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# Pituitary Adenylyl Cyclase-Activating Polypeptide Stimulates DNA Synthesis But Delays Maturation of Oligodendrocyte Progenitors

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The neuropeptide pituitary adenylyl cyclase-activating peptide (PACAP) and one of its receptors ($PAC_1$) are expressed in embryonic neural tube, where they appear to regulate neurogenesis and patterning. We now show that $PAC_1$ gene expression is also present in neonatal rats in the ventricular and subventricular zones and in the optic chiasm, areas that are rich in oligodendrocyte (OL) progenitors (OLP). Because actions of PACAP on OLP have not been reported, we examined the effects of PACAP on the proliferation of purified OLP in culture and on myelinogenesis in cerebellar slices. Northern analyses on total RNA from purified glial cell subtypes revealed an abundant 7 kb hybridizing transcript in OLP, which was confirmed to correspond to the $PAC_1$ receptor by reverse transcription-PCR. The presence of this receptor was also corroborated by radioligand binding and cAMP assay. In cultured OL, receptor density decreased during maturation but was partially counterbalanced by the appearance of sites that bound both PACAP and the related peptide vasoactive intestinal peptide. PACAP increased DNA synthesis in OLP cultures almost twofold and increased the bromodeoxyuridine-labeling index in O4-positive OLP. PACAP treatment also resulted in decreased sulfate incorporation into sulfatide in cultures of differentiating OL. The PACAP effect on sulfatide synthesis was fully reproduced in a cerebellar explant model. These findings indicate that PACAP may act at two stages during OL development to (1) stimulate proliferation and (2) delay maturation and/or myelinogenesis.

*Key words: PACAP receptors ($PAC_1$); PACAP; oligodendrocytes; proliferation; cAMP; myelination*

The neuropeptide pituitary adenylyl cyclase-activating peptide (PACAP) belongs to a peptide family that includes secretin, glucagon, growth hormone-releasing factor, and vasoactive intestinal peptide (VIP) (Arimura, 1998). PACAP is known to interact via seven transmembrane-spanning domain G-protein-coupled receptors (Rawlings, 1994; Harmar et al., 1998). Two PACAP receptors ($VPAC_1$ and $VPAC_2$) have high affinity for both VIP and PACAP, whereas a third PACAP receptor ($PAC_1$) binds only PACAP with high affinity. Activation of PACAP and VIP receptors typically leads to a robust $G_s$-mediated cAMP elevation (Arimura, 1998). However, $PAC_1$ receptors are in many cases linked to other signaling pathways such as phospholipase C, phosphoinositol 3-kinase, mitogen-activated protein kinase, and calcium mobilization (Rawlings, 1994). This may be explained in part by the presence of eight known $PAC_1$ receptor splice variants that couple alternatively to $G_i$ and $G_q$ but also exhibit different affinities for various PACAP-related ligands (Spengler et al., 1993; Chatterjee, 1996; Pantaloni et al., 1996; Dautzenberg et al., 1999).

Neuropeptides in the PACAP family and their receptors are highly conserved from protochordates to humans and are widely expressed in the nervous system (McRory and Sherwood, 1997; Hu et al., 2000a,b). It is well accepted that neuropeptides released from specific axonal terminals act as neurotransmitters, neuromodulators, or autocrine/paracrine factors that interact with various neuronal, glial, neuroendocrine, and immune cell populations (Martin et al., 1992; De la Fuente et al., 1996; Legradi et al., 1998; Shibuya et al., 1998; Otto et al., 1999). Interaction of peptides with receptors on target cells may result in modulation of membrane potentials and activation of intracellular signaling systems leading to physiological responses such as release of neurohormones or neurotransmitters (Rawlings and Hezareh, 1996).

Recent data suggest that PACAP exerts developmental actions. PACAP gene expression and PACAP immunoreactivity are widely distributed in neurons within the embryonic and neonatal rat brain (Nielsen et al., 1998a,b; Waschek et al., 1998; Skoglosa et al., 1999). Activation of VIP and PACAP receptors has been shown to regulate proliferation of developing neuroblasts *in vitro* and *in vivo* (Pincus et al., 1990; Lu and DiCicco-Bloom, 1997; Waschek et al., 1998; Vaudry et al., 1999; DiCicco-Bloom et al., 2000). In addition, neuroprotective roles for VIP and PACAP have been proposed (for review, see Waschek, 1996; Lindholm et al., 1998). For example, VIP and/or PACAP have been shown to stimulate neuronal survival directly (Pincus et al., 1990; DiCicco-Bloom et al., 2000) and also to influence survival via the glial cell

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release of neurotrophic factors (Brenneman et al., 1987). Yet other studies suggest that VIP/PACAP receptors might be involved in astroglial development (Zupan et al., 1998) and in microglia function (Kong et al., 1999; Kim et al., 2000). To support these findings, $PAC_1$ receptor gene expression has been detected by reverse transcription (RT)-PCR in purified astrocytes (Grimaldi and Cavallaro, 1999) and microglia cultures (Kim et al., 2000). Furthermore, glial tumor cells (including astroglioma and oligodendroglioma cells) express $PAC_1$ receptors (Vertongen et al., 1995). Despite these data, neither $PAC_1$ receptors nor PACAP actions have yet been reported in the oligodendrocytes (OL) or OL progenitors (OLP). The results reported here provide the first evidence that PACAP receptors are present on OLP and that PACAP plays a role in OL development and myelinogenesis.

## MATERIALS AND METHODS

### *Cell and tissue preparations*

Purified OLP cultures were prepared from neonatal Wistar rats (<24 hr old) as described previously (McCarthy and de Vellis, 1980) and as modified by Cole and de Vellis (1989). Briefly, OLP were purified by differential adhesion and plated in poly-D-lysine-coated culture flasks. Cells were cultured in DMEM and Ham's F12 medium (supplemented with 1.2 gm/l $NaHCO_3$, pH 7.4, 15 mM HEPES buffer, and 10% fetal bovine serum) for specified time periods (1, 2, 5, and 10 d) to examine OLP at various developmental stages. In longer-term cultures, the medium was changed every 3 d. At the specified time period, cells were directly harvested for RNA extraction or replated into 24-well tissue culture plates at $\sim 0.1 \times 10^6$ cells/well (50,000 cells/cm$^2$) for other studies. That OLP differentiated appropriately into OL under these conditions was confirmed by the fact that >95% of cells stained positive for myelin basic protein (antibody provided by Dr. A. Campagnoni, University of California, Los Angeles) after 10 d in culture. In addition, MBP levels increased >10-fold over the 10 d period as determined by Western blot (data not shown).

For slice cultures, cerebella were dissected from neonatal Wistar rat pups (<24 hr old) (Notterpek et al., 1993). Meninges were carefully removed, and tissue was incubated in Liebovitz's medium (Life Technologies, Gaithersburg, MD). Cerebella were mounted on a sterile Aclar square and placed on a McIlwain tissue chopper for sectioning. Sagittal slices of 300–400 $\mu$m thickness (12–16 slices per cerebellum) were cut, avoiding the buds of the lateral lobes. Isolated slices were plated into poly-D-lysine-coated four-well plates and incubated in DMEM–F12 media containing 10% heat-inactivated horse serum, 20% fetal bovine serum, glucose (4.1 mg/ml), and insulin (15.6 $\mu$g/ml). Slices were cultured for 3 weeks in decreasing concentrations of fetal bovine serum according to the protocol of Notterpek et al. (1993).

### In situ *hybridization*

One-day-old neonatal rat brains were perfused and then fixed with 4% paraformaldehyde (freshly prepared in DEPC-treated $H_2O$). For cryoprotection, tissues were equilibrated in 30% sucrose (PBS) and then frozen in OCT. Tissue was sectioned sagittally at 10–12 $\mu$m thickness, mounted on Superfrost Plus slides (Fisher Scientific, Houston, TX), and then stored at −70°C until use. Preparation of the sense and antisense $^{33}$P-labeled riboprobes from the $PAC_1$ receptor cDNA (Pisegna and Wank, 1993) and *in situ* detection were performed as described previously (Waschek et al., 1998). Slides were dipped in Kodak NTB2 emulsion. After development, slides were examined with a Zeiss Axiovert 135M microscope equipped with the Spot Cooled Color Digital Camera (Diagnostic Instruments, Inc.).

### *Northern analyses*

Cultures of OLP, astrocytes, and microglia with a purity >95% were prepared from mixed glial cultures by differential plating as described previously (McCarthy and de Vellis, 1980; Cole and de Vellis, 1989). Purified OLP and microglia were harvested on the sixth day after plating. The remaining astrocytes were also harvested at this time. RNA was extracted from these cultures by the method of Chomczynski and Sacchi (1987). Total RNA (30 $\mu$g/lane) was subjected to electrophoresis (1.2% agarose, 3-[*N*-morpholino]propanesulfonic acid, and 2% formaldehyde gel) and transferred to a nylon membrane (Magna; Micron Separations, Inc.). The membrane was baked at 80°C for 30 min and then UV cross-linked. Sequential hybridizations were performed with the following cDNA probes: rat $PAC_1$ (Waschek at al, 1998), rat 2′3′ cyclic nucleotide 3′ phosphodiesterase (CNPase) (Bernier et al., 1987), and mouse GFAP (Kashima et al., 1993). Between hybridizations, the blot was stripped by incubating twice in a solution containing 96% formamide, 10 mM Tris, pH 8.0, and 10 mM EDTA for 30 min at 65°C. Prehybridizations were performed at 42°C for 30 min in ULTRAhyb (Ambion); hybridizations were overnight at 42°C in the same buffer but containing $0.2 \times 10^6$ cpm/ml probe, labeled with [$\alpha$-$^{32}$P]dCTP to a specific activity of $2 \times 10^9$ cpm/$\mu$g using the Random primers DNA Labeling System (Life Technologies). After hybridization, the blot was washed in 2× SSC and 0.1% SDS (twice for 5 min at 42°C) and then in 0.2× SCC and 0.1% SDS (twice for 15 min at 42°C) and then exposed for 24–48 hr in a PhosphorImager screen (Molecular Dynamics, Sunnyvale, CA). Signals were analyzed with ImageQuant software.

### *Binding studies*

Binding assays were performed in triplicate as described by Lelièvre et al. (1996). Briefly, cells plated in 24-well plates were washed and preincubated with fresh medium for 15 min at 37°C. Culture medium was removed and replaced with 270 $\mu$l of 4°C binding medium (DMEM, HEPES, BSA, and protease inhibitors) containing 30,000 cpm of radiotracer ($^{125}$I-PACAP; 2200 Ci/mmol; NEN Life Sciences) and 30 $\mu$l of native peptides at the specified concentrations. Cells were incubated at 4°C for 2 hr, washed with a solution of 0.1% BSA in PBS (0.05 M), pH 7.4, and then lysed with 300 $\mu$l of NaOH (0.5 M) and transferred into 5 ml tubes and counted in a gamma counter (WALLAC Oy, Turku, Finland).

### *cAMP measurements*

OLP and differentiated OL were plated in 24-well plates at a density of 100,000 cells/well (50,000 cells/cm$^2$). After 12 hr, cells were pretreated with 10 $\mu$M isobutylmethylxanthine for 20 min. Neuropeptides were then added for another 20 min at 37°C. Cells were then lysed, and the cAMP radioimmunoassay (NEN Life Sciences) was performed as described previously (Vertongen et al., 1996).

### *[$^3$H]Thymidine incorporation assay*

Freshly prepared OLP cultures were initially plated into 75 cm$^2$ flasks. On the following day, OLP were replated into 24-well plates at a density of 50,000 cells/well (25,000 cells/cm$^2$). The replating used the same medium that was used in other experiments but that contained only 2% fetal bovine serum (the concentration of serum was lowered to reduce the basal proliferation). On the following and subsequent 2 d, neuropeptides were added at the specified concentrations. The last peptide addition was followed 1 hr later with the addition of 1 $\mu$Ci of [$^3$H]thymidine to each well. Fourteen hours later, cells were harvested. Incorporated [$^3$H]thymidine (DuPont NEN) was precipitated by TCA and assayed as described previously (Lelièvre et al., 1998).

### *Determination of labeling index*

*Bromodeoxyuridine labeling.* Freshly isolated OLP were plated on poly-D-lysine-coated (P−6407; Sigma, St. Louis, MO) coverslips in 10% fetal bovine serum. Two hours later, the medium was diluted with an equal volume of glial development medium (GDM) (Yonemasu et al., 1998). This adjustment was made to increase cell adhesion to coverslips. After an additional 4 hr, 10 $\mu$M bromodeoxyuridine (BrdU; Boehringer Mannheim, Indianapolis, IN) was added along with either PACAP (100 nM) or vehicle.

*Colocalization of BrdU with specific glial cell markers.* After 18 hr of treatment, cells were fixed with 3.7% formaldehyde (Fisher Scientific) in PBS for 10 min and then stored at 4°C. For immunofluorescence detection, OLP were washed three times with 4°C PBS and then incubated in −20°C methanol for 20 min. Cells were then washed three times with PBS, treated with 1% Triton X-100 in PBS for 20 min at 37°C, and then transferred to room temperature for 10 min. Primary antibodies to glial markers were added along with the anti-BRDU antibody as follows: O4 [1:15; supernatant of hybridoma (provided by Dr. S. Pfeiffer, University of Connecticut)], polyclonal anti-GFAP (1:100; catalog #G-9269; Sigma), and anti-ED-1 (1:100; catalog #MCA341; Serotec, Indianapolis, IN). Visualization of O4 was performed using goat anti-mouse IgM AMCA (catalog #1 110 075 075; Jackson ImmunoResearch, West Grove, PA). Anti-GFAP and anti-ED-1 used goat anti-rabbit Texas Red (catalog

#1 111 075 075; Jackson ImmunoResearch) and goat anti-mouse IgG FITC (catalog #F-9006; Sigma), respectively. Detection of BrdU was performed according to the manufacturer's protocols (BrdU Labeling and Detection Kit 1, catalog #1296 736; Boehringer Mannheim). Images were acquired on a Zeiss microscope equipped with a Hamamatsu C5810 Color Camera.

*Calculation of labeling index in OLP precursors.* The number of BrdU+/O4+ and BrdU−/O4+ cells was counted manually in 10 random fields in both control and PACAP-treated cultures. The OLP-labeling index in each field was calculated as the number of BrdU+/O4+ cells divided by the total number of O4+ cells (i.e., sum of BrdU+/O4+ and BrdU−/O4+ cells). Mean values for control and PACAP-treated cultures were compared using the *t* test.

### In vitro *cerebellar myelination assay*

Seven-day-old OL cultures and cerebellar slice cultures were prepared and treated with peptides or drugs for the next 14 d and assayed for sulfatide synthesis (Rome et al., 1986; Notterpek et al., 1993). Medium was changed every 2 d during treatments. Before harvest, cultures were maintained for 48 hr in complete low-sulfate DMEM and F12 growth media in the presence of 5 μCi/well [$^{35}$S]$NaSO_4$ (43 Ci/mg; ICN). Then, sulfolipids were extracted and assayed as described previously (Cardwell and Rome, 1988). This assay was shown by TLC to measure sulfate incorporation specifically into sulfatide, excluding other molecular species such as gangliosides (data not shown). As an experimental control, a mouse monoclonal galactocerebroside/sulfatide (GC) antibody, shown previously to inhibit differentiation (and therefore sulfate incorporation) (Ranscht et al., 1982; Notterpek et al., 1993), was added at 15 μl/well to some cultures. For each experimental time point, four cultures were used, and each time point was repeated at least three times.

## RESULTS

### Expression of $PAC_1$ mRNA in areas rich in OLP

*In situ* hybridization for the $PAC_1$ receptor was performed on frozen sagittal brain sections of rats on postnatal day 0.5 (P0.5), a time when neurogenesis is virtually complete. The analyses were focused on regions containing the ventricular and subventricular zones (VZ and SVZ, respectively) and on the optic chiasm and adjoining optic nerve. Cells in the VZ at this stage appear to generate primarily glial progenitors that then populate the SVZ (Parnavelas, 1999). The SVZ, on the other hand, contains high numbers of OLP, which proliferate extensively in this region before migrating to the cerebral cortex (Paterson et al., 1973; Levison and Goldman, 1993; Parnavelas, 1999). The optic chiasm and adjoining optic nerve contain proliferating OLPs that are believed to give rise to the oligodendrocytes in the optic nerve (Skoff et al., 1980; Raff et al., 1984; Small et al., 1987).

As shown previously at earlier stages in embryonic mice (Waschek et al., 1998), $PAC_1$ gene transcripts in postnatal rats were abundant and uniformly distributed in the VZ (Fig. 1*A–D*). $PAC_1$ signals were also uniformly distributed over the SVZ in postnatal rats, but at a lower level (Fig. 1*B,D*). Moreover, dense accumulations of silver grains appeared to be present over some cells in the SVZ (Fig. 1*B,D*, *arrows*). In contrast, no specific labeling was observed in sections hybridized with a sense probe (Fig. 1*E,F*). Near the base of the forebrain, $PAC_1$ gene transcripts were clearly seen to be clustered over individual cells in the optic chiasm and adjoining optic nerve (Fig. 2*A,B*). Again, no specific labeling was observed in nearby sections hybridized with a sense probe (Fig. 2*C,D*). Overall the data indicate that specific $PAC_1$ receptor gene transcripts are present in regions of the neonatal brain known to give rise to OL.

### Expression of the $PAC_1$ receptor gene in purified OLP

To determine whether $PAC_1$ receptors are expressed specifically in OLP, we isolated and cultured OLP, astrocytes, and microglia from P0.5 rat cortices by methodology established previously and validated in this laboratory (McCarthy and de Vellis, 1980; Cole and de Vellis, 1989; Liva et al., 1999). The bipolar morphology of OLP after 24 hr in culture is shown in Figure 3*A* and is essentially the same as that reported by Reynolds and Wilkin (1990) under similar conditions. Northern analyses were performed on total RNA prepared from various purified glial cell subtypes using the $PAC_1$ receptor cDNA, as well as cDNA markers for OLP (CNPase) and astrocytes (GFAP). A transcript hybridizing to the $PAC_1$ probe was clearly detected in cultured OLP at a size of 7 kb (Fig. 4, *top*), similar to that reported in the adult rat brain and other tissues (Hashimoto et al., 1993). On the other hand, the $PAC_1$ mRNA was barely detected in total RNA from purified astrocytes and was not detectable in microglia by this analysis. To confirm that these cultures were highly enriched in the desired cell types, the blot was subsequently hybridized to specific glial cDNA markers. A cDNA probe for CNPase, a relatively specific OLP marker, hybridized strongly and specifically to RNA from purified OLPs, whereas a GFAP cDNA probe, an astrocyte-specific marker, hybridized strongly and specifically to RNA from astrocytes (Fig. 4, *middle*, *bottom*). We were unable to identify a cDNA probe specific for microglia; however, these microglial cultures exhibited very weak GFAP and CNPase mRNA signals and were 95–99% pure as judged by immunohistochemical staining with the microglia marker ED-1, as shown previously by Liva et al. (1999). Thus, $PAC_1$ gene transcripts were abundant in OLP cultures, and their detection was not likely because of contamination of OLP cultures with astrocytes or microglia. To confirm that the hybridizing band in OLP RNA represented an authentic $PAC_1$ gene transcript, RNA from OLP was also analyzed by RT-PCR. Primers were selected that spanned the N terminal (containing the primary ligand-binding sequences) of the receptor. These primers are capable of detecting the known splice variants in this region (Dautzenberg et al., 1999). PCR-amplified products were subjected to Southern blot analysis using a $^{32}$P-labeled oligonucleotide probe designed to recognize all splice variants in this region. This revealed a single band at the size expected for the prototype N terminal of the receptor (data not shown). This was confirmed by DNA sequencing of PCR products.

To determine whether OLP express high-affinity PACAP-binding sites and to investigate their characteristics, the ligand-binding profile was determined on intact cells. Cultured OLP were incubated with $^{125}$I-PACAP-27 in the presence and absence of increasing ligand concentrations of PACAP-27, PACAP-38, and VIP. Specific binding of $^{125}$I-PACAP-27 was ~1500 cpm/100,000 cells in these cultures. PACAP-38 was found to be the most potent displacer ($IC_{50}$, 1.2 pM; Fig. 5*A*). PACAP-27 exhibited a >100-fold lower affinity than did PACAP-38 ($IC_{50}$, 0.3 nM), whereas VIP was unable to displace $^{1\,25}$I-PACAP ($IC_{50} > 1$ μM). These observations indicate that OLP express a high-affinity PACAP-preferring binding site conforming to the $PAC_1$ receptor (Hashimoto et al., 1993).

To determine whether OLP express PACAP receptors that are functionally coupled to a cAMP second messenger system, cells were treated with increasing concentrations of neuropeptides in the presence of the phosphodiesterase inhibitor IBMX (10 μM). Under these conditions, PACAP-38 elevated cAMP levels to a maximum fivefold induction at 1 nM (Fig. 6*A*). VIP also increased cAMP levels, but only at concentrations of 100 nM or higher. Overall, the analyses by Northern blot, RT-PCR, radioligand binding, and cAMP assay indicate that freshly purified OLP

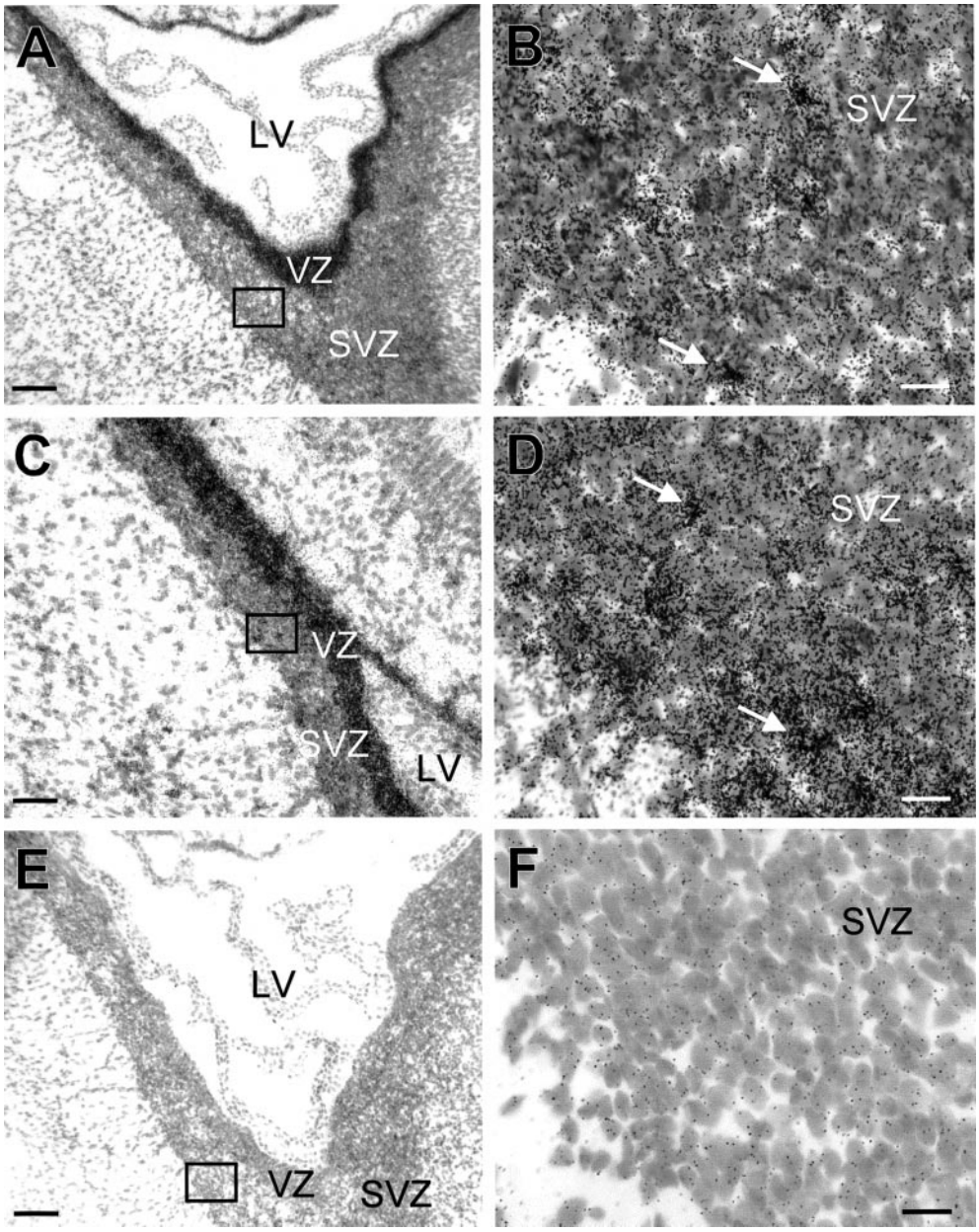


*Figure 1.* *A–D*, *In situ* hybridization of $PAC_1$ receptor mRNA in sagittal sections containing the anterior regions of ventricular and subventricular zones of the lateral ventricle. *A, C*, Low magnification of $PAC_1$ mRNA signals. *B, D*, High magnification of *boxed regions* of *A* and *C*, respectively, showing localized clusterings of silver grains (*arrows*). *E, F*, Low and high magnifications, respectively, of a nearby section showing hybridization to a $PAC_1$ receptor sense probe. The *boxed region* of *E* is shown in *F*. *LV*, Lateral ventricle. Scale bars: *A*, *C*, *E*, 50 μm; *B*, *D*, *F*, 16 μm.

express a functional PACAP-preferring $PAC_1$ receptor that couples moderately to a cAMP messenger system.

### PACAP receptor expression during oligodendrocyte maturation in culture

Purified OLP mature in culture with a time course similar to that observed *in vivo* based on their morphology and their temporal expression of OL stage-specific markers (Reynolds and Wilkin, 1990; Hardy and Reynolds, 1993). After initial overnight culture, cells show a bipolar phenotype without obvious differentiation features (Fig. 3*A*). Twenty-four hours later (day 2), primary extension processes have been initiated in a majority of cells. These cells are referred to as "multipolar pre-OL" (Fig. 3*B*). On day 5, a majority of cells bore multipolar processes with primary and secondary branching (Fig. 3*C*, "immature OL"). After 10 d in culture, most cells are "mature OL," exhibiting primary, secondary, and tertiary processes, and become highly refringent under the microscope (Fig. 3*D*).

During this course of OLP maturation, PACAP receptors were examined by radioligand binding and displacement and by cAMP assay. As bipolar progenitors progressed to the multipolar pre-OL stage, a significant 30% reduction of the total available $^{125}$I-PACAP-binding sites per 100,000 cells was observed (Fig. 5*B*). In addition, the affinity of PACAP-27 increased dramatically ($IC_{50}$, 300 pM after initial overnight culture vs 11 pM after 48 hr). VIP was still incapable of displacing $^{125}$I-PACAP binding at this stage. As OL reached a more differentiated stage (day 5), the number of total $^{125}$I-PACAP-binding sites was reduced further and exhibited similar high affinity for PACAP-38 and PACAP-27 ($IC_{50} \sim 1$ pM). At this stage, VIP first became capable of displacing a small portion of the total PACAP-binding sites (Fig. 5*C*). Later, in 10-d-old OL cultures, VIP displaced ~45% of the total binding sites, whereas 55% of the binding sites were specific for PACAP ligands (Fig. 5*D*).

To determine whether these changes in PACAP-binding char-

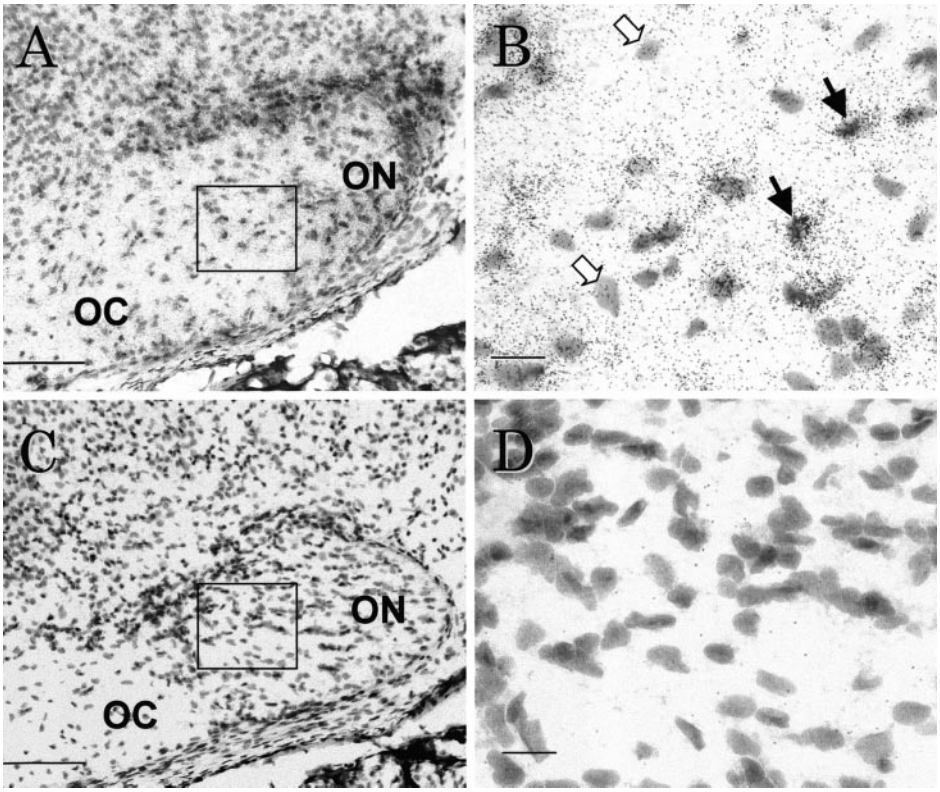


*Figure 2.* *In situ* hybridization of a sagittal section showing clusterings of $PAC_1$ receptor gene transcripts over individual cells in an area containing the optic chiasm (*OC*) and adjoining optic nerve (*ON*). *A*, Low-magnification photomicrograph of a section hybridized with the $PAC_1$ riboprobe. *B*, High magnification of the *boxed area* in *A*. *Solid arrows* point to dense accumulations of silver grains over individual cells around the junction of the optic chiasm and optic nerve. *Open arrows* point to cells in the same area that lack specific hybridization signals. *C, D*, Low and high magnifications, respectively, of a nearby section showing hybridization to a $PAC_1$ receptor sense probe. The *boxed area* in *C* is shown in *D*. Scale bars: *A, C*, 50 μm; *B, D*, 16 μm.

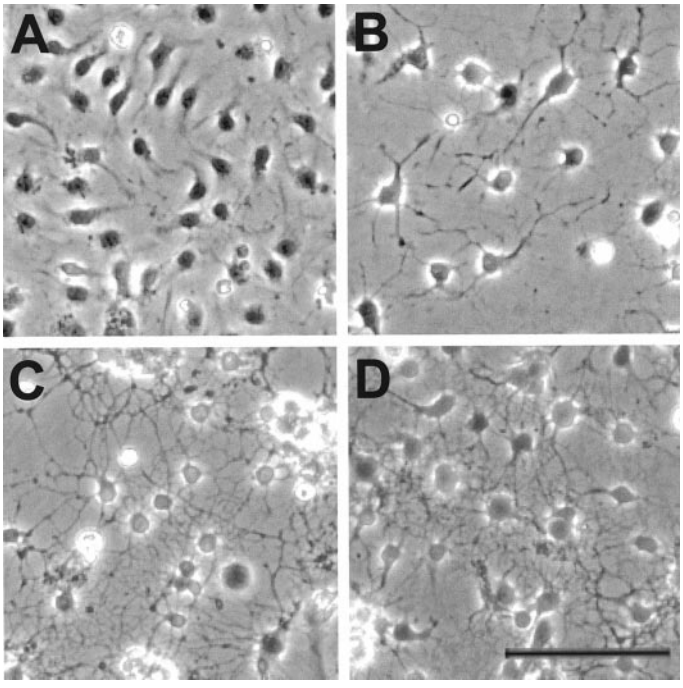


*Figure 3.* Phase-contrast photomicrographs illustrating morphological changes in cultured developing OL. Cells were cultured in DMEM and F12 containing 10% fetal bovine serum for 1 d (OLP; *A*), 2 d (multipolar pre-OL; *B*), 5 d (immature OL; *C*), and 10 d (mature OL; *D*). Scale bar, 50 μm.

acteristics were reflected in the ability of peptides to stimulate adenylate cyclase, peptide-induced changes in cAMP levels were determined in more-differentiated OL and compared with that in less-differentiated OLP. PACAP-38 still exhibited a dose-dependent increase in cAMP levels in 10-d-old cultures, but the potency and maximum degree of stimulation were much lower than that in progenitors (Fig. 6*B*). The radioligand-binding and cAMP induction data indicate that there is a change in the characteristics of VIP and PACAP receptors as OL mature. OLP express essentially a PACAP-preferring $PAC_1$ receptor, with moderate coupling to cAMP production, whereas more-differentiated OL begin to express receptors for VIP and PACAP that couple poorly to cAMP production. We also performed the RT-PCR analysis on the N-terminal (ligand-binding) domain of the $PAC_1$ receptor in differentiated OL. Like in OLP, this did not reveal splice variants shown to also bind VIP with high affinity (Dautzenberg et al., 1999) (data not shown). Thus, the emergence of VIP-binding sites in differentiated OL does not appear to be explained by splice variants in this region of the $PAC_1$ receptor.

### Effect of PACAP on DNA synthesis

Freshly isolated OLP undergo proliferation for ~48–72 hr in the presence of 10% fetal bovine serum. Initial experiments using these culture conditions indicated that overnight treatment with PACAP-38 significantly induced proliferation, but only at concentrations of $10^{-7}$ M or higher (data not shown). In subsequent experiments, the concentration of serum was lowered to 2% (to decrease basal proliferation), and the PACAP treatment period was extended for a total of 3 d. These conditions unmasked a much more potent action of PACAP on DNA synthesis, with significant stimulation observed at $10^{-10}$ M (Fig. 7). A maximum 80% increase in [$^3$H]thymidine incorporation was observed in the presence of 100 nM PACAP-38. FGF-2, a known OLP mitogen (McKinnon et al., 1990), also increased DNA synthesis approximately twofold under these conditions.

To determine whether PACAP specifically stimulated DNA synthesis in OLP rather than in contaminating cell types in

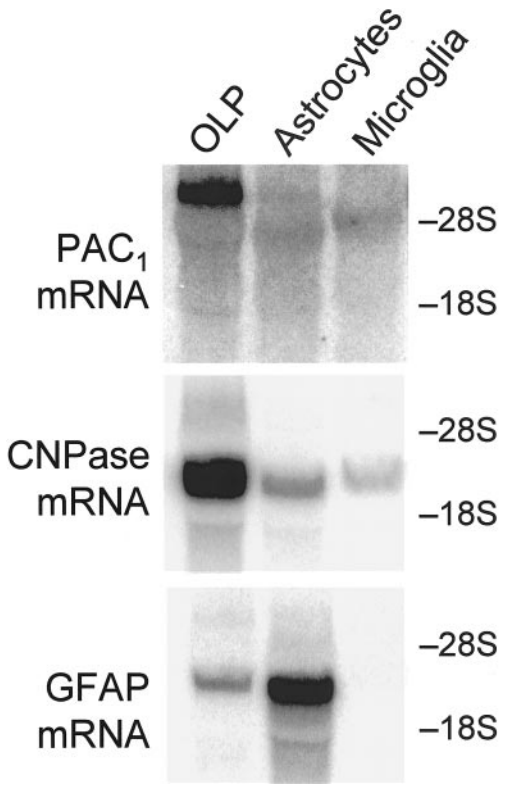


*Figure 4.* $PAC_1$ receptor gene expression in purified cultures of OLP, astrocytes, and microglia. A Northern blot was prepared containing 30 $\mu$g of total RNA from cultures of each cell type. The blot was sequentially hybridized with the following $^{32}$P-labeled cDNA probes: $PAC_1$ cDNA (*top*), CNPase (OLP marker; *middle*), and GFAP (astrocyte marker; *bottom*). The strong and weak signals for CNPase and GFAP, respectively, in *lane 1* (*left lane*) indicate that OLP cultures were highly enriched for OLP and contained few contaminating astrocytes. In contrast to the strong signal for $PAC_1$ mRNA in OLP, cultures of purified astrocytes expressed barely detectable levels of $PAC_1$ mRNA (*lane 2*). The location of the 28 S and 18 S ribosomal RNA bands, visualized by UV transillumination of the ethidium bromide-stained gel, is indicated to estimate approximately the sizes of the hybridizing transcripts

these cultures, a double-labeled immunocytochemistry analysis was performed. The BrdU-labeling index was determined in OLP cells treated for 18 hr with either 100 nM PACAP or control medium in the presence of 10% fetal bovine serum. OLP were detected in this assay with O4, whereas astrocytes were detected with anti-GFAP. Under control conditions, approximately half of O4-staining cells (OLP) incorporated BrdU (Fig. 8*A–C*), whereas in PACAP-treated cultures, a much higher proportion of O4+ cells incorporated BrdU (Fig. 8*D–F*). Very few astrocytes incorporated BrdU either in control cultures (Fig. 8*J,K*) or in those treated with PACAP (data not shown). ED-1-labeled microglia were extremely rare. Counting of individual cells after drug treatment indicated that PACAP treatment increased the percent of O4+ cells incorporating BrdU from 52 ± 6 to 79 ± 4% (mean ± SD; $p < 0.001$). This corresponded to a 52% increase in DNA synthesis over control. Similar to the untreated control cells, very few GFAP+ cells and virtually no ED-1+ microglia incorporated BrdU after PACAP treatment (data not shown). This precluded a reliable determination of the labeling index in astrocyte progenitors or microglia.

### PACAP effects on myelinogenesis: *in vitro* differentiated OL cultures and *ex vivo* cerebellar slices

Myelinogenesis was examined by measuring the incorporation of radiolabeled sulfate into sulfatides (an essential lipid in the formation of myelin membrane) during the final 2 d of a 14 d treatment period with peptides. Two models for myelinogenesis were used in these experiments: (1) differentiated OL in culture (*in vitro* assay) and (2) cerebellar slices (*ex vivo*). For *in vitro* assays, OLP were precultured for 10 d to allow them to mature into multipolar OL. Immunohistochemical analysis (data not shown) and previous work have demonstrated that OL at this stage are immunopositive for myelin basic protein (Reynolds and Wilkin, 1990). These cultures were treated daily with different concentrations of PACAP for 14 d. Anti-GC antibodies, shown previously to inhibit myelinogenesis in cultured cells and slice preparations (Rome et al., 1986; Notterpek et al., 1993), and PDGF were used as controls. PACAP-38, at concentrations of 1 nM and 0.1 $\mu$M, was found to reduce membrane sulfatide synthesis by ~40 and 50%, respectively (Fig. 9*A*). Anti-GC also decreased sulfate incorporation, whereas treatment with PDGF (10 ng/ml) resulted in increased sulfatide synthesis.

To confirm the peptide effects on sulfatide synthesis in purified OL, studies were repeated using the cerebellar explant system (Fig. 9*B*). As observed in isolated cells, sulfate incorporation was stimulated by PDGF and inhibited by anti-GC antibodies. PACAP-38 was found to trigger a potent inhibition of sulfatide formation in cerebellar slices. A treatment with PACAP during the final week only still reduced sulfate incorporation (data not shown), suggesting that PACAP acted on OL at a later developmental stage in slice cultures. Surprisingly, the 2 week treatment with PACAP was not able to block the stimulatory effect of PDGF (data not shown).

## DISCUSSION

The studies reported here implicate the neuropeptide PACAP as a newly defined factor potentially involved in OL development in the neonatal rat brain. That PACAP might be involved in OL development was suggested by the fact that this peptide is widely expressed in neurons in the early postnatal rat brain (Nielsen et al., 1998b; Skoglosa et al., 1999). We found that gene expression for a PACAP-specific receptor ($PAC_1$) was abundant in the postnatal VZ and SVZ, germinal regions that give rise primarily to oligodendrocytes and astrocytes (Paterson et al., 1973; Levison and Goldman, 1993; Parnavelas, 1999), and that $PAC_1$ gene transcripts were localized over specific cells in the optic chiasm and adjoining optic nerve (Fig. 2). OLP in the latter structures are believed to give rise to the oligodendrocyte lineage in the optic nerve (Small et al., 1987). Although a number of groups have examined possible roles for PACAP in astroglial function, no studies have appeared so far that indicate that PACAP receptors are expressed in OL or OLP or that suggest a role for PACAP in OL development. Using purified cultures of specific glial cell subtypes, we showed by Northern analysis, radioligand binding, and second messenger assay that OLP express functional PACAP-preferring $PAC_1$ receptors. These were found to be moderately coupled to cAMP production. Then, using both cultured OLP and cerebellar explant models, we showed that PACAP regulated two aspects of oligodendrocyte development. First, it enhanced DNA synthesis, and second, it inhibited sulfatide synthesis. These findings suggest two potential actions of PACAP in the temporal and spatial control of OL proliferation and myelin production in the developing brain.

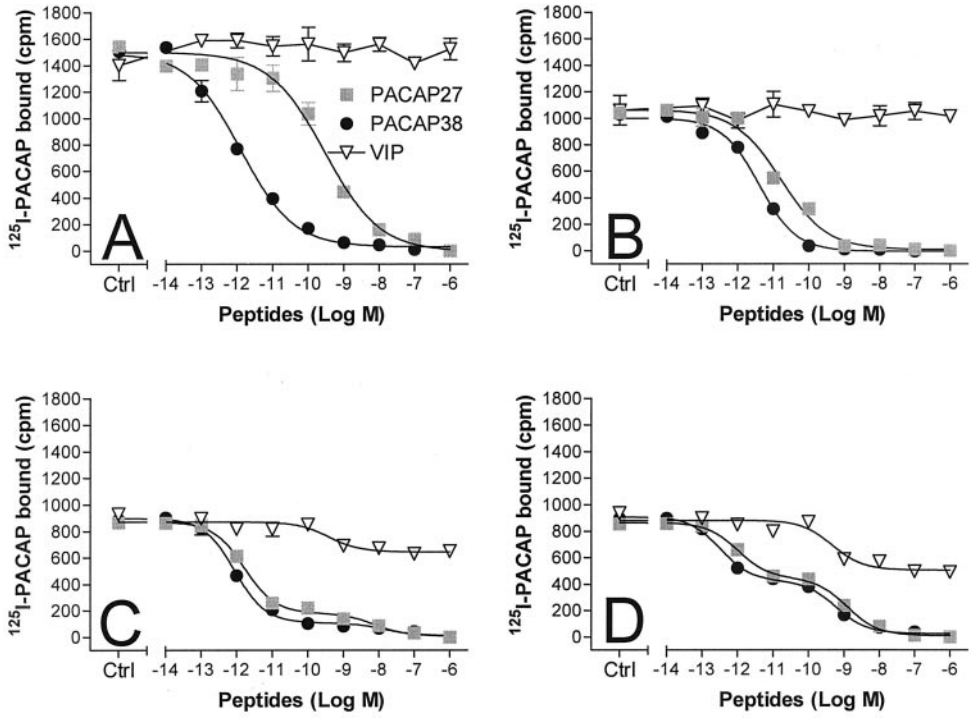


*Figure 5.* Displacement of $^{125}$I-PACAP binding by PACAP analogs on isolated cells at different stages of maturation (corresponding to the stages in Fig. 3). *A*, OLP. *B*, Multipolar pre-OL. *C*, Immature OL. *D*, Mature multipolar OL. Displacements were performed in triplicate as indicated in Materials and Methods. At least three independent experiments were performed for each of the four maturation stages. Cell number was determined in each set of experiments to convert the raw binding data into counts per minute bound per 100,000 cells. Binding curves were estimated using sigmoidal or two-site-displacement equations and computerized with Graphprism software. *Ctrl*, Control.

Although PACAP was able to induce cAMP synthesis in freshly isolated OLP, the signaling pathway used by PACAP to stimulate OLP proliferation is still uncertain. Low concentrations of cAMP analogs were reported to be mitogenic in cultured Schwann cells (Raff et al., 1978). However, similar studies by the same group did not reveal mitogenic actions of cAMP analogs on OL precursors isolated from neonatal rat optic nerve (Hart et al., 1989). In addition to the cAMP pathway, $PAC_1$ receptors are known to regulate cell proliferation and/or survival via MAP kinase, phospholipase D, and phosphatidylinositol 3-kinase and other signaling cascades (Morisset et al., 1995; Villalba et al., 1997; Lelièvre et al., 1998). Interestingly, PACAP has been shown to either stimulate or inhibit proliferation, depending on the cell type. For example, although we showed here that PACAP simulated OLP proliferation, we have shown previously that PACAP

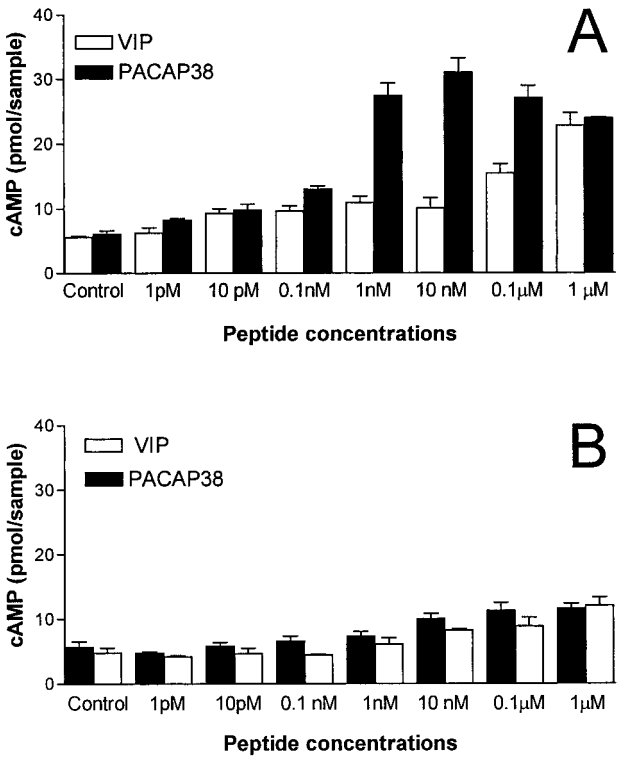


*Figure 6.* Neuropeptide-induced cAMP levels in OLP (*A*) and in OL allowed to differentiate for 10 d in culture (*B*). Cells were pretreated with IBMX (10 $\mu$M) for 20 min followed by neuropeptide treatment for another 20 min. PACAP-38 and VIP were used at the specified concentrations from 1 pM to 1 $\mu$M.

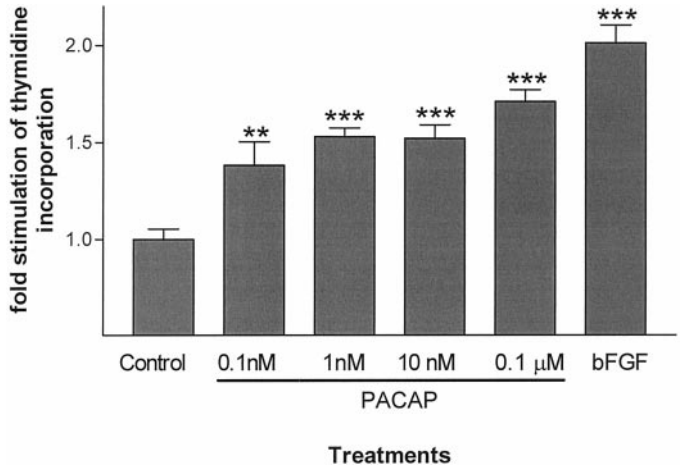


*Figure 7.* PACAP and FGF-2-induced DNA synthesis in cultured OLP. Freshly isolated cultures of OLP were preincubated for 1 d in medium containing 2% fetal bovine serum and then treated for 3 d with PACAP-38 at the indicated concentrations and FGF-2 (10 ng/ml). [$^3$H]Thymidine was added during the final 14 hr of peptide treatment. [$^3$H]Thymidine incorporation in control cultures was ~7000 cpm/well. Data were analyzed by ANOVA (** and ***, values significantly different from control at $p < 0.05$ and 0.005, respectively).

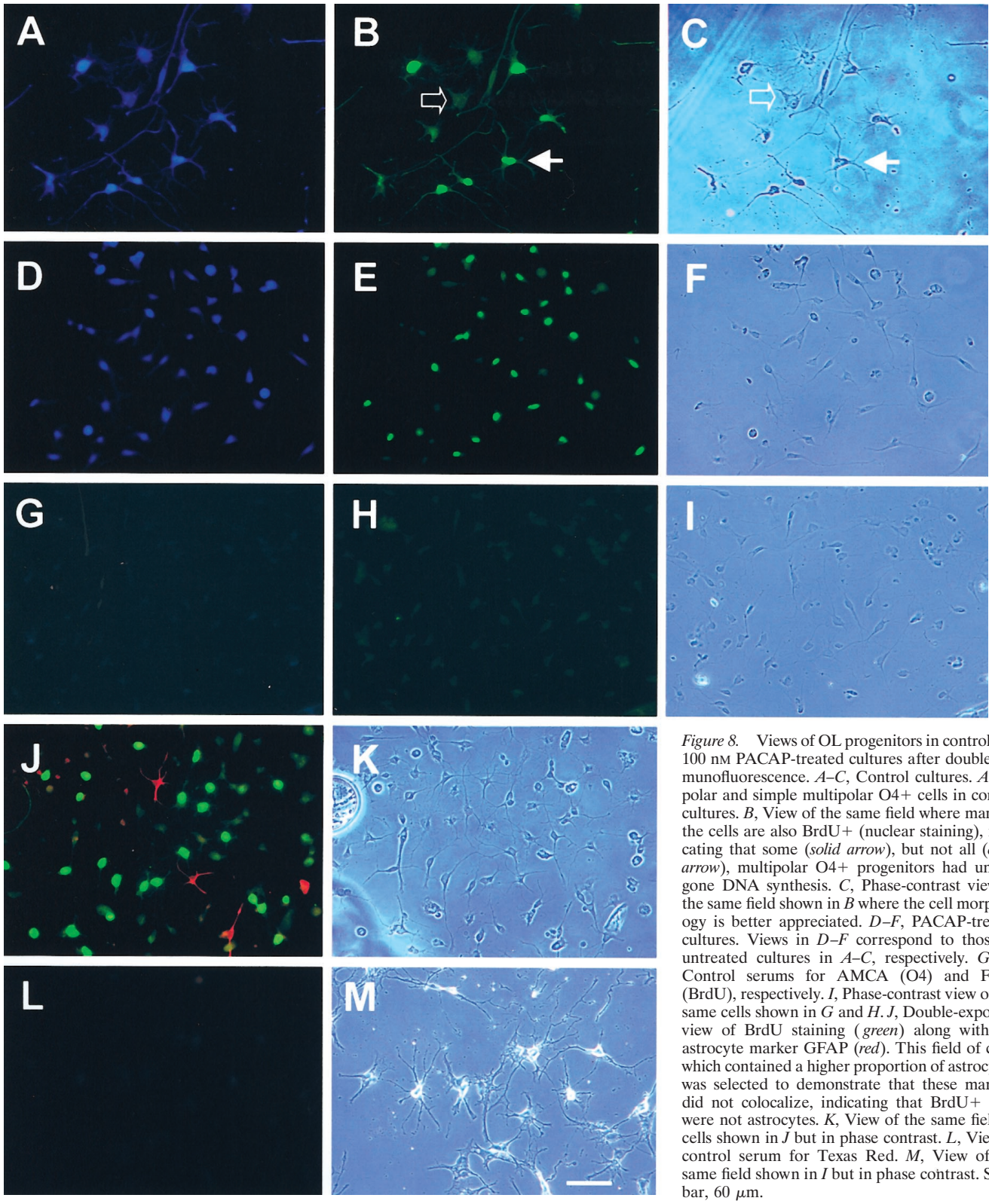


*Figure 8.* Views of OL progenitors in control and 100 nM PACAP-treated cultures after double immunofluorescence. *A–C*, Control cultures. *A*, Bipolar and simple multipolar O4+ cells in control cultures. *B*, View of the same field where many of the cells are also BrdU+ (nuclear staining), indicating that some (*solid arrow*), but not all (*open arrow*), multipolar O4+ progenitors had undergone DNA synthesis. *C*, Phase-contrast view of the same field shown in *B* where the cell morphology is better appreciated. *D–F*, PACAP-treated cultures. Views in *D–F* correspond to those of untreated cultures in *A–C*, respectively. *G, H*, Control serums for AMCA (O4) and FITC (BrdU), respectively. *I*, Phase-contrast view of the same cells shown in *G* and *H*. *J*, Double-exposure view of BrdU staining (*green*) along with the astrocyte marker GFAP (*red*). This field of cells, which contained a higher proportion of astrocytes, was selected to demonstrate that these markers did not colocalize, indicating that BrdU+ cells were not astrocytes. *K*, View of the same field of cells shown in *J* but in phase contrast. *L*, View of control serum for Texas Red. *M*, View of the same field shown in *I* but in phase contrast. Scale bar, 60 μm.

inhibited DNA synthesis in neural precursors isolated from the hindbrain portion of the neural tube of embryonic day 10.5 (E10.5) mice (Waschek et al., 1998). Another group showed that PACAP stimulated the proliferation of E15.5 rat sympathetic neuroblasts, whereas it inhibited the proliferation of E13.5 cortical neuroblasts (Lu and DiCicco-Bloom, 1997; DiCicco-Bloom et al., 2000). Cell-specific intracellular signaling systems and receptor-coupling mechanisms may thus play an important role in regulating the proliferative actions of PACAP.

The ability of PACAP to inhibit myelinogenesis was demonstrated in initial experiments using cultured OLP undergoing differentiation. It has been shown previously that as OLP differ-

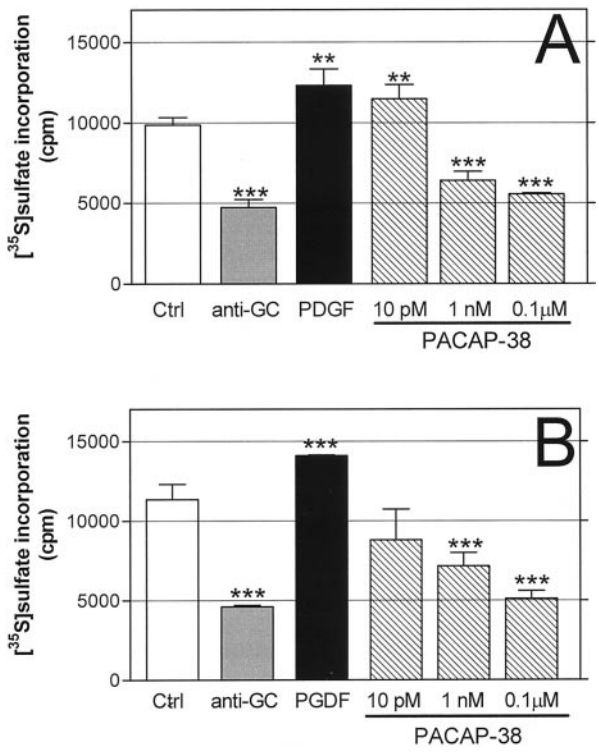


*Figure 9.* Modulation of myelinogenesis by PACAP and various treatments in differentiating OL in culture (*A*) and in cerebellar slices (*B*). After 7 d of differentiation in culture, the following were added: PACAP (at the indicated concentrations), PDGF (10 ng/ml), and anti-GC antibody (15 μl/ml). Drugs additions were repeated every other day for a period of 2 weeks. Myelinogenesis was examined during the final 2 d of treatment by measuring sulfate incorporation into sulfatides as described in Materials and Methods. Three independent experiments were performed, each in triplicate. Statistical analysis was by ANOVA (** and ***, values significantly different from respective control at $p < 0.05$ and 0.005, respectively).

entiate in culture, they show the characteristic morphological changes shown here (Fig. 3D), as well as increased expression of markers characteristic of myelinating cells (GC and MBP), and reduced expression of GD3 (characteristic of immature precursors) (Hardy and Reynolds, 1991). To confirm the data obtained in cultured OL, similar experiments were conducted on cerebellar slice cultures in which myelination of axons has been demonstrated previously (Notterpek et al., 1993). Again, PACAP potently inhibited sulfate incorporation. Likewise, the reported effect of GC antibodies on sulfate incorporation was recapitulated in the cerebellar slice cultures. The fact that PACAP inhibited sulfatide synthesis in purified OL cultures suggests that PACAP acted in slice cultures directly on OL rather than indirectly on another cell type.

It was interesting that PDGF stimulated sulfatide synthesis in OL cultures and cerebellar slices. PDGF also stimulated DNA synthesis in our OLP cultures (data not shown). PDGF is a well known mitogen but also permits OL differentiation (Bögler et al., 1990). One possible explanation for this apparent dual action of PDGF is that developing OL change their response to PDGF with time. The differing actions of PACAP and PDGF on myelinogenesis might thus reflect differences in modulation by external factors or, alternatively, by intrinsic differences in the precise mechanisms of their mitotic actions, for example, promotion of symmetric versus asymmetric division (Ibarrola et al., 1996). A third possibility is that these peptides have separate actions on oligodendrocyte maturation that are unrelated to their effects on proliferation. A separate inhibitory action of PACAP on maturation is supported by the fact that treatment of cerebellar slices with PACAP during the final week only (i.e., during the third week *in vitro*) was still able to inhibit sulfatide synthesis (data not shown). It seems likely that OLP proliferation would be minimal during this time.

The intracellular signaling pathway used by PACAP to inhibit myelinogenesis is also uncertain. PACAP moderately increased cAMP production in freshly isolated OLPs, but the degree of induction clearly decreased with maturation in culture. The relative loss in the ability of PACAP to increase cAMP production in differentiated OL was accompanied by a reduction in receptor number and a change in binding characteristics. Progenitors expressed high-affinity binding sites that specifically bound PACAP-38, whereas differentiated OL exhibited a lower level of PACAP-binding sites that did not discriminate between PACAP-27 and PACAP-38. In addition, VIP did not compete with radioiodinated PACAP binding in OLP, whereas in differentiated OL, almost half of the available $^{125}$I-PACAP-binding sites were displaced by subnanomolar concentrations of VIP. Specifically, a switch from predominantly PACAP-preferring receptors to VIP and PACAP receptors was observed as OLP differentiated into more mature OL. This observation is reminiscent of previous findings of VIP and PACAP receptor shifts during neuroblastoma cell differentiation (Lelièvre et al., 1996; Waschek et al., 1997). A differential expression of receptors for thyroid hormone (T3) during OL maturation has also been described previously (Carre et al., 1998). Briefly, OLP expressed primarily a T3 receptor variant ($\alpha 2$) that did not bind T3, whereas a different receptor form ($\beta 1$) that binds T3 became expressed as OL matured.

The significance of the apparent shift in PACAP receptor pharmacology with OLP maturation in culture and the mechanism mediating the shift are unknown. We found that the $PAC_1$ receptor gene continued to be expressed as OL differentiated (data not shown), suggesting that at least some of the binding was caused by expression of the same $PAC_1$ gene. Because the $PAC_1$ receptor has at least eight different splice variants, differing in their coupling and relative affinities for PACAP analogs, one can imagine that a shift in splice form might result in an enhanced affinity for VIP and/or activation of alternate signaling pathways. We did not detect in OL cultures any of the splice variants in the N-terminal extracellular part of $PAC_1$. This receptor domain has been shown to regulate the relative affinity for VIP and PACAP agonists (Dautzenberg et al., 1999). We have not yet attempted to detect splice variants in either the third intracellular loop or the fourth transmembrane domain. These variants have been shown to couple the $PAC_1$ receptor to alternative signaling pathways, although they do not seem to change the receptor affinity for VIP (Spengler et al., 1993; Chatterjee et al., 1996). Thus, several questions remain regarding the receptor forms and signaling pathways that mediate the actions of PACAP in OL development.

It is worth noting that although $PAC_1$ gene expression was easily detectable in cultured OLP by Northern analysis on total RNA, the same assay did not clearly reveal detectable $PAC_1$ gene expression in equivalent amounts of RNA from cultured astrocytes or microglia (Fig. 4). However, as discussed previously, others have detected $PAC_1$ mRNA in these other glial cell types by more-sensitive RT-PCR assays (Grimaldi and Cavallaro, 1999; Kim et al., 2000). Furthermore, $PAC_1$ receptors appear to medi-

ate some of the actions of PACAP on cultured astrocytes (Figiel and Engele, 2000) and on cultured microglia (Kim et al., 2000). Thus, our results do not imply that $PAC_1$ receptors are not present in these other glial cell types.

What is the importance of the proposed PACAP actions on OLP proliferation and myelinogenesis in normal development and disease? We speculate that the inhibitory action of PACAP on myelinogenesis is necessary to delay myelination, for example, to allow time for synaptic contacts to be established and mature. Interestingly, the expression of PACAP is strongly upregulated in several models of nerve regeneration (Zhang et al., 1996; Zhou et al., 1999; for review, see Zigmond, 1997). PACAP might thus be a factor used to stimulate OLP proliferation after nerve injury (Gensert and Goldman, 1997; McTigue et al., 1998). Subsequently, PACAP might delay myelinogenesis to allow time for the nerve to reinnervate its targets. Finally, the proliferative action of PACAP on OLP may be relevant in CNS tumorigenesis and tumor progression because glioblastomas, including oligodendrocytomas, commonly express PAC and VIP receptors (Vertongen et al., 1995).

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