Neurotransmitter receptor activation triggers $p27^{Kip1}$ and $p21^{CIP1}$ accumulation and G1 cell cycle arrest in oligodendrocyte progenitors

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SUMMARY

We examined the pathways that link neurotransmitter receptor activation and cell cycle arrest in oligodendrocyte progenitors. We had previously demonstrated that glutamate receptor activation inhibits oligodendrocyte progenitor proliferation and lineage progression. Here, using purified oligodendrocyte progenitors and cerebellar slice cultures, we show that norepinephrine and the $\beta$-adrenergic receptor agonist isoproterenol also inhibited the proliferation, but in contrast to glutamate, isoproterenol stimulated progenitor lineage progression, as determined by O4 and O1 antibody staining. This antiproliferative effect was specifically attributable to a $\beta$-adrenoceptor-mediated increase in cyclic adenosine monophosphate, since analogs of this cyclic nucleotide mimicked the effects of isoproterenol on oligodendrocyte progenitor proliferation, while $\alpha$-adrenoceptor agonists were ineffective. Despite the opposite effects on lineage progression, both isoproterenol and the glutamate receptor agonist kainate caused accumulation of the cyclin-dependent kinase inhibitors $p27^{Kip1}$ and $p21^{CIP1}$, and $G_1$ arrest. Studies with oligodendrocyte progenitor cells from INK4a−/− mice indicated that the $G_1$ cyclin kinase inhibitor p16INK4a as well as p19ARF were not required for agonist-stimulated proliferation arrest. Our results demonstrate that $\beta$-adrenergic and glutamatergic receptor activation inhibit oligodendrocyte progenitor proliferation through a mechanism that may involve $p27^{Kip1}$ and $p21^{CIP1}$; but while neurotransmitter-induced accumulation of $p27^{Kip1}$ is associated with cell cycle arrest, it does not by itself promote oligodendrocyte progenitor differentiation.

Key words: Norepinephrine, O-2A cells, Glia, Cyclin kinase inhibitors, Glutamate receptors, cAMP, Voltage-dependent K+ channels

INTRODUCTION

The early appearance of monoaminergic systems in the mammalian central nervous system has led to speculation that these transmitters may have a regulatory function in brain development (Levitt and Moore, 1979; Thomas et al., 1995; Zhou et al., 1995). The monoamine content of the brain sharply increases during the last week of gestation, and continues to increase during the first postnatal week (Coyle and Henry, 1973). Mammalian glial cells are likely to be a primary target of neuronally released norepinephrine (NE) in vivo (see Stone and Ariano, 1989 for review), and this transmitter could potentially modulate macroglial development. For example, in vivo studies in the rat optic nerve have demonstrated that NE regulates astrocyte proliferation through the activation of $\alpha$-adrenergic receptors (Hodges-Savola et al., 1996).

Ultrastructural analysis of the rat visual cortex has revealed that noradrenergic terminals make direct contacts with oligodendrocytes and astrocytes (Aoki, 1992; Paspalas and Papadopoulos, 1996). Both cell types display physiological responses to NE that are mediated through distinct receptor subtypes belonging to the $\alpha$- and $\beta$-subfamily (McCarthy and deVellis, 1978; McCarthy, 1983; Dave et al., 1991; Wigginton and Minneman, 1991; Cohen and Almazan, 1993; Roy and Sontheimer, 1995; Bernstein et al., 1996; Rohrer and Kabilka, 1998). Activation of $\beta$-adrenoceptors inhibits astrocyte proliferation through an increase in cAMP production and inhibition of MAP kinase translocation (Kurino et al., 1996). Changes in intracellular cAMP levels also modulate glial cell differentiation, as shown by the enhancing effects of cAMP analogs on the rate of oligodendrocyte maturation (Raible and McMorris, 1989; 1993). Taken together, these studies indicate that NE may affect glial cell development through the activation of $\beta$-adrenoceptors.

Oligodendrocytes develop from progenitor cells (OPs) (McCarthy and deVellis, 1980; Raff et al., 1983; Levi et al., 1986; Barres et al., 1990; Gard and Pfieffer, 1990; Fulton et al., 1992; Levine et al., 1993; Patneau et al., 1994; Gallo and Armstrong, 1995), which express a variety of neurotransmitter receptors and membrane ion channels both in vitro and in vivo (see Steinhauser and Gallo, 1996 for review). In the mammalian brain, OPs proliferate, migrate and differentiate...
postnatally (Reynolds and Wilkin, 1988; Warrington and Pfeiffer, 1992; Levison and Goldman, 1993; Luskin and McDermott, 1994; Zerlin et al., 1995) and are consequently exposed to a variety of neuronal signals, including neurotransmitters. We have previously studied the effects of the excitatory transmitter glutamate, and demonstrated that activation of glutamate receptors (GluRs) inhibits OP proliferation and prevents their differentiation (Gallo et al., 1996; Knutson et al., 1997; Yuan et al., 1998).

In the present study, we analyze the physiological effects of NE, and show that this neurotransmitter is an antiproliferative signal which, in contrast to glutamate, accelerates OP cell differentiation. The biological effects of NE are mediated through selective activation of β-adrenoceptors and an increase in intracellular cAMP. Because of its antiproliferative effects, we analyzed the effects of β-adrenoceptor activation on proteins known to be involved in cell cycle arrest, i.e. cyclin-dependent kinase inhibitors (cdkis). We show that β-adrenoceptor agonists increase the levels of p27Kip1 (Polyak et al., 1994a,b), a cdki involved in the constitutive arrest of OP cell proliferation during development (Casaccia-Bonnefil et al., 1997; Durand et al., 1997), and of p21CIP1, a protein that plays a crucial role in the regulation of cell proliferation and terminal differentiation in distinct lineages (Macleod et al., 1995; Parker et al., 1995; Skapek et al., 1995). Finally, we demonstrate that GluR agonists also trigger accumulation of p27Kip1 and p21CIP1.

MATERIALS AND METHODS

Materials
Platelet-derived growth factor (PDGF; human AB, heterodimer form) and basic fibroblast growth factor (bFGF; human) were both from Upstate Biotechnology (Lake Placid, NY). Norepinephrine, isoproterenol, kainic acid, 8Br-cAMP and dibutyryl-cAMP (db-cAMP) were all from Sigma (St. Louis, MO). Phenylephrine and clonidine were from Research Biochemicals International (Natick, MA). α-aminoo-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) was from Tocris Cookson (Bristol, UK). Anti-cyclin D antibodies (anti-human, polyclonal) were from Upstate Biotechnology. Anti-p27Kip1, anti-p15INK4b and anti-p21CIP1 antibodies (all rabbit polyclonals) were from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell cultures
Purified cortical OP cell cultures were prepared by modifications of previously described methods (Armstrong et al., 1990; McKinnon et al., 1990; Gallo and Armstrong, 1995; Gallo et al., 1996). Briefly, E20 Sprague-Dawley rats were killed following the NIH Animal Welfare guidelines, cortices were removed, mechanically dissociated, suspended in Dulbecco Modified Eagle Medium (DMEM; Life Technologies, Gaithersburg, MD) containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT), and plated in plastic T75 flasks. After 12 days in culture, OP cells growing on top of a confluent monolayer of astrocytes were detached by shaking overnight (McCarthy and de Vellis, 1980). Contaminating microglial cells were further eliminated by plating this fraction on plastic culture dishes for 1 hour. The OP cells, which do not attach well to plastic, were collected by gently washing the dishes, replated (3×10^4 cells/cm^2) onto poly-D-ornithine-coated plates (0.1 mg/ml) and cultured in DME-N1 biotin-containing medium. After 2 hours, either PDGF (10 ng/ml), or bFGF (10 ng/ml) or PDGF+bFGF were added to the culture medium. OP cells were cultured for 1-2 days and treated every 24 hours with PDGF and/or bFGF. Synchronization of OP cells was accomplished by culturing the cells in DME-N1 biotin-containing medium for 1-2 days before treating with growth factors (PDGF or bFGF). Differentiation into O4+ pre-oligodendrocytes was promoted by withdrawing the mitogenic factors and growing the OP cells for an additional 2 days in DME-N1 medium containing 0.5% FBS.

 Cultures prepared from mice carrying the Ink4a deletion (Serrano et al., 1996) were prepared from P1 pups. Cortical OP cells from these mice were prepared and cultured following the same protocol used for the rat progenitor cells.

Purified rat and mouse OP cells used for immunostaining were grown on glass coverslips pre-coated with poly-D-ornithine. In our previous studies in rat OP cultures (Gallo and Armstrong, 1995; Gallo et al., 1996), we demonstrated that 100% of the cells expressed nestin and more than 90% of the nestin+ cells were GD3+ or A2B5+. Fewer than 5% O4+ cells and no O1+ cells were found in the rat cultures (Gallo and Armstrong, 1995; Gallo et al., 1996).

Immunocytochemical characterization of the cortical Ink4a-/- mouse cultures demonstrated that more than 95% of the cells were OPs, based on the following criteria: (i) positive staining with an antisera against the NG2 proteoglycan (Stallcup and Beasley, 1987; Durand et al., 1998); (ii) positive staining with anti-GAP-43 antibodies (Curtis et al., 1991; Fanarraga et al., 1995); (iii) nestin expression, as detected with anti-nestin antibodies (Gallo and Armstrong, 1995); (iv) small percentage (fewer than 5%) of O4+ cells (Fanarraga et al., 1995; Gallo and Armstrong, 1995); and (v) bipolar or monopolar morphology (Fanarraga et al., 1995; Gallo and Armstrong, 1995). In agreement with previous reports (Fanarraga et al., 1995; Durand et al., 1998), the majority of cortical mouse OP cells were not stained with A2B5 or anti-GD3 antibodies. Finally, as previously demonstrated by Fanarraga et al. (1995) for cultured wild-type mouse OPs, also in the Ink4a-/- mouse cultures GAP-43 expression was down-regulated in the small percentage of O4+ cells present, as compared to OPs. No GFAP+ cells were detected in the purified mouse Ink4a-/- OP cells.

Cerebellar organotypic slice cultures and cell dissociation
Cerebellar organotypic slice cultures were prepared from postnatal day 6 Sprague-Dawley rats and processed as previously described (Yuan et al., 1998). Groups of 4-6 cerebellar slices were placed on each filter and maintained in culture for a total of 48-72 hours. Isoproterenol (50 μM) was added to the slices for 48 hours. Bromodeoxyuridine (BrdU, 50 μM; Sigma) was added to the slices for the last 24 hours. After 72 hours in culture, cerebellar slices were treated with protease and with trypsin inhibitor. Cells were then dissociated by triturating through a Pasteur pipette and plated on poly-D-ornithine-coated coverslips for immunocytochemistry. Cells were stained and analyzed 2 hours after plating.

Cell proliferation assays in cell cultures
Purified cortical OP cells were plated in DME-N1 biotin-containing medium with 0.5% FBS in 24 multiwell plates at a density of 3×10^4 cells/cm^2. After 2 hours, PDGF and/or bFGF, and either norepinephrine, isoproterenol, phenylephrine, clonidine, 8Br-cAMP, db-cAMP, or kainic acid were added to the cultures along with methyl-[3H]thymidine (0.5 μCi/ml; 85 Ci/mmol; Amersham Life Science, Arlington Heights, IL). Unless otherwise stated, after 22 hours cells were lysed and [3H]thymidine incorporation was measured by precipitation with 10% trichloroacetic acid and scintillation counting. Pretreatment of the cells with PDGF or bFGF for 24 hours had no effect on the response to treatment with the agonists (see also Gallo et al., 1996).

[3H]Thymidine incorporation assays in synchronized OP cells were performed by culturing the cells without growth factors for 48 hours and then treating them with either PDGF or bFGF (both 10 ng/ml). Isoproterenol or kainate were added with the growth factors, or between 22 and 24 hours after. All cells were labeled with [3H]thymidine at 12 hours after adding PDGF or bFGF, and harvested after 18 hours to measure [3H]thymidine incorporation.
Immunocytochemistry and counting of cell cultures and dissociated cells

The following primary antibodies were used: LB1 (Levi et al., 1986; Curtis et al., 1988), NG2 (Stallcup, 1981), A2B5 (Eisenbarth et al., 1979), nestin (Tohyama et al., 1992; Gallo and Armstrong, 1995), O4 (Sommer and Schachner, 1981), O1 (Sommer and Schachner, 1981) and anti-BrDU (Dako Corp., Carpinteria, CA). All secondary fluorochrome-conjugated antibodies were from Cappel-Organon Teknika (Durham, NC). Double indirect immunofluorescence experiments in rat and mouse cultures were performed as previously described (Gallo and Armstrong, 1995; Gallo et al., 1996; Yuan et al., 1998). For cell counting, 10-20 microscopic fields (Zeiss Axioshot fluorescence microscope; 40× objective) were counted for each coverslip, and 2 coverslips for each experiment were analyzed. At least 3 independent experiments were performed for each antibody, corresponding to a total of several thousands of cells counted (see figure legends). Data are presented as averages ± s.e.m.

cAMP assay

OP cells were cultured in PDGF+bFGF for 2 days. Cultures enriched in O4 pre-oligodendrocytes were obtained as described above. Cells were stimulated with drugs for 10 minutes and harvested in PBS containing 4 mM EDTA. cAMP levels were assayed using a Cyclic AMP [3H] assay system kit (Amersham) according to the manufacturers recommendations.

Western blot analysis

After treatment with growth factors and drugs, 2×10^6 cells were washed twice and harvested in cold PBS. The cells were resuspended and lysed by sonication in 300 μl sample buffer (150 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM NaVO₄, 1 mM NaF, 0.25% Na-deoxycholate, 10 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM AEBSF). The lysate was centrifuged at 5,000 g for 15 minutes and the supernatant collected. An aliquot was taken for protein determination using the Pierce BCA* protein assay kit (Rockford, IL), and the supernatant was used for Western blotting. Membranes (Schleicher & Schuell, Keene, NH) were blocked in 5% non-fat dry milk in PBST (17 mM KH₂PO₄, 50 mM NaF, pH 7.4, 0.25% Na-deoxycholate, 10 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM AEBSF). Blots were blocked with 5% non-fat dry milk in PBST (17 mM KH₂PO₄, 50 mM NaHPO₄, 1.5 M NaCl, pH 7.4±0.05% Tween 20) for 1 hour at room temperature, then incubated at room temperature for 2 hours in PBST and 5% non-fat dry milk containing anti-cyclin D, anti-p15/KIP1, anti-p27/KIP1 or anti-p21/CIP1 antibodies. Protein bands were detected using the Amersham ECL kit with horseradish peroxidase-conjugated secondary antibodies. Relative intensities of the protein bands were quantified by scanning densitometry (ScanWizard Plug-In, Microtek, Redondo Beach, CA).

TUNEL assays

Apoptotic cell death was determined by fluorescence microscopy by using the TUNEL assay (Boehringer Mannheim). Synchronized cultured OP cells were fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100, 0.1% sodium citrate (2 minutes at 4°C), and stained with TUNEL according to the manufacturer’s instructions. Apoptotic OP cells were brightly fluorescent. For cell counting, 10 microscopic fields (Zeiss Axioshot fluorescence microscope; 40× objective) were counted for each coverslip, and 2 coverslips were analyzed for each experiment (see Table 1). A total of 3 independent experiments were performed.

Electrophysiology

For electrophysiological experiments, cells were cultured with 10 ng/ml PDGF (proliferating OP cells). Cells were perfused with medium of the following composition (in mM): NaCl 160; KCl 2.5; CaCl₂ 1.5; MgSO₄ 1.5; glucose 10; HEPES 10; tetrodotoxin 0.5-1 μM. Tight seal (>5 Gohm) whole cell recordings were made from LB1+ (GD3+) OPs. Patch electrodes had resistances of 2-6 Mohm when filled with (in mM) potassium glutonate 130; NaCl 10; Na₂ATP 2; NaGTP 0.3; HEPES 10; ethylene glycol-bis-β-aminoethylether, N,N,N′,N′-tetraacetic acid (EGTA) 0.6; glutathione (5 mM); buffered to pH 7.4 and approx. 275 mOsm. Cells were voltage clamped between −70 and +40 mV and test pulses delivered to −60 to +70 mV (0.1 Hz). Records were filtered at 2 kHz and digitized at 5-10 kHz. Linear leak current and capacitative artifacts were digitally subtracted off-line using Clampfit (Axon Instruments, Burlingame CA, USA). The series resistances were in the range 5-15 Mohm (Mean 8.8±0.9 Mohm; n=25) and were compensated to at least 85%. Cell capacitance was measured directly from the amplifier. Plots of the voltage-dependence of current activation were fitted using a Boltzmann equation of the form:

\[ g/g_{max} = [1 + \exp \{V_{1/2} - V/k]\]^{-1} \]

where \( g/g_{max} \) is the normalized conductance, \( V \) is the membrane potential, \( V_{1/2} \) is the membrane potential at which the current amplitude is half maximum and \( k \) is a constant. For the construction of the majority of activation curves, the sum of two independent Boltzmann equations were used. All data are expressed as the mean ± s.e.m.

RESULTS

OP cells express functional adrenergic receptors

β-adrenergic receptors are positively coupled to adenylate cyclase in a variety of cell types, including neurons and glia (McCarthy and deVellis, 1978; Baker et al., 1986; Stone and Ariano, 1989; Stone et al., 1990). Treatment of OP cells with the β-receptor agonist isoproterenol caused a 15- to 40-fold stimulation of intracellular cAMP levels (Fig. 1A). The effect of isoproterenol was dose-dependent and reached a plateau at a concentration of 10 μM (Fig. 1A). Treatment of O4 pre-oligodendrocytes with similar concentrations of isoproterenol had no significant effect on intracellular cAMP (Fig. 1A). In a parallel set of experiments in OP cells (Fig. 1B), it was found that norepinephrine (NE) also significantly stimulated cAMP formation (25- to 30-fold increase over control). The α₁-adrenoceptor agonist phenylephrine displayed only modest effects, whereas the α₂-agonist clonidine was ineffective (Fig. 1B).

Activation of adrenergic receptors increases [Ca²⁺], in different types of neural cells (Dave et al., 1991; Cohen and Almazan, 1993; Duffy and MacVicar, 1995; Bernstein et al., 1996). In the present study, we confirmed that, in the majority of cortical OP cells, NE and the α₁-agonist phenylephrine caused persisting oscillatory Ca²⁺ responses of variable amplitudes (Simpson and Russell, personal communication). Responses to the α₂-agonist clonidine were transient and very small in amplitude, whereas the β-agonist isoproterenol was ineffective (Simpson and Russell, personal communication). Taken together, these data indicate that proliferating OP cells express functional α- and β-adrenergic receptors, and that activation of β-subtypes is selectively coupled to cAMP formation.

Activation of β-adrenergic receptors selectively inhibits OP cell proliferation

Purified cortical OP cells cultured in the presence of PDGF or bFGF for 24 hours displayed a 2- to 4-fold increase in [³H]thymidine incorporation compared to cells cultured in the absence of growth factors (Gallo and Armstrong, 1995; Gallo et al., 1996; Knutson et al., 1997). In the presence of either
mitogen, NE significantly inhibited [3H]thymidine incorporation in OP cells in a concentration-dependent fashion (Fig. 2A). IC50 values for NE were 144.9±20.4 and 136.8±7.6 μM (n=6) in PDGF and bFGF, respectively. The antiproliferative effects of NE were mimicked by the selective α1-adrenergic receptor agonist phenylephrine (PHE; 100 μM) did not modify intracellular cAMP levels (A). The selective α2-adrenergic receptor agonist phenylephrine (PHE; 100 μM) displayed a small effect, while activation of α2-receptors by clonidine (CLO; 100 μM) was ineffective (B). Data are averages of 3-6 independent experiments ± s.e.m. *P<0.01; **P<0.05 vs. control (Student’s t-test).

Activation of β-adrenergic receptors stimulates OP cell differentiation

It has been previously demonstrated that an elevation of intracellular cAMP in cells of the oligodendrocyte lineage promotes their differentiation (Pleasure et al., 1986; Raible and McMorris, 1989, 1993). Therefore, we analyzed the effects of isoproterenol on OP cell development in culture by studying staining characteristics with the antibodies O4 and O1, which identify more mature stages of the oligodendrocyte lineage (Sommer and Schachner, 1981). In cells treated with PDGF for 48 hours, isoproterenol significantly stimulated OP cell lineage progression. A 2-fold increase in the percentage of O4+ cells was observed in cells maintained in the presence of PDGF and isoproterenol, compared to cells cultured without the β-adrenergic agonist (Fig. 4A). Consistent with its lack of effect on cAMP formation in cells cultured without mitogens (see Fig. 1A), isoproterenol did not promote OP differentiation in cells cultured in N1+0.5% FBS without growth factors (Fig. 4A). The β-adrenergic agonist also increased the percentage of O1+ cells in cultures maintained with PDGF, and had only a modest effect in cells cultured in N1+0.5% FBS without PDGF (Fig. 4B).

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<tr>
<th>Time after PDGF (hours)</th>
<th>% of TUNEL-positive cells</th>
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<tr>
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<td>Control</td>
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<td>6</td>
<td>6.4±0.7</td>
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<tr>
<td>12</td>
<td>7.2±1.0</td>
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<tr>
<td>18</td>
<td>6.3±0.7</td>
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<td>24</td>
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Cortical OP cells were synchronized by culturing in the absence of growth factors for 48 hours, and treated with PDGF (control, 10 ng/ml) in the presence or in the absence of isoproterenol (50 μM) or kainate (100 μM). Cells were stained at different times after re-adding the growth factor and the receptor agonists (6-24 hours). A total of 6 coverslips (10 microscopic fields/cover slip) from 3 independent experiments were analyzed. Total cells counted from phase contrast imaging for each treatment at each time were as follows: between 11,100 and 17,363 for PDGF alone, between 10,220 and 14,529 for PDGF+isoproterenol, and between 9,872 and 13,866 for PDGF+kainate. Data are averages ± s.e.m. (n=6). At time 0 (i.e. before adding PDGF), 7.9±0.9% of the total cells were TUNEL-positive (total cells counted = 13,464). Treatment with neither isoproterenol nor kainate significantly modified the percentage of TUNEL-positive cells compared to PDGF alone at any of the times tested.
Fig. 2. Norepinephrine inhibits OP cell proliferation through selective activation of β-adrenergic receptors. [3H]thymidine incorporation assays. Purified rat OP cells were cultured in DME-N1+0.5% FBS and PDGF or bFGF. Cells were plated in 24-well plates. After 2 hours, PDGF or bFGF (both 10 ng/ml), in combination with either norepinephrine, isoproterenol (ISO), phenylephrine (PHE), clonidine (CLO), kainate (KAI), 8Br-cAMP or db-cAMP were added to the cultures with [3H]thymidine (0.5 μCi/ml). After 22 hours, [3H]thymidine incorporation was measured by trichloroacetic acid precipitation and scintillation counting. Cells cultured with PDGF or bFGF displayed a 2- to 4-fold increase in [3H]thymidine incorporation, compared to cells cultured in the absence of growth factors. In C isoproterenol concentration was 25 μM and kainate was 100 μM. In D, isoproterenol concentration was 50 μM. (A-C) Cells in N1 medium without growth factors incorporated 10.75±1.023 cpm/well/22 hours (average ± s.e.m., n=27; 30,000 cells/well). Averages of 3-9 experiments in triplicate ± s.e.m. are shown. A and C: *P<0.001, **P<0.005, ***P<0.05 versus control. B: *P<0.0005, **P<0.005 vs. control (Student’s t-test). D: Cells in N1 medium without growth factors incorporated 7,225±306 cpm/well/22 hours (average ± s.e.m., n=24; 30,000 cells/well). Averages of 8 experiments in triplicate ± s.e.m. are shown. **P<0.005, ***P<0.05 versus control (Student’s t-test).

These results, taken together with the cAMP data shown in Fig. 1A, indicate that isoproterenol stimulates OP cell lineage progression most likely through a cAMP-dependent mechanism.

Activation of β-adrenergic receptors inhibits OP cell proliferation and stimulates their differentiation in slice cultures

In a previous study, we demonstrated that oligodendrocyte progenitor development can be studied in cerebellar slice cultures (Yuan et al., 1998). Since the cerebellar cortex is rich in noradrenergic innervation (Astor-Jones et al., 1995), we wanted to determine whether activation of β-adrenergic receptors also affected OP development in cerebellar slice cultures. Organotypic cultures were therefore treated with isoproterenol and BrdU, and cells were identified after dissociation. The antibodies NG2 and O1 were used as markers for OPs and oligodendrocytes, respectively (Yuan et al., 1998). Isoproterenol caused a 3-fold decrease in the percentage of NG2+ cells (Fig. 5A) and a 50% decrease in BrdU labeling index (Fig. 5B). The β-adrenergic receptor agonist also triggered a corresponding 3-fold increase in the percentage of O1+ oligodendrocytes (Fig. 5C). These results are in agreement not only with the data obtained in cultured cortical OP cells (Figs 2, 4), but also with experiments performed in cultured cerebellar OP cells, in which isoproterenol inhibited, by more than 75%, [3H]thymidine incorporation (Yuan and Gallo, unpublished observations).

In conclusion, the experiments performed in cerebellar slices show that in a cytotoarchitecturally intact system activation of β-adrenergic receptors inhibits OP proliferation and stimulates their differentiation. Therefore, the effects of activation of these receptors on OP development are not an artifact of cell dissociation, but rather represent a possible developmental role for the noradrenergic innervation in a cytotoarchitecturally intact system.

Activation of β-adrenergic receptors causes G1 arrest in OP cell cycle and prevents entry into S phase

We next wanted to determine whether activation of β-adrenoceptors affected OP cell proliferation by causing arrest in a specific phase of the cell cycle. Since we previously demonstrated that activation of GluRs also inhibited OP proliferation (Gallo et al., 1996; Knutson et al., 1997), we also wanted to compare the effects of β-adrenoceptor and GluR agonists on the cell cycle. OP cells were synchronized by culturing them in the absence of growth factors for 48 hours, and then adding PDGF or bFGF, allowing them to re-enter the cell cycle. The expression of cyclin D1, a marker of G1 phase, was analyzed at different times of incubation with the growth factors (Ross, 1996). Fig. 6A shows that cyclin D1 protein expression was low and almost undetectable before incubation with PDGF (time 0). At this time cells did not incorporate BrdU or [3H]thymidine (data not shown). Cyclin D1 levels increased up to 18 hours and then decreased again (to 40±16% of maximum at 24 hours; Fig. 6A). Parallel BrdU incorporation experiments demonstrated that after adding PDGF or bFGF to the culture medium 94.0±4.0 and 97.3±2.6% respectively of the OPs re-entered the cell cycle within 18 hours (average ± s.e.m.; 3 duplicate experiments). These results are consistent with the view that OP cells are predominantly in G0 prior to
treatment with PDGF, and enter G1/S after treatment with the mitogen. Treatment with isoproterenol or kainate did not modify cyclin D1 accumulation for the first 18 hours (Fig. 6A), indicating that OP cell entry into G1 phase was not affected by the receptor agonists.

Our findings that OPs re-enter the cell cycle after a 48 hour mitogen withdrawal are at variance with those in optic nerve progenitors, which irreversibly differentiate within 24 hours in the absence of mitogenic factors (Durand et al., 1997). This is likely due to differences in the culture medium. We maintained cortical OPs without growth factors (i.e. thyroid hormone, ciliary neurotrophic factor, NT-3 and forskolin; Durand et al., 1997) for 48 hours.

We then reasoned that if β-adrenergic receptor activation specifically interfered with a phase of the cell cycle that precedes S phase, we would detect differential inhibitory effects when isoproterenol was added at different times to synchronized OP cells. Fig. 6B shows that isoproterenol prevented OP cells from entering S-phase when added to the cultures within 6 hours after PDGF, but had no significant inhibitory effects when added between 12 and 24 hours after the growth factor. Identical results were obtained in OP cells cultured with bFGF (Ghiani and Gallo, unpublished observation). Interestingly, isoproterenol was significantly less effective when added 6 hours after PDGF (Fig. 6B) or bFGF (Ghiani and Gallo, unpublished observation) than at the same time as the mitogens.

The GluR agonist kainate prevented OP cells from entering S phase when added to the cultures within 12 hours after PDGF (Fig. 6C) or bFGF (Ghiani and Gallo, unpublished observation). After 12 hours the effect of kainate was either very small or absent (Fig. 6C). Neither isoproterenol nor kainate caused apoptosis in synchronized OP cells, as determined by TUNEL assays (Table 1). Taken together, these results indicate that: (i) both isoproterenol and kainate prevent OP cells from entering S phase, and (ii) both receptor agonists cause arrest in G1 phase of the OP cell cycle, rather than G0 arrest or apoptosis. The time schedule of isoproterenol- and

Fig. 3. The anti-proliferative effects of β-receptor agonists and cAMP analogs are reversible. Rat OP cells were cultured in PDGF (10 ng/ml) in the absence or in the presence of norepinephrine (NE, 100 μM; A), isoproterenol (ISO, 30 μM; B), 8Br-cAMP (1 mM; C) or db-cAMP (1 mM; D). After 22 hours, all cells were placed in fresh culture medium without agonists or cAMP analogs, but containing PDGF (10 ng/ml) and [3H]thymidine (0.5 μCi/ml). Cells were harvested after 6, 12 and 24 hours of agonist-free medium, and [3H]thymidine incorporation was determined by trichloroacetic acid precipitation and scintillation counting. Averages ± s.e.m. (n=3) are shown.

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Fig. 4. Activation of β-adrenergic receptors promotes OP lineage progression and stimulates differentiation. Immunohistochemical staining of rat oligodendrocyte lineage cells with the monoclonal antibodies O4 and O1. OP cells were purified and cultured in DMEM-N1 medium+0.5% FBS with PDGF (10 ng/ml). Isoproterenol (ISO; 30 μM) was added to the culture medium 2 hours after plating. CTR: control, no isoproterenol. After 46 hours, cells were immunostained with O4 or O1 antibodies and counted. Averages ± s.e.m. from 3–4 experiments (n=40–50 microscopic fields counted/antibody) are shown. The total number of cells counted for each culture condition ranged between 3,231 and 11,281. *P<0.0001, **P<0.005 versus control (Student’s t-test).
kainate-induced arrest in proliferation is also consistent with a block in G1 phase, based on an OP cell cycle of 24 hours in PDGF (Barres et al., 1994; Vick et al., 1992; Gao and Raff, 1997).

**Activation of β-adrenergic receptors increases p27kip1 and p21cip1 levels**

The activity of several cyclin-dependent kinases and related inhibitory proteins (cdk inhibitors) regulates eukaryotic cell cycle progression (Ross, 1996). Two families of mammalian cdk inhibitors that act in G1 have so far been identified in animal cells. The INK4 family comprises p15, p16, p18 and p19, whereas the KIP/CIP family comprises p21, p27 and p57 (Martin-Castellanos and Moreno, 1997). Anti-proliferative signals negatively regulate G1 phase progression through the induction of one or more of these proteins (Martin-Castellanos and Moreno, 1997). In order to determine the pathways of neurotransmitter-induced cell cycle arrest, we chose to

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**Fig. 5.** Activation of β-adrenergic receptors inhibits oligodendrocyte progenitor proliferation and stimulates their differentiation in cerebellar slice cultures. Quantitative analysis of cerebellar cells dissociated from P6 rat slice cultures treated for 48 hours with isoproterenol (ISO, 50 μM). CTR: control, no isoproterenol. All tissue slices were treated with BrdU for 24 hours. Cells were dissociated and stained with NG2+anti-BrdU (A and B) or O1+anti-BrdU antibodies (C). As previously demonstrated (Yuan et al., 1998), none of the O1+ cells were BrdU+. A total of six coverslips (10 fields/coverslip) were counted for each antibody staining from 3 independent experiments. A total of 78,461 and 45,460 cells were counted for NG2+BrdU and O1+anti-BrdU staining, respectively, in 3 independent experiments. *

**Fig. 6.** The anti-proliferative effect of isoproterenol is due to G1-arrest in OP cell cycle. (A) Time course of cyclin D expression as determined by western blot analysis. Rat progenitor cells were synchronized by culturing in the absence of growth factors for 48 hours, and treated with PDGF (10 ng/ml) in the presence or in the absence of isoproterenol (ISO; 50 μM) or kainate (KAI; 100 μM). Cells were harvested at different times after re-adding the growth factor and the receptor agonists (0-24 hours). Cyclin D expression was analyzed with anti-cyclin D polyclonal antibodies that bind to cyclin D1 and D2. The major band identified by the antibody is cyclin D1 (36 kDa), whereas the lower molecular mass band is cyclin D2 (34 kDa). p.c. = positive control, purified cyclin D proteins co-migrate with the cyclin D from OP cells. 20 μg of total proteins were loaded on the gel for each sample. (B,C) Isoproterenol and kainate lose their anti-proliferative effects as rat OP cells proceed through the cell cycle. [3H]thymidine incorporation assays were performed on synchronized OP cells that were pulsed with PDGF (10 ng/ml). Isoproterenol (50 μM; B) or kainate (100 μM; C) were added at the same time as the growth factors, or 6-24 hours later. [3H]Thymidine (0.5 μCi/ml) was added to cells from 12 hours to 30 hours after PDGF addition. Cells were then harvested to measure [3H]thymidine incorporation by trichloroacetic acid precipitation and scintillation counting. Data represent averages of 2-3 experiments ± s.e.m. run in triplicate (n=6-9 wells). B: *P<0.0005 vs. PDGF; C: *P<0.001, **P<0.05 versus PDGF. n.s. = not significant.
examine the role of different cdk inhibitors in OP cell proliferation.

Mice carrying a targeted deletion of the INK4a locus are deficient for both the p16\(^{INK4a}\) and the p19\(^{ARF}\) gene products (Serrano et al., 1996). Cell proliferation assays were performed in purified OP cultures prepared from INK4a\(^{-/-}\) mice (Fig. 7). Similar to the cellular composition of rat OP cultures, the mouse cultures comprised cells that displayed the morphological and antigenic properties previously described for wild-type mouse OPs (Stallcup and Beasley, 1987; Curtis et al., 1991; Gallo and Armstrong, 1995; Fanarraga et al., 1995; Durand et al., 1998). The vast majority (more than 95%) of the cells were NG2\(^+\)/nestin\(^+\)/GAP-43\(^+\) (Ghiani and Gallo, unpublished observations; see Materials and Methods for immunocytochemical characterization of these cultures). \(\text{[^3]H}\)thymidine incorporation assays in INK4a\(^{-/-}\) OPs demonstrated that both isoproterenol and kainate significantly inhibited PDGF- (Fig. 7A) and bFGF-stimulated (Fig. 7B) cell proliferation, indicating that neither p16\(^{INK4a}\) or p19\(^{ARF}\) are necessarily involved in the intracellular pathways leading to cell cycle arrest. The selective AMPA receptor agonist AMPA also decreased OP cell proliferation by approximately 40% in cells cultured with PDGF or bFGF (Fig. 7A,B), indicating that also in INK4a\(^{-/-}\) cells AMPA receptors are involved in the...
regulation of OP proliferation. Finally, expression of the INK4 cyclin-dependent kinase inhibitor p15INK4b in OP cells cultured with PDGF was low and not modified by either isoproterenol or kainate (3 experiments, Ghiani and Gallo, unpublished observations).

Recent studies have demonstrated that the arrest in OP cell proliferation that occurs during development is due to accumulation of the cdk inhibitor p27Kip1 (Casaccia-Bonnefil et al., 1997; Durand et al., 1997; Tikoo et al., 1997, 1998). We therefore studied whether p27Kip1 induction occurred during isoproterenol- and kainate-induced arrest in OP cell proliferation. Fig. 8A shows a western blot analysis of p27Kip1 expression in non-synchronized rat OP cells (i.e. in cultures treated with PDGF from the time of cell plating). After 48 hours of treatment, isoproterenol and kainate respectively increased p27Kip1 expression by 315±47% (average ± s.e.m.; n=3) and 232±41% (n=3) over the levels observed in cells cultured in PDGF alone. Importantly, the effect of isoproterenol was mimicked by the cAMP analogs 8Br-cAMP and db-cAMP, which respectively increased p27Kip1 expression by 199±13% (average ± s.e.m.; n=5) and 191±23% (n=5) over PDGF alone (Fig. 8A).

In synchronized rat OP cells (Fig. 8B), a 24-hour treatment with isoproterenol and kainate did not significantly modify p27Kip1 levels, compared to cells treated only with PDGF. After 2 days in culture, PDGF decreased the levels of p27Kip1 as compared to cells grown without growth factors (N1; Fig. 8B). At this time, treatment with either isoproterenol or kainate caused a significant accumulation in p27Kip1 above the levels measured in OP cells cultured with PDGF (Fig. 8B), confirming the previous observation in non-synchronized cells at 48 hours. In cells cultured without mitogens, p27Kip1 levels were 141±17% (average ± s.e.m.; n=4) of those measured in the presence of PDGF. Isoproterenol increased p27Kip1 levels by 242±33% (n=4), and kainate by 174±14% (n=4), compared to cells cultured in PDGF alone.

In parallel experiments in non-synchronized OP cells (Fig. 9), it was found that the protein levels of the cdk inhibitor p21CIP1 were also increased by isoproterenol (307±14% increase over PDGF alone; n=3) and by kainate (207±37% increase over PDGF alone; n=3) (Fig. 9A). The stimulatory effect of isoproterenol on p21CIP1 accumulation was also mimicked by 8Br-cAMP (240±20% increase over PDGF alone; n=4) and db-cAMP (218±16% increase over PDGF alone; n=4) (Fig. 9B).

Taken together, these results show that both β-adrenergic and glutamatergic receptor activation trigger p27Kip1 and p21CIP1 accumulation in dividing OP cells. Importantly, p27Kip1 levels in isoproterenol- and kainate-treated cells are even higher than those measured in cells that spontaneously accumulate p27Kip1 and stop dividing in the absence of mitogens (Fig. 8B). Finally, the stimulatory effects of isoproterenol on p27Kip1 and p21CIP1 accumulation occur at least in part through a cAMP-dependent pathway.

**Fig. 9.** Activation of β-adrenergic and glutamatergic receptors stimulates p21CIP1 accumulation in OP cells. Western blot analysis of p21CIP1 expression in non-synchronized rat OP cells. Cells were plated in PDGF (10 ng/ml) in the presence or in the absence of either isoproterenol (ISO; 50 μM), kainate (KAI; 100 μM), 8Br-cAMP (1 mM) or db-cAMP (1 mM). Cells were harvested after 48 hours and 40-50 μg of total proteins were loaded on the gel for each sample. Histograms represent relative levels of p21CIP1 determined by densitometric analysis of autoradiographs from western blots. Values are expressed as ratios of cells treated with PDGF alone and are mean ± s.e.m. of 3 (A) or 4 (B) separate experiments. A: *P<0.02 versus PDGF alone; B: *P<0.001 versus PDGF alone (Student’s t-test).


$1086\mu M$ for 24-48 hours. Fig. 10 shows that the properties of Kv currents were markedly altered in cells chronically treated with isoproterenol, compared to control cells. The voltage dependence of activation of the sustained current was shifted to the right in cells treated with isoproterenol. This modulation by isoproterenol also resulted in a shift of the activation threshold to more depolarized potentials, consequently no current was detected until test pulses to $-20$ mV (cfr. control currents which activated close to $-40$ mV; Knutson et al., 1997). We have previously shown that the sustained current phenotype in OP cells consists of two temporally overlapping sustained currents with distinct voltage-dependent activation profiles (Knutson et al., 1997). Separation of the Boltzmann function, used to describe the voltage-dependence of activation, into the two current components revealed that isoproterenol caused a shift to the right of both current components (Fig. 10B). The fractional contribution of the two overlapping currents to the total current was also modified. In control cells, the low threshold current contributed 38% of the maximal current, while in isoproterenol-treated cells this current contributed 47%. In 12 representative cells, the resting membrane parameters of the cell (i.e. resting membrane potential, input resistance and membrane capacitance) were unaltered following chronic treatment with isoproterenol (data not shown). Similarly, although currents were somewhat smaller in isoproterenol-treated cells, there was no statistical difference in the sustained current density when measured at +70 mV (Fig. 10D and data not shown).

Like isoproterenol, chronic treatment of OP cells with 8Br-cAMP (1 mM) also shifted the threshold for outward potassium current activation approx. +20 mV (data not shown). The voltage-dependence of potassium current activation was also shifted to more depolarizing potentials (data not shown). In contrast to isoproterenol, however, the effects of 8Br-cAMP were limited to the lower threshold current component, such that the half activation was approx. 15 mV more positive ($-4.5\pm1.5$ mV; $n=16$).

In conclusion, these electrophysiological experiments demonstrate that, unlike GluR agonists, two anti-proliferative signals, isoproterenol and 8Br-cAMP, do not cause a direct block of K+ channels in OP cells, but trigger long-term modification of K+ channel function.

**DISCUSSION**

In previous studies in purified OP cells and in cerebellar slice cultures, we demonstrated that activation of GluRs of the AMPA subtype causes inhibition of OP cell proliferation and prevents their differentiation (Gallo et al., 1996; Yuan et al., 1998). One of the central findings of the present report is that norepinephrine also inhibits OP cell proliferation, but

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**Fig. 10.** Chronic treatment of OP cells with isoproterenol shifts the voltage dependence of activation of the sustained outward current. Rat OP cells were cultured in the presence of PDGF. (A) Representative sustained current families activated from a prepulse of $-40$ mV to +70 mV in 10 mV increments (see inset). Untreated control cells were derived from the same culture batch, but cultured in the absence of isoproterenol (30 $\mu$M). (B) Construction of the voltage dependence of activation plots from 13 representative cells demonstrate that chronic treatment with isoproterenol (30 $\mu$M) shifts the voltage dependence of activation of the sustained outward current to more positive potentials. Conductance-voltage plots were best fit by the sum of two Boltzmann functions in both control and isoproterenol treated cells, suggesting that the total current observed consists of two temporally overlapping sustained current components. Treatment with isoproterenol also shifted the activation threshold to more depolarized potentials, consequently outward currents were not detected until test pulses $\sim20$ mV (cfr. control currents which activated close to $-40$ mV). (C) Separation of the total outward current into the two components revealed that isoproterenol (30 $\mu$M) shifted the voltage dependence of activation of both components. Following chronic treatment with isoproterenol the contribution of the lower threshold current to the total current increased to 47% compared to 38% in control. The dashed lines indicate the half-maximal voltage for control untreated cells. (D) Isoproterenol (30 $\mu$M), despite significantly decreasing the current amplitude measured at +70 mV ($P<0.05$), was without effect on the current density measured at the same test potential. **$P<0.05$ versus control.**
stimulates differentiation through selective activation of β-adrenergic receptors.

In accordance with the results obtained in dissociated cultured cells, we demonstrate that β-adrenergic receptor activation with the selective agonist isoproterenol inhibits OP proliferation and stimulates their differentiation in a cytoarchitecturally intact system, i.e. in cerebellar slice cultures. Oligodendrocyte development in slice cultures is similar to that in situ and myelination occurs within the first 2 weeks in vitro (Berger and Frotscher, 1994; see also Yuan et al., 1998). Our findings confirm that β-adrenergic receptors are expressed in OP cells in situ (see also Bernstein et al., 1996), and indicate that the intracellular signaling cascade that links β-adrenergic receptor activation and cell cycle arrest in OPs is not an artifact of dissociated cell cultures.

Recent studies demonstrated that arrest in OP cell proliferation occurs as a result of p27Kip1 accumulation after several cell divisions, and that the levels of this cyclin-dependent kinase inhibitor increase in differentiating oligodendrocytes (Casaccia-Bonnefil et al., 1997; Durand et al., 1997; Friessen et al., 1997). Overexpression of p27Kip1 in OP cells after adenoviral infection caused cell cycle arrest (Tikoo et al., 1998). Furthermore, an increased number of glial cells was found in the optic nerve of p27Kip1−/− knockout mice (Casaccia-Bonnefil et al., 1997). These findings demonstrate that p27Kip1 is one of the key molecular components of the mechanism underlying cell cycle progression or withdrawal in the oligodendrocyte lineage.

Additional cell cycle proteins, in particular cyclin-dependent kinase inhibitors that regulate G1 cyclins, are likely to be implicated in withdrawal from the OP cell cycle. Among these, p21CIP1 is involved in cell cycle exit and terminal differentiation in distinct cell lineages (Macleod et al., 1995; Parker et al., 1995; Skapek et al., 1995). Cells isolated from mutant mice lacking p21CIP1 are defective in G1 checkpoint control (Brugarolas et al., 1995; Deng et al., 1995), and p21CIP1 levels are higher in differentiated oligodendrocytes than in progenitor cells (Casaccia-Bonnefil et al., 1997).

In agreement with the findings described above, our results show that neurotransmitter receptor-mediated arrest in OP cell proliferation is likely to involve accumulation of the G1 cyclin-dependent kinase inhibitors p27Kip1 and p21CIP1, because activation of both glutamate and norepinephrine receptors caused a significant increase in p27Kip1 and p21CIP1 protein levels, and cell cycle arrest in G1. Both isoproterenol and kainate still inhibited proliferation of OP cells isolated from INK4a−/− mice lacking two other genes involved in G1/S transition, p16INK4a and p19ARF. The recent finding that p19ARF binds to MDM2 causing activation of the tumor suppressor gene p53 (Pomerantz et al., 1998; Zhang et al., 1998) also indicates that p53 does not participate in isoproterenol- and kainate-induced growth suppression. This is further supported by our analysis demonstrating that neither receptor agonists induced excitotoxic stimulation and apoptosis, a process that involves p53 production in neural cells (Sakhi et al., 1994; Jordan et al., 1997). Finally, neither isoproterenol or kainate modified p15INK4b levels in OP cells (data not shown).

These data suggest that p27Kip1 and p21CIP1 induction are part of the adrenergic and glutamatergic receptor-mediated pathways that prevent OP cells from entering S-phase. Therefore, these cyclin-dependent kinase inhibitors are key components of the molecular machinery that causes arrest in OP cell proliferation either constitutively (Casaccia-Bonnefil et al., 1997; Durand et al., 1997; Friessen et al., 1997; Tikoo et al., 1997; 1998) or in response to physiological signals (present study). Our findings are also consistent with the study by Kato et al. (1994) showing that cAMP-dependent induction of p27Kip1 in macrophages caused G1 arrest in the cell cycle. In OP cells, the cAMP analogs db-cAMP and 8Br-cAMP triggered p27Kip1 and p21CIP1 accumulation and inhibited OP proliferation. Future experiments will determine whether glutamatergic and β-adrenergic receptor agonists induce p27Kip1 and p21CIP1 accumulation through separate intracellular pathways, or whether these are at least in part overlapping.

We have previously shown that GluR agonists inhibit OP cell differentiation, i.e. decreased the percentage of O4+ cells under conditions that favored lineage progression (Gallo et al., 1996; Yuan et al., 1998). In the present study, we demonstrate that the β-adrenergic receptor agonist isoproterenol exerted opposite effects, compared to GluR activation, on OP cell differentiation by increasing the percentage of O4+ and O1+ cells in the cultures. These findings suggest that: (i) neurotransmitter receptor-mediated arrest in OP cell proliferation and differentiation are uncoupled events; (ii) neurotransmitter receptor-mediated induction of p27Kip1 and p21CIP1 expression is involved in proliferation arrest, but not in the initiation of cell differentiation, and (iii) different neurotransmitter receptors are coupled to distinct and possibly converging pathways that independently regulate OP cell proliferation and differentiation. Consistent with our interpretation, two independent lines of evidence support the notion that accumulation of p27Kip1 by itself is not sufficient to initiate oligodendrocyte differentiation. First, in p27Kip1−/− cultures, OP cells were still capable of differentiating (Casaccia-Bonnefil et al., 1997). Second, overexpression of p27Kip1 in OP cells caused cell cycle arrest, but did not induce differentiation (Tikoo et al., 1998).

Clearly, additional pathways need to be activated in order to initiate neurotransmitter-stimulated oligodendrocyte differentiation. Elevation of intracellular cAMP by membrane-permeable analogs was previously shown to accelerate the onset of oligodendrocyte differentiation (McMorris, 1983; Pleasure et al., 1986; Raible and McMorris, 1989, 1993). Our data show that β-adrenergic receptor activation with isoproterenol increased intracellular cAMP and stimulated OP cell lineage progression. It is very likely, therefore, that isoproterenol promotes oligodendrocyte differentiation through a cAMP-dependent mechanism.

G1 progression and cell proliferation are also regulated by K+ channel activity in a variety of eukaryotic cells (Nilius and Droogmans, 1994; Wonderlin and Strobl, 1996). In non-dividing oligodendrocyte lineage cells, only inwardly rectifying K+ currents can be detected, whereas in proliferating cells induction of both sustained and transient outward K+ currents was observed (Sontheimer et al., 1989; Barres et al., 1990; Borges and Kettenmann, 1995; Chvatal et al., 1995; Gallo et al., 1996; Knutson et al., 1997). Direct blockage of these outward currents with tetraethylammonium, or Na+-dependent blockage by GluR activation or depolarization, inhibited OP cell proliferation and lineage progression (Gallo...
dependence of K+ channel activation, but more importantly proliferation. This was associated with a shift in the voltage-dependence of K+ channel activation, but more importantly moved the activation threshold to more depolarizing potentials. The shift in activation threshold will consequently require a much larger depolarizing stimulus to activate a similar fraction of current to that seen in control cells. The mechanism by which isoproterenol and 8Br-cAMP modify outward K+ channel function in OP cells is still unknown and under investigation, but is likely to involve activation of protein kinase A and/or inhibition of growth factor-mediated stimulation of MAP kinase (Kurino et al., 1996; Pende et al., 1997).

Oligodendrocyte development takes place in vivo after neuronal maturation has occurred (Reynolds and Wilkin, 1988; Warrington and Pfeiffer, 1992; Levison and Goldman, 1993; Luskin and McDermott, 1994; Zerlin et al., 1995). The finding that noradrenergic terminals make direct contacts with oligodendrocytes in vivo (Paspalas and Papadopoulos, 1996) and that adrenergic receptors are expressed by oligodendrocyte lineage cells in situ (Bernstein et al., 1996) indicates that development of these cells may be modulated by noradrenergic signaling during development. The observations that β-adrenergic receptor-mediated cAMP formation does not occur in pre-oligodendrocytes (Fig. 1A) and that isoproterenol does not stimulate OP cell differentiation in cells cultured without mitogens (Fig. 4) suggest that β-adrenergic receptor-mediated signaling may be restricted to proliferative phases of oligodendrocyte development, and dismantled after arrest in cell proliferation has occurred.

In conclusion, our studies demonstrate that oligodendrocyte development can be modulated by norepinephrine (present study) and glutamate (Gallo et al., 1996; Knutson et al., 1997; Yuan et al., 1998). These antiproliferative signals may play an important role in regulating oligodendrocyte number and differentiation. In addition, we have identified p27Kip1, p21Cip1 and K+ channels as common targets of the signaling pathways triggered by glutamate and norepinephrine in OP cells. Thus, different neurotransmitters can play similar inhibitory roles on oligodendrocyte progenitor proliferation, but they can exert distinct effects on cell differentiation. These findings further dissociate the link between cessation of cell proliferation and the promotion of differentiation in the oligodendrocyte lineage.

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