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5-Lipoxygenase is required for proliferation of immature cerebellar granule neurons in vitro

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Abstract

Primary cultures of rat cerebellar granule neurons express 5-lipoxygenase, an enzyme from the inflammatory pathway of arachidonic acid. Outside the central nervous system (CNS) 5-lipoxygenase participates in cell proliferation. We hypothesized that 5-lipoxygenase is needed for proliferation of immature cerebellar granule neurons. Using cultures prepared from 7-day-old rat pups, we confirmed in vitro neurogenesis by immunocytolabeling with 2-bromo-5-deoxyuridine and β-tubulin isotype III and quantified the rate of cell proliferation by assaying [^{3}H]thymidine incorporation. We found that immature cerebellar granule neurons express large amounts of 5-lipoxygenase, and that treatment with a 5-lipoxygenase antisense, to reduce expression of this gene, decreased significantly (by 60%) the content of 5-lipoxygenase protein and effectively reduced cell proliferation. [^{3}H]thymidine incorporation was significantly reduced by each of the three 5-lipoxygenase inhibitors we tested: AA-861 [2-(12-hydroxydodeca-5, 10-diynyl)-3,5,6-trimethyl-1,4-benzoquinone], MK-886 ($C_{27}H_{33}ClNO_2S \cdot Na$), and L-655,238 [α-penyl-3-(2-quinolinylmethoxy)-benzenemethanol]. Their anti-proliferative effect was reversible. We propose that neuronal expression of 5-lipoxygenase is crucial for neurogenesis in vitro, and possibly also in vivo.

Keywords: 5-Lipoxygenase; Neurogenesis; Antisense; AA-861; MK-886; L-655,238

1. Introduction

5-Lipoxygense is an enzyme involved in dioxygenation of arachidonic acid into 5-hydroperoxyeicosatetraenoic acid. 5-Lipoxygenase is typically studied because of its ability to generate inflammatory leukotrienes; however, there are indications that this protein may be involved in numerous other, non-enzymatic, intracellular actions, and that it is also expressed in central nervous system (CNS) neurons (for review, Manev et al., 2000). It has been established rather consistently that, outside the CNS, inhibitors of 5-lipoxygenase reduce the in vitro proliferation of bone-marrow cells and of cell lines from various other sources. For example, the fairly selective 5-lipoxygenase inhibitor MK-886 ($C_{27}H_{33}ClNO_2S \cdot Na$) reduced proliferation and induced apoptotic cell death in cultures of prostate PC-3 cells (Anderson et al., 1998); MK-886 or 2-(12-hydroxydodeca-5, 10-diynyl)-3,5,6-trimethyl-1,4-benzoquinone (AA-861) (another selective 5-lipoxygenase inhibitor) inhibited the proliferation of prostate cancer cells (Ghosh and Myers, 1997). 5-Lipoxygenase inhibitors also reduced the proliferation of pancreatic cancer cells; interestingly, only cancerous, but not normal, pancreatic cells exhibited high 5-lipoxygenase expression (Ding et al., 1999). In this work, we postulated that possibly *neuronal* expression of 5-lipoxygenase also participates in cell proliferation, i.e., in mitotic neurogenesis.

In addition to developmental role of neurogenesis, the importance of postnatal neurogenesis for the proper functioning of the adult brain has recently been emphasized by the findings that the primate and the human brain retains its ability to generate neurons throughout life (Eriksson et al., 1998; Gould et al., 1999; Roy et al., 2000). Thus, it was apparent that some of the widely used drugs might exert negative effect on mitotic neurogenesis. For example, an effect of opiates on neurogenesis in the adult hippocampus may be responsible for drug of abuse-induced alterations of CNS functioning (Eisch et al., 2000). Also pertinent is whether pharmacological regulation of postnatal neurogenesis could lead to therapeutic effects. For example, mitotic neurogenesis was induced in the adult rat

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hippocampus by peripheral administration of insulin-like growth factor I (IGF-I) (Aberg et al., 2000) or with chronic administration of antidepressant drugs (Jacobs and Fornal, 1999; Malberg et al., 1999; Manev et al., 2001). Clearly, a better understanding of the mechanisms involved in neuronal proliferation is needed.

Because proliferation of cerebellar granule neurons continues postnatally during the period lasting up to the end of the third postnatal week (Bayer et al., 1993), primary cultures of rat cerebellar granule neurons (typically prepared from 7-day-old pups) have been used as an in vitro model to study the mechanisms of neurogenesis (Lin and Bulleit, 1997; Fiszman et al., 1999; Wechsler-Reya and Scott, 1999). Thus, it was observed that opioids (e.g., morphine) inhibit neurogenesis, both in this culture in vitro (Hauser et al., 2000) and in the adult hippocampus in vivo (Eisch et al., 2000). Among the proliferatory factors that are effective in stimulating neurogenesis in such cultures are the IGF-I (Lin and Bulleit, 1997), which also stimulates neurogenesis in the adult hippocampus (Aberg et al., 2000), the Sonic Hedgehog protein (Wechsler-Reya and Scott, 1999), and the neurotransmitter γ-aminobutyric acid (GABA) (Fiszman et al., 1999).

Typically, in cerebellar granule neuron cultures, proliferation is observed during the first several days in vitro; thereafter, cerebellar granule neurons differentiate and acquire mature neuronal phenotypes. Recently, we found that immature cerebellar granule neurons express larger amounts of 5-lipoxygenase mRNA and protein than the mature differentiated cultures (Manev and Uz, 1999). This observation and the above-discussed findings prompted us to hypothesize that 5-lipoxygenase may be related to cerebellar granule neuron proliferation and, consequently, to mitotic neurogenesis. We tested and confirmed our hypothesis in proliferating rat cerebellar granule neuron cultures using an antisense oligonucleotide that effectively reduces 5-lipoxygenase protein expression or selective 5-lipoxygenase inhibitors [AA-861, MK-886, L-655,238 (α-penyl-3-(2-quinolinylmethoxy)-benzenemethanol)].

2. Materials and methods

2.1. Primary cultures of cerebellar granule neurons and treatments

The cerebellar granule neuron cultures were prepared from 7-day-old rat pups (Sprague–Dawley; Harlan) as described elsewhere (Manev et al., 1989; Brewer, 1995). The experimental protocol was approved by the Institutional Animal Care and Use Committee. Cells were grown in a serum-free medium with the B27 supplement (Neurobasal medium; B27 supplement, 10 ml/500 ml medium [Gibco]; 25 mM KCl). This medium does not support the proliferation of non-neuronal cells; thus; the addition of an antimitotic, necessary for cultures grown with 10% serum, is not needed (Brewer, 1995; Fiszman et al., 1999). Cultures were grown in polylysine-coated 10-cm dishes (for mRNA and Western blot assays), 35-mm dishes (microscopy), or 24-well dishes ([^{3}H]thymidine incorporation assay of cell proliferation); the volumes were 10, 2 and 0.5 ml, respectively. The concentration of cells was 600,000/ml. For immunostaining, cultures were grown on polylysine-coated glass circles (20 mm). Cultures were maintained at 37°C and 5% CO_2. 5-Lipoxygenase inhibitors (AA-861, MK-886, or L-655,238; Biomol, Plymouth Meeting, PA) were dissolved in dimethylsulfoxide (DMSO); inhibitors or DMSO (control) were added to cultures: 1μl/0.5 ml medium/well. Oligonucleotides were designed according to the published rat 5-lipoxygenase sequence (Balcarek et al., 1988) (antisense: TCT GGG AAG TGA GCG CTG; scramble: TCA GTG ACT GCG AGT GGG) and added to cultures: 1 μl/0.5 ml to result in a final concentration of 10 μM.

2.2. Time-lapse microscopy

Two hours after plating, a 35-mm culture dish was placed onto an inverted Nikon microscope equipped with an enclosed temperature-controlled stage (37°C, saturated with 5% CO_2). A randomly chosen field was continuously video recorded for 24 h using a time-lapse recording system.

2.3. Immunocytochemistry

2-Bromo-5-deoxyuridine (BrdU; Sigma, St. Louis, MO, USA) labeling was used to visualize the DNA of dividing cells, thus identifying cells that underwent proliferation while BrdU was present in the medium. BrdU (10 μM) was added to the culture for a period of 24 h, starting 18 h after plating. Thereafter, BrdU was washed out, and cultures were returned to a fresh medium and left undisturbed for 9 days, when they were fixed with 4% paraformaldehyde and stained with a monoclonal rat anti-BrdU antibody (Accurate; 1:300; 2 h at room temperature) and colabeled with mouse anti-β-tubulin isotype III antibody (Sigma; 1:500; 2 h at room temperature) to visualize neuronal cell bodies and neurites (Roy et al., 2000). Corresponding biotinylated secondary antibodies [anti-rat and anti-mouse immunoglobulin G (IgG); Vector; 4 μg/ml of phosphate-buffered saline (PBS) + 1% normal goat serum; 1 h at room temperature] and the ABC Elite Kit (Vector; 1 h at room temperature) were applied to visualize the specific staining of BrdU (blue color, with an imidazol acetate buffer) and of β-tubulin (brown color, with a Tris buffer). In addition to neuronal labeling with anti-β-tubulin antibody, anti-GFAP antibody (anti-glial fibrillary acidic

protein; Sigma; 1:500 at room temperature) was used to label non-neuronal/glia cells.

2.4. Western immunoblotting

Cells from six 10-cm dishes/group were scraped into a homogenizing buffer containing 20 mM Tris–HCl, 5 mM EGTA, and 5 mM EDTA. After centrifugation at 14,000 rpm, 30 min, the pellet was resuspended in the homogenizing buffer containing 1 mM benzamidine–HCl and 0.5 mg/ml leupeptin. Equal volumes of protein samples (10 to 60 μg protein) and gel loading solution (50 mM Tris–HCl, 8% β-mercaptoethanol, 10% sodium dodecylsulfate [SDS], 18% glycerol, and a trace amount of bromphenol blue) were mixed and the mixtures were boiled. Equal amounts of proteins from each experimental group were run onto a 7.5% (w/v) acrylamide gel and were subsequently transferred electrophoretically to a Hybond enhanced chemoluminescence (ECL) nitrocellulose membrane (Amersham). The blots were blocked with 5% (w/v) powdered nonfat milk in Tris-buffered saline Tween (TBST), 2 ml nonidet P-40, and 0.02% (w/v) SDS (pH 8.0). They were incubated overnight with the primary anti-5-lipoxygenase antibody (rabbit polyclonal; Cayman No. 160402) at a dilution of 1:1000. The blots were then washed with TBST and incubated with a horseradish-peroxidase-linked secondary antibody (anti-rabbit IgG; 1:3000) for 2 h at room temperature and processed with the Amersham ECL kit (Amersham); blots were again washed with TBST and exposed to ECL film. To normalize our data, we simultaneously measured β-actin immunoreactivity using the monoclonal primary antibody (Sigma, 1:1000, overnight) and anti-mouse IgG (1:3000 for 2 h) as the secondary antibody. The optical densities of the bands on the autoradiograms were quantified using the Loats Image Analysis System (Westminster, MD, USA); the optical density of the 5-lipoxygenase signal was corrected by the optical density of the corresponding β-actin band Uz et al., 1999.

2.5. Quantitative reverse transcription-polymerase chain reaction (RT-PCR) assay of 5-lipoxygenase and cyclophilin mRNAs

This was performed as described in detail elsewhere (Uz et al., 1997; Manev and Uz, 1999). Briefly, decreasing concentrations of 5-lipoxygenase or cyclophilin (cyc), internal standard, cRNA were added to 1 μg of the total RNA. The RNA/cRNA mixtures were denaturated at 80°C for 6 min and then reverse transcribed with cloned Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Gibco, BRL; 200 U) in reverse transcription buffer containing 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, and 1 mM deoxynucleotide triphosphates (dNTPs) (Gibco) using random hexamers (Pharmacia Biotech; 5 mM) and a ribonuclease inhibitor (human placental ribonuclease inhibitor) (Amersham; 28 U) in a volume of 20 μl. The reverse transcription mixture was incubated at 37°C for 60 min to promote cDNA synthesis. The reaction was terminated by heating the samples at 98°C for 5 min, and the mixture was quick-chilled on ice. *Competitive PCR amplification*: After termination of the reverse transcription reaction, cDNA aliquots containing reverse transcribed material were amplified with Hot *Tub* DNA polymerase (Amersham) in the Thermal Cycler (Perkin-Elmer, 9600). The amplification mixture contained cDNA, 0.5 μM specific primer pairs, 200 μM dNTPs, 1.5 mM $MgCl_2$, 50 mM Tris–HCl (pH 9.0), 20 mM ammonium sulfate, 15 mM KCl, and 1.5 U of Hot *Tub* polymerase in a 100-μl volume. Trace amounts of [^{32}P]dCTP (Amersham; 0.5–1 μCi/sample) were included during the PCR step for subsequent quantification. The PCR mixture was amplified for 30 cycles with denaturation (94°C, 15 s), annealing (60°C, 30 s), and elongation (72°C, 30 s) amplification steps. The reaction was terminated with a 5-min final elongation step. *Quantitation*: Following amplification, aliquots were digested with *Bgl*II overnight in triplicates and run by agarose gel electrophoresis. To quantitate the amounts of the products corresponding to the amplified mRNA, the ethidium bromide-stained bands were excised and the radioactivity was determined by Cerenkov counting. The results were calculated as attomoles of 5-lipoxygenase or cyc mRNA per 1μg of total RNA. Results are expressed as attomoles of 5-lipoxygenase/μg total RNA. Correction by the content of cyclophilin mRNA did not alter the results (not shown).

2.6. [^{3}H]thymidine incorporation to quantify the rate of cell proliferation

Cultures were grown in 24-multiwell dishes (300,000 cells/dish) and were treated with 1 μCi/ml [^{3}H]thymidine (Amersham; TRK120). After incubation (time indicated for each particular experiment), the medium with radioactive thymidine was removed and cultures were washed twice with Locke's buffer (Locke's buffer described in Manev et al., 1989). Cells were detached using a trypsin/EDTA solution (Sigma), collected in individual tubes, and processed on a cell harvester equipped with Whatman GF/C filters. Samples were washed six times. Filters were allowed to dry and the radioactivity was counted in a liquid scintillation β counter.

2.7. Trypan-blue exclusion assay

Cultures were incubated with 0.4% trypan-blue solution (Sigma) for 3 min (Manev et al., 1997). Only cells with a damaged cell membrane are permeable to trypan blue. The numbers of trypan-blue-positive and trypan-blue-negative (viable) cells were counted (20× objective) in two ran-

domly chosen fields per culture dish. Data are expressed as percentage of trypan-blue-negative cells.

2.8. Statistics

Analysis of variance (ANOVA) was followed by the Student's *t*-test or the Dunnett's test for multiple comparison with control. $^{*}P < 0.05$ was taken as significant.

3. Results

It was established earlier in slightly different experimental conditions that cultures prepared from the postnatal rat cerebellum contain immature neurons and proliferative neuroblasts, and that they proliferate in vitro, generating new neurons (Kane et al., 1996; Wolf et al., 1997; Fiszman et al., 1999). We confirmed these observations under our

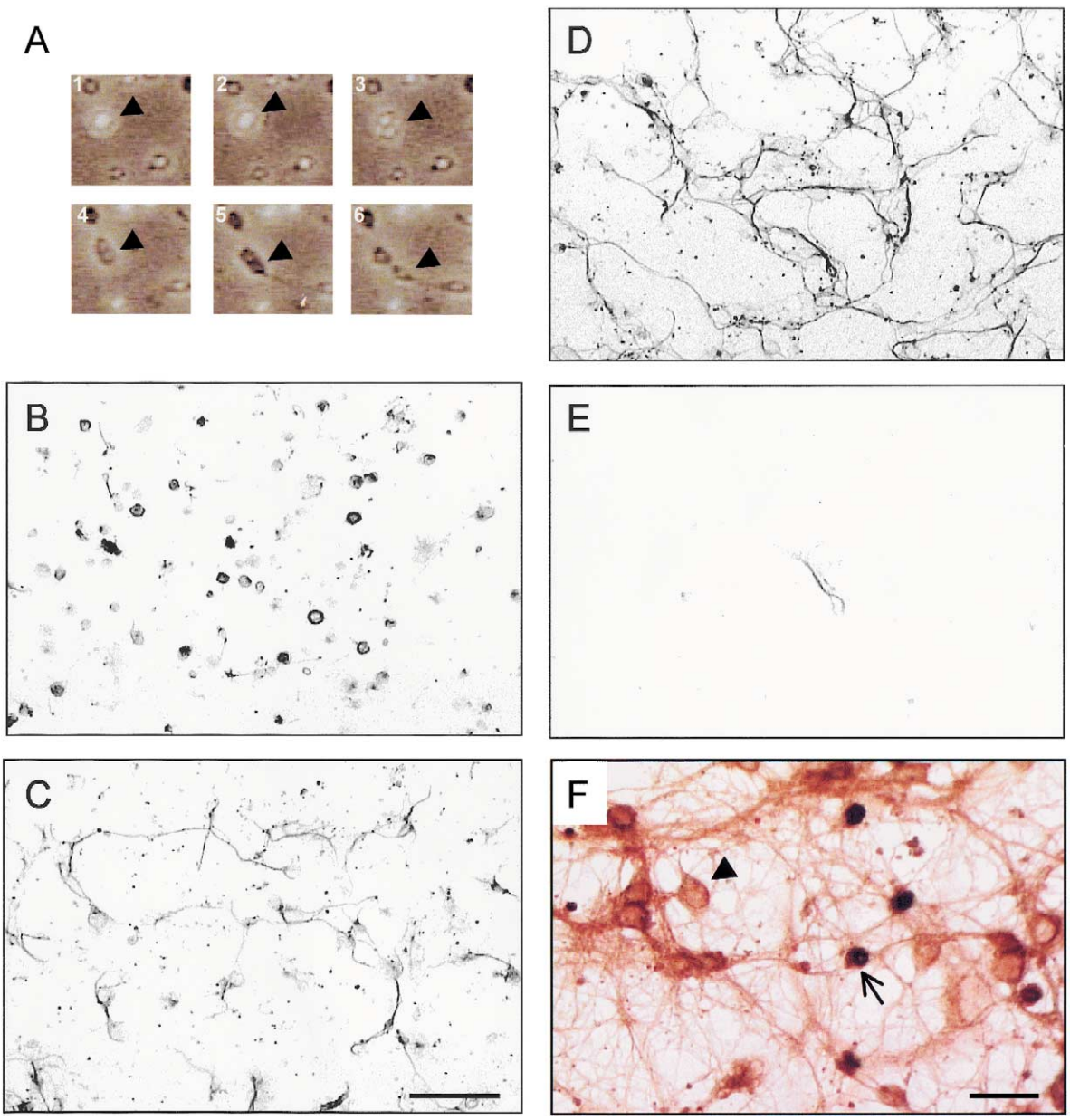

Fig. 1. Neurogenesis in cerebellar granule neuron cultures. (A) Time-lapse microscopy: cells were prepared from 7-day-old pups and plated onto a 35-mm culture dish; the same field was monitored and recorded using time-lapse microscopy over a period of 24 h, starting 2 h after plating The triangle in A points to a cell that divided and acquired the phenotype of a neuron. Within first 24 h in vitro, the majority of cells express a neuronal marker, β-tubulin isotype III (B–D), and only few are positive for a glia marker, GFAP (E). Panels B, C, and D show cultures at 3, 6, and 24 h in vitro, respectively; panel E = 24 h in vitro (scale bar B–E = 50 μm). The long-term survival and neuronal differentiation of newly formed, BrdU-positive, neurons is demonstrated in panel F. To this end, BrdU (10 μM) was added to the culture for a period of 24 h, starting 18 h after plating. Thereafter, BrdU was washed out, and cultures were returned to a fresh medium and left undisturbed for 9 days, when they were fixed with paraformaldehyde and stained with an anti-BrdU antibody and with an anti-β-tubulin isotype III antibody. Note the dark anti-BrdU nuclear staining of some (newly formed) neurons (arrow) and its absence in other neurons (arowhead) (F). Also shown in F is the β-tubulin-positive neural network (scale bar = 20 μm).

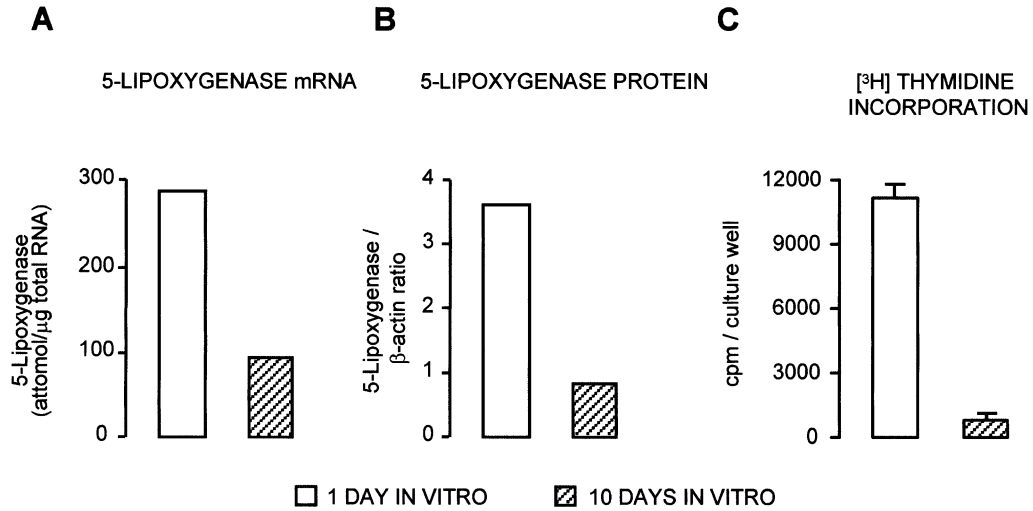

Fig. 2. Immature (1 day in vitro) cerebellar granule neuron cultures express higher levels of 5-lipoxygenase mRNA and protein and proliferate at a higher rate than more mature cerebellar granule neuron cultures (10 days in vitro). Cultures were grown in a serum-free medium without any inhibitors of cell proliferation (Brewer, 1995). Cultures were maintained either in 10-cm dishes (A, B) or in 24-well dishes (C). Cells were harvested for mRNA (RT-PCR) and protein (Western blot) assays 24 h after plating (1 day in vitro) or 10 days after plating (10 days in vitro). Shown in A and B are results obtained from sister cultures from the same preparation (each sample was a pool of 5 to 6 10-cm dishes). In C, [^{3}H]thymidine was added to 1 day in vitro or 10 days in vitro cultures and the reaction was stopped after 4 h. Results (mean ± S.E.M., $n = 6$) are expressed as counts per min (cpm)/well ($P < 0.001$; t-test).

experimental conditions (Fig. 1). Using time-lapse microscopy we verified that in primary rat cerebellar granule neuron cultures, cells indeed proliferate during the first few days in vitro, and that proliferating cells acquire a neuron-like phenotype (Fig. 1A). Within the first 24 h in vitro, most cells expressed a neuronal marker β-tubulin isotype III (Fig. 1B–D), whereas only few (0–3 per field) were positive for a glia marker GFAP (Fig. 1E). Using a method of double-immunolabeling for BrdU (a marker of proliferation) and for β-tubulin isotype III, we investigated whether cerebellar granule neurons that proliferate at 1 day in vitro survive as mature neurons, e.g., up to 10 days in vitro. A pulse labeling with BrdU for 24 h at 1 day in vitro resulted in labeling that was still retained by neurons 9 days later (Fig. 1F).

As previously observed in cerebellar granule neuron cultures grown in the presence of 10% serum (Manev and Uz, 1999), also in cultures maintained in a medium without serum and in the absence of an antimitotic, the contents of 5-lipoxygenase mRNA and 5-lipoxygenase protein were greater in immature (1 day in vitro) than in mature (10 days in vitro) cultures (Fig. 2A,B). As expected, we also found a high rate of [^{3}H]thymidine incorporation in cultures at 1 day in vitro, but not at 10 days in vitro (Fig. 2C).

Treating immature cultures with an antisense oligonucleotide against 5-lipoxygenase, to reduce expression of this gene, lowered significantly (by 60%) the content of 5-lipoxygenase protein (Fig. 3A,B). The 5-lipoxygenase antisense treatment, but not the treatment with the corre-

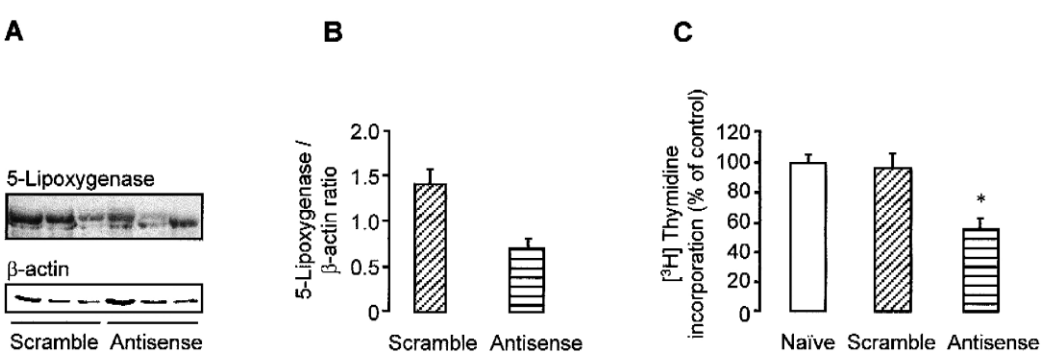

Fig. 3. 5-Lipoxygenase antisense reduces 5-lipoxygenase protein content (A, B) along with reducing cell proliferation (C) in 1-day-old cerebellar granule neuron cultures. Oligonucleotides (antisense and the corresponding scramble oligonucleotide) were added to cultures (1 μl/0.5 ml; 10 μM final) 18 h after plating, and the addition was repeated 6 h later. The experiment was terminated 6 h after the second oligonucleotide application ([^{3}H]thymidine was added for the last 6 h). For Western blotting, three dilutions (60, 40, and 20 μg protein) of each sample were assayed with a 5-lipoxygenase antibody and re-probed with a β-actin antibody (A). 5-Lipoxygenase/β-actin ratios were calculated for each dilution, and their averages (mean ± S.E.M.) are shown in B. [^{3}H]thymidine incorporation was measured in 6 wells/group; results (mean ± S.E.M.) are expressed as percent of naïve control (100%); * $P < 0.001$ vs. naïve and scramble.

sponding scramble oligonucleotide, effectively reduced the proliferation of immature cerebellar granule neurons (Fig. 3C).

The incorporation of [^{3}H]thymidine was significantly and concentration dependently reduced by treatment of immature cerebellar granule neurons with any of the three 5-lipoxygenase inhibitors we tested (AA-861, MK-886, L-655,238) (Fig. 4A); the most efficacious in inhibiting cell proliferation was AA-861. The two 5-lipoxygenase-activating protein inhibitors, MK-886 and L-655,238, exhibited similar, albeit lower, efficacy (5-lipoxygenase-activating protein, which promotes 5-lipoxygenase enzymatic activity, is also expressed in cerebellar granule neuron cultures; Manev and Uz, 1999). Treatment of cultures for 4 h with the anti-proliferative concentrations of 5-lipoxygenase inhibitors did not significantly decrease the numbers of trypan-blue-negative (viable) cells/field; their percentage was: vehicle = 73 ± 1.6; AA-861 (5 μM) = 76 ± 4.9; L-655,238 (25 μM) = 76 ± 2.9; (n = 4). Moreover, the anti-proliferative effects of the 5-lipoxygenase inhibitors were reversible; [^{3}H]thymidine incorporation reverted to a control-like rate after they were removed from the culture medium (Fig. 4B).

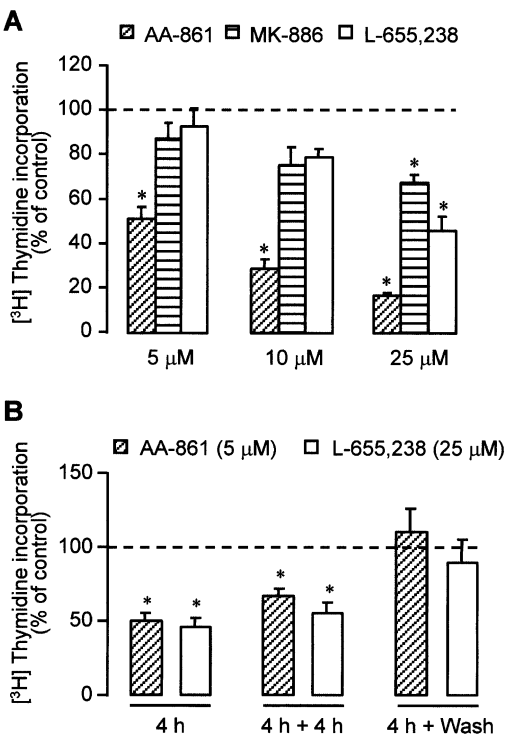

Fig. 4. 5-Lipoxygenase inhibitors reversibly reduce cell proliferation in immature cerebellar granule neuron cultures. Illustrated is concentration-dependent inhibitory action of 5-lipoxygenase inhibitors (AA-861, MK-886, L-655,238) on cell proliferation (A) and the reversibility of their action if the drugs are washed out from the cerebellar granule neuron cultures (B). Cerebellar granule neurons (300,000 cells/well) were grown in a serum-free medium in 24-well dishes. Drug or vehicle (dimethylsulfoxide; 1 μl/0.5 ml medium/well) was added 18 h after plating; 4 h later 1 μl/well [^{3}H]thymidine was added and the reaction was stopped after 4 h. In B, 5 μM AA-861 or 25 μM L-655,238 was used; [^{3}H]thymidine was added either during the 4-h incubation with drug/vehicle or for 4 h following the 4-h drug incubation and washout (3 × 0.5 ml of medium/well). Results (mean ± S.E.M., n = 6–12) are expressed as percent of control ($^*P < 0.01$ vs. control; Dunnett's test). Similar results were obtained in at least two different culture preparations.

4. Discussion

In this work, we have demonstrated that in immature neurons/neuroblasts, the presence of 5-lipoxygenase is essential for maintaining normal proliferation. Earlier, it was observed that an inborn error in the 5-lipoxygenase pathway that results in deficient leukotriene synthesis is also accompanied by lethal neurological symptoms (Mayatepek, 2000; Mayatepek and Flock, 1998). Since work by others suggests that 5-lipoxygenase also participates in tumor proliferation (Ghosh and Myers, 1997; Anderson et al., 1998), it appears that the 5-lipoxygenase pathway is a crucial mechanism involved in cell proliferation.

In accordance with previously published results by others (Kane et al., 1996; Lin and Bulleit, 1997; Wolf et al., 1997; Fiszman et al., 1999), we have confirmed that during the initial days in vitro primary rat cerebellar granule neuron cultures generate new neurons. These new neurons incorporate BrdU labeling and survive and differentiate (e.g., at least to 10 days in vitro, when the cultures no longer show high rates of proliferation, i.e., [^{3}H]thymidine incorporation). The highly expressed 5-lipoxygenase mRNA and protein in proliferating immature cultures (e.g., at 1 day in vitro) appear to be involved in cell proliferation/neurogenesis; namely, we observed that reducing the content of neuronal 5-lipoxygenase protein with a 5-lipoxygenase-specific antisense oligonucleotide significantly reduced cell proliferation. Similar results were obtained in human pancreatic cells (Ding et al., 1999). These authors found that pancreatic cancer cell lines, but not normal pancreatic cells, express 5-lipoxygenase, and that 5-lipoxygenase antisense markedly inhibits cell proliferation. In contrast to antisense studies, studies with 5-lipoxygenase *knockout* mice, as reviewed by Anderson et al. (1999), did not produce clear-cut results in terms of cell proliferation and/or development. The explanation could lie in the compensatory mechanisms that are usually set in motion by permanent knockout procedures and in other, poorly understood genetic factors (Goulet et al., 2000). One possibility is that 12-lipoxygenase pathway, which also is expressed in neurons (Palluy et al., 1994; Li et al., 1997), may become activated to compensate for an inherited artificially knocked-down 5-lipoxygenase functioning; namely, in pancreatic cells, 12-lipoxygenase antisense was also capable of reducing cell proliferation (Ding et al., 1999), which suggests that 5-lipoxygenase and 12-lipoxygenase might exert complementary actions. Neverthe-

less, when bone-marrow-derived mast cells from 5-lipoxygenase-deficient knockout mice were grown in culture, it was observed that their life span was shortened, along with markedly increased programmed cell death (Chen and Funk, 1998). The functional significance of neuronal 12-lipoxygenase is not fully understood; for example, 12-lipoxygenase has been shown capable of influencing processes leading to neuronal death (Li et al., 1997); however, it does not appear to be capable of preventing selective 5-lipoxygenase inhibitors/antisense from reducing neurogenesis in our in vitro model. Whether neuronal 12-lipoxygenase may exert some influence on neurogenesis in its own right cannot be concluded from our experiments.

There are at least two possible mechanisms by which 5-lipoxygenase protein could affect neurogenesis: (a) enzymatic action, and (b) non-enzymatic action. The enzymatic action of 5-lipoxygenase includes its participation in the conversion of arachidonic acid (20-carbon omega-6 fatty acid) into biologically active inflammatory leukotrienes. These and other metabolites of arachidonic acid (commonly named eicosanoids) are known to play an important role in cancerogenesis. The system of mitogen-activated protein kinase (MAPK), which is a dominant pathway involved in cell growth, appears to govern the activation and nuclear translocation of 5-lipoxygenase (Lepley and Fitzpatrick, 1996). The exact nature of the involvement of the various eicosanoids in cell proliferation appears to be complex and tissue/cell specific. Typically, drugs that inhibit 5-lipoxygenase activity have been found effective in inhibiting cell proliferation (Anderson et al., 1998; Ding et al., 1999). The three different 5-lipoxygenase inhibitors we studied, AA-861, MK-886, and L-655,238, were effective in inhibiting neurogenesis in cultures of cerebellar granule neurons even during a rather short incubation time (e.g., 4 h). Because their antiproliferative effect was reversible, it is unlikely that it resulted from simple neurotoxicity and cell loss, and because both the antisense and the pharmacological approach resulted in inhibition of cell proliferation, we conclude that this effect is due to inhibition of the 5-lipoxygenase pathway. Nevertheless, it is certainly possible that 5-lipoxygenase inhibitors could exert other, 5-lipoxygenase-unrelated, effects. For example, it has been suggested that MK-886, which affects 5-lipoxygenase by its inhibitory action on 5-lipoxygenase-activating protein, can cause apoptosis independently of 5-lipoxygenase-activating protein and, thus, independently of its action on 5-lipoxygenase (Datta et al., 1999). Alternately, 5-lipoxygenase inhibitors could interfere with the non-enzymatic actions of 5-lipoxygenase. The latter have only recently became known, and it is still too early to suggest their role in neurogenesis. Nevertheless, it is tempting to speculate that cell proliferation could be affected by non-enzymatic interactions of 5-lipoxygenase protein with cytoskeletal proteins or with growth factor receptor-bound protein 2 (Grb2) (Lepley and Fitzpatrick, 1994). The role of adaptor proteins, such as Grb2 in the developing mammalian brain, has recently been emphasized (Cattaneo and Pelicci, 1998).

Although 5-lipoxygenase inhibitors are still being considered for development as new anti-cancer therapies (Ding et al., 1999; Myers and Ghosh, 1999), clinically available compounds with 5-lipoxygenase-inhibiting activity, such as zileuton (McGill and Busse, 1996) and mizolastine (Pichat et al., 1998), are already being used as anti-inflammatory drugs. As yet, it is not clear whether they penetrate into the brain sufficiently to affect neurogenesis. Our in vitro results suggest that further research is needed to fully characterize the functional significance of neuronal 5-lipoxygenase and its possible involvement in neurogenesis in vivo.

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