Functional *N*-Methyl-D-Aspartate Receptors in O-2A Glial Precursor Cells: A Critical Role in Regulating Polysialic Acid–Neural Cell Adhesion Molecule Expression and Cell Migration

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Abstract. The capacity for long-distance migration of the oligodendrocyte precursor cell, oligondendrocyte-type 2 astrocyte (O-2A), is essential for myelin formation. To study the molecular mechanisms that control this process, we used an in vitro migration assay that uses neurohypophysial explants. We provide evidence that O-2A cells in these preparations express functional *N*-mehtyl-D-aspartate (NMDA) receptors, most likely as homomeric complexes of the NR1 subunit. We show that NMDA evokes an increase in cytosolic Ca^{2+} that can be blocked by the NMDA receptor antagonist AP-5 and by Mg^{2+}. Blocking the activity of these receptors dramatically diminished O-2A cell migration from explants. We also show that NMDA receptor activity is necessary for the expression by O-2A cells of the highly sialylated polysialic acid–neural cell adhesion molecule (PSA-NCAM) that is required for their migration. Thus, glutamate or glutamate receptor ligands may regulate O-2A cell migration by modulating expression of PSA-NCAM. These studies demonstrate how interactions between ionotropic receptors, intracellular signaling, and cell adhesion molecule expression influence cell surface properties, which in turn are critical determinants of cell migration.

DURING development of the vertebrate brain, precursors of oligodendrocytes or oligodendrocyte-type 2 astrocyte (O-2A)[1] cells migrate along nerve fibers, and this movement is essential for the process of myelination (Lachapelle et al., 1984; Levison et al., 1993; Small et al., 1987; Kiernan and ffrench-Constant, 1993). While the capacity of O-2A cells to migrate over a long distance has been described extensively, the underlying cellular and molecular regulatory events are not well understood. Results from several studies suggest that growth factors such as PDGF and basic fibroblast growth factor may play an important role in O-2A cell migration (Noble et al., 1988; Richardson et al., 1988). PDGF provided by type 1 astrocytes or by neurons is believed to maintain O-2A cells in a bipolar migratory state and to guide their migration, whereas basic FGF appears to transform these cells into a nonmigratory multipolar form (McKinnon et al., 1993). In addition, PDGF appears to be a potent chemoattractant of O-2A cells (Armstrong et al., 1990).

Apart from growth factors, adhesion molecules such as integrins (Milner and ffrench-Constant, 1994) and the neural cell adhesion molecule (NCAM), in particular its polysialylated isoforms (Trotter et al., 1989; Wang et al., 1994), have also been implicated in O-2A migration. NCAM is known to serve as a homophilic receptor promoting cell–cell adhesion (Edelman, 1986). However, it is also expressed in an alternative form containing long chains of α2-8 linked polysialic acid (PSA) (Finne et al., 1983). PSA has been shown to convert NCAM into an antiadhesive agent (Rutishauser, 1992; Sadoul et al., 1983). The pattern of expression of PSA on NCAM is highly regulated during

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1. *Abbreviations used in this paper*: aa, amino acid; Ab, antibody; AMPA, DL-α-amino-3-hydroxy-5-methyl-4-isoxazoepropionic acid; BrdU, bromodeoxyuridine; $[Ca^{2+}]_i$, free intracellular calcium concentration; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; d-AP5, 2-D-L-aminophosphonovaleric acid; Endo N, endoneuroaminidase N; GalC, galactocerebroside; GFAP, glial fibrillary acidic protein; MK-801, (+)-5-methyl-10,11-dihydro-5H-dibenzo [a,b] cyclohepten-5,10-imine maleate; NCAM, neural cell adhesion molecule; NMDA, *N*-methyl-D-aspartate; NT3, neurotrophin-3; O-2A, oligodendrocyte-type 2 astrocyte; PSA, polysialic acid; RT, reverse transcriptase; TTX, tetrodotoxin.

development, and the resulting attenuation of cell interactions is critical to a variety of morphogenetic events including cell migration, transformation of cell shape, and axonal growth (Acheson et al., 1991; Doherty et al., 1992; Rougon et al., 1993). Consistent with this view, O-2A cells express the polysialylated isoforms of NCAM, whereas mature oligodendrocytes or type 2 astrocytes generated by these precursors exhibit the adult, less sialylated isoforms (Trotter et al., 1989; Wang et al., 1994).

Recently, using an in vitro migration assay that consists of explants of newborn rat neurohypophysis, we have provided direct evidence for the role of PSA on NCAM in O-2A cell migration (Wang et al., 1994). We have demonstrated that neurohypophysial explants from newborn rats generate cellular outgrowths in which virtually all migrating glial cells belong to the O-2A cell lineage (Wang et al., 1994). Treatment of O-2A cells with the enzyme endoneuraminidase (Endo N), which specifically removes PSA from the cell surface, produced a complete blockade of the dispersion of the O-2A cell population from the explant. This effect was reversible upon cessation of Endo N treatment (Wang et al., 1994). Since Endo N does not affect the protein core of the NCAM molecule, it is conceivable that it is PSA rather than NCAM itself that is essential for the migration of O-2A cells. Thus, one might expect that factors that modulate the degree of sialylation of NCAM play a decisive role in O-2A cell motility.

Recent studies suggest that calcium ion influx may have a critical role in PSA-NCAM expression in neurons, pancreatic β cells (Kiss et al., 1994), and myotubes (Rafuse and Landmesser, 1996). Ca^{2+} may also be involved in controlling PSA expression in O-2A cells. Ca^{2+} entry into neurons as well as glial cells can be mediated by ligand-gated ion channels such as glutamate receptors. Indeed, L-glutamate has been identified as an effective stimulus to trigger Ca^{2+} influx in O-2A cell lineage cells (Fulton et al., 1992; Holzwarth et al., 1994; Kastritsis and McCarthy, 1993; Pende et al., 1994). In vivo, O-2A cells migrate along axons that are able to release glutamate in an activity-dependent manner (Chiu and Kriegler, 1994), and it has been proposed that glutamate could act as a developmental factor that regulates the migration as well as the proliferation of O-2A cells (Pende et al., 1994). The main glutamate receptor that controls Ca^{2+} entry in neurons is of the NMDA type, and the NMDA receptor–mediated Ca^{2+} influx is thought to be involved in neuronal plasticity (Bliss and Collinbridge, 1993) and morphogenetic events (Komuro and Rakic, 1993; Scheetz and Constantine-Platon, 1994).

In the present study, we tested the hypothesis that NMDA receptors are also present on O-2A cells and that Ca^{2+} influx plays a role in the migration process. We present direct evidence for the existence of NMDA-type glutamate receptors in O-2A cells from previously characterized (Wang et al., 1994) neurohypophysial explants. We demonstrate that their blockade inhibits the migration of these cells. We also show that the activity of this receptor is necessary for the expression of PSA-NCAM, which is needed for the migration of O-2A cells. We thus provide experimental support for a role of NMDA-type glutamate receptor activity in O-2A cell migration and a basis for the underlying molecular mechanism.

Materials and Methods

Materials

Recombinant, human PDGF (PDGF-AA homodimer form) and neurotrophin-3 (NT3) were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN) and Regeneron Pharmaceuticals (Tarrytown, NY), respectively. 2-D-L-aminophosphonovaleric acid (d-AP5), the competitive antagonist of the NMDA receptor, and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), the DL-α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and kainate receptor antagonist and NMDA, were purchased from Tocris Neuramin (Bristol, UK); [+]-5-methyl-10,11-dihydro-5H-dibenzo [a,b] cyclohepten-5,10-imine maleate (MK-801), the noncompetitive NMDA receptor ionchannel blocker, was purchased from Research Biochemical Inc. (Natick, MA); the Na^+ channel antagonist tetrodotoxin (TTX), the antagonist to the L-type voltage-sensitive Ca^{2+} channel nifedipin, cyclopiazonic acid that depletes IP_3-sensitive Ca^{2+} stores without inhibiting L-channel activity (Nelson et al., 1994), and bromodeoxyuridine (BrdU) were purchased from Sigma Chemical Co. (St. Louis, MO). We used the enzyme Endo N, purified in our laboratory from phage K1 (Wang et al., 1994).

Neurohypophysial Explant Cultures and Migration Assay

Explant cultures of the neurohypophysis were prepared from newborn (postnatal day 0) rats (Sprague-Dawley; Sivz, Zürich, Switzerland) as described previously (Wang et al., 1994). To avoid contamination by neuroendocrine cells from the intermediate lobe and the adenohypophysis, the peripheral parts of the neurohypophysis were carefully eliminated. The absence of such cells in our preparations was verified by immunostaining with antibodies directed against proopiomelanocortin-derived peptides.

Cultures were maintained under three basic conditions: (*a*) in serum-free medium (Bottenstein, 1984) complemented with PDGF/NT3 (10 ng/ml); (*b*) in serum-free conditioned medium (Wang et al., 1994); and (*c*) in serum-complemented medium (10% FCS; GIBCO BRL, Gaithersburg, MD).

For the migration assay, cultures were divided into four groups: (*a*) controls maintained in serum-complemented or serum-free medium for 3 d; (*b*) as (*a*) with the addition of different antagonists and agonists of glutamate receptors and other ion channels; (*c*) serum-complemented medium with 3.5 mM Mg^{2+}; (*d*) as (*b*) followed by an additional 3 d without antagonists. The extent of migration of O-2A lineage cells was assessed by counting the number of A2B5/glial fibrillary acidic protein (GFAP) (medium with serum) and O4/galactocerebroside GalC (serum-free medium) immunopositive cells present in the outgrowth after 3 d of culture (Wang et al., 1994) on at least four coverslips for each group in three to five independent experiments.

Culture of Cortical Neurons

Primary mixed cultures of neuronal and glial cells were prepared from newborn cerebral cortex as described (Kiss et al., 1994) and used for PCR amplification of mRNA encoding NMDA receptor subunits (NR2A-D) and intracellular free calcium measurements (see below) 7 d after plating.

Antibodies and Immunocytochemistry

A rabbit polyclonal antibody (Ab) (Dakopatts, Copenhagen, Denmark) to GFAP was used (1:200 dilution) to identify astrocytes. Mouse mAb A2B5 hybridoma supernatant (American Type Culture Collection, Rockville, MD; 1:5 dilution) (Eisenbarth et al., 1979) and GD3 Ab (1:200 dilution) (Curtis et al., 1988) specific to cell surface ganglioside epitopes were used to label O-2A progenitor cells. O4 mAb (hybridoma supernatant, 1:5 dilution) (Eisenbarth et al., 1979) was used to identify undifferentiated oligodendrocytes and anti-GalC (Ranscht et al., 1982) mAb (culture supernatant) for differentiated oligodendrocytes. Anti-PSA (Meningococcus group B [Men B]) is a mouse IgM Ab (1:400 dilution of ascites fluid) that recognizes specifically α 2-8–linked PSA with chain length superior to 12 residues (Hayrinen et al., 1995; Rougon et al., 1986). The rabbit antiserum directed against the NCAM protein core was a site-directed Ab recognizing the seven NH_2-terminal residues of NCAM (1:1,000 dilution) (Rougon and Marshak, 1986). Rabbit anti-NMDAR1 is a polyclonal antiserum (Chemicon Intl., Inc., Temecula, CA) that reacts with splice variants NR1-1a, NR1-1b, NR1-2a, and NR1-2b expressed in rat brain (1:100

dilution) (Petralia et al., 1994). Neurons were identified with a mouse anti-MAP2 mAb (1:200 dilution) (Sigma Chemical Co.) and proliferating cells with an mAb directed to BrdU (Boehringer Mannheim Biochemicals) (1:50 dilution).

For double labeling, Abs directed against surface antigens (PSA, NCAM, A2B5, O4, GalC) were applied onto live cells for 30 min; cells were then fixed in cold (4°C) 4% paraformaldehyde in 0.1 M phosphate buffer, permeabilized with a solution of PBS/0.5% BSA/0.3% Triton X-100, and incubated with the GFAP antibody for 1 h at room temperature. Bound antibodies were revealed with fluorescein-conjugated sheep anti–mouse Ig (dilution 1:80; Boehringer Mannheim Biochemicals) or rhodamine-conjugated sheep anti–rabbit IgG (dilution 1:40; Boehringer Mannheim Biochemicals) secondary antibodies (diluted in PBS/0.5% BSA solution). For immunostaining with the anti-NR1 Ab, cultures were fixed with periodate-lysine-paraformaldehyde (McLean's) fixative. Cultures were examined with a fluorescence microscope (Axiophot; Zeiss, Oberlochen, Germany). Controls treated with nonspecific mouse IgM, IgG preimmune sera, or secondary antibodies alone showed no staining. In double-immunolabeling experiments, the use of only one primary Ab followed by the addition of both anti–mouse FITC- and anti–rabbit TRITC-conjugated secondary antibodies resulted in only single labeling. Endo N treatment completely abolished PSA immunostaining (see Fig. 2) without affecting NCAM or GFAP staining.

Immunocytochemical Assay of PSA Surface Expression

To assess the relationship between NMDA receptor activity and PSA surface expression, neurohypophysial explants were maintained for 52 h in serum-free medium complemented with PDGF (10 ng/ml) and NT3 (10 ng/ml). Then the explants were removed, and the experiments were performed on the remaining cell population. Cells were treated with Endo N (0.5 U/ml) at 37°C for 1 h to remove preexisting PSA from the cell surface, as described (Wang et al., 1994). After treatment, cultures were rinsed in serum-free medium containing 0.5% BSA and divided into three groups: (*a*) fixed immediately after the enzymatic treatment; (*b*) allowed to recover either in serum-free medium with PDGF/NT3 or serum-complemented medium for 16 h; (*c*) as (*b*) with addition of d-AP5 (100 μM) to the medium. An additional group (*d*) of cultures was maintained in serum-free medium in the presence of PDGF/NT3 for 2 d, and then incubated in the presence or absence of d-AP5 (100 μM) for 5 d. Subsequently, all preparations were double labeled with anti-PSA/GFAP and anti-PSA/NCAM antibodies and examined with either an Axiophot microscope (Zeiss) equipped with an epifluorescence device or a confocal laser scanning microscope system (MCR 600; BioRad Microscience Ltd., Hertfordshire, UK) attached to an Axiophot microscope. The laser source was a krypton/argon mixed gas laser that emits three strong beams of light in exact coalignment at wavelengths of 488, 568, and 647 nm (model 5425; Ion Laser Technologies, Salt Lake City, UT). To avoid any interference in fluorescence emission, FITC and TRITC images of double-labeled cells were taken consecutively.

Quantitation of PSA surface fluorescence was performed using confocal microscopy and the Comos software package, version 4.62 (BioRad Microscience Ltd.), as described (Kiss et al., 1994). Briefly, cells double labeled for PSA and NCAM or PSA and GFAP were randomly sampled on the basis of NCAM or GFAP staining (TRITC) using a ×63 oil immersion Plan-l objective. Images of PSA (FITC) of each selected cell were collected (5–10 cells for each culture). The intensity of fluorescent excitation of cells, gain, and black levels were kept constant for each session of measurement. Fluorescent intensity was measured by random sampling of the cell surface using a frame of 12 × 8 pixels (2.28 × 1.52 μm). Five independent measures of peak intensities were recorded and averaged for each cell. The pixel intensity threshold was adjusted so that the background corresponded to level O (maximum in arbitrary units was 255). The peak intensities measured over individual O-2A–like cells were averaged for each culture, and then normalized to those obtained from group 1 cultures (fixed immediately after the treatment of Endo N) that had been processed in the same experiment and measured in the same session.

Bromodeoxyuridine Incorporation In Vitro

BrdU (Sigma Chemical Co.) was added to cultures at a final concentration of 10 μM. Explants were fixed in 70% ethanol at 41°C for 2 h, treated with 1 N HCl for 15 min to denature the DNA, and double labeled with anti-BrdU mAb and anti-GFAP polyclonal Ab, as described above.

In Situ Hybridization

Oligonucleotide probes complementary to the mRNAs encoding glutamate receptor subunits were selected on the basis of the cloned cDNA sequences and synthesized on a DNA synthesizer (394; Applied Biosystems, Foster City, CA). The NR1 probe is complementary to a sequence encoding amino acid (aa) residues 566–580 of the mature NR1 polypeptide, NR2A (aa 567–579), and NR2B (aa 557–572). They were 3'-end labeled by incubation with [^{35}S]deoxy-ATP (New England Nuclear, Boston, MA) and terminal deoxynucleotidyl transferase (Pharmacia Fine Chemicals, Piscataway, NJ) to attain specific activities of ~5–8 × 10^8 cpm/μg. The specificity of the probes has been previously described (Monyer et al., 1992). Explant cultures of the neurohypophyses maintained in serum-free medium in the presence of PDGF and NT3 for 3 d were rinsed in PBS, fixed in 4% paraformaldehyde, and processed for in situ hybridization as described previously (Bartanusz et al., 1993). After an overnight hybridization at 41°C, cultures were washed successively in 4 ×, 1 ×, and 0.1 × SSC, quickly dehydrated in ethanol (70%), and air dried. Autoradiography was achieved using NTB3 emulsion (Eastman Kodak Co., Rochester, NY), and slides were exposed 4–5 wk at 4°C. Analysis of in situ hybridization autoradiographs was accomplished on hematoxylin-eosin–counterstained sections. No in situ hybridization signal was detected in cultures in the presence of an excess amount (50-fold) of unlabeled probes. Furthermore, the specificity of all probes has been controlled as to their distinct cellular pattern of expression throughout the rat brain within the same experiments.

PCR Amplification of mRNA Encoding NMDA Receptor Subunits and Southern Blot Analysis

Extraction of RNA. Total RNA was isolated from neurohypophysial explants, cells in the migratory pool, and primary cultures of cortical neurons using RNAzol B (Cinna/Biotecx Laboratory, Inc., Houston, TX) containing guanidium thiocyanate and phenol, as previously described (Chomczynski and Sacchi, 1987). cDNA was synthesized using the reverse transcriptase (Promega, Madison, WI) system. The enzyme was heat denatured at 95°C for 5 min. Amplification of DNA sequences was performed essentially as described (Saiki et al., 1988). Briefly, 6 μl of cDNA template were added to a reaction mixture containing 10 mM Tris-HCl, 0.1 mM EDTA, 1 mM DTT, 100 mM KCl, 0.1% Triton X-100, 0.2 mM dNTP, 0.2 mg/ml activated DNA, 0.2 mM of each primer (sequences are given below), and 12.5 U of DNAzyme (Finnzymes OY, Espoo, Finland) in a total volume of 50 μl. Amplification conditions were: 30 cycles of 1 min at 95°C, 1.5 min at 62°C, and 1.5 min at 72°C, followed by a final extension step at 72°C for 7 min.

Primer sequences: NR1-PCR1: 5'CATGGCGAAACCAGCCCACACCAT (corresponds to aa 634–641); NR1-PCR2: 5'GGAGTGGAACGGAATGATGGGCGAG (corresponds to aa 495–504); NR2A-PCR1: 5'CAATCTGACTGGATCACAGAGC (corresponds to aa 887–893); NR2A-PCR2: 5'CTGTCCTTCCCTTGAAAGGATC (corresponds to aa 947–951); NR2B-PCR1: 5'CAATAACCCACCCTGTGAGG (corresponds to aa 948–956); NR2B-PCR2: 5'GGTGGGTTGTCACAGTCATAG (corresponds to aa 1,016–1,022); NR2C-PCR1: 5'CCATTCTGGCCCTCGGTGCCC (corresponds to aa 308–314); NR2C-PCR2: 5'TTAGATCCCCGCTGCTGAAGG (corresponds to aa 453–460); NR2D-PCR1: 5'GGTGGTGGCCAGAGGTGCCC (corresponds to aa 322–329); NR2D-PCR2: 5'GATGCAAGTGCCGCTGATGG (corresponds to aa 447–454).

Southern Blot Analysis. PCR reaction products were analyzed on a 1% agarose gel, and DNA bands were visualized by ethidium bromide staining. After electrophoresis, DNA fragments were transferred onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH) as described (Sambrook et al., 1989) and hybridized with the following oligonucleotide probes, labeled at the 5' end with T4 polynucleotide kinase (Boehringer Mannheim Biochemicals).

Hybridization was performed at 42°C, overnight in 6 × SSC, 5 × Denhardt's, 0.5% SDS and 100 μg/ml denatured salmon sperm DNA, and 2 × 10^6 cpm ^{32}P-labeled probes. After hybridization, membranes were washed for 2 h at 42°C, followed by 20-min washes at 50°, 55°, and 60°C. Autoradiography was done on x-ray films (XAR; Eastman Kodak Co.).

Sequences of the probes used for Southern blotting are as follows: NR-1: (see in situ hybridization); NR-2A: 5'GTAGCTCTTTTAGGTGAGTCC (corresponds to aa 915–921); NR-2B: 5'CCTTCAGCTGCAGGTTACC (corresponds to aa 979–976); NR-2C: 5'ACAGGACTC CATGGTCCCACCGTCCC (corresponds to aa 384–393); NR-2D: 5'CCAGTGAAGGGTTCACCAGAAAGCC (corresponds to aa 375–384).

Patch-Clamp Recordings

Membrane currents were recorded with the patch-clamp technique in whole-cell configuration with an axopatch 200A amplifier (Axon Instruments, Inc., Burlingame, CA). Patch electrodes were pulled from thick-walled borosilicate glass (Clark Electromedical Instruments, Pangbourn, UK) and had an open resistance of 4–7 MΩ. The access resistance after breaking the patch was below 10 MΩ, otherwise the cell was rejected. Series resistance compensation was always used and set to 85%. The cells were continuously perfused, permitting application of drugs. Current and voltage traces were sampled at 50 Hz or 1 KHz by an interface (SICMU) connected to an IBM computer and stored on its hard disk. All recordings were performed at 22°C.

The external medium had the following composition (mM): 154 NaCl, 5 KCl, 2 $CaCl_2$, 0.1 $MgCl_2$, 5 glucose, and buffered with 10 Hepes. pH adjustment to 7.4 was obtained with NaOH. Pipettes were filled with a solution containing (mM): 120 KCl, 33 KOH, 2 $MgCl_2$, 0.1 $CaCl_2$, 2 MgATP, and 11 EGTA. In the Cs^+-containing solution, KCl and KOH were substituted with CsCl and CsOH.

Intracellular Free Calcium Measurements

Cultures were loaded with fura-2 acetoxymethyl ester (1 μM final concentration) for 30 min at 37°C in culture medium. Ratiometric measurement was performed in a new spectrofluorimeter Specsys® developed at the Geneva University Medical Centre (Geneva, Switzerland). The excitation source is provided by a xenon lamp coupled to quartz light guides and pairs of synchronous spinning wheels that allow the excitation and emission wavelengths to be changed. For fura-2 assessment, 340/510-nm and 380/510-nm pairs were selected. The coverslips with cultures of neural lobe explant were sealed with silicon grease over the central hole of a petri dish that served as a superfusion chamber installed on an inverted microscope (F-100; Nikon Inc., Garden City, NY). Cells were observed and monitored in the epifluorescence mode. The emitted light was cut off at ~400 nm and directed into a high sensitivity photomultiplier tube kept refrigerated at 9°C. UV intensity of the excitation light was adjusted by a specific diaphragm to prevent unnecessary cell irradiation. Signals from the photomultiplier were digitized and computer processed. The preparations were continuously superfused (1 ml/min) at 37°C with the following modified Krebs-Ringer bicarbonate salt solution buffered with Hepes and gassed with air (mM): 158 NaCl, 5.6 KCl, 2.5 $CaCl_2$, and 5 $NaHCO_3$; when not indicated, $MgCl_2$ was 0.5; Hepes, 10.0; glucose, 10.0; pH 7.4. Stimulating solutions were introduced by side-infusion through a micropipette (internal diam ~50 μm) placed in the vicinity of the cell under study, as already described (Pralong et al., 1990).

Results

Neurohypophysial O-2A cells are bipotential precursors that can give rise to either oligodendrocytes or type 2 astrocytes, depending on the factors present in their environment (Wang et al., 1994). If explant cultures were maintained in serum-free medium complemented with NT3/PDGF (10 ng/ml), the differentiation of O-2A precursors was prevented (Barres et al., 1994). This allowed us to obtain a large number of migrating and proliferating O-2A cells around the explant (Fig. 1, *A* and *B*, and Fig. 2 *A*). These cells were immunostained with antibodies against established markers of oligodendrocyte precursors such as A2B5 (Fig. 1 *A*), GD3 (Fig. 1 *B*), O4 (Fig. 2 *G*), and PSA-NCAM (see Fig. 6). The percentage of A2B5/GD3/O4-positive cells varied from experiment to experiment, ranging from 80–95% of the total migrating population. In these preparations, MAP2- or neurofilament- (neuron-specific markers) positive cells could not be detected (data not shown), which is consistent with the notion that there are no neurons in the neurohypophysis in vivo (Hatton, 1990). When the precursor cells were allowed to differentiate in serum-free conditioned medium (without PDGF/NT3), GalC+ oligodendrocytes became detectable (Fig. 1, *C* and *D*). In contrast, in serum-containing medium, type 2 astrocytes but no oligodendrocytes were discernible in the migratory pool (Fig. 1, *E* and *F*; see Fig. 8). We have previously demonstrated that under this condition, virtually all GFAP + astrocytes display A2B5 immunoreactivity, and thus belong to the O-2A lineage (Wang et al., 1994). In all three conditions, the migrating cells were the O-2A progenitors; type 2 astrocytes and oligodendrocytes were not motile (Small et al., 1987).

Presence of NMDA Receptor Subunit RNA and Protein Immunoreactivity in Cells of the O-2A Lineage

The NMDA receptor is a pentameric transmembrane protein that is composed of different subunits (Moriyoshi et al., 1991; Seeburg et al., 1995). Five known receptor subunit genes have been identified and sequenced (Moriyoshi et al., 1991; Seeburg et al., 1995). The NR1 subunit is essential for function, while members of the NR2 subunit class, produced by four different genes (NR2A–NR2D), can be considered modulatory (Moriyoshi et al., 1991; Seeburg et al., 1995). We first assayed for the presence of NR1 RNA transcripts and immunoreactivity in neurohypophysial O-2A cells using in situ hybridization analysis, immunocytochemistry, and analysis of the NR1 transcripts by reverse transcriptase followed by sequence amplification (RT-PCR). The last approach was also used to assay for the presence of NR2A–D RNA transcripts. Neurohypophysial cultures were kept in serum-free medium complemented with NT3/PDGF (10 ng/ml) for 3 d. To delineate the cellular origin of NR1 subunit mRNA, in situ hybridization was performed using ^{35}S-labeled NR1-specific oligonucleotide probes (Fig. 2, *B* and *C*). An accumulation of silver grains was observed over most cells with thin processes and a bipolar or multipolar morphology (Fig. 2 *B*). There was no signal on large epitheloid cells, presumably fibroblasts (Fig. 2 *B*), or on pure fibroblast cultures that were processed concomitantly with neurohypophysial cultures (not shown). Control hybridization with excess amount (50-fold) of unlabeled probe gave no signal (Fig. 2 *C*). These results strongly suggested that the NR1 subunit of the NMDA receptor was present in O-2A cells. Additional support for this conclusion was provided by the results of immunocytochemical analysis using a polyclonal NR1-specific Ab. NR1 immunoreactivity was localized specifically on process-bearing A2B5+ (Fig. 2, *D–F*) or O4+ (Fig. 2 *G* and *I*) cells. Some of these cells were negative for NR1. We have never observed NR1 immunostaining in A2B5-/$O4^-$ cells (e.g., in fibroblasts) (Fig. 2, *D–I*). Thus, NR1 subunit expression in these cultures appears to be exclusive to cells of the O-2A lineage.

The presence of NR1-specific mRNA was further assessed by PCR amplification using NR1-specific primers. After 6 d in vitro, explants and the cellular outgrowths were harvested separately (see Materials and Methods). cDNA was then synthesized from total RNA. Results shown in Fig. 3 *I* demonstrate the presence in both explants and migrating cells of an amplified DNA fragment with a size corresponding to that predicted from the NR1 mRNA sequence (Fig. 3 *I*, *A*). Further analysis of the fragments by Southern blotting using a labeled NR1-specific probe (Fig. 3 *I*, *B*) and by restriction enzyme digestion (not shown)

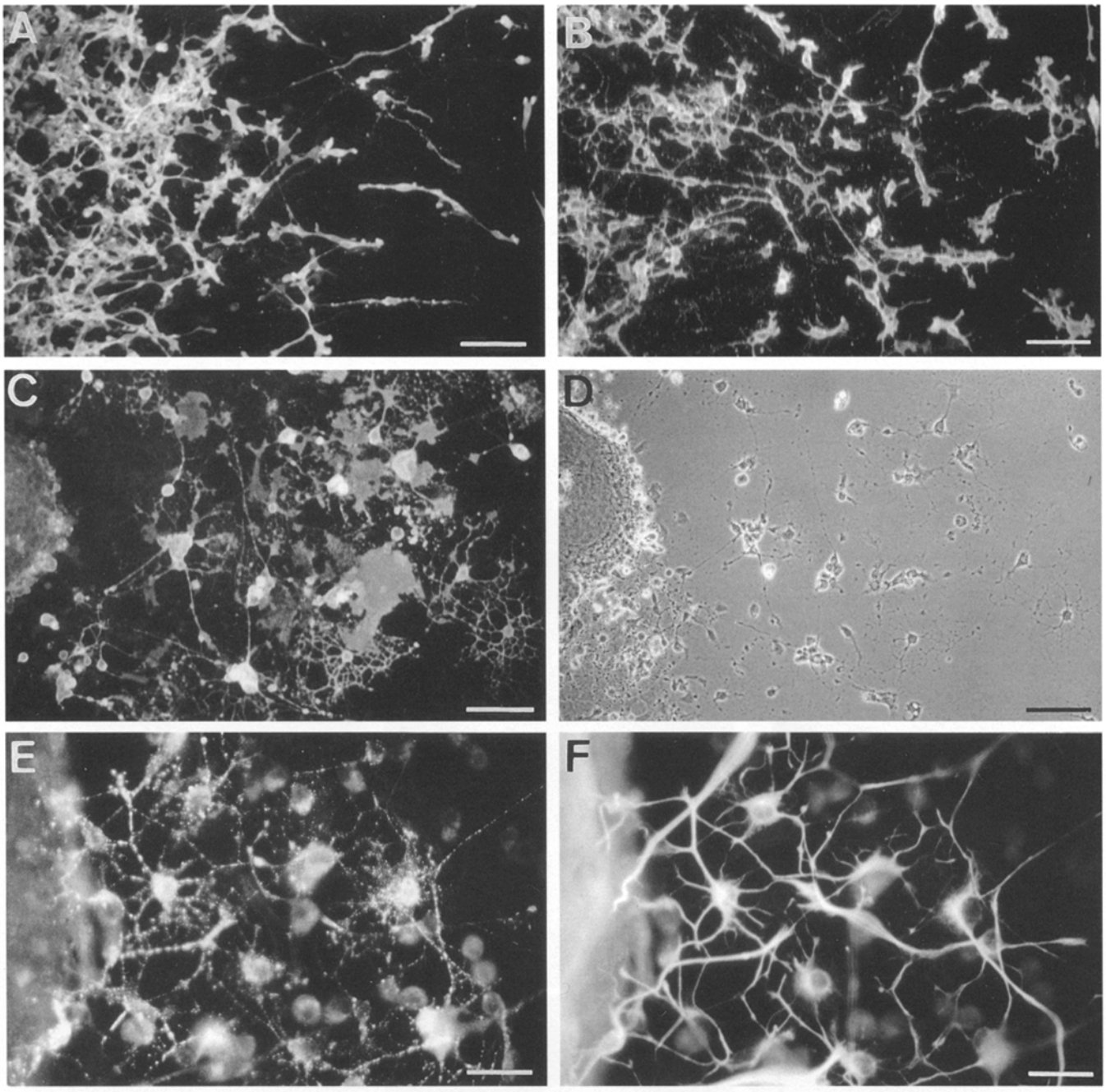
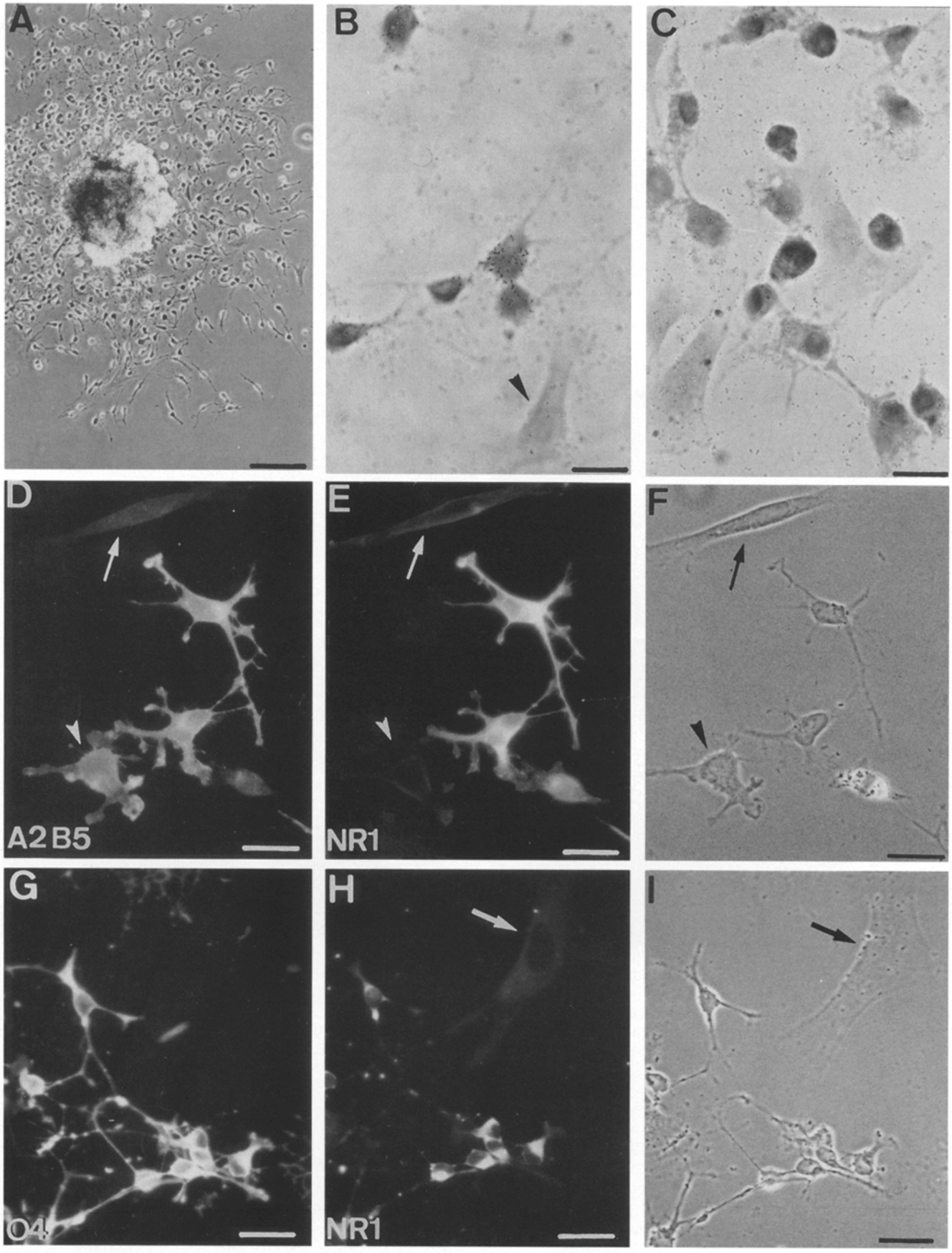


Figure 1. O-2A lineage cells in the cellular outgrowth of neurohypophysial explants. (*A* and *B*) A2B5 (*A*) and GD3 (*B*) immunostained O-2A cells generated in serum-free medium complemented with PDGF/NT3 (10 ng/ml). These cells also exhibit O4 (see Fig. 2 *G*) and PSA-NCAM (see Fig. 6) immunoreactivity, but not that of GFAP and the neuronal marker MAP2. (*C* and *D*) GalC-positive oligodendrocytes after 3 d in serum-free conditioned medium without PDGF/NT3. (*E* and *F*) A2B5/GFAP immunoreactive type 2 astrocytes after 3 d in serum-complemented medium. Bars: (*A–D*) 75 μm; (*E* and *F*) 30 μm.

confirmed that the amplified DNA fragments were identical to those obtained from cortical neurons (Fig. 3 *II*). From these results, we infer that explant and migratory cells contain NR1-specific mRNA.

We also investigated whether O-2A cells were expressing mRNA encoding NR-2A and NR-2B subunits of the NR-2 class. Results of in situ hybridization (not shown) and RT-PCR amplification (Fig. 3 *II*) were negative for either mRNA in the neurohypophysis, whereas both mRNAs were easily detected in rat brain sections (not shown) or cultures of cortical neurons (Fig. 3 *II*) used as positive controls. RT-PCR amplification followed by Southern blotting was finally performed to explore for the presence of mRNAs encoding NR-2C and NR-2D subunits. Neither mRNA could be detected in explants or in migrating cells (data not shown). These results suggest that O-2A cells express


A
B
C
D
A2B5
E
NR1
F
G
O4
H
NR1
I

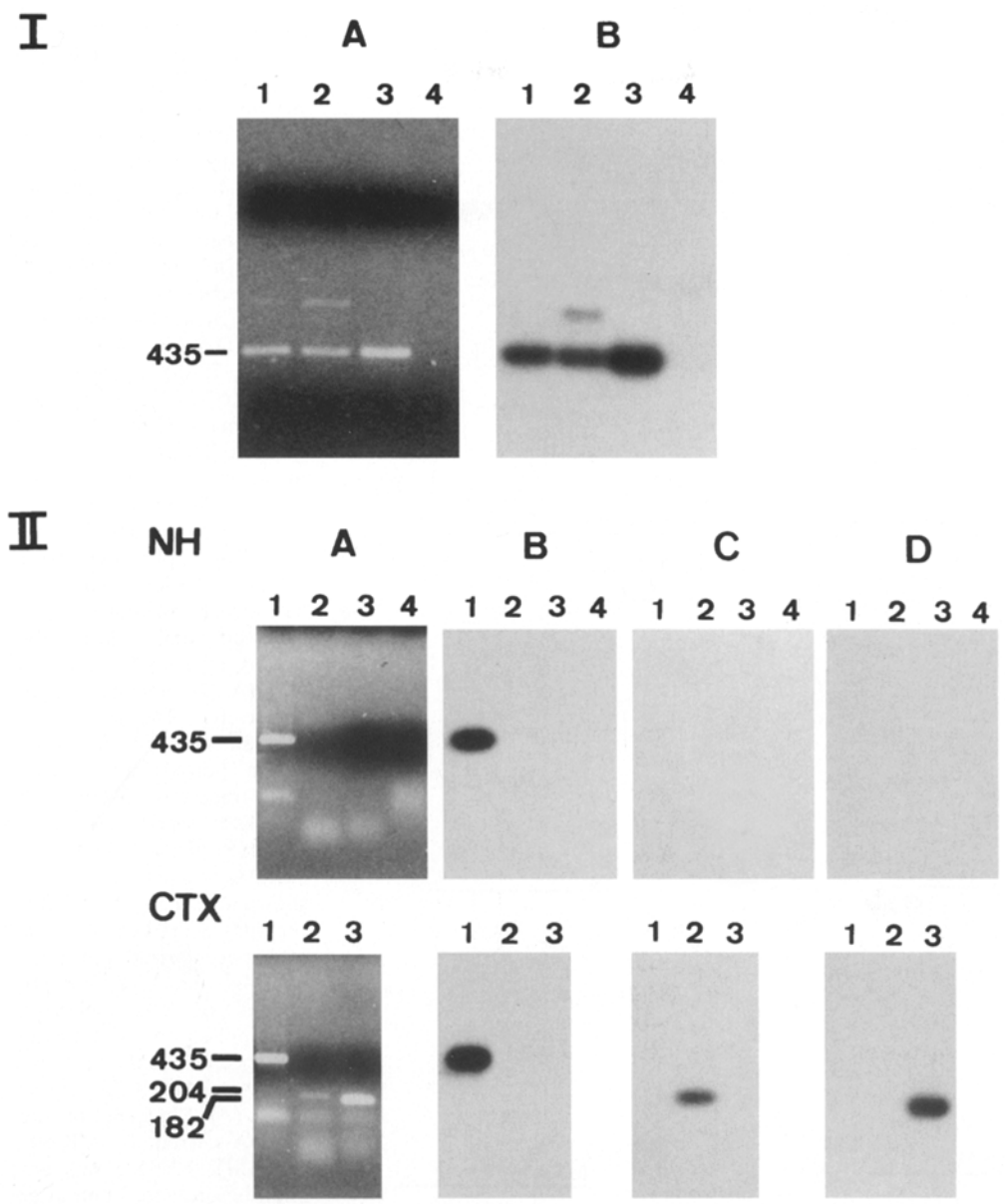


Figure 3. (*I*) Presence of mRNA encoding the NR1 subunit of the NMDA receptor in neurohypophysial explants. cDNA synthesized from total RNA isolated from explants and from migrating cells was used for the amplification by PCR of NR1 sequences, using oligonucleotide primers PCR1 and PCR2 (see Materials and Methods). Amplified fragments were then analyzed on a 1% agarose gel followed by Southern blotting. (*A*) Ethidium bromide staining of NR1-specific PCR-amplified fragments from cells in explants (lanes *1* and *3*) and from migrating cells (lane *2*). (Lane *4*) Control amplification in the absence of primers. (*B*) Results of a Southern blotting of DNA amplified with NR1-specific oligonucleotide primers. DNA fragments were transferred onto nitrocellulose filters and hybridized with ^{32}P-labeled NR1-specific oligonucleotides (see Materials and Methods). (Lanes *1–4*) Same as in *A*. (*II*) Analysis of the presence of NR1, NR2A, and NR2B subunits of NMDA receptors in NH explant cultures using PCR amplification. NR1-, NR2A-, and NR2B-specific oligonucleotide primers (see Materials and Methods) were used in PCR amplifications on cDNA from explant cultures of NH and cortex (*CTX*; positive control). (Lanes *1–4*) PCR amplifications using NR1, NR2A, NR2B primers, and no cDNA, respectively. (*A*) Ethidium bromide staining of PCR amplified fragments. (*B–D*) Results of Southern blots using ^{32}P-labeled oligonucleotide probes specific for NR1, NR2A, and NR2B, respectively.

mRNA corresponding to the NR1 subunit but none of the other subunits (NR2A–D) known to be present in neurons.

Patch-Clamp Recordings

To demonstrate that NMDA receptors in O-2A cells are functional, we have performed a series of whole-cell patch-clamp recording experiments on cultures maintained in serum-free medium with or without PDGF/NT3. The cells included in the study were small unipolar or bipolar cells with thin processes. They were close (within 20 μm) to the explant, and some still attached to it with a process. The mean cell capacitance determined by proper cancellation of the capacitive transient artifacts was in the range of 2.5–

Figure 2. Localization in O-2A cells of mRNA and protein immunoreactivity for the NR1 subunit. (*A*) Overview of an explant culture maintained in serum-free medium supplemented with PDGF and NT3 (10 ng/ml). Under these conditions, the percentage of bipolar or multipolar A2B5/O4-positive O-2A–like cells varies from experiment to experiment, ranging from 80–95% of the total migrating cell population. (*B*) In situ hybridization labeling associated with bipolar O-2A–like cells. Notice that there is no specific labeling over epitheloid fibroblast-like cells (*arrow*). (*C*) No specific hybridization signal is seen in the presence of a 50-fold excess of unlabeled oligonucleotide probe. (*D–I*) NR1 immunostaining (*E* and *H*) in A2B5-positive (*D*) and O4-positive (*G*) O-2A cells. Antibody binding was visualized with an FITC-labeled secondary antibody. The A2B5-positive cell (*arrowhead*) and fibroblast-like cells (*arrows*) are negative. Bars: (*A*) 100 μm; (*B* and *C*) 10 μm; (*D–F*) 20 μm; (*G–I*) 30 μm.

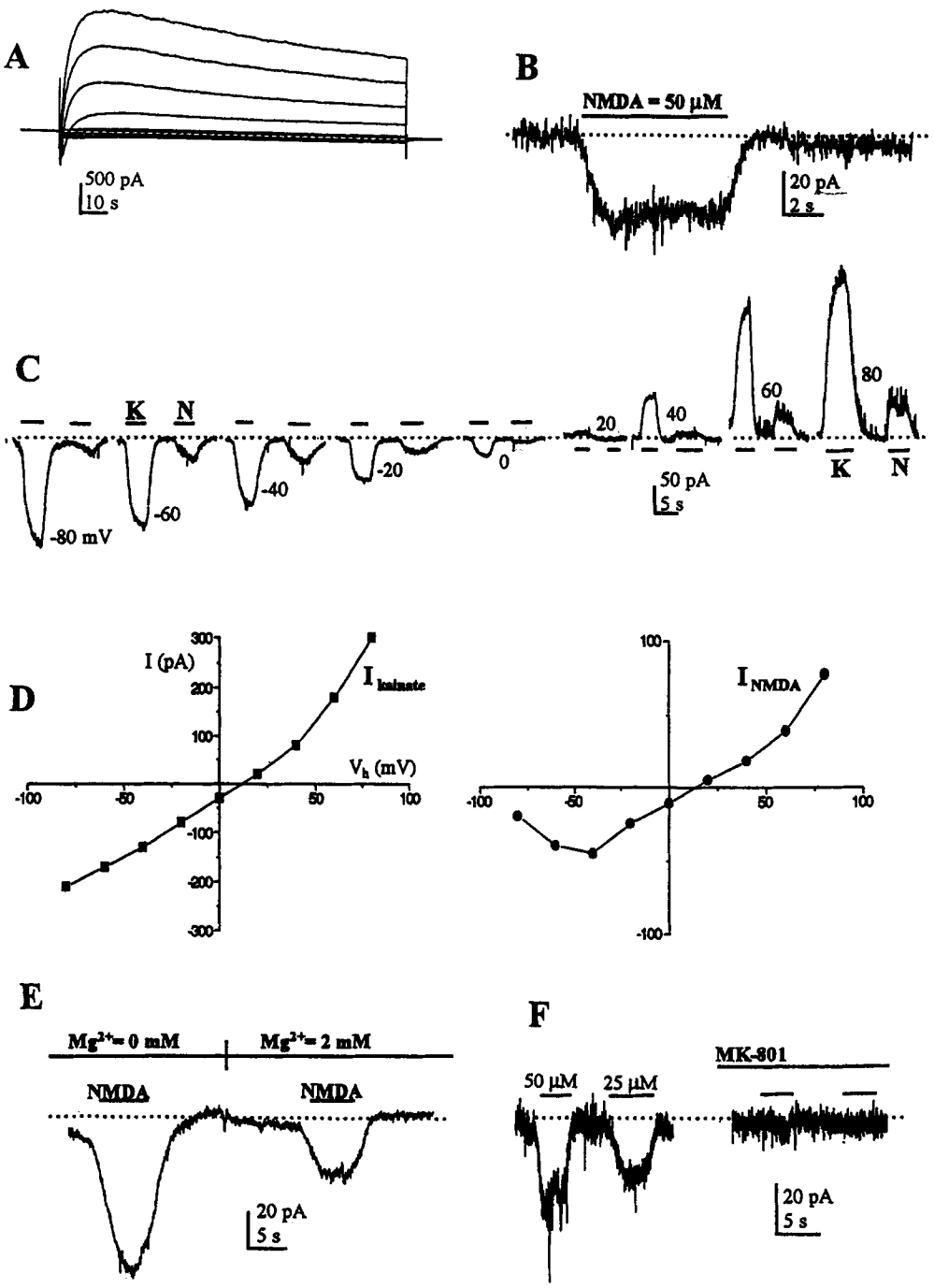


Figure 4. NMDA current in the glial precursor cells. (*A*) Typical pattern of voltage-activated currents in a glial precursor cell. Starting from a holding potential of −80 mV (V_h), the membrane was stepped for 100 ms to potentials ranging from −120 mV to +40 mV by 20-mV increment pulses. (*B*) Current activated by a 20-s application of NMDA (*50 μM*) from a V_h of −70 mV (same cell as in *A*). (*C*) Currents activated by kainate (100 μM) and NMDA (50 μM) application at various V_h recorded with a Cs^+-containing pipette. V_h was changed at least 30 s before drug application and is indicated beside each trace. The two horizontal bars for each potential indicate the period of kainate (*K*) and NMDA (*N*) application, respectively. The trace was split, and only the drug application periods are shown. Each trace was offset on the base line (*dotted line*). (*D*) I/V curves of the kainate and NMDA current (n = 4) shown in *C*. The NMDA current has a marked decrease at hyperpolarized potential. (*E*) Mg^{2+} block at hyperpolarized potential. NMDA was applied on a cell in the presence (*2 mM*) and absence of Mg^{2+} (V_h = −80 mV). (*F*) Blockade of the NMDA current by MK-801 (10 μM). NMDA was applied before and 3 min after MK-801 application. The horizontal bars on the top of the trace indicate the applications of NMDA with the corresponding concentration. MK-801 resulted in a complete block of the NMDA current.

10 pFa (7.7 pFa ± 1.7; n = 25), which corresponds to a sphere of ~11-μm diam. In KCl recording solution, these cells displayed the typical pattern of voltage-activated currents of the O-2A glial precursors (Fig. 4 *A*) (Barres et al., 1990; Blankenfeld et al., 1992). The principal current was an outwardly rectifying K^+ current, both of the non-deactivating and the deactivating type. A TTX-sensitive Na^+ current was recorded in about half of the cells, and about a third expressed a small highly Ba^{2+}-sensitive inward rectifier current. To assay for NMDA receptors, the ligand NMDA (50 μM) in the presence of 100 μM external Mg^{2+} was added to the external solution. On all 14 cells tested, NMDA activated an inward current (Fig. 4 *B*) at V_h (−70 mV) in the range of 20–100 pA (50.5 pA ± 13) that slowly inactivated upon prolonged application. To avoid desensitization, repetitive applications were done at intervals of at least 30 s. Responses could be obtained with 5 μM NMDA and maximized around 100 μM (data not shown). In Fig. 4 *C*, we present a typical recording where both kainate and NMDA were applied to the same cell that was recorded with a Cs^+-containing pipette solution. Fig. 4 *D* demonstrates that the I/V curve obtained from the cell shown in Fig. 4 *C* displayed the typical block in the hyperpolarized potentials (Mayer and Westbrook, 1987). Some degree

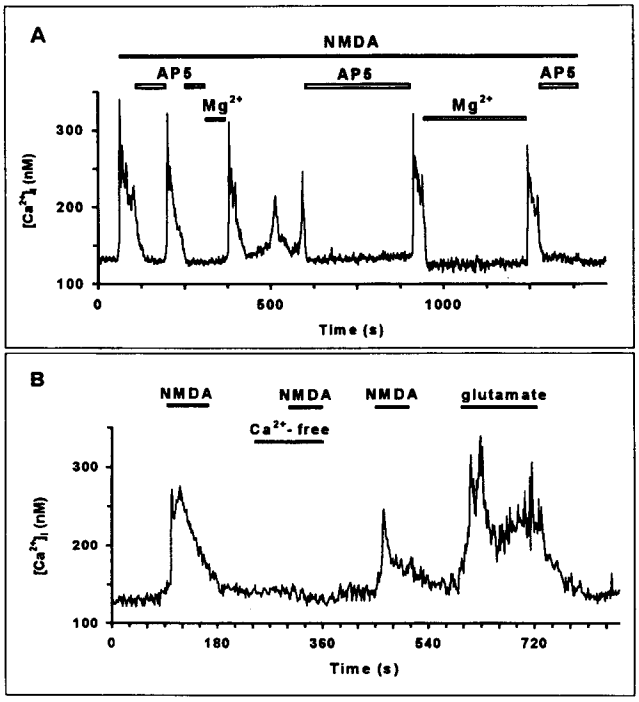


Figure 5. Changes in $[Ca^{2+}]_i$ in fura-2–loaded O-2A cells evoked by NMDA stimulation: effects of the receptor antagonist d-AP5, extracellular Mg^{2+} addition, or extracellular Ca^{2+} omission. (*A*) Inhibition of NMDA-induced (50 μM) $[Ca^{2+}]_i$ rise by d-AP5 (*AP5*; 100 μM) and high extracellular Mg^{2+} (3.5 mM) tested sequentially on the same cell. (*B*) Effects of extracellular Ca^{2+} omission (Ca^{2+}-free, nominally free + 100 μM EGTA) on NMDA-evoked Ca^{2+} rise and comparison with the receptor agonist glutamate (25 μM). When not indicated, NMDA is applied simultaneously with nominally free Mg^{2+} + 100 μM glycine. We noticed that prolonged preperifusions with glycine tended to desensitize the NMDA-induced responses. The traces shown in *A* and *B* are representative of at least five independent experiments.

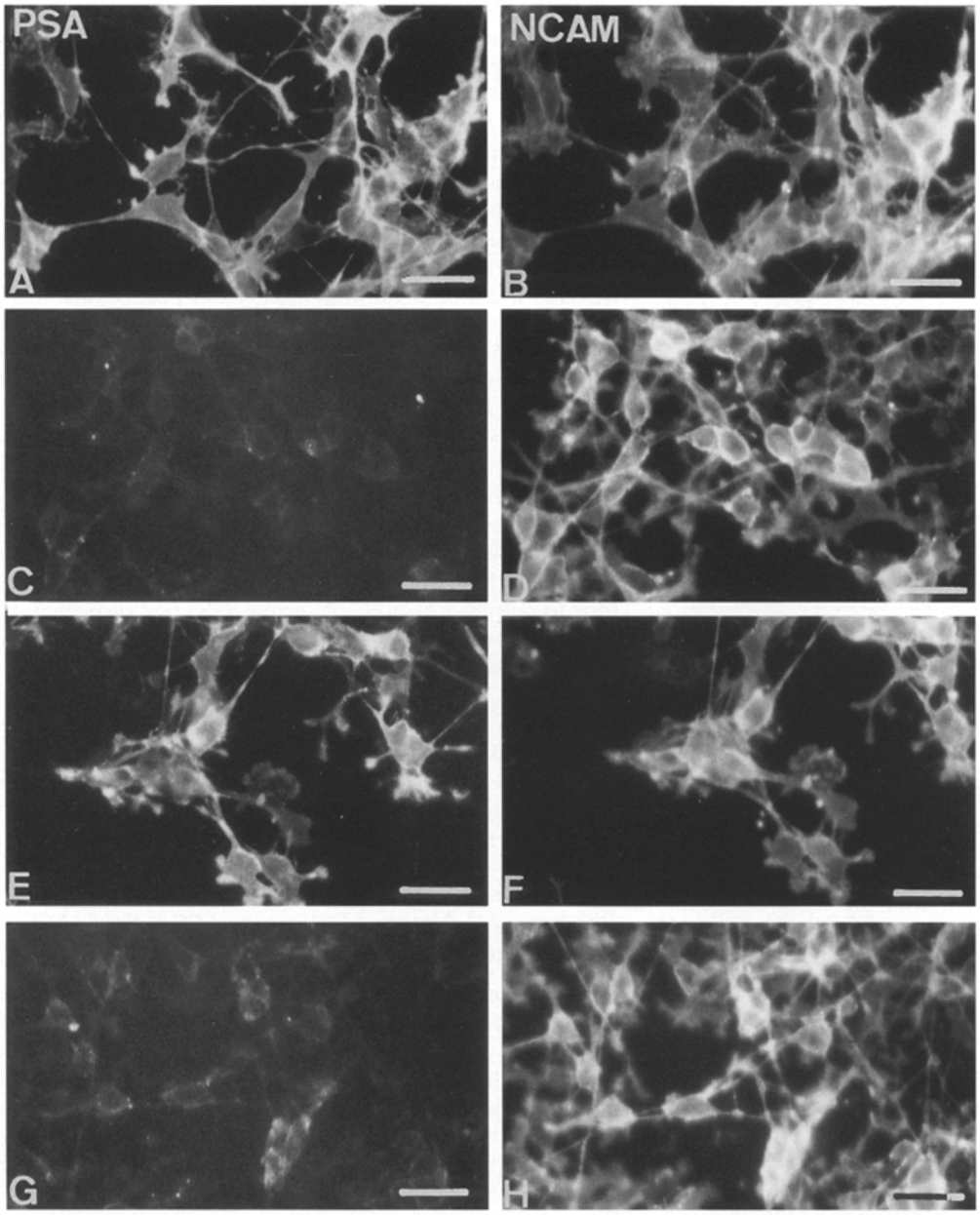
of rectification in the hyperpolarized range was always present, although it was highly variable. The current amplitude was systematically smaller at −80 mV than at −40 mV, and the mean ratio of I(−80)/I(−40) was 0.76 ± 0.2 (mean ± SD, n = 5). This block could, however, be relieved by omitting Mg^{2+} in the superfusate (see Fig. 4 *E*). The current in the absence of Mg^{2+} was increased by a factor of 2.3 ± 0.8 (n = 4) as compared with that recorded in the 2 mM Mg^{2+} solution. Fig. 4 *F* shows that the NMDA current was completely abolished by MK-801. These results, i.e., the range of the activating concentrations, the block by MK801, and the Mg^{2+}-sensitive rectification of the I/V curve in the hyperpolarized potentials, show conclusively that the application of the ligand NMDA on O-2A precursors activates a current that has similarities with the neuronal NMDA current.

Intracellular Calcium Measurements

Since NMDA receptors are known to be permeable to Ca^{2+}, we tested whether changes in free intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) could be induced in fura-2–loaded O-2A–like cells by NMDA (Fig. 5). Explants used for this study were maintained in serum-free medium with or without PDGF/NT3. NMDA application on a typical bipolar migrating cell, together with glycine (100 μM) in a Mg^{2+}-free medium, caused an immediate elevation of intracellular free Ca^{2+} (Fig. 5 *A*). The specificity of the response was demonstrated by the application of the NMDA receptor antagonist d-AP5 (100 μM) that obliterated reversibly and completely the $[Ca^{2+}]_i$ rise caused by the NMDA stimulation. Perifusion with high Mg^{2+} had similar effects as demonstrated on the same cell. To test whether NMDA did not cause primarily an elevation of $[Ca^{2+}]_i$ through the mobilization of Ca^{2+} from intracellular Ca^{2+} stores, NMDA was applied in the absence of extracellular Ca^{2+} (Fig. 5 *B*). Under such conditions, no NMDA-evoked $[Ca^{2+}]_i$ rise was observed. Readdition of extracellular Ca^{2+} resulted in the restoration of the NMDA-induced $[Ca^{2+}]_i$ elevation. As a control, 25 μM glutamate, a concentration known to stimulate NMDA receptors preferentially, was tested on the same cell. This maneuver caused a similar Ca^{2+} increase as observed previously with NMDA (Fig. 5 *B*). It is noteworthy that the proportion of cells demonstrating NMDA-evoked $[Ca^{2+}]_i$ responses varied from culture to culture (20–50% of tested cells) and displayed different response patterns. The NMDA-evoked response was in general of smaller amplitude (Δ $[Ca^{2+}]_i$ <250 nM) than that evoked in cortical neurons in primary culture tested under the same conditions (Δ $[Ca^{2+}]_i$ ~400 nM; not shown). Taken together, these observations are consistent with the view that activation of functional NMDA receptors on migrating O-2A–like cells generates small $[Ca^{2+}]_i$ elevations that arise primarily from Ca^{2+} influx.

The Pharmacological Blockade of NMDA Receptors Inhibits the Expression of PSA-NCAM in O-2A Lineage Cells

We then tested whether NMDA receptor activity was linked to PSA-NCAM expression in O-2A cells. We used a previously described experimental strategy to explore this possibility (Kiss et al., 1994). O-2A cells were allowed to migrate in the presence of NT3/PDGF for 52 h; the explant was then carefully removed to prevent further outgrowth, and the remaining population of cells was treated with Endo N to remove PSA from their surface. Cultures were allowed to recover in either serum-complemented or serum-free medium (with NT3/PDGF) in the presence or absence of d-AP5 (100 μM), and then were fixed and double immunostained for PSA/GFAP or PSA/NCAM. Fig. 6, *A* and *B*, illustrates the localization of PSA and NCAM immunostaining in O-2A cells. Endo N treatment completely removed PSA immunoreactivity from the cell surface (Fig. 6, *C* and *D*). An initial series of experiments allowed us to establish that PSA staining recovered significantly 16 h after removal of the enzyme (Fig. 6, *E* and *F*). Hence, we used this time point for the quantitative analysis of PSA surface staining. Fig. 6, *G–I*, shows that the pharmacological blockade of NMDA receptors by d-AP5 inhibited PSA recovery on the surface of O-2A cells by ~90% compared with controls. This effect was identical in the presence or absence of serum. PSA recovery was not influenced by depolarizing conditions (in the presence of 50 mM K^+). Thus, the pharmacological blockade of NMDA receptors prevents the reexpression of PSA on the O-2A cell surface after Endo N treatment.



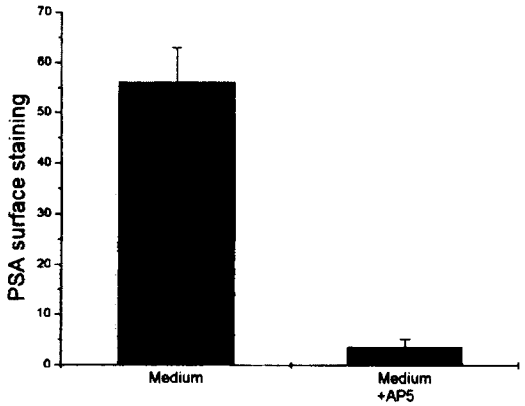


Figure 6. Recovery of PSA immunoreactivity on the surface of neurohypophysial O-2A cells after Endo N treatment as evaluated by quantitative confocal microscopy. Cultures maintained in serum-free medium with PDGF/NT3 for 52 h were treated with Endo N for 1 h, rinsed, allowed to recover in the absence or presence of 100 μM d-AP5 for 16 h, and then double immunostained for PSA and NCAM. (*A* and *B*) PSA-NCAM immunostaining in a control culture. (*C* and *D*) Cells fixed and double immunostained immediately after Endo N treatment. Notice that PSA immunoreactivity has virtually disappeared, whereas NCAM immunostaining is not affected by the treatment. (*E* and *F*) Endo N–treated cells were allowed to recover for 16 h in serum-free medium complemented with PDGF/NT3. Cells display an intense PSA staining. (*G* and *H*) This recovery is almost completely blocked with d-AP5 treatment. (*E*) PSA surface staining was evaluated by quantitative confocal microscopy as described in Materials and Methods. The results show relative values in arbitrary units (means ± SEM of $n = 3$ independent experiments from 110 cells), normalized to that obtained at the end of Endo N treatment (group 1). Bars, 30 μm.

Based on these observations, one could expect that long-term blockade of NMDA receptors would also down-regulate PSA on O-2A cells. To test this hypothesis, O-2A cells were allowed to migrate in serum-free medium complemented with PDGF and NT3 for 2 d, and then further cultured in the presence or absence of 100 μM d-AP5 for 5 d. The antagonist produced a dramatic decrease in PSA staining on the surface of O-2A cells compared with controls (Fig. 7). Interestingly, NCAM immunostaining was not influenced by the treatment. Other features such as cell shape or A2B5 immunoreactivity were not influenced by d-AP5. The addition of an AMPA receptor channel antagonist, CNQX (10 μM), to the medium was without effect (not shown). These experiments indicate that the activity of the NMDA receptor is required for PSA but not for NCAM expression on O-2A cells.

Antagonists to the NMDA Type of Glutamate Receptors Block the Migration of O-2A Lineage Cells from Neurohypophysial Explants

The migration of O-2A–like cells from neurohypophysial explants critically depends on the presence of PSA-NCAM (Wang et al., 1994). We thus tested the role of NMDA receptor activation in the regulation of this process. Cell migration was assayed under two conditions: in serum-containing medium and in serum-free medium without growth factors. O-2A cells were allowed to migrate for 3 d and to differentiate into type 2 astrocytes or GalC+ oligodendrocytes either in the presence or absence of antagonists to NMDA or non-NMDA receptors, and then were fixed and processed for immunocytochemistry. O-2A lineage cells have been identified on the basis of their double immunostaining with anti-A2B5 and -GFAP Abs (Raff, 1989) (serum-containing medium) or with anti-GalC Ab (serum-free medium), and migration was assessed by counting cells in the zone of the cellular outgrowth. In the presence of serum without antagonists, ~50–60 GFAP+/A2B5+ cells were detected around each explant (Fig. 8); this number is significantly lower than that in serum-free medium with PDGF/NT3. Migration was dramatically inhibited by blocking NMDA receptors either with d-AP5 (100 μM), a competitive antagonist, or by the noncompetitive antagonist, MK-801 (1 μM) (Fig. 8 *E*). These effects were dose dependent (Fig. 8 *F*). Inclusion to the culture medium of 20 μM CNQX caused a significantly weaker inhibition (Fig. 8 *E*). Negligeable effects were observed after the application of the Na^+ channel antagonist TTX (1 μM), the antagonist to the L-type voltage-sensitive channels nifedipin (10 μM), and cyclopiazonic acid (10 μM) that depletes IP_3-sensitive Ca^{2+} stores (Fig. 8 *E*). Importantly, the presence of NMDA receptor antagonists did not influence the migration of GFAP-negative, fibroblast-like cells (Fig. 8 *D*), which is consistent with the lack of NR1 immunoreactivity and mRNA in these cells (Fig. 2). Identical results were obtained in the absence of serum. Under control conditions (without NMDA antagonist), an average of 24 ± 6 GalC+ cells per explant were counted in the cellular outgrowth. In the presence of d-AP5 (100 μM), no GalC+ oligodendrocytes could be detected in the outgrowth. This effect was reproducible in three independent experiments that involved the analysis of 19 individual cultures.

The possible involvement of NMDA receptor in O-2A cell migration was further suggested by the effect of high concentrations of Mg^{2+}. Since extracellular Mg^{2+} blocks NMDA receptor activation in a voltage-dependent manner (Nowak et al., 1984), it is expected to influence cell migration. Indeed, explants maintained in medium containing 3.5 mM Mg^{2+}, known to effectively block NMDA receptors, resulted in a significantly smaller number of migrating cells (Fig. 8 *E*).

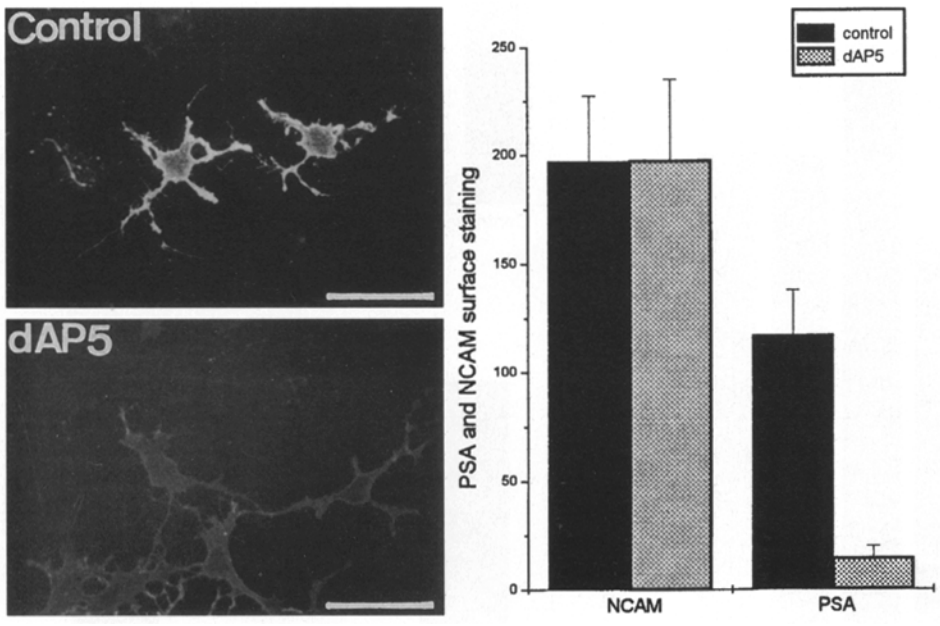
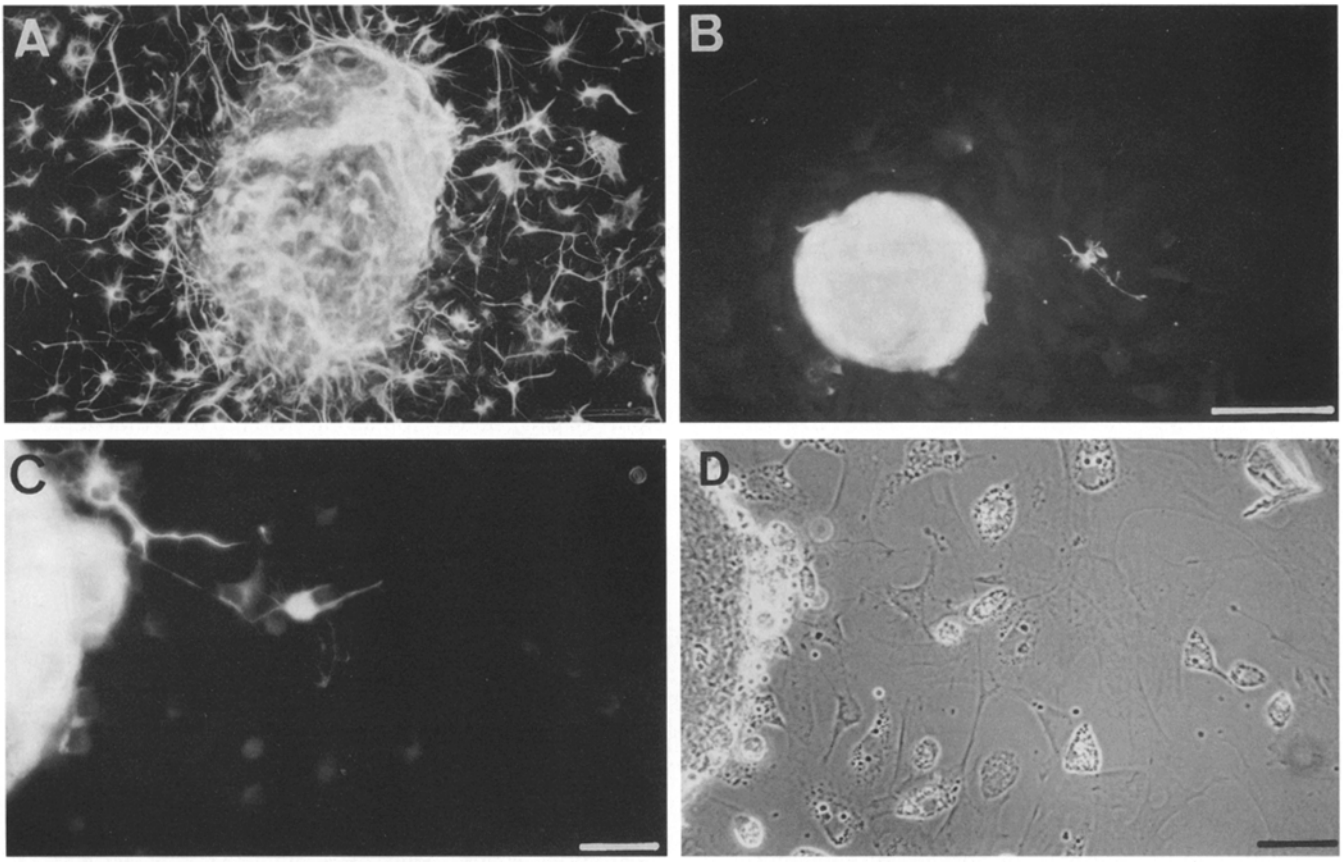
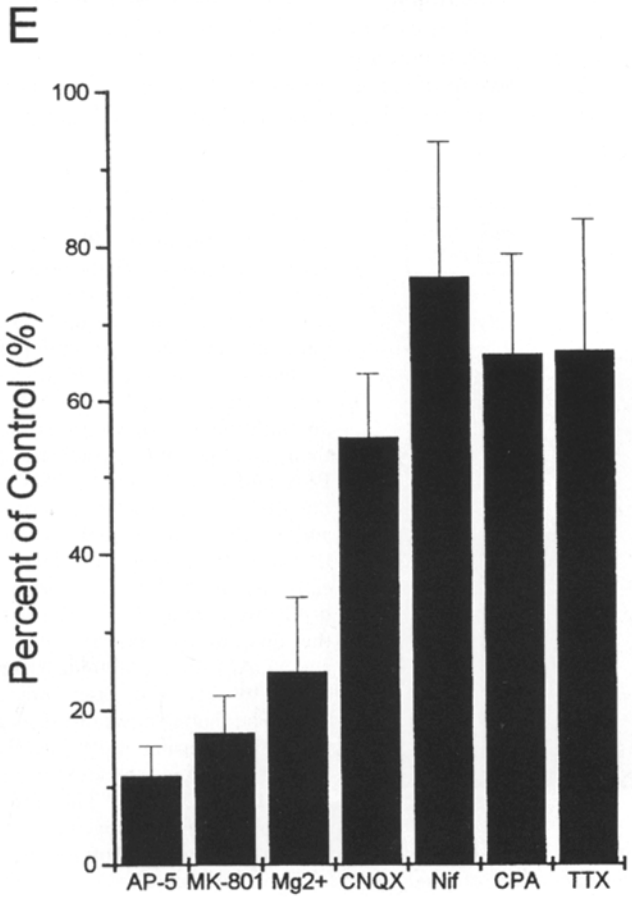
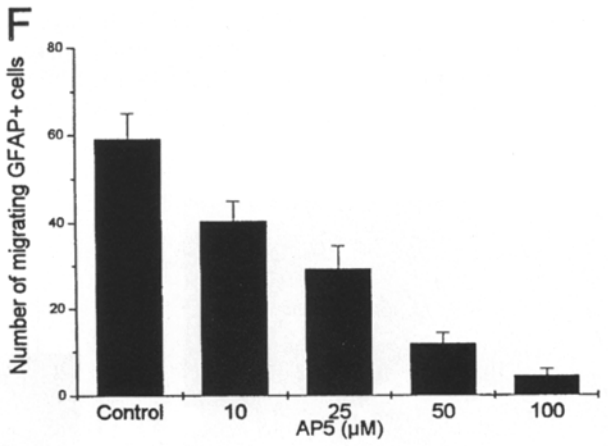
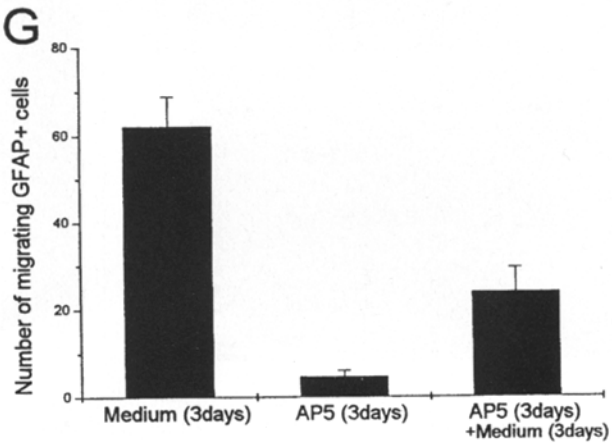


Figure 7. The effect of long-term d-AP5 treatment on PSA-NCAM surface immunoreactivity of neurohypophysial O-2A cells. O-2A cells were allowed to migrate in the presence of PDGF/NT3 (10 ng/ml) for 2 d and were maintained in the presence or absence of 100 μM d-AP5 for an additional 5 d. Cultures were then double immunostained for PSA and NCAM. (*Left*) PSA immunoreactive cells from control and d-AP5–treated cultures. Note that the PSA immunostaining of cells is markedly decreased by d-AP5 treatment. (*Right*) Results of the quantitative analysis of PSA and NCAM surface staining as evaluated by confocal laser microscopy. The figure shows the absolute values in arbitrary units (mean ± SEM of $n = 5$ independent experiments from 120 cells). The statistical comparison of average intensities of PSA and NCAM immunoreactivity from five independent experiments indicates no significant differences between homologous experimental groups (interexperimental variations are <10%); thus, absolute values obtained in different experiments are comparable. Bars, 50 μm.


A
B
C
D


E
Percent of Control (%)
100
80
60
40
20
0
AP-5
MK-801
Mg2+
CNQX
Nif
CPA
TTX


F
Number of migrating GFAP+ cells
80
60
40
20
0
Control
10
25
50
100
AP5 (μM)


G
Number of migrating GFAP+ cells
80
60
40
20
0
Medium (3days)
AP5 (3days)
AP5 (3days)
+Medium (3days)

To exclude the possibility that selective cell death is responsible for the inhibition of cell migration, we tested the reversibility of the effect of NMDA receptor antagonists. Explants were cultured in the presence of 100 μM d-AP5 for 3 d, followed by an additional 3 d without d-AP5. The number of migrating cells in these cultures increased by five times as compared with that measured at the end of the 3-d d-AP5 treatment (Fig. 8 *G*). However, this number was smaller than that measured with untreated controls, confirming our previous observations that the peak of cell migration takes place during the first 3 d in vitro (Wang et al., 1994). It appears thus that the viability of O-2A lineage cells was not significantly influenced by d-AP5. This conclusion is further supported by experiments in which O-2A cell populations were kept in the presence d-AP5 for 5 d (see below) without noticeable cell death. In another series of experiments, we tested the effect of NMDA antagonists on the differentiation of O-2A cells into GFAP+ type 2 astrocytes. O-2A cells were allowed to migrate in the presence of PDGF and NT3, and then differentiation was induced by addition of serum. The appearance of GFAP+ type 2 astrocytes was not affected by the presence of NMDA antagonists (not shown). None of the above-mentioned antagonists seemed to influence GFAP or A2B5 immunolabeling in migrating cells.

To determine whether NMDA receptor antagonists influence the proliferation rate of O-2A lineage cells, explants were maintained in the presence of PDGF/NT3 and exposed to BrdU during the whole culture period (3 d). The number of BrdU-positive cells in the outgrowth zone was not different in the presence or absence of d-AP5 (180 ± 22 and 173 ± 21 BrdU-labeled cells per explant without and with d-AP5, respectively. Hence, it is unlikely that NMDA antagonists reduce the number of migrating cells by influencing their proliferation rate. This conclusion is consistent with our previous results (Wang et al., 1994), indicating that the majority of migrating O-2A lineage cells do not incorporate BrdU in serum-containing medium.

Taken together, these results point to a critical role for NMDA receptor activation in O-2A cell migration from neurohypophysial explants.

Discussion

Our results provide direct evidence for the expression of functional NMDA receptors in O-2A lineage cells. Moreover, they strongly suggest that the activity of these receptors participate in the regulation of cell migration. This may occur via the modulation of the expression of PSA on NCAM known to play a critical role in O-2A cell motility. This constitutes an example of how interactions between ionotropic receptors, intracellular signaling, and cell adhesion molecule expression influence properties of the cell surface, which, in turn, are a critical determinant of cell migration (Rakic et al., 1994). Most importantly, these studies suggest a potential mechanism through which neuronal activity, via neuromediator (glutamate) release, may affect glial cell motility during morphogenesis.

The experiments described in this paper provide several lines of evidence for the expression of functional NMDA receptors on neurohypophysial O-2A cells. First, we have demonstrated the presence of RNA transcripts and immunoreactivity for the NR1 subunit, known to be essential for function. NR1 immunoreactivity is exclusively localized in A2B5+/O4 + cells that displayed the typical O-2A cell morphology. Similarly, the in situ hybridization signal was specific to this cell type. In contrast, A2B5/O4-negative, epitheloid cells (fibroblasts) were consistently negative. We have shown previously that all A2B5+ cells in these cultures belong to the O-2A cell lineage (Wang et al., 1994). Second, using whole-cell patch-clamp recording, we have found that application of NMDA induced an inward current that had properties similar to the current of the neuronal NMDA receptor. These include the range of activating concentrations of NMDA (5–100 μM), the block by MK-801, and the Mg^{2+}-sensitive rectification of the I/V curve in the hyperpolarized potentials. Third, the application of NMDA elicits increases in intracellular Ca^{2+}, which could be blocked by the specific antagonist d-AP5 as well as by perfusing cultures with Ca^{2+}-free solutions or high Mg^{2+}. These properties are different from those described in Bergman glia cells in which the NMDA-induced current required a high concentration of agonist (1 mM) and displayed a linear current voltage relationship, and the activation was not accompanied by detectable changes in cytosolic Ca^{2+} (Muller et al., 1993). Since NMDA receptors in O-2A lineage cells have similar properties to the neuronal type of NMDA receptors, it is important to exclude the possible contamination by neurons. It must be emphasized that there are no neurons in the neurohypophysis in vivo (Hatton, 1990), and we could not detect neurons in these cultures. We cannot formally exclude the existence of pluripotent, neuron/glia precursors in our cultures.

Figure 8. Effect of pharmacological blockade of ionotropic glutamate receptors on the migration of O-2A lineage cells. Cultures were maintained in serum-complemented medium for 3 d, fixed, and double immunostained for GFAP and A2B5 as described in Materials and Methods. (*A*) Low power photomicrograph of a control explant with GFAP-immunolabeled type 2 astrocytes in the outgrowth. These cells also display A2B5 immunoreactivity (not shown here, but see Fig. 1, *E* and *F*). (*B*) The addition of the NMDA receptor antagonist, d-AP5 (100 μM), to the culture medium almost completely blocks the migration of GFAP-positive cells. (*C* and *D*) High power photomicrographs of a d-AP5–treated culture. The presence of d-AP5 does not affect the outgrowth of non-GFAP–positive cells. (*E*) Total number of GFAP-positive cells in the migrating population under different experimental conditions: in the presence of d-AP5 (100 μM); MK-801 (1 μM); CNQX (20 μM); Mg^{2+} (3.5 mM); nifedipin (*Nif*) (10 μM); TTX (1 μM); and cyclopiazonic acid (*CPA*) (10 μM). Each value is the mean ± SEM from n = 3–5 independent experiments expressed as a percentage of the control value (100% = 51 ± 12 cells per explant). (*F*) The dose-dependent effect of NMDA receptor antagonist d-AP5 on cell migration. Values are the mean ± SEM, from n = 3 independent experiments. Bars: (*A* and *B*) 100 μm; (*C* and *D*) 25 μm. (*G*) Recovery from d-AP-5 treatment. Explants treated with d-AP5 for 3 d were allowed to recover in the absence of the antagonist for an additional 3 d. The cultures were double immunostained for GFAP and A2B5 as described in Materials and Methods. The results show the absolute number of GFAP-positive cells in the migratory population, and each value is the mean ± SEM of n = 3 independent experiments.

However, since neurons cannot be detected, we propose that, if present, these multipotent precursor cells are likely to adopt the O-2A lineage cell fate. Moreover, cells that diplayed NMDA-induced currents had a morphology and pattern of voltage-activated currents characteristic for O-2A cells (Barres et al., 1990; Blankenfeld et al., 1992). Fourth, the pharmacological blockade of NMDA receptors specifically inhibited the migration of O-2A cells.

The expression of NMDA receptors on O-2A cells is unexpected since previous studies failed to demonstrate (but could not exclude) NMDA receptor expression in these cells (Barres et al., 1990; Fulton et al., 1992; Patneau et al., 1994; Usowicz et al., 1989; Wyllie et al., 1991; Gallo et al., 1994). One possible explanation for this discrepancy is that NMDA receptor expression is lineage stage dependent, and O-2A cells obtained from explants are in a different developmental stage compared with other preparations such as dispersed cell cultures. This hypothesis is currently being tested in our laboratory. The stage-dependent expression of voltage-dependent calcium channels in the O-2A cell lineage has been demonstrated (Sontheimer et al., 1989; Blankenfeld et al., 1992). It has also been suggested that the enzymatic isolation of cells used for the preparation of dispersed cell cultures selectively destroys NMDA receptors (Barres et al., 1990). It is uncertain whether O-2A cells from neurohypophysis differ from those obtained from the optic nerve and from other brain regions. Although neurohypophysial O-2A cells are similar to O-2A cells from other sources in many respects (Wang et al., 1994), most O-2A cells from the neurohypophysis express a low level of GFAP immunoreactivity in serum-containing medium, in contrast with O-2A cells from other structures, such as the optic nerve (Dubois-Dalcq, 1987; Raff, 1989; Wang et al., 1994). We have previously proposed that pituicytes, the resident astrocytes in the neurohypophysis, may develop from O-2A–like cells; thus, GFAP expression of the O-2A cell may be an indication of an in vivo commitment toward the astrocytic phenotype (Wang et al., 1994). Importantly, our in situ hybridization studies indicate that the NR1 subunit mRNA is present in the neurohypophysis in vivo (Kiss, J.Z., and C. Wang, unpublished observations). Clearly, further studies are required to establish whether the expression of functional NMDA receptors is a feature of oligodendrocyte precursors in general and, if so, whether they are involved in the regulation of cell migration in vivo.

Our results raise the possibility that NMDA receptors expressed in neurohypophysial cells may represent homomeric complexes of the NR1 subunits. Previous studies have established that functional characteristics of the NMDA receptor are determined by its subunit composition (Moriyoshi et al., 1991; Seeburg et al., 1995). Expression studies in oocytes suggest that homomeric complexes of the NR1 subunit form functional receptors (Monyer et al., 1992; Moriyoshi et al., 1991; Seeburg et al., 1995), which may also be present in vivo (Durand et al., 1993; Zhang et al., 1994). However, the efficiency of this homomeric receptor was found to be very low (Moriyoshi et al., 1991). While the NR1 subunit RNA and immunoreactivity were clearly detectable in O-2A cells of the neurohypophysis, we were unable to demonstrate the presence of the NR2 subunit RNAs in these cells, even using a sensitive technique such as RT-PCR analysis. The possibility that the homomeric NR1 receptor type is expressed in O-2A cells is consistent with the very low increase of intracellular Ca^{2+} elicited by NMDA, comparable to that obtained with the homomeric NR1 receptors reconstituted in oocytes (Moriyoshi et al., 1991). Nevertheless, we cannot exclude the possibility that the NR1 subunit is combined with a yet unknown subunit to form a heteromeric receptor.

Our study provides evidence for the presence of functional NMDA receptors on O-2A cells, but we have not carried out a detailed pharmacological and molecular characterization of the receptor. It is known, for example, that at least seven NR1 splice variants (NR1A–G) are expressed in the brain, which differ in their spatial and temporal expression patterns (Laurie and Seeburg, 1994), their cellular distribution (Ehlers et al., 1995), and their sensitivity to polyamines, phorbol esters, and Zn^{2+} (Durand et al., 1993; Hollmann et al., 1993). The probes we used in this study recognize a fragment common to all splice variants; thus, the possibility exists that different spliced isoforms of the NR1 subunit are present in O-2A cells.

Despite moderate increases in cytosolic Ca^{2+} concentration relative to neurons that follows their activation, the NMDA receptors on O-2A cells appear to have a critical role in the regulation of cell migration. These cells are known to express other Ca^{2+} channels such as voltage-dependent Ca^{2+} channels and non-NMDA, AMPA/kainate receptors that were shown to be Ca^{2+} permeable (Barres et al., 1990; Borges et al., 1994; Patneau et al., 1994; Pende et al., 1994; Wyllie et al., 1991). The presence of these channels has also been confirmed by patch-clamp recordings as well as by the intracellular calcium measurements in this study. However, the application of the non-NMDA receptor antagonist, CNQX, only caused a moderate (~40%) decrease of cell migration as opposed to the strong inhibition (~90%) obtained with the selective NMDA antagonist, d-AP5. This is consistent with the observation that CNQX, in contrast with d-AP5, was ineffective in preventing PSA-NCAM expression in these cells. Similarly, nifedipin, an antagonist to the L-type voltage-sensitive calcium channels and cyclopidazonic acid that depletes IP_3-sensitive Ca^{2+} stores without affecting Ca^{2+} channels (Nelson et al., 1994), produced only a small inhibition of O-2A cell migration. It appears, thus, that although the activation of AMPA/kainate receptors, voltage-sensitive calcium channels, and the release of Ca^{2+} from intracellular stores may contribute to the migration of O-2A cells, NMDA receptor–related mechanisms are hierarchically more important. Evaluating the NMDA-induced current and increase in cytosolic Ca^{2+}, we cannot exclude the possibility that local changes in calcium concentration are underestimated by the whole-cell measurements of mean intracellular calcium with Fura-2. NMDA receptors may be located at specific sites in the cell membrane of the O-2A cells. Whatever the interpretation, our observations provide further support for the idea that in addition to signal transduction, the NMDA class of glutamate receptors plays a critical role in morphogenetic events in the nervous system, such as synaptic plasticity associated with long-term potentiation (Bliss and Collinbridge, 1993; Bourne and Nicoll, 1993), migration of cere-

bellar granule cells (Komuro and Rakic, 1993), and axonal growth (Scheetz and Constantine-Paton, 1994).

How does the NMDA receptor affect cell migration? One possibility is that PSA-NCAM expression is a target of the signal transduction cascade that follows NMDA receptor activation. This cell adhesion molecule has been implicated in a number of morphogenetic events such as neuroblast migration (Ono et al., 1994), axonal growth (Doherty et al., 1990; Zhang et al., 1992), myelination and remyelination (Oumesmar et al., 1995), activity-dependent synaptic plasticity, and regeneration (Muller et al., 1994). It is of interest that PSA-NCAM expression is associated not only with morphogenetic events taking place during development but also with brain structures that undergo activity-induced morphological remodeling in the adult (Rougon and Figarella-Branger, 1993; Theodosis and Poulain, 1993). Thus, PSA-NCAM may represent a crucial link between cell activation, transmembrane signal transduction, and rapid remodeling of the cell surface. Earlier we have demonstrated that PSA-NCAM can be readily translocated to the cell surface upon cell activation through the Ca^{2+}-dependent exocytotic pathway (Kiss et al., 1994). In this study we have shown in O-2A cells that the expression of PSA on NCAM depends on NMDA receptor activation. The recovery of PSA immunostaining on the surface of O-2A cells after Endo N treatment requires ~16 h as opposed to 1–2 h for the recovery in neurons and β cells (Kiss et al., 1994). Most likely, different aspects of PSA-NCAM metabolism are involved in the two cases. The rapid reexpression of PSA on the surface of neurons upon activation of the regulated exocytotic pathway probably reflects the translocation of a readily mobilizable intracellular pool of PSA-NCAM that may involve secretory vesicles (Kiss et al., 1994). In the case of O-2A cells, the time course of recovery is more compatible with the biosynthesis of new PSA-NCAM. Thus, it is reasonable to propose that NMDA receptor activity is linked to the biosynthesis of PSA-NCAM in progenitor cells. We observed that if the long-term blockade of NMDA receptors resulted in a dramatic decrease in expression of PSA, this did not modify the immunolabeling for NCAM. Therefore, it seems unlikely that changes in the transcription of NCAM protein backbone are primarily involved in this process. The hypothesis that NMDA receptor activation is involved in the polysialylation of NCAM is consistent with recent published data suggesting that Ca^{2+} ions and protein kinase C play a role in NCAM isoform synthesis and polysialylation (Rafuse and Landmesser, 1996). A potential target for the observed regulation of PSA-NCAM expression is the enzyme polysialyltransferase, recently cloned in hamster (Eckhardt et al., 1995) and human (Nakayama et al., 1995), whose RNA transcripts seem to correlate well with the formation of PSA in various tissues. The availability of probes for detection of the enzyme will allow us to test this hypothesis.

Taken as a whole, our results have interesting implications for the physiological role of extracellular glutamate in O-2A cell migration. In vivo, these cells migrate along axons, which are able to release glutamate in an activity-dependent manner (Chiu and Kriegler, 1994). The affinity of NMDA receptors in O-2A cells for agonist is within the range of expected baseline extracellular glutamate concentrations. In the neurohypophysis, glial cells are in close contact with neurosecretory axons, and the activation of these fibers was shown to influence the differentiation and motility of neurohypophysial astrocytes (Hatton, 1990; Theodosis and Poulain, 1993). Since neurosecretory terminals were shown to contain glutamate immunoreactive small vesicles (Meeker et al., 1991), glutamate appears to be a good candidate to mediate neuron to glia communication and to regulate glial cell movement through the activation of NMDA receptors and PSA-NCAM expression. This hypothesis is consistent with our previous results in pituicytes, showing that the expression of PSA, but not that of NCAM, requires the presence of neurosecretory axons in the neurohypophysis (Kiss et al., 1993).

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