranes, and probed with $^{32}$P-labeled internal oligonucleotides specific for each type of receptor.
isoform. Rec denotes a receptor probe that does not discriminate among different isoforms of the PCA1 receptor, as indicated in A. C, Cyclic AMP stimulation alters the expression of alternatively spliced isoforms of PAC1 receptors. RT-PCR/Southern blot hybridization carried out with total RNA (10 μg) purified from primary cortical cells induced to differentiate into astrocytes by treatment with 8Br-cAMP (1 mM) after withdrawal of bFGF. PCR primers and oligonucleotide probes correspond to those indicated in A.

**Figure 7.** A, Production of cAMP in cortical precursor cells after treatment with PACAP or VIP. Cells were plated on 35 mm dishes and cultured in serum-free defined medium with 20 ng/ml bFGF. Basic FGF was withdrawn from the medium and cells were treated with IMBX (2 mM) alone (-) or with IBMX and VIP (10 or 100 nM) or PACAP (10 or 100 mM) for 15 minutes. The medium was aspirated and cAMP was extracted and determined by radioimmunoassay. Values represent mean ± s.e.m. of three independent experiments carried out in duplicates. B, Inhibition of PACAP-induced astrocyte differentiation by the cAMP antagonist Rp-cAMPS (10 μM). The cAMP antagonist was added to cells 5 minutes before PACAP (100 nM). Cells were fixed and processed for immunocytochemistry with a monoclonal anti-GFAP antibody 2 days after treatment. The percentage of GFAP-positive cells relative to the total number of cells per field of vision (fov) is depicted. Values represent mean ± s.e.m. of data gathered from three experiments.
Figure 1A
Figure 2A
Figure 2B
Figure 6A
Figure 6B
Figure 6C
Figure 7A

Graph showing the effect of PACAP and VIP on [cAMP] pmol/ml. The x-axis represents peptide concentration ranging from 0 to 100 nM, with points at 10 nM and 100 nM. The y-axis represents [cAMP] pmol/ml, ranging from 0 to 50 pmol/ml. Two lines are plotted: one for PACAP and another for VIP. The PACAP line shows a more steep increase compared to the VIP line.
Figure 7B

% GFAP-labeled cells/fov

PACAP

PACAP + Rp-cAMPS

Figure 7B