A role for glutamate and its receptors in the regulation of oligodendrocyte development in cerebellar tissue slices

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SUMMARY

We tested the hypothesis that the neurotransmitter glutamate would influence glial proliferation and differentiation in a cytoarchitecturally intact system. Postnatal day 6 cerebellar slices were maintained in organotypic culture and treated with glutamate receptor agonists or antagonists. After dissociation, cells were stained with antibodies for different oligodendrocyte developmentally regulated antigens. Treatment of the slices with the glutamate receptor agonists kainate or \( \alpha \)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid significantly decreased the percentage of \( \text{LB1}^+ \), \( \text{NG2}^+ \) and \( \text{O4}^+ \) cells, and their bromodeoxyuridine labeling index. The non-N-methyl-D-aspartate glutamate receptor antagonist 6,7-dinitroquinoxaline-2,3-dione increased the percentage and bromodeoxyuridine labeling of \( \text{LB1}^+ \), \( \text{NG2}^+ \) and \( \text{O4}^+ \) cells. In intact slices, RNA levels of the oligodendrocyte gene for 2',3'-cyclic nucleotide 3'-phosphodiesterase were decreased by kainate and \( \alpha \)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, and increased by 6,7-dinitroquinoxaline-2,3-dione. The percentage of astrocytes was not modified by kainate, \( \alpha \)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid or 6,7-dinitroquinoxaline-2,3-dione. Treatment with the N-methyl-D-aspartate receptor antagonist 2-amino-5-phosphonopentanoic acid did not alter the percentage of \( \text{O4}^+ \) cells, nor their proliferation. Incubation with the \( \gamma \)-aminobutyric acid receptor antagonist bicuculline did not modify the percentage of \( \text{LB1}^+ \), \( \text{A2B5}^+ \) and \( \text{O4}^+ \) cells. In purified cerebellar oligodendrocyte progenitor cells, glutamate receptor agonists blocked K\(^+\) currents, and inhibited cell proliferation and lineage progression. The K\(^+\) channel blocker tetrathylammonium also inhibited oligodendrocyte progenitor cell proliferation. These findings indicate that in rat cerebellar tissue slices: (i) glutamate specifically modulates oligodendrocyte but not astrocyte development through selective activation of \( \alpha \)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors, and (ii) cell depolarization and blockage of voltage-dependent K\(^+\) channels is likely to be the triggering mechanism.

Key words: Glutamate receptors, O-2A cells, Glia, Voltage-dependent K\(^+\) channels, Tetrathylammonium, Cyclic nucleotide phosphodiesterase

INTRODUCTION

Oligodendrocytes originate from progenitor cells (OP) which display a high proliferative potential before they terminally differentiate (Raff et al., 1988; Bogler et al., 1990; Gard and Pfeiffer, 1990; McKinnon et al., 1990; Barres and Raff, 1993; Hardy and Reynolds, 1993a; Gallo and Armstrong, 1995; Canoll et al., 1996; Zhang and Miller, 1996). In the rat optic nerve, the final number of oligodendrocytes is determined by the proliferative rate of the progenitors and by the process of programmed cell death that occurs during development (Barres et al., 1992; Raff, 1992). Several cellular factors regulate OP cell proliferation and oligodendrocyte death (see Raff, 1989; Goldman, 1992; Pfeiffer et al., 1993; Barres and Raff, 1993; Miller, 1996 for reviews). Because oligodendrocytes differentiate later than all other neural cell types, it is likely that OP development is influenced by signals deriving from other CNS cells, including neurons (Hardy and Reynolds, 1993b; Dutly and Schwab, 1991). Furthermore, in view of their migratory behavior (Armstrong et al., 1990a; Orentas and Miller, 1996; Milner et al., 1997; Schmidt et al., 1997), OP cells are likely to be exposed to a changing extracellular microenvironment that may affect proliferation and differentiation at critical stages.

In proliferating oligodendrocyte lineage cells, extracellular signals can be decoded through a variety of ligand- and voltage-gated ionic channels which are expressed in culture and in vivo (Sontheimer et al., 1989; Barres et al., 1990; von Blankenfeld et al., 1991; Wyllie et al., 1991; Fulton et al., 1992; Borges et al., 1994; Patneau et al., 1994; Berger, 1995; Borges and Kettenmann, 1995; Gallo et al., 1996; Steinhauser and Gallo, 1996). Activation of glutamate and \( \gamma \)-aminobutyric acid (GABA) receptors causes depolarization of the OP cell membrane (Barres et al., 1990; von Blankenfeld et al., 1991; Wyllie et al., 1991; Fulton et al., 1992; Patneau et al., 1994). We have previously demonstrated, using purified cortical OP cells grown in primary culture, that non-N-methyl-D-aspartate (non-NMDA) glutamate receptor agonists inhibit cell proliferation (Gallo et al., 1996).
Several crucial questions arise concerning the involvement of neurotransmitter receptors and membrane channels in the regulation of glial cell proliferation and differentiation. Firstly, whether the neurotransmitter receptor-mediated effects are relevant to glial development in systems that retain the cytoarchitectural integrity and the complexity of cellular interactions of the tissue of origin, and ultimately in the intact brain. Secondly, whether activation of different receptor subtypes can affect distinct glial cell populations (Steinhauser and Gallo, 1996). Thirdly, whether even within the same population of glial cells separate phases of development (proliferation, migration and differentiation) can be modulated by different receptor systems (Gallo et al., 1996; Wang et al., 1996; Steinhauser and Gallo, 1996).

Procedures required to prepare and maintain glial cells in culture may modify cell membrane properties and intracellular signaling systems (Duffy and MacVicar, 1995; kimelberg et al., 1997). Also, in dissociated cultures, cells are maintained in a proliferative state by exogenous mitogens, and only interactions among a limited number of cell types are established. In organotypic slice cultures, both neurons and glial cells continue to develop with a time schedule similar to that in vivo (Gahwiler et al., 1997). In particular, the morphology, distribution and differentiation of oligodendrocytes in slice cultures are similar to those in situ, and axons are fully myelinated within the first 2 weeks in vitro (Berger and Frotscher, 1994).

In the present study, we used rat cerebellar organotypic slice cultures to analyze the influence of amino acid neurotransmitters on oligodendrocyte development in a cytoarchitecturally intact system. The vast majority of neuronal cell types in the cerebellar cortex release amino acids (glutamate or GABA) as neurotransmitters (Balazs, 1992), and cell types in the cerebellar cortex release amino acids (glutamate or GABA) as neurotransmitters (Balazs, 1992). Alternatively, GABA and muscimol were from Sigma (St Louis, MO). AMPA and 6,7-dinitroquinoxaline-2,3-dione (DNQX) were from Tocris Cookson (Bristol, UK). 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 (from Streptomyces griseus, 1.5 mg/ml; Sigma, catalog no. P6911) for 5 minutes at 37°C and then treated with trypsin/EDTA (0.05% trypsin/0.5% EDTA; Sigma) for 5 minutes at 4°C. Cells were then dissociated by trituration through a Pasteur pipette (35 strokes) and plated on poly-D-ornithine-coated 25 mm diameter coverslips at a density of 2×10^5 cells/coverslip in 200 μl of DMEM-N1+10% FBS for immunocytochemistry. Cells were stained and analyzed 2 hours after plating.

**Cultures of purified oligodendrocyte progenitor cells**

Purified cerebellar OP cultures were prepared as previously described from postnatal day 2 Sprague-Dawley rat cerebella following the NIH Animal Welfare guidelines (McCarthy and de Vellis, 1980; Armstrong et al., 1990b; McKinnon et al. 1990; Gallo et al., 1996). Cells were cultured on poly-D-ornithine-coated (0.1 mg/ml) plates or glass coverslips, and cultured in DMEM-N1 medium. After 2 hours, either platelet-derived growth factor (PDGF; human AB, heterodimer form; 10 ng/ml), or basic fibroblast growth factor (bFGF; human; 10 ng/ml), or PDGF+bFGF (both from Upstate Biotechnology, Inc., Lake Placid, NY) was added to the culture medium.

In the cerebellar cultures treated with growth factors (Figs 9 and 10), more than 90% of the cells were OP cells, as determined by staining with the LB1 (anti-GD3) antibody within the first 24 hours after plating (Levi et al., 1986; Yuen and Gallo, unpublished observations).

**Cell proliferation assays in cell cultures**

Cell proliferation assays were performed as previously described (Gallo et al., 1996; Knutson et al., 1997). Purified cerebellar OP cells were cultured with DMEM-N1+5% FBS. Two hours after plating, PDGF and/or bFGF and kainic acid, or AMPA, or veratridine (Sigma, St. Louis, MO), or tetraethylammonium (TEA; Sigma) or a medium containing 45 mM KCl (Knutson et al., 1997) were added to the culture medium together with [methyl-3H]thymidine (0.5 μCi/ml; 85 Ci/mmol; Amersham Life Science, Arlington Heights, IL). After 22 hours, cells were lysed and [3H]thymidine incorporation was measured by precipitation with 10% trichloroacetic acid and scintillation counting.

**Immunocytochemistry and cell counting**

Cells dissociated from organotypic slice cultures were plated on coverslips and stained after 2 hours as previously described (Gallo and Armstrong, 1995; Gallo et al., 1996). Primary cultures of cerebellar oligodendrocyte lineage cells were stained 1-2 days after plating. The following primary antibodies were used: LB1 (Levi et al., 1986; Curtis et al., 1988); A2B5 (Eisenbarth et al., 1979); NG2 (Stallcup, 1981); O4 (Sommer and Schachner, 1981); O1 (Sommer and Schachner, 1981); TUJ-1 (anti-neuron-specific β-tubulin; BABC0, Richmond, CA); anti-β III tubulin (β III-tubulin; Sigma); anti-glial fibrillary acidic protein (GFAP) (Boehringer, Mannheim, or Chemicon Int. Inc., Temecula, CA) and OX-42 (Serotec, Oxford, UK). All secondary fluorochrome-conjugated...
antibodies were from Cappel-Organon Teknika (Durham, NC). For double staining with antibodies to cell surface components (LB1, NG2, A2B5, O4, O1 and OX-42) and anti-BrdU antibodies, live cells were incubated for 60 minutes with primary antibodies diluted in DMEM, followed by fluorescein-conjugated goat anti-mouse (GAM) IgG (for LB1 and OX-42) or IgM (for A2B5, O4 and O1) for 30 minutes. A fluorescein-conjugated anti-rabbit antibody was used for NG2. Cells were then fixed in 4% paraformaldehyde for 10 minutes at room temperature and incubated with 0.07 N NaOH for 10 minutes at room temperature. After washing in PBS, cells were fixed in 4% paraformaldehyde for 10 minutes and permeabilized in 0.1% Triton X-100 in PBS for 10 minutes at room temperature. After washing in PBS, cells were incubated with 10% heat-inactivated goat serum for 15 minutes, with monoclonal anti-BrdU antibodies (1:20; Dako Corp., Carpinteria, CA) for 16 hours at 4°C and with rhodamine-conjugated goat anti-mouse (GAM) IgG for 30 minutes at room temperature. Cells were mounted in Vectashield™ (Vector Laboratories, Burlingame, CA) and counted as described below.

For double staining with LB1 or NG2 or A2B5 and anti-GFAP or TUJ-1, cells were first stained with LB1 or NG2 or A2B5 and then fixed in 4% paraformaldehyde (pH 7.4, in PBS) for 15 minutes. After permeabilization in 95% ethanol/5% acetic acid for 10 minutes at −20°C, cells were incubated with a rabbit anti-human GFAP antibody (1:300, overnight at 4°C; Chemicon Int. Inc.) or TUJ-1 (1:500, 1-2 hours at room temperature), and incubated for 30 minutes with a rhodamine-conjugated goat anti-rabbit (GAR), or a rhodamine-conjugated mouse anti-IgG, respectively. For single staining with anti-GFAP antibody, cells were fixed and permeabilized, and then incubated with anti-GFAP antibody (Boehringer), followed by fluorescein-conjugated GAR. For double staining with LB1 and Dil-Ac-LDL, or OX-42 and Dil-Ac-LDL, cells were first incubated with Dil-Ac-LDL (10 μg/ml; Biomedical Technologies Inc., Stoughton, MA) for 3-4 hours at 37°C and extensively washed before staining with LB1 or OX-42 as described above. All cells processed for immunocytochemistry were mounted in Vectashield (Vector Laboratories, Burlingame, CA). Controls for antibody specificity were performed by sequentially omitting each of the primary antibodies in the immunostaining protocols. The immunofluorescence micrographs presented are representative of at least three experiments and were taken on a Zeiss Axioshot fluorescence microscope (40× and 100× Neofluar objectives).

For cell counting, 10-20 microscopic fields were counted for each coverslip, and 1-2 coverslips for each experiment were analyzed. At least 2 independent experiments were performed for each antibody staining. All data are presented as averages ± s.e.m.

Cell viability and apoptosis assays
For all assays, cells dissociated from cerebellar slices were plated on coverslips for 1-2 hours and counted using fluorescein and rhodamine filters on a Zeiss Axioshot fluorescence microscope. To evaluate cell lysis/necrosis, cells were incubated with fluorescein diacetate (Sigma; 10 μg/ml) and propidium iodide (Sigma; 5 μg/ml) for 5 minutes. Viable OP cells appeared bright fluorescent green, whereas non-viable cells were stained red (Jones and Senft, 1985).

Apoptotic cell death was determined by fluorescence microscopy by using the TUNEL assay (Boehringer Mannheim) or with the nuclear dye Hoechst 33258 (Calbiochem, La Jolla, CA). For TUNEL staining, dissociated 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 Hoechst 33258 staining, dissociated cells were fixed in methanol/acetic acid (3:1) for 30 minutes. After incubation for 15 minutes at 37°C with 0.4 μg/ml Hoechst 33258, fluorescence was examined using a 100× Neofluor objective. Apoptotic OP cells were identified by nuclear condensation and/or fragmented chromatin.

Two independent experiments were performed for each assay. In each experiment, 8-10 microscopic fields were counted for each coverslip, and 2 coverslips were analyzed. All data are presented as averages ± s.e.m.

RNA isolation and Northern blot analysis
Total RNA was isolated from P6 cerebellar slices using a single-step procedure (RNAzol™; Tel-test, Friendswood, TX) (Chomczynski and Sacchi, 1987). A total of 4 independent experiments were performed (see Fig. 12). All RNA samples (20 μg/lane) were resolved by electrophoresis through a 1.5% agarose-formaldehyde denaturing gel, electrotransferred onto Hybond™ (Schleicher and Schuell; Keene, NH) membranes, cross-linked to the membranes by UV irradiation (Stratalinker, Stratagene, CA) and hybridized with a CDP EcoRI/HindIII 1.5 kb cDNA fragment (Gravel et al., 1994). The probe was labeled at approximately 10⁶ cpm/μg DNA. Blots were hybridized in 6x SSC, 5x Denhardt’s and 0.5% SDS at 65°C, washed at high stringency with 0.2x SSC, 0.1% SDS at 65°C, and exposed to film for 16-72 hours. CNP transcripts were quantified using a phosphorimager system (Molecular Dynamics, Sunnyvale, CA) and were normalized by staining the membranes with 0.03% methylene blue in 0.3 M sodium acetate to compare RNA loading in each lane.

Electrophysiology
Tight seal (>5 Gohm) whole cell recordings were made from GD3+ OP cells cultured with 10 ng/ml of PDGF. Cells were perfused with media 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. Patch electrodes had resistances of 2-6 Mohm when filled (in Na⁺) with 200-250 mM KCl + 1-3 mg/ml biocytin. Whole cell recordings were made from GD3 + cells with a patch pipette in the Ω-mode at −70 mV. Cells were held at −50 mV and then voltage clamped to −70 mV and −100 mV. The clamp current was stored digitally and then lowpass filtered at 1 kHz. The current was filtered at 2 kHz and digitized at 5-10 kHz. Series resistances were in the range 7-12 Mohm (Mean 9.2±0.3 Mohm; n=13) and were compensated by at least 85%.

RESULTS

Immunocytochemical characterization of neural cells dissociated from cerebellar slice cultures
Initial experiments were designed to demonstrate the utility and fidelity of the slice culture system with respect to cell content and antigenic characterization of the cell fraction isolated after protease treatment of the tissue. To estimate total cell recovery after the tissue dissociation procedure the total amount of DNA was measured in tissue and in isolated cells, and converted into cell numbers (6.5 μg DNA=10⁶ cells; Ausubel et al., 1995). Whole P6 cerebella were sliced and cultured for 3 days. Total DNA was then measured in slices that were not treated with protease and in slices that were incubated with the enzyme and dissociated. A total of 1.9±10⁷ and 0.07 cells/cerebellum (n=3) was measured in untreated tissue, whereas 1.5±10²±0.17 cells/cerebellum (n=3) were obtained from cerebellar slices treated with protease, corresponding to an 80±6.9% recovery. These results indicate that cell recovery after protease treatment was high.

We then wanted to determine what percentage of the dissociated cells represented OPs. Cerebellar slices were kept in culture for a total of 3 days, cells were dissociated with protease and characterized by immunocytochemistry. Anti-
Fig. 1. Oligodendrocyte progenitors from cerebellar slice cultures stained with NG2, LB1 and A2B5 antibodies. Immunofluorescence of dissociated cerebellar cells (P6 slice cultures) stained with NG2 (A, phase contrast; B and D, fluorescence), LB1 (C) and A2B5 (E) antibodies. All tissue slices were pulsed with BrdU for 24 hours, and dissociated cells were double-stained with anti-BrdU antibodies (rhodamine). A significant proportion of cells labeled with the antibodies are also dividing, as shown by their BrdU nuclear staining. Note the preserved bipolar morphology of some LB1-positive cells (C). Scale bar, 40 μm in A and B; 15 μm in C–E.

GD3 (monoclonals LB1 and R24; Curtis et al., 1988; Goldman et al., 1984), NG-2 (Stallcup, 1981) and A2B5 (Eisenbarth et al., 1979) antibodies were used. Fig. 1 shows cells dissociated from P6 slices and immunostained with NG2 (Fig. 1A,B,D), LB1 (Fig. 1C) and A2B5 (Fig. 1E) antibodies. In tissue or mixed cultures, these antibodies display some cross-reactivity with other cell populations, in particular immature granule cell neurons and microglia (Goldman and Reynolds, 1996). We found no evidence for cross-reactivity of LB1, NG2 and A2B5 with neurons in our dissociated cell population, most likely due to a loss of neuronal immunoreactivity after 3 days in vitro (Goldman and Reynolds, 1996). Double staining experiments with the anti-neuron-specific β-tubulin monoclonal antibody TUJ-1 (Lee et al., 1990) demonstrated that a very small number of LB1+, NG2+ or A2B5+ cells were stained with the neuron-selective antibody. In a total of 2,083 LB1+ cells analyzed (3 independent experiments), only 22 (i.e. 1%) were double-stained with TUJ-1 antibodies. Similarly, out of 1,561 NG2+ cells, only 22 (i.e. 1.4%) were also TUJ-1+. The larger size (between 10 and 15 μm; Fig. 1) of the cells stained with LB1, NG2 or A2B5 further indicated that these antibodies did not label the small (5-8 μm) and numerous granule cells, the major proliferative neuronal population of the postnatal cerebellum (Altman, 1972).

In double immunostaining experiments, it was found that, in cells isolated from P6 slices, 30.7±2.3% (n=33 microscopic fields; 6,361 total cells counted) of the NG2+ cells were also O4+, as was previously found in cerebellar cultures (Levine, 1989; Levine et al., 1993).

Cells with an LB1+/GFAP+, NG2+/GFAP+ and an A2B5+/GFAP+ phenotype (type-2 astrocytes) have been described in culture (Raff et al., 1983; Levi et al., 1986; Levine and Stallcup, 1987; Curtis et al., 1988), and the LB1 antibody

Fig. 2. Cells from cerebellar slice cultures stained with LB1 or NG2 antibodies are not astrocytes or microglia. Quantitative analysis of dissociated cerebellar cells (P6 slice cultures) stained with LB1 and GFAP (A), NG2 and GFAP (B), LB1 and LDL (C), and OX-42 and LDL (D). After staining, a total of 4–6 coverslips (10 fields/cover slip) were counted for each antibody staining from 2–3 independent experiments. Between 3,884 and 11,388 cells were counted for each antibody combination from phase contrast images. LB1-positive or NG2-positive cells were all GFAP-negative. n.d., not detectable. The OX-42-positive cell population entirely overlapped with the LDL-positive one. Data represent averages ± s.e.m. obtained from 2–3 independent experiments (n=40–60 fields counted/antibody combination).
has been shown to cross react with microglial cells in tissue slices (Wolsijik, 1995). Fig. 2 summarizes a set of double immunostaining experiments which demonstrated that in our experimental system: (i) LB1, A2B5 and NG2 antibodies did not stain astrocytes, and (ii) that microglial cells were not labeled by LB1. None of the LB1+ or NG2+ cells were GFAP+ (Fig. 2A,B), in agreement with previous findings that cells displaying the type-2 astrocyte phenotype could not be found in cerebellum in vivo (Reynolds and Wilkin, 1988; Curtis et al., 1988). Similarly, no A2B5+/GFAP+ cells were found (3,884 total cells counted).

Microglial cells were identified based either on their immunoreactivity to the monoclonal antibody OX-42 (Perry et al., 1985), or their capacity of accumulating low density lipoprotein (Dil-Ac-LDL) by phagocytosis. The cerebellar dissociated cell suspension included a small percentage of OX-42+ cells (Fig. 2D). The vast majority of the OX-42+ cells were Dil-Ac-LDL+ (Fig. 2D), and all the Dil-Ac-LDL+ cells were OX-42+ (Fig. 2D). None of the LB1+ cells, or the NG2+ and A2B5+ cells accumulated Dil-Ac-LDL (Fig. 2C; Yuan and Gallo, unpublished observations). This indicates that none of the OX-42+ cells are LB1+, i.e. that LB1 does not cross-react with OX-42/Dil-Ac-LDL+ microglia in these cell preparations.

In conclusion, the cells stained with the LB1, NG2 or A2B5 antibody represent cells of the oligodendrocyte lineage, and represent 3-4% of the total cell population. In cell suspensions isolated from P2, P6 and P10 slice cultures, it was found that: (i) the number of LB1+, NG2+ and A2B5+ cells/cerebellum increased between P2 and P6, but slightly decreased between P6 and P10, and (ii) their BrdU index decreased by 4-5 fold between P2 and P10 (Yuan and Gallo, unpublished observations). These results are consistent with previous in vivo observations (Reynolds and Wilkin, 1988; Warrington and Pfeiffer, 1992; Levine et al., 1993; Nishiyama et al., 1996).

**Glutamate receptor activation inhibits oligodendrocyte progenitor cell proliferation in cerebellar slice cultures**

The GluR agonists kainate and AMPA caused a significant (approximately 50%) and uniform decrease in the percentage of LB1+, NG2+ and A2B5+ cells in P6 slices (Fig. 3A,C,E). Preventing GluR activation with the antagonist 6,7-dinitroquinolinic acid 2,3-dione (DNQX) increased the percentage of OP cells by approximately 40% (Fig. 3A,C,E). OP cell proliferation, as measured by double staining with anti-BrdU antibodies after pulse-labeling, was significantly decreased by kainate and AMPA, but increased by DNQX (Fig. 3B,D,F).

The effect of kainate on NG2+ cells and on their BrdU index was dose-dependent in the concentration range of 1-300 μM and saturated at a concentration of 200 μM for both parameters (Fig. 4A,B).

The initial concentration of glutamate in the slice culture medium, as determined by HPLC, was 113.6±13.4 μM (average ± s.e.m.; n=3), and after 3 days in culture it decreased to 16.8±13.4 μM (n=3), presumably due to uptake into the tissue and degradation. The concentration of glutamate in the tissue microenvironment of OP cells is not known, but the proliferative effects of DNQX indicate that glutamate levels in the slices are within the concentration range of AMPA receptor activation in oligodendrocyte lineage cells (Patneau et al., 1994; Gallo et al., 1996).

Different cell viability assays demonstrated that the decrease in LB1+, NG2+ and A2B5+ cells observed with kainate or AMPA was not due to a neurotoxic effect of the agonists. Propidium iodide (PI) uptake was used to evaluate cell lysis/necrosis after a 48 hour exposure to GluR agonists. The percentage of viable cells was evaluated with fluorescein diacetate (FDA) staining. More than 95% of the cells were viable under all culture conditions (Table 1). The percentage of apoptotic cells was determined by the TUNEL assay and by staining with the Hoechst 33258 dye. Neither kainate nor AMPA increased the rate of apoptosis in the tissue slices (Table 1).

![Fig. 3. Activation of non-NMDA glutamate receptors inhibits oligodendrocyte progenitor proliferation in cerebellar slice cultures.](image-url)
Fig. 4. Dose-response of the effects of kainate on oligodendrocyte progenitor and pro-oligodendroblast number and proliferation in cerebellar slice cultures. Quantitative analysis of cerebellar cells dissociated from P6 slice cultures treated for 48 hours with kainate (1-300 μM). All tissue slices were pulsed with BrdU for 24 hours. Cells were dissociated and stained with NG2-anti-BrdU (A,B) and O4-anti-BrdU (C,D) antibodies. A total of 20 microscopic fields were counted for each kainate concentration and each antibody combination. Between 11,779 and 24,097 cells were counted for staining with NG2 for each concentration from phase contrast images. Between 4,267 and 10,036 cells were counted for staining with O4 for each concentration. Data represent averages ± s.e.m. A and B: *P<0.005, ***P<0.0001; C and D: *P<0.05; **P<0.01; ***P<0.001, compared with their respective controls (Student’s t-test). In A-C, 200 μM kainate was not significantly different from 300 μM, indicating that the effect of the agonist saturated at a concentration of 200 μM for both NG2+ and O4+ cells.

Taken together, these results indicate that: (i) activation of GluRs inhibits OP cell proliferation in cerebellar slices; (ii) endogenous levels of glutamate in the tissue are sufficient to activate GluRs and inhibit OP cell proliferation, and (iii) a decreased rate of proliferation results in a significant decrease in the total number of OP cells in the tissue.

Effects of glutamate receptor activation on pro-oligodendroblasts and oligodendrocytes in cerebellar slice cultures

The monoclonal antibodies O4 and O1 distinguish pro-oligodendroblasts and differentiated oligodendrocytes, respectively (Sommer and Schachner, 1981; Gard and Pfeiffer, 1990; Gallo and Armstrong, 1995; Gallo et al., 1996). Fig. 5 shows cells dissociated from P2-P10 cerebellar slices double-stained with the monoclonal antibody O4 and anti-BrdU. A significant number of O4+ cells were found at all developmental stages (Fig. 5B). These cells displayed a multipolar morphology with a rather complex array of radially oriented processes (Fig. 5C-E). At P2 and P6, many O4+ cells were also BrdU+, indicating that their proliferative potential was maintained in the slices. Consequently, BrdU+ pairs of O4+ cells were frequently found (Fig. 5F,G).

A significant number of O1+ cells was also detected in the dissociated cellular fraction at P2-P10 (Fig. 6A-D). O1+ cells displayed a diverse and complex process-bearing morphology at all ages analyzed (Fig. 6E-G). No strongly stained O1+ cells were BrdU+ at P2 as well as the later developmental stages studied (Fig. 6).

The total number of O4+ and O1+ cells was determined in cerebellar slices cultured from P2, P6 and P10 cerebella. Consistent with in vivo data (Reynolds and Wilkin, 1988; Warrington and Pfeiffer, 1992), the largest number of O4+ and O1+ cells was found in P10 slices. The total number of O4+ cells/cerebellum increased from 132±7 ×10³ (n=3) at P2 to 5.9±0.08 ×10⁶ (n=3) at P10. The number of O1+ cells/cerebellum increased from 25±0.1 ×10³ (n=3) at P2 to 3.6±0.05 ×10⁶ in P10. A higher percentage (15-20%) of dividing O4+ cells was detected in P2 and P6 slices, whereas at P10 only approximately 3% of the O4+ cells were BrdU+ (Yuan and Gallo, unpublished observations).

Incubation of P6 cerebellar slices with GluR agonists and antagonists significantly modified the percentage of O4+ cells and their proliferation (Fig. 7A,B). Kainate decreased the percentage of O4+ cells (Fig. 7A) as well as their BrdU index (Fig. 7B), whereas the antagonist DNQX had opposite effects (Fig. 7A,B). Interestingly, the NMDA GluR antagonist 2-amino-5-phosphonopentanoic acid (APV) did not modify the percentage or the BrdU index of O4+ cells (Fig. 7A,B). Consistent with these findings, also the percentage of O1+ cells was reduced by kainate and AMPA, and increased by DNQX (Fig. 7C).

The effect of kainate on O4+ cells and on their BrdU index was dose-dependent in the concentration range of 1-300 μM.

Table 1. Cell viability in cerebellar slice cultures

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<tr>
<th>Assay</th>
<th>% of non-viable cells</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
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<tr>
<td>Propidium iodide</td>
<td>2.9±0.38</td>
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<tr>
<td>TUNEL</td>
<td>2.8±0.001</td>
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<tr>
<td>Hoechst 33258</td>
<td>0.12±0.04</td>
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Cerebellar slice cultures were prepared from rat cerebella at postnatal day 6 and maintained in culture for a total of 72 hours. Cerebellar slices were treated with either kainate (200 μM), AMPA (200 μM) or DNQX (30 μM) for the last 48 hours. Cells were dissociated and processed for staining as described in Materials and Methods. A total of 4 coverslips (10 microscopic fields/coverslip) from 2 independent experiments were analyzed. In the Hoechst 33258 assay, only cells with condensed chromatin were counted. Total cells counted from phase contrats imaging for each treatment were as follows: between 22,215 and 39,388 in the fluorescein diacetate/propidium iodide assay, between 72,428 and 87,858 in the TUNEL assay, and between 6.587 and 8.207 in the Hoechst assay. Data are averages ± s.e.m. (n=40 fields counted in each assay). *P<0.001; **P<0.05 compared with control (Student’s t-test).
and, similar to the effects on NG2+ cells, saturated at a concentration of 200 μM for both parameters (Fig. 4C,D). The minimal antiproliferative concentration of kainate was however one order of magnitude higher for O4+ than for NG2+ cells (Fig. 4B,D).

These data indicate that activation of GluR also affects the proliferation and number of O4+ pro-oligodendroblasts.

The regulation of OP development is receptor-selective and is not observed in astrocytes

Previous studies have demonstrated that OP cells also express GABA A receptors (von Blankenfeld et al., 1991). Incubation of the cerebellar slices with the GABA A receptor antagonist bicuculline did not modify either the percentage of LB1+ or O4+ cells, or their proliferation (Table 2). However, treatment of the slices with the GABA A agonist muscimol significantly decreased the percentage of LB1+ and O4+ cells, as well as their BrdU incorporation (Table 2). Finally, neither Glu nor GABA A receptor agonists nor antagonists affected astrocytes in P6 cerebellar slices. Both the percentage and the almost undetectable proliferative index (see legend of Fig. 8) of GFAP+ cells were unaltered after treatment with kainate, DNQX, muscimol or bicuculline (Fig. 8).

Altogether, these results indicate that endogenous activation of GABA A receptors is not a major mechanism in the regulation of cerebellar OP development, and that the effects of GluR activation in the postnatal cerebellum are oligodendrocyte-specific.

Glutamate receptor activation regulates RNA expression of an oligodendrocyte gene

In the CNS, transcripts of the CNP gene are selectively expressed in oligodendrocytes (Trapp et al., 1988). Since CNP enzymatic activity is at its highest during myelination (Kurihara and Tsukada, 1967; Waehneldt, 1975), mRNA levels for this gene are likely to reflect oligodendrocyte maturation in vivo. We therefore exploited the cell-specific expression of CNP gene as a molecular marker to study the effect of GluR activation on OP maturation. We predicted that under conditions that prevented OP lineage progression CNP mRNA would be down-regulated, whereas the opposite would occur under conditions that favored OP development.

Two alternatively spliced transcripts of the CNP gene, CNP1 and CNP2, have previously been described (Gravel et al., 1994). Fig. 9A shows that significant levels of both CNP1 and CNP2 mRNA (2.6 and 2.4 kb, respectively) could be detected in P6 cerebellar slice cultures by using a C-terminal cDNA probe that hybridized to both splice isoforms. Treatment of the cerebellar slices with kainate or AMPA caused a 55 and 37% decrease, respectively, in the levels of CNP mRNAs (Fig. 9B). In contrast, treatment with the GluR antagonist DNQX significantly increased CNP RNA levels by 32% (Fig. 9B).

Glutamate receptor agonists and depolarizing agents prevent cerebellar OP proliferation and lineage progression in culture

In a previous study, we demonstrated that GluR activation is linked by K+ channel activity to the control of proliferation of cortical OP cells (Gallo et al., 1996). To define a possible mechanism of GluR-mediated modulation of cerebellar oligodendrocyte development, we analyzed purified cerebellar OP cells and the effects of GluR agonists and agents that reduced K+ channel activity on cell proliferation and lineage progression. Fig. 10A-C shows that cultured cerebellar OP cells were stimulated to proliferate by PDGF and bFGF, as shown by a 2-fold increase in [3H]thymidine incorporation, compared to cells cultured in the absence of growth factors. Micromolar concentrations of the GluR agonists kainate and AMPA significantly inhibited [3H]thyidine incorporation into OP cells. Agents that blocked or reduced voltage-gated K+ currents produced similar effects on cell proliferation. The K+ channel blocker tetraethylammonium (TEA), the Na+ channel opener veratridine, or concentrations of K+ that attenuated outward K+ currents (see below and Fig. 11) also decreased cerebellar OP proliferation (Fig. 10A-C).

We next analyzed the consequences of proliferation arrest on cerebellar OP lineage progression. Fig. 10D shows that under conditions that favor lineage progression (i.e. N1, PDGF or bFGF) kainate inhibited acquisition of the O4 phenotype, whereas DNQX significantly increased the percentage of O4+ pro-oligodendroblasts. Conversely, in conditions that maintained the pre-OP (GD3+/nestin+) or OP phenotype (GD3+/nestin+) (i.e. cells cultured with PDGF+bFGF; Gallo and Armstrong, 1995; Gallo et al., 1996) the percentage of O4+ cells was unaltered by kainate or DNQX.

These data complement and extend our previous analysis in cortical OP cells, because: (i) they demonstrate that proliferation and differentiation of OP cells isolated from a distinct part of the brain is also regulated by glutamate and its receptors; (ii) they indicate that membrane depolarization is a common mechanism in cortical and cerebellar OP cells underlying the anti-proliferative effects of glutamate; (iii) they suggest that, since glutamate is a ubiquitous transmitter in the mammalian brain, it is likely that activation of its receptors regulate oligodendrocyte development in many parts of the mammalian central nervous system.

Glutamate receptor agonists and depolarizing agents attenuate outward K+ currents in cultured cerebellar OP cells

Voltage-dependent K+ currents were analyzed in cerebellar OP cells, to determine whether activation of GluRs or cell

<p>| Table 2. GABA receptors and cerebellar oligodendrocyte development |</p>
<table>
<thead>
<tr>
<th>% of total cells</th>
<th>% of LB1+ or O4+ cells</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>LB1+</td>
</tr>
<tr>
<td>Control</td>
<td>3.0±0.2</td>
</tr>
<tr>
<td>Bicuculline</td>
<td>3.4±0.3</td>
</tr>
<tr>
<td>Muscimol</td>
<td>1.9±0.2</td>
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Cerebellar slice cultures were prepared from postnatal day 6 rat cerebella. The slices were maintained in culture for 72 hours, treated with bicuculline (1 μM) and muscimol (100 μM) for the last 48 hours in culture, and pulsed with bromodeoxyuridine (BrdU) for the last 24 hours. Cells were acutely dissociated and stained with LB1 and O4 antibodies. A total of 6-8 coverslips from 3-4 independent experiments were counted for each treatment and for each antibody. Total cells counted from phase contrast imaging for each treatment and for each antibody ranged between 7,084 and 16,060. Data are averages ± s.e.m. (n=60-80 fields counted/antibody combination). *P<0.0001; **P<0.001 compared with control (Student’s t-test).
depolarization similarly blocked voltage-dependent K+ channels as was seen in cortical OP cells (Gallo et al., 1996; Knutson et al., 1997). Fig. 11 shows that test pulses to +60 mV (Vhold = −40 mV) activated a sustained outward current. Sustained currents were blocked by 57.8±4.9% (n=7) by the K+ channel blocker tetraethylammonium (TEA 10 mM; Fig. 11A). Addition of the GluR agonist kainate (200 μM), at a concentration which blocked proliferation by 70%, reduced the sustained current by 61.5±9.3% (n=6; Fig. 11B). Previously we had demonstrated that non-NMDA receptor activation attenuated the sustained potassium current by a mechanism involving an increase in intracellular Na+ ion concentration (Knutson et al., 1997). We next demonstrated that a direct increase in [Na+]i by veratridine (50 μM) blocked K+ currents by 38.8±3.8% (n=6; Fig. 11C). Finally, depolarization of OP cells by an elevation of [K+]o (45 mM), which inhibited proliferation by 60% (Fig. 10), reduced K+ currents by 50.2±9.4% (n=5) compared to those recorded in 2.5 mM [K+]o (Fig. 11D). This is a value close to that predicted from the Nernst equation (56%).

The electrophysiological data demonstrate that the K+ current complement is similar in cerebellar and cortical OP cells and that voltage-dependent K+ channels are involved in the regulation of OP proliferation in different brain areas.

**DISCUSSION**

Organotypic slice cultures have been widely used to study development and physiology of neural cells (see Gahwiler et al., 1997 for review). In the present study, we demonstrate that cerebellar slice cultures are an appropriate system to manipulate and examine oligodendrocyte development. Firstly, cells of the oligodendrocyte lineage (either NG2+/LB1+ progenitors or O4+ pro-oligodendroblasts) display a significant proliferative potential in the slice...
Regulation of oligodendrocyte development

cultures. Secondly, the number and proliferation index of oligodendrocyte lineage cells after dissociation from the tissue is consistent with previous findings in vivo (Reynolds and Wilkin, 1988; Warrington and Pfeiffer, 1992; Levine et al., 1993; Nishiyama et al., 1996). Thirdly, the lack of O1+/BrdU+ cells in slice cultures is consistent with the finding that postmitragatory and actively myelinating O1+ oligodendrocytes are not dividing in vivo, whereas O4+ pro-oligodendroblasts are still capable of proliferating (Warrington and Pfeiffer, 1992). Our experiments in slice cultures further demonstrate that O4+/GalC− cells represent the most mature stage of oligodendrocyte development with a significant proliferative potential (Warrington and Pfeiffer, 1992). It can be concluded that all the proliferative stages of the oligodendrocyte lineage in vivo are found in the slices, where cells move along their pathway of differentiation consistent with their time schedule in vivo.

The central findings of this work represent an advancement in our understanding of the role of neurotransmitters and their receptors in the regulation of glial cell development in the brain. This study to our knowledge is the first demonstration that in a cytoarchitecturally intact system the neurotransmitter glutamate and its receptors play a significant role in regulating OP proliferation and differentiation. Furthermore, we show that the anti-proliferative effects of glutamate are extended to a more differentiated (but still dividing) stage of the oligodendrocyte lineage, i.e. to O4+ pro-oligodendroblasts, and that endogenous activation of NMDA or GABA receptors does not affect oligodendrocyte development. Finally, we demonstrate that cerebellar astrocyte number and proliferation are not influenced by GluR agonists and antagonists, indicating that at the developmental stages analyzed the anti-proliferative effects of GluR activation are cell-specific.

The effects of kainate and AMPA on OP proliferation and development are not due to GluR-mediated toxicity, as determined by 3 distinct assays, which were used to quantitate the percentage of necrotic and apoptotic cells in our slice cultures. Importantly, the finding that the antagonist DNQX did not significantly modify the percentage of non-viable cells indicates that only negligible endogenous GluR-mediated toxicity occurs under basal conditions.

The non-NMDA receptor agonists kainate and AMPA inhibited the proliferation and reduced the total number of cerebellar NG2+/LB1+ OP cells. The receptor-selective agonist

Fig. 6. Oligodendrocytes from cerebellar slice cultures are stained with the monoclonal antibody O1. Immunofluorescence of dissociated cerebellar cells (P2, P6 and P10 slice cultures) stained with O1 (fluorescein) and anti-BrdU (rhodamine) antibodies. Cerebellar slice cultures were pulsed with BrdU for 24 hours. Several O1-positive cells were found in the dissociated cell suspension at P6 (A, phase contrast; B, fluorescence) and at P10 (C, phase contrast; D, fluorescence). None of the O1-positive cells was BrdU-positive. The O1-positive cells displayed a process-bearing morphology at P2 (E), P6 (F) and P10 (G). Scale bar, 40 μm in A-D; 15 μm in E-G.
AMP A produced similar effects to kainate on cell proliferation and lineage progression, indicating that AMPA receptors are the major subtype involved. We have previously demonstrated that the anti-proliferative effect of GluR agonists in cultured cortical OP cells is mimicked by voltage-dependent K+ channel blockers or cell depolarization, and is likely to be due to a Na+-dependent blockage of K+ channels (Gallo et al., 1996; Knutson et al., 1997). In the present study, we show that in purified cerebellar OP cells TEA and depolarizing agents also reduced currents through K+ channels and blocked proliferation. The mechanism by which activation of non-NMDA receptors results in inhibition of OP proliferation in cerebellar tissue slices is therefore likely to involve cell depolarization, increase in intracellular Na+ and blockage of voltage-dependent K+ channels. Consistent with this

**Fig. 7.** Activation of non-NMDA glutamate receptors decreases pro-oligodendroblast number and proliferation in cerebellar slice cultures. Quantitative analysis of cerebellar cells dissociated from P6 slice cultures treated for 48 hours with DNQX (30 μM), kainate (KAI; 200 μM), AMPA (200 μM) or APV (100 μM), CTR, control. All tissue slices were pulsed with BrdU for 24 hours, and cells were stained with O4, O1 and anti-BrdU antibodies. (A) O4-positive cells. (B) O4/BrdU-positive cells. (C) O1-positive cells. The percentage of O1-positive cells was not modified by treatment with APV (1 EXP.). No O1+/BrdU+ cells were detected under any of the conditions examined. After staining, a total of six coverslips (10 fields/coverslip) were counted from 3 independent experiments for each condition. Between 2,343 and 20,838 cells were counted for each condition from phase contrast images. Data represent averages ± s.e.m. (n=60 fields counted/antibody combination). *P<0.001; **P<0.0005 compared with their respective controls (Student’s t-test).

**Fig. 8.** Glutamate receptor agonists do not modify astrocyte number or proliferation. Quantitative analysis of cerebellar cells dissociated from P6 slice cultures treated for 48 hours with DNQX (30 μM), kainate (KAI; 200 μM) or muscimol (MUSC; 100 μM) or bicuculline (BIC; 1 μM), CTR, control. All tissue slices were pulsed with BrdU for 24 hours, and cells were stained with GFAP+anti-BrdU. After staining, a total of six coverslips (10 fields/coverslip) were counted from 3 independent experiments. Between 9,474 and 12,909 cells were counted for each condition from phase contrast images. Only a total of 1-5 GFAP-positive cells were found to be BrdU-positive under any treatment at this developmental stage. Data represent averages ± s.e.m. (n=60 fields counted/antibody combination). No significant difference in the percentage of GFAP-positive cells was found after any treatment (Student’s t-test).

**Fig. 9.** Activation of non-NMDA glutamate receptors attenuates CNP gene expression. Northern blot analysis of total RNA (25 μg/lane) isolated from P6 cerebellar slice cultures treated for 48 hours with DNQX (30 μM), kainate (KAI; 200 μM) or AMPA (200 μM). (A) representative autoradiograph obtained after a 12 hours exposure of the blot. Numbers on the left represent molecular sizes in kilobases, as derived from an RNA standard. Two different CNP gene transcripts (CNP1 and CNP2; Gravel et al., 1994) were detected. Blots were stained with methylene blue to control for uniform RNA loading (bottom of A). (B) histograms derived from Phosphor-Imager (Molecular Dynamics) analysis of CNP1+CNP2 RNAs of 4 different blots from 4 independent experiments. Data are expressed as ratios over CNP RNA levels in untreated cerebellar slices, and are normalized for RNA loading. Data represent averages ± s.e.m. (n=4). *P<0.005 vs. control (untreated slices; Student’s t-test).
hypothosis, glial precursor cells have been found to express functional GluRs and K+ channels also in situ (Fulton et al., 1992; Berger, 1995), and electrophysiological analysis in brain slices has revealed that activation of non-NMDA receptors causes blockade of K+ channels in these cells (Berger, 1995). Furthermore, agents that block voltage-dependent K+ channels were found to prevent myelination in a slice culture preparation from mouse spinal cord (Shrager and Novakovic, 1995).

In cultured cortical OP cells, GluR agonists prevented lineage progression to the pro-oligodendroblast stage of the lineage, as determined by a significant decrease in the percentage of O4+ cells (Gallo et al., 1996). It was hypothesized that this effect was due to the inhibition of the cellular mechanism that counts the number of divisions necessary to differentiate (Gallo et al., 1996). In cerebellar slice cultures, kainate and AMPA exert an additional and significant anti-proliferative effect on O4+ pro-oligodendroblasts. Our previous electrophysiological analysis demonstrated that O4+ cells do indeed display non-NMDA receptor-mediated currents (Patneau et al., 1994). Since a significant proportion of these cells (approximately 25%) divide at P6 in the tissue slices, it can be concluded that non-NMDA receptor agonists directly inhibit proliferation of dividing O4+ cells. This is confirmed by our findings in purified cerebellar OP cells cultured with bFGF, in which kainate decreased the percentage of O4+/BrdU+ cells by approximately 50% (Yuan and Gallo, unpublished observations). The analysis in cerebellar slices shows that, in a more intact system, non-NMDA receptor agonists likely inhibit oligodendrocyte lineage progression by two distinct mechanisms: (i) inhibition of OP proliferation, which prevents cells from undergoing the number of divisions necessary to differentiate; and (ii) direct inhibition of O4+ cell proliferation.

The highest levels of CNP enzymatic activity have been found in oligodendrocytes and Schwann cells, and in the brain the CNP gene is selectively expressed in oligodendrocytes (McMorris, 1983; Vogel and Thompson, 1988; Trapp et al., 1988). The molecular analysis of CNP transcript expression in cerebellar slice cultures strongly indicates that non-NMDA receptors are involved in the regulation of oligodendrocyte development, and the CNP gene can be used as a molecular marker to monitor lineage progression of the oligodendrocyte cell population within the cerebellar slices. Furthermore, the regulation of CNP transcripts in cerebellar slice cultures shows that the effects of non-NMDA receptor agonists and antagonists observed are not consequences of cell dissociation and staining. It is not yet understood whether the changes in CNP transcript expression are due to altered levels of mRNA per cell, to changes in numbers of cells expressing the CNP gene, or both.

The present study also shows that under our experimental conditions the effects of glutamate on oligodendrocyte lineage cells are receptor-specific. The NMDA receptor antagonist APV did not affect O4+ cell number and proliferation, indicating that NMDA receptors are not involved in the regulation of oligodendrocyte development. Previous experiments in OP cells from different brain areas have failed to demonstrate expression of functional NMDA-activated channels (Wyllie et al., 1991; Patneau et al., 1994). In a recent report, however, NR1 mRNA transcripts and NMDA-mediated membrane currents were found in OP cells from neurohypophysial explants (Wang et al., 1996). In these cultures, NMDA receptors were described to regulate OP cell migration (Wang et al., 1996). Therefore, OP cells from different brain areas may be heterogeneous in terms of ligand-gated channel expression, and distinct subtypes of GluRs may be responsible for differentiative and anti-proliferative effects.
regulate their development at different levels (Steinhauser and Gallo, 1996).

Functional GABA_A receptors are expressed in oligodendrocyte progenitors and opening of GABA-activated channels causes cell depolarization (von Blankenfeld, 1991). In the present study, incubation with the exogenous GABA_A receptor agonist muscimol significantly decreased the percentage of LB1+ and O4+ cells, as well as their proliferation. However, treatment with the antagonist bicuculline did not modify oligodendrocyte lineage cell development. These findings indicate that, (i) endogenous GABA levels in the cerebellar slices are insufficient to activate the receptors and to affect oligodendrocyte development, and (ii) the effects of endogenous glutamate on oligodendrocyte progenitor cell proliferation and development are not saturating, but can be increased by exogenous application of a different depolarizing agent, i.e. a GABA_A agonist.

In conclusion, non-NMDA receptors play an important role in the regulation of oligodendrocyte development at proliferative stages of the lineage. Glutamate and its receptors are likely to be part of the complex network of signals that regulate oligodendrocyte development in vivo. Our present study indicates that in a system in which complex cellular interactions and signaling are maintained, the excitatory neurotransmitter is effective in regulating proliferation and differentiation of oligodendrocyte lineage cells. In regions rich in glutamatergic neurons, such as the cerebellar cortex, glutamate released from axons (Kriegler and Chiu, 1993; Weinreich et al., 1975; Wheeler et al., 1966) in the developing white matter could be one of the many signals that regulate the final number of oligodendrocytes. The anti-proliferative effects of glutamate could also have neuropathological implications under conditions that involve breakdown of the blood-brain barrier (Cancilla et al., 1993). Finally, in view of the differences between immature versus mature oligodendrocytes to glutamate receptor agonists, higher levels of glutamate could induce oligodendrocyte cell death under pathological conditions (Matute et al., 1997; McDonald et al., 1998).

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