

# Neuromodulin (GAP43): A Neuronal Protein Kinase C Substrate Is Also Present in 0-2A Glial Cell Lineage.

## Characterization of Neuromodulin in Secondary Cultures of Oligodendrocytes and Comparison with the Neuronal Antigen

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**Abstract.** Neuromodulin (also called GAP43, B50, F1, pp46), a neural-specific calmodulin binding protein, is a major protein kinase C substrate found in developing and regenerating neurons. Here, we report the immunocytochemical characterization of neuromodulin in cultured 0-2A bipotential glial precursor cells obtained from newborn rat brain. Neuromodulin is also present in oligodendrocytes and type 2 astrocytes (stellate-shaped astrocytes), which are both derived from the bipotential glial 0-2A progenitor cells, but is absent of type 1 astrocytes (flat protoplasmic astrocytes). These results support the hypothesis of a common cell lineage for neurons and bipotential 0-2A progenitor cells and suggest that neuromodulin plays a more general role in plasticity during development of the central nervous system. The expression of neuromodulin in secondary cultures of newborn rat oligodendrocytes and its absence in type 1 astrocytes was confirmed by Northern blot analysis of isolated total RNA from these different types of cells using a cDNA probe for the neuromodulin mRNA and by Western blot analysis of the cell extracts using polyclonal antibodies against neuromodulin. The properties of the neuromodulin protein in cultured oligodendrocytes and neuronal cells have been compared. Although neuromodulin in

oligodendrocytes is soluble in 2.5% perchloric acid like the neuronal counterpart it migrates essentially as a single protein spot on two-dimensional gel electrophoresis whereas the neuronal antigen can be resolved into at least three distinct protein spots. To obtain precise alignments of the different neuromodulin spots from these two cell types, oligodendrocyte and neuronal cell extracts were mixed together and run on the same two-dimensional gel electrophoresis system. Oligodendroglial neuromodulin migrates with a pI identical to the basic forms of the neuronal protein in isoelectric focusing gel. However, the glial neuromodulin shows a slightly lower mobility in the second dimensional lithium dodecyl sulfate-PAGE than its neuronal counterpart. As measured by <sup>32</sup>Pi incorporation, neuromodulin phosphorylation in oligodendrocytes is dramatically increased after short-term phorbol ester treatments, which activate protein kinase C, and is totally inhibited by long-term phorbol ester treatments, which downregulates protein kinase C, thus confirming its probable specific *in vivo* phosphorylation by protein kinase C. In primary cultures of neuronal cells, two of the three neuromodulin spots were observed to be phosphorylated with an apparent preferential phosphorylation of the more acid forms.

**N**EUROMODULIN (NM<sup>1</sup>; also called GAP43, B50, F1, pp46) is a membrane-associated, protein kinase C (PKC) substrate found in neuronal growth cones (Skene and Virag, 1989). Although the cellular role of NM has not been clearly established, its synthesis in fetal rat brain is strongly correlated with axonal outgrowth (Kalil and

1. *Abbreviations used in this paper:* As, astrocyte; GFAP, glial fibrillary acidic protein; LDS, lithium dodecyl sulfate; NM, neuromodulin; OL, oligodendrocyte; PKC, protein kinase C.

Skene, 1986; Perrone-Bizzozero et al., 1986; Meiri et al., 1986). Expression of NM in nonneuronal cells transfected with an expression vector encoding NM induces filipodial processes, suggesting that NM is involved in the general mechanisms that control cell shape and membrane modeling (Zuber et al., 1989). Several other putative functions have been proposed for NM such as sequestration of calmodulin along the cytoplasmic surface of appropriate membrane domains (Alexander et al., 1988) and regulation of phosphati-

dylinositol turnover (Van Hooff et al., 1988). The correlation between the translocation of PKC to the membrane, phosphorylation of NM, and long-term potentiation indicate that NM may play a role both in normal neurite growth during brain development and in synaptic plasticity in the adult (Nelson et al., 1989). Although NM normally is a membrane-bound protein, analysis of the protein's sequence shows no potential membrane-spanning domains. Attachment to the membrane is likely to occur after posttranslational fatty acylation of the protein (Skene and Virag, 1989).

Since a transient association of NM with the plasma membrane of cultured astroglial cells has recently been reported (Vitkovic et al., 1988), we examined the presence of NM in mixed and pure glial cell cultures. In dissociated cell cultures derived from either the developing optic nerve or the brain, and grown in the presence of serum, two populations of astrocytes have been distinguished on the basis of morphological, antigenic, and functional criteria: the epithelioid type 1 astrocyte (type 1 As) and the stellate type 2 astrocyte (type 2 As) (Raff et al., 1983, 1984; Ffrench-Constant and Raff, 1986; Levi et al., 1986; Aloisi et al., 1988a; Behar et al., 1988; Sontheimer et al., 1989). These two populations belong to two different cell lineages. Type 2 As originates from bipotential glial O-2A progenitor cells. The latter may also differentiate into oligodendrocytes (OL) in serum-free medium (Raff et al., 1983; Levi et al., 1987; Behar et al., 1988). In this paper, we demonstrate that NM is a phenotype that characterizes O-2A glial cell lineage, and have compared its properties with those of NM from neuronal cells.

## Materials and Methods

### Chemicals

All electrophoretic chemicals were from Bio-Rad Laboratories (Richmond, CA). Pharmacia LKB (Uppsala, Sweden) ampholine pH 3.5–10 or pH 3.5–9.5 and ampholine pH 3–10 (Serva Fine Biochemicals Inc., Garden City Park, NY) were used as carrier ampholytes.  $^{32}\text{P}$ i (40 mCi/ml) was from Amersham International (Amersham, UK). The Immobilon and nitrocellulose blotting membranes were from Millipore Continental Water Systems (Bedford, MA). PMA was from Sigma Chemical Co. (St. Louis, MO). Waymouth's MD 705/1 medium was from Flow Laboratories, Inc. (McLean, VA); DME, calf serum, and FCS were from Gibco Laboratories (Grand Island, NY). Rhodamine-conjugated sheep anti-mouse IgG and fluorescein-conjugated sheep anti-rabbit IgG were from Biosys (Compiègne, France). Alkaline phosphatase-conjugated goat anti-rabbit IgG was from Bio-Rad Laboratories. Mouse mAbs from mouse-mouse-hybrid cells (clone G-A-5) against glial fibrillary acidic protein (GFAP) were from Boehringer Mannheim Biochemicals (Indianapolis, IN). The monoclonal A2B5 antibody was generously provided by G. Rougon (Institut de Chimie Biologique, Université d'Aix-Marseille, France).

### Cell Cultures

Type 1-like astrocyte cultures were prepared by a modification of the method of Booher and Sensenbrenner (1972). Cerebral hemispheres of newborn rats were dissected, cleaned of their meningeal membranes, and dissociated by passage through a 2-mm diameter needle into a small volume of nutrient medium. This medium was Waymouth's MD 705/1 medium supplemented with 110 mg/l sodium pyruvate, 50 U/ml penicillin, 50  $\mu\text{g}/\text{ml}$  streptomycin, and 10% FCS (Pettmann et al., 1981). The cell suspension was added to a suitable volume of complete nutrient medium to obtain a final volume of 60 ml for one brain. 4 ml of the cell suspension were plated per Falcon Petri dish (60-mm diameter,  $5 \times 10^3$  cells per  $\text{cm}^2$ ). Cultures were incubated at 37°C in a 5%  $\text{CO}_2$  humidified atmosphere. Culture medium was changed after 5 d and then twice a week. After 10 d in culture 85–95% cells were positively stained for GFAP.

Mixed glial primary cultures and secondary cultures of oligodendrocytes

were prepared by the method of Besnard et al. (1989). Briefly, primary mixed glial cell cultures were prepared by dissociating newborn rat cerebral hemispheres as described above (except for a final dilution of one brain for 30 ml) and maintained in Waymouth's MD 705/1 medium supplemented with 10% calf serum. After 18–20 d, these primary cultures consist of astroglial and oligodendroglial cells, but also contain A2B5-positive cells (present results) which correspond to the O-2A progenitor glial cells already described by other authors (Raff and Miller, 1984). However, in 10-d cultures, mostly astroglial cells and O-2A progenitor glial cells are present. To obtain secondary cultures of oligodendrocytes, the oligodendroglial cells were dislodged from 18–20-d cultures by gently syringing the culture medium on the cell layer with a 2-mm diameter needle. The detached cells were sedimented by centrifugation and the cell pellet was dissociated by 10 passages through the same needle. Cells were replated in 60-mm Falcon Petri dishes precoated with poly-L-lysine ( $3 \times 10^3$  cells per  $\text{cm}^2$ ). After 24 h, the serum-containing medium was removed and a chemically defined medium was used (Besnard et al., 1989).

Secondary cultures of type 2-like astrocytes were prepared using high cell density cultures maintained in Waymouth's medium supplemented with 10% FCS as described by Aloisi et al. (1988b). Primary cultures of neuronal cells from cerebral hemispheres of 14-d-old rat embryos were prepared by a previously described method (Gensburger et al., 1986).

### Immunocytochemical Procedures

For immunocytochemical studies cells were plated onto polylysine-treated glass coverslips in 35-mm Falcon Petri dishes. For immunostaining with NM polyclonal antibodies (diluted 1:200) (prepared in our laboratory, see below) or with GFAP mAbs (diluted 1:4), cells were fixed with 4% paraformaldehyde in PBS for 15 min followed by 30% methanol for 5 min at room temperature. After treatment with fluorescein-conjugated sheep anti-rabbit IgG (diluted 1:100) preparations were observed with a Zeiss fluorescence microscope.

For double-immunofluorescence staining with A2B5 and NM antibodies, cells were fixed with 4% paraformaldehyde in PBS for 15 min, then incubated with A2B5 mouse mAbs (culture supernatant diluted 1:2) followed by rhodamine-conjugated sheep anti-mouse IgG, and then, after fixation with 30% methanol for 5 min preparations were incubated with rabbit anti-NM antibodies followed by fluorescein-conjugated sheep anti-rabbit IgG.

### Western Blot Analysis

**Production of NM Antibodies.** Antibodies against purified bovine brain neuromodulin (Baudier et al., 1989) were prepared in New Zealand rabbits. The protein (300–500  $\mu\text{g}$ ) was emulsified in complete Freund's adjuvant and injected intradermally at multiple dorsal sites. At 2-wk intervals, the rabbits were given two booster injections of 150–250  $\mu\text{g}$  of protein in Freund's adjuvant. The rabbits were bled repeatedly, and the antibody titers determined by Western blotting. Antisera that showed significant activity at 1:1,000–1:2,000 dilution were collected.

**Immunoblotting.** Cell extracts (see below) were electrophoresed (see legends of figures) and then transferred electrophoretically to an Immobilon or nitrocellulose blotting membrane. After transfer the membrane was incubated 30 min in 3% gelatin in TBS buffer. After being washed with TBS, the blotting membrane was incubated overnight at room temperature with neuromodulin antibodies diluted 1:1,000 in 1% gelatin in TTBS buffer (TBS plus 0.05% Tween 20) followed by incubation with alkaline phosphatase-conjugated goat anti-rabbit IgG (diluted 1:3,000) in 1% gelatin in TTBS buffer for 2 h. Immunoreactive proteins were detected by using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium in 40 mM sodium carbonate, pH 9.8, and 5 mM  $\text{MgCl}_2$ .

### RNA Extraction and Northern Blot Analysis

Total cytoplasmic RNA was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi, 1987). The RNAs were electrophoresed on agarose gels in the presence of formaldehyde, and transferred onto nitrocellulose membranes. Prehybridization was performed for 4 h at 42°C in 50% formamide, 2.5  $\times$  Denhardt's solution, 10 mM sodium phosphate, pH 7.5, 10 mM Tris-HCl, pH 7.5, 5 $\times$  SSC (1 $\times$  SSC: 0.15 M NaCl, 15 mM sodium citrate, pH 7.0), 10  $\mu\text{g}/\text{ml}$  yeast tRNA, and 250  $\mu\text{g}/\text{ml}$  single-stranded salmon sperm DNA. After 18 h hybridization at 42°C with a cDNA for NM, blots were washed first in 2 $\times$  SSC, 0.1% SDS for 15 min, then in 0.2 $\times$  SSC, 0.1% SDS for 15 min at 20°C, and in 2 $\times$  SSC, 0.1% SDS for 15 min at 50°C. Blots were exposed to Kodak x-ray

films (X-AR5) for 24 h. NM cDNA probe (Cimler et al., 1987) was  $^{32}\text{P}$ -labeled by random primer method, to a specific activity of  $2 \times 10^8$  cpm/ $\mu\text{g}$ .

### Labeling of Cells with $^{32}\text{P}$ and Preparation of Cell Extracts

Primary cultures of type 1-like astrocytes (type 1 As), secondary cultures of oligodendrocytes (OL), and primary cultures of neurons in 60-mm dishes were incubated for 30 min with phosphate-depleted Waymouth's medium, and the cells were incubated with 2 ml of the same medium containing 1 mCi of  $^{32}\text{P}$ i for 2 h at  $37^\circ\text{C}$ . Then, the cells were nontreated or treated with PMA for 15–20 min as indicated in each experiment. The reaction was stopped by removing the medium and rapidly washing the cultures twice with ice-cold PBS. The cells were immediately extracted with 800  $\mu\text{l}$  of 2.5% perchloric acid, 1% Triton X-100, 150 mM NaCl, transferred into Eppendorf tubes, and sonicated for 15 min in a water bath. After centrifugation for 10 min at 15,000 g, the supernatants were collected. The perchloric acid-soluble proteins were precipitated with 20% TCA. At this stage Triton X-100 also precipitated. After centrifugation for 40 min at 15,000 g, the pellets were washed twice with 1 ml cold ethanol, briefly sonicated in a water bath and centrifuged at 15,000 g for 40 min. Proteins were resuspended in 100  $\mu\text{l}$  of lysis buffer containing 2.5% Ampholine, pH 3.5–10, and 2.5% Ampholine, pH 3–10, 9.5 M urea, 2% (wt/vol) CHAPS, 20 mM DTT.

For one-dimensional PAGE and Western blot analysis of the NM protein in type 1 As, OL, and neurons, cells were rinsed three times with PBS. The cells were disrupted by brief sonication in 1 ml of 100 mM Tris-HCl, pH 7.4, 100 mM NaCl, 2 mM EDTA, 2 mM DTT, and 1% Triton X-100 and the extracts were cleared by centrifugation. For the perchloric acid-treated cell extracts, the supernatant was adjusted to 2.5% perchloric acid and immediately centrifuged for 15 min at 15,000 g. The supernatants were then dialyzed against 40 mM Tris-HCl, pH 6.9, 50 mM NaCl, 2 mM DTT.

### Two-dimensional Gel Electrophoresis

Two-dimensional PAGE was performed essentially as described by O'Farrell (1975) and O'Farrell et al. (1977). First dimensional IEF gels (1.5 mm  $\times$  14 cm) contained a mixture of 2.5% Ampholine, pH 3.5–10, and 2.5% Ampholine, pH 3–10, or a mixture of 2.5% Ampholine, pH 3.5–9.5, and 2.5% Ampholine, pH 3–10, when indicated. Gels were prerun with 20  $\mu\text{l}$  of lysis buffer at 300 V for 1 h. Cell extracts (50 ml) in lysis buffer were then loaded and IEF was performed at 1,000 V for 18 h.

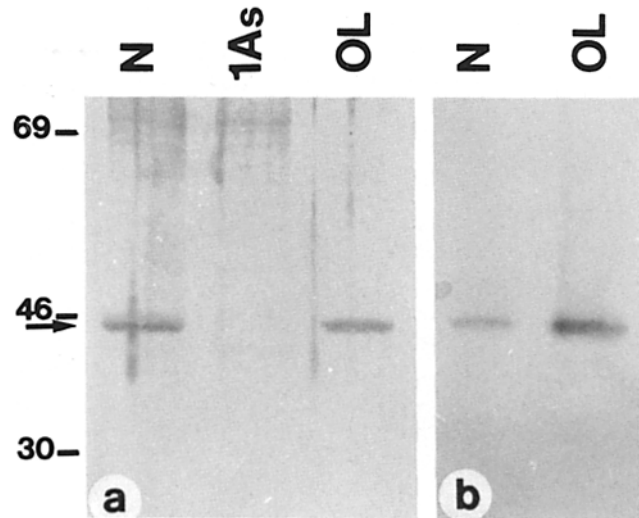
First dimensional NEPHGE gels (1.5 mm  $\times$  14 cm) contained the same mixture of Ampholines as IEF gels. Cell extracts (50 ml) in lysis buffer were loaded and electrophoresed at 500 V for 4 h.

For the second dimension 0.1% lithium dodecyl sulfate (LDS)-12% or 12.5% PAGE was used as indicated below in the text. Gels were run at 12 mA/gel until the dye front reached the bottom. For autoradiography the gels were then soaked in 30% methanol, 2.5% glycerol for 5 or 15 h, dried, and exposed to Hyperfilm MP (Amersham International) at  $-70^\circ\text{C}$  for 24–48 h. For silver staining the dried gels were first rehydrated in 30% methanol overnight and then silver stained.

## Results

### Immunocytochemical Characterization of NM in O-2A Progenitor Glial Cells, Type 2 As, and OLs

Recently, authors reported a transient expression of NM in cultured astrocytes from neonatal rat brain seeded at high density (Vitkovic et al., 1988). In an attempt to confirm this observation we first compared the expression of NM in 1% Triton X-100 solubilized extracts of cultured type 1 As, OL, and neurons from rat cerebrum by Western blot analysis (Fig. 1 a). Only OL and neurons were found to express significant NM-like protein. Further biochemical comparisons of the NM proteins in neurons and OL confirmed the identity of the protein in both cell types (see below and Fig. 1 b). Since we did not detect NM immunoreactivity on the immunoblot in type 1 As throughout their development in culture, we suppose that the observed transient expression of

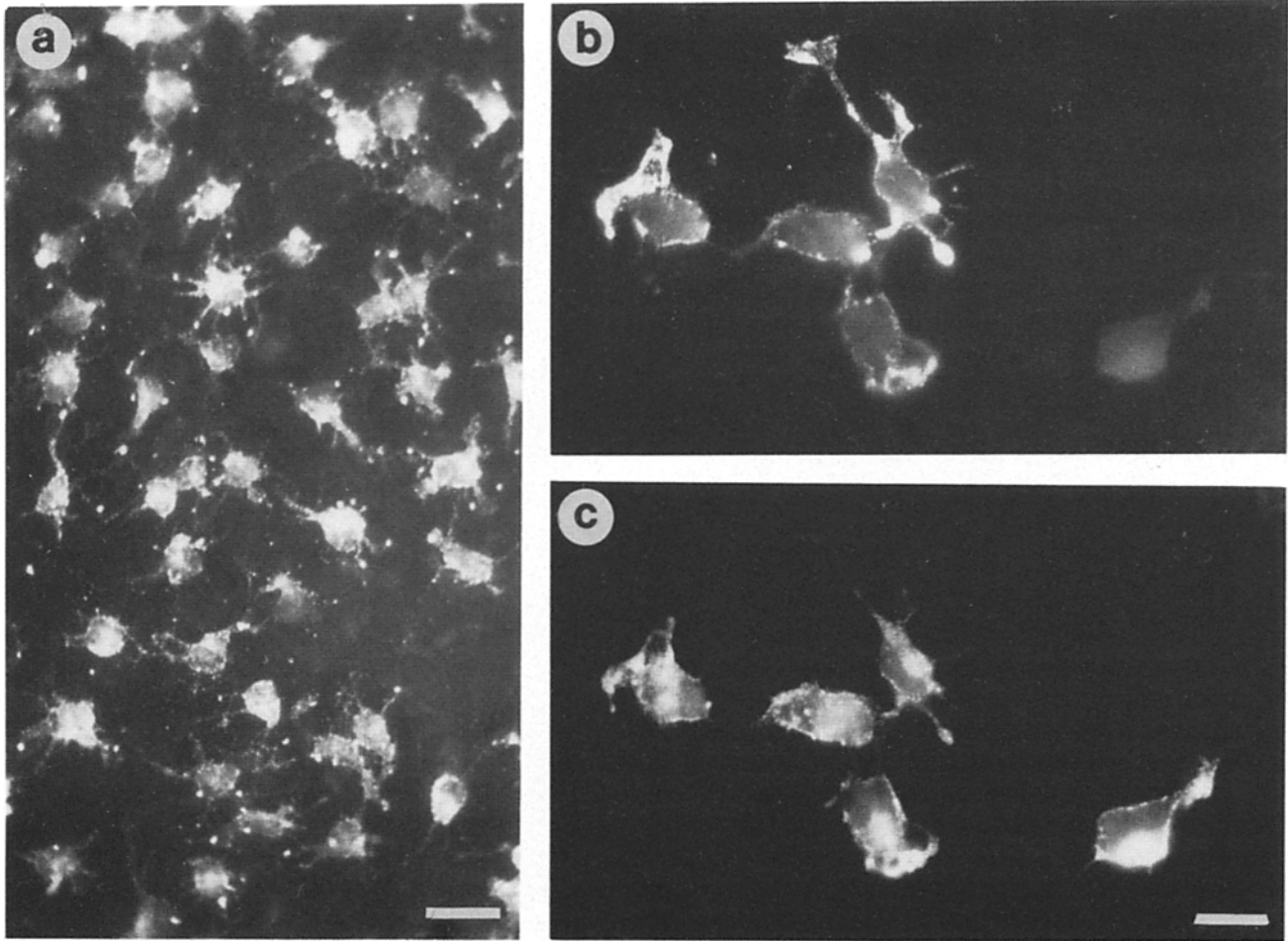


**Figure 1.** Protein blot analysis of NM in rat brain cell cultures. (a) NM is detected in Triton X-100-solubilized extracts of neurons (N) and OL but not in type 1 As. (b) NM in neurons (N) and OL is soluble in 2.5% perchloric acid. Cell extracts were prepared as described under Materials and Methods. The proteins in neuronal (20  $\mu\text{g}$ ), OL (50  $\mu\text{g}$ ), and type 1 As (50  $\mu\text{g}$ ) crude extracts and in the corresponding perchloric acid-soluble extracts were run on a 0.1% SDS-9% PAGE and transferred to an Immobilon blotting membrane for Western blot analysis using NM antibodies (dilution 1:1,000) as described under Materials and Methods. The position of NM is indicated by an arrow.

NM in high density astrocyte cell cultures, observed by the above mentioned authors, may result from the presence of a population of astrocytes (GFAP $^+$ ) in their cultures that do not correspond to the type 1 As and do express NM phenotype at one stage of their development. Using immunocytochemistry to investigate this possibility, we examined the presence of NM in glial O-2A progenitor cells and type 2 As, which may transiently contaminate type 1 As cultured at high cell densities.

Primary cultures of glial cells derived from newborn rat cerebrum, seeded at high cell density, and grown in medium supplemented with 10% calf serum are composed of an underlayer of type 1-like As and flat small dark cells growing on top of this layer (Besnard et al., 1989). The small cells have been previously identified as O-2A bipotential precursors of OL and type 2 As (Behar et al., 1988; Aloisi et al., 1988a,b). In Fig. 2 a, an 8-d-old primary culture of glial cells labeled with NM polyclonal antibodies is shown. The confluent underlayer of type 1 As was totally devoid of NM immunoreactivity but all of the overlying, small cells were characterized as NM $^+$ . NM immunoreactivity accumulated in the somata but also in the short growing processes, which could not be distinguished using phase-contrast optics (not shown).

O-2A progenitor cells express on their surface neuronal gangliosides which bind the mAb A2B5 (Eisenbarth et al., 1979; Behar et al., 1988). The A2B5 phenotype disappears when the O-2A cells differentiate into OL but is transiently expressed in cultured type 2 As. Double labeling of the O-2A progenitor cells using A2B5 mAbs and NM antibodies revealed that most of the NM $^+$  cells were also A2B5 $^+$  (Fig. 2, b and c) thus pointing out the common expression of these



**Figure 2.** Immunofluorescence staining for NM of mixed glial primary cultures. (a) Cells were maintained in Waymouth's medium supplemented with 10% calf serum for 8 d. All the small cells growing upon the monolayer of type 1 As were NM<sup>+</sup>. (b and c) Double-immunofluorescence staining of cells grown for 11 d for localization of A2B5 and NM. Four A2B5<sup>+</sup> (b) NM<sup>+</sup> (c) O-2A progenitor cells are seen. Note that the cell body and processes of an adjacent cell on the right of the photograph are NM<sup>+</sup> but A2B5<sup>-</sup>, suggesting that this cell has already been oriented toward an OL differentiation. Bars: (a) 25  $\mu$ m; (b and c) 17  $\mu$ m.

markers in O-2A progenitor cells. There were no cells A2B5<sup>+</sup>/NM<sup>-</sup> but some cells were found A2B5<sup>-</sup>/NM<sup>+</sup> suggesting that some cells have already been oriented towards an OL differentiation. In separate immunostaining experiments, we found that about 10% of the small cells only expressed the OL-specific galactocerebroside antigen (data not shown). Here again, neither A2B5 nor NM immunoreactivity was detected in the underlayer of type 1 As, confirming the restriction of these phenotypes to the O-2A progenitor cells.

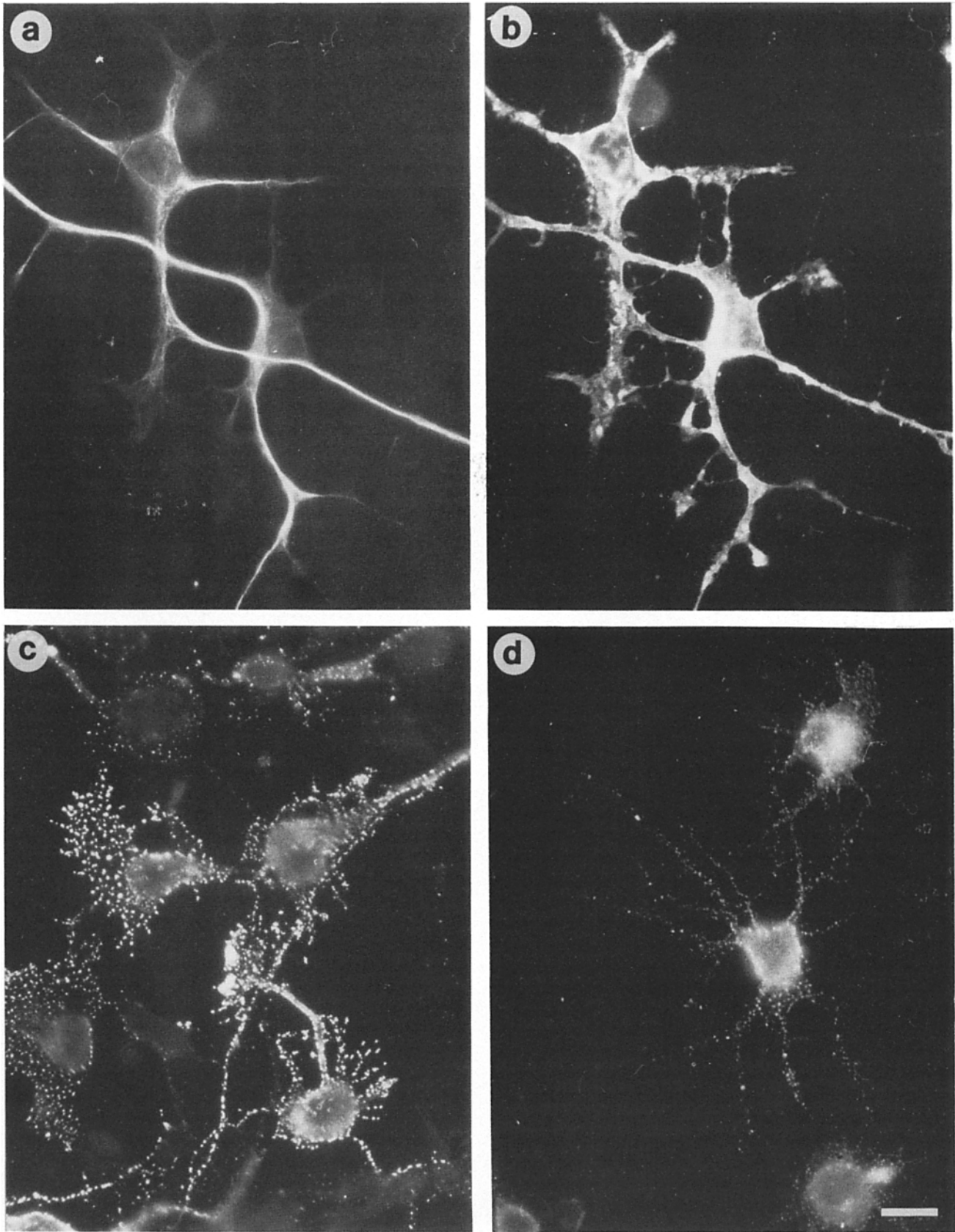
Cultures enriched in type 2 As were obtained by subculturing the bipotential glial precursor at high cell density in the presence of 10% FCS, as previously described by Aloisi et al., (1988b). After 7 d, the subcultures were composed mainly of a population of stellate type 2 As, which coexpressed GFAP<sup>+</sup> and the NM antigen (Fig. 3, a and b).

When the bipotential O-2A glial precursor cells were subcultured in a serum-free medium the cells differentiate into OL (Raff et al., 1983; Aloisi et al., 1988a; Behar et al., 1988) that express the galactocerebroside (GC) antigen (data not shown). After 8 d in secondary culture most of the GC<sup>+</sup> cells also expressed NM (Fig. 3 d). The labeling of OL with

NM antibodies was very similar to that observed for neurons (Fig. 3 c). The reaction product was characterized by intense, discontinuous, punctate staining of the processes. In contrast, in the type 2 As, the NM immunoreactivity was uniformly distributed in the somata and processes and showed no punctate pattern.

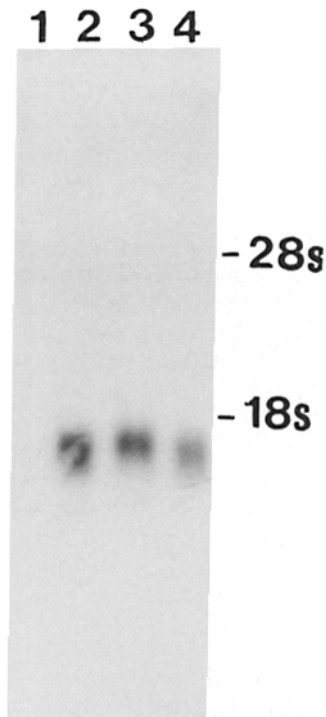
#### **Expression of NM mRNA in O-2A Progenitor Cell, OL, and Neuronal Cell Cultures**

To confirm the immunoblot and immunocytochemical data showing the presence of NM in O-2A progenitor cells and OL, we compared the expression of NM mRNA in O-2A progenitor cells and secondary cultures of OL, primary cultures of type 1 As, and primary cultures of neuronal cells. Northern blot analysis of isolated total cellular RNA from these different cell types revealed that only O-2A progenitor cells, OL, and neuronal cells synthesized significant NM mRNA levels that hybridized to a NM cDNA probe (Fig. 4). The hybridized band in the three positive cell types migrated at the same position as the NM mRNA from fetal rat brain which was run as a positive control (not shown).



**Figure 3.** NM immunoreactivity in cultured type 2 As, OLs, and neurons. *a* and *b* show two astrocytes of type 2 double labeled with GFAP (*a*) and NM antibodies (*b*) after 1 wk in secondary cultures. In *c*, a primary culture of neuronal cells was labeled with NM antibodies 5 d after plating. In *d*, OLs were labeled with NM antibodies after 8 d in secondary cultures. Note the punctate staining of the processes in NM<sup>+</sup> OL and neuronal cells. Bar, 17  $\mu$ m.





**Figure 4.** Expression of NM mRNA in 0-2A progenitor cells, OLs, and neuronal cells. The expression of NM mRNA was studied by Northern blot analysis of total RNA (15  $\mu$ g per sample) of primary cultures of type 1 As maintained for 17 d in culture (lane 1), secondary cultures of OL maintained in serum-free medium for 6 d (lane 2), neuronal cells after 5 d in culture (lane 3), and 0-2A progenitor cells dislodged at 10 d from mixed glial cell cultures as described under Materials and Methods.

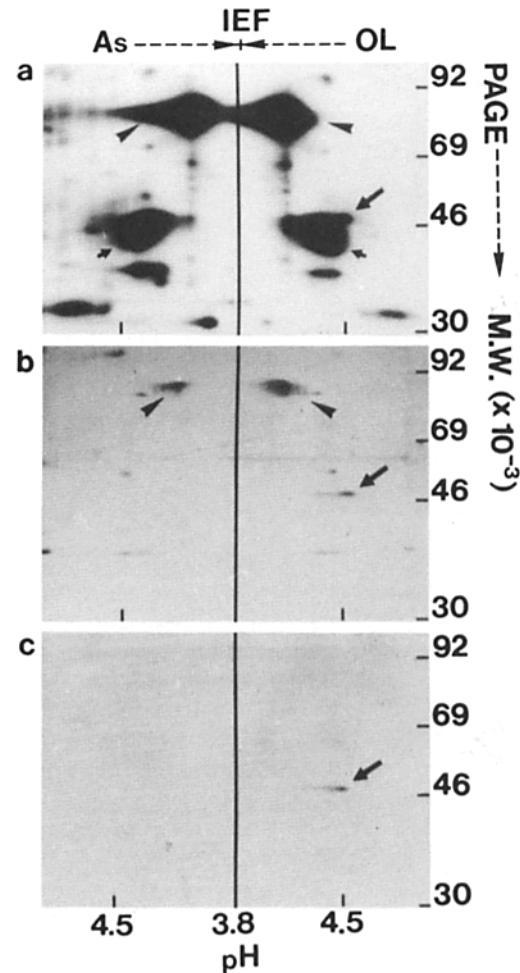
### Characterization of Neuromodulin in OLs and Neurons

**NM of OLs and Neurons Is Soluble in 2.5% Perchloric Acid.** We recently observed, that although solubility in 2.5% perchloric acid is generally uncommon among proteins, NM, and several other PKC substrates share this property, suggesting that perchloric acid solubility might be a common property of a class of PKC substrates (Baudier et al., 1989). In agreement with the hybridization data and immunocytochemistry data, Western blot analysis of 1% Triton X-100 solubilized extracts of cultured neurons, type 1 As, and OL revealed with neuromodulin polyclonal antibodies showed that only neurons and OL, expressed detectable amounts of NM (Fig. 1 *a*). Western blot analysis of the soluble proteins obtained from the same Triton X-100 solubilized extracts but further treated with 2.5% perchloric acid indicated that both neuronal and OL NM proteins were soluble in 2.5% perchloric acid (Fig. 1 *b*). Moreover, the electrophoretic mobility of both OL and neuronal NM was observed to decrease when increasing percentages of polyacrylamide were used in the one-dimensional PAGE (data not shown), as previously reported for purified NM from bovine brain (Baudier et al., 1989). In this system, no difference in the mobility of the OL and neuronal NM was evident, but a slightly lower mobility of the oligodendroglial protein compared with its neuronal counterpart (see below and Fig. 7) was detected by two-dimensional gel electrophoresis.

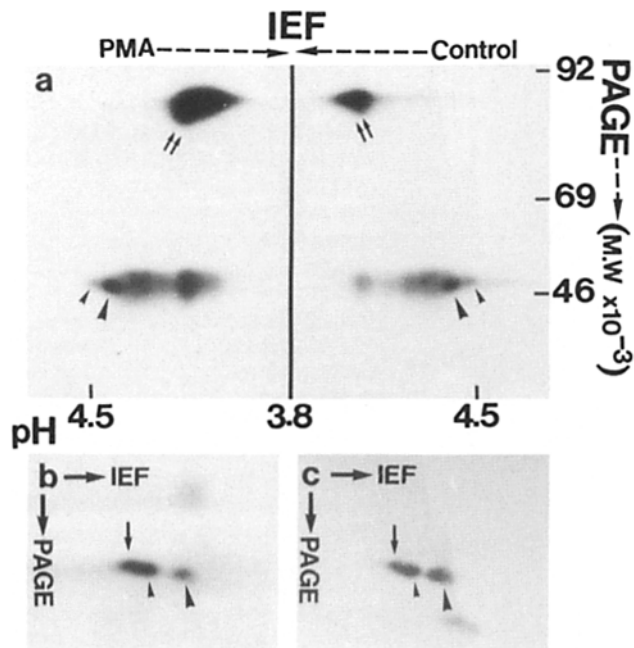
### Comparison of the NM Antigen in OLs and Neurons by Two-dimensional Gel Electrophoresis

We analyzed the 2.5% perchloric acid-soluble proteins of cell extracts from cultured type 1 As, OL, and neurons by two-dimensional PAGE. In Fig. 5, *a-c* the 2.5% perchloric acid-soluble proteins of OL and type 1 As extracts are compared. In Fig. 5 *a* are shown OL and type 1 As cultures which

were incubated 2 h with  $^{32}$ Pi followed by 20 min treatment with PMA before protein extraction and the second dimensional gel was submitted to autoradiography. Fig. 5 *b* shows silver staining of the same gel and Fig. 5 *c* shows the immu-



**Figure 5.** Two-dimensional gel analysis of the 2.5% perchloric acid-soluble proteins in OL and type 1 astrocyte extracts. Secondary cultures of OL at day 8 and primary cultures of type 1 As at day 20 were used. Cell extracts were prepared as described under Materials and Methods and perchloric acid-soluble proteins were separated by two-dimensional IEF gels. The acidic halves of the first dimensional IEF gels (*IEF*) were cut and run in tandem (the acidic ends facing each other) on the same second dimensional 0.1% LDS-12% PAGE (*PAGE*). In *a*, OL (right side of the figure) and type 1 As (left side of the figure) cultures were incubated 2 h with  $^{32}$ Pi (0.5 mCi/ml) followed by 15 min treatment with 0.1  $\mu$ M PMA before protein extraction, and the second dimensional gel was submitted to autoradiography. *b* shows the silver staining of the same gel. *c* presents the immunoblot analysis of the protein after transfer to an Immobilon blotting membrane using NM antibodies (1:1,000) and alkaline phosphatase-conjugated second antibodies. The NM antigen is indicated by a large arrow. Arrowheads indicate the position of the MARCKS protein and the small arrow indicates the position of an unidentified PKC substrate present in both OL and type 1 As that do not stain with silver. The line drawn through the gel represents the position where the acidic end of the two IEF gels came into contact and corresponds approximately to pH 3.8. In the margins arrows indicate the direction of the protein migration in the two electrophoretic systems.

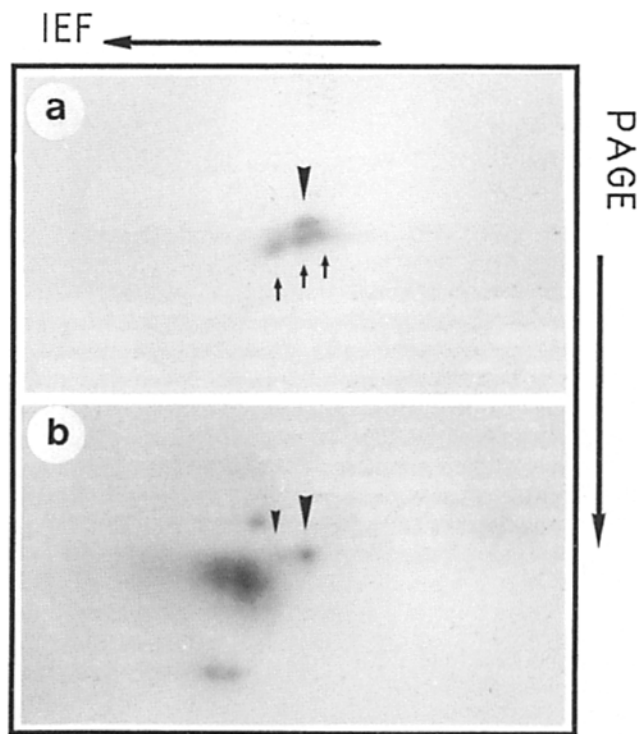


**Figure 6.** Two-dimensional gel analysis of NM in the 2.5% perchloric acid-soluble extracts of neuronal cells. Neuronal cell extracts were prepared as described under Materials and Methods and proteins were separated in the same two-dimensional electrophoresis system as described in Fig. 5. In *a*, neurons were incubated 2 h with  $^{32}\text{P}$ i (0.5 mCi/ml) and were stimulated (PMA) or not (control) with 0.1  $\mu\text{M}$  PMA for 15 min before extraction and the second dimensional gel was submitted to autoradiography. *b* and *c* show an enlargement of the silver staining pattern and immunostaining pattern of the NM antigen in neuronal extracts, respectively, and separation by two-dimensional gel electrophoresis. In *b* and *c* the arrow indicates the position of the primary translation product, and the arrowheads in *a-c* indicate the positions of the two acidic post-translational maturation products. Double arrows in *a* indicate the position of the MARCKS protein. Note in *c* the presence of a slight immunoreactive trace under the more acidic NM spot. This immunoreactive product was also found in bovine brain NM preparation after *in vitro* phosphorylation by PKC.

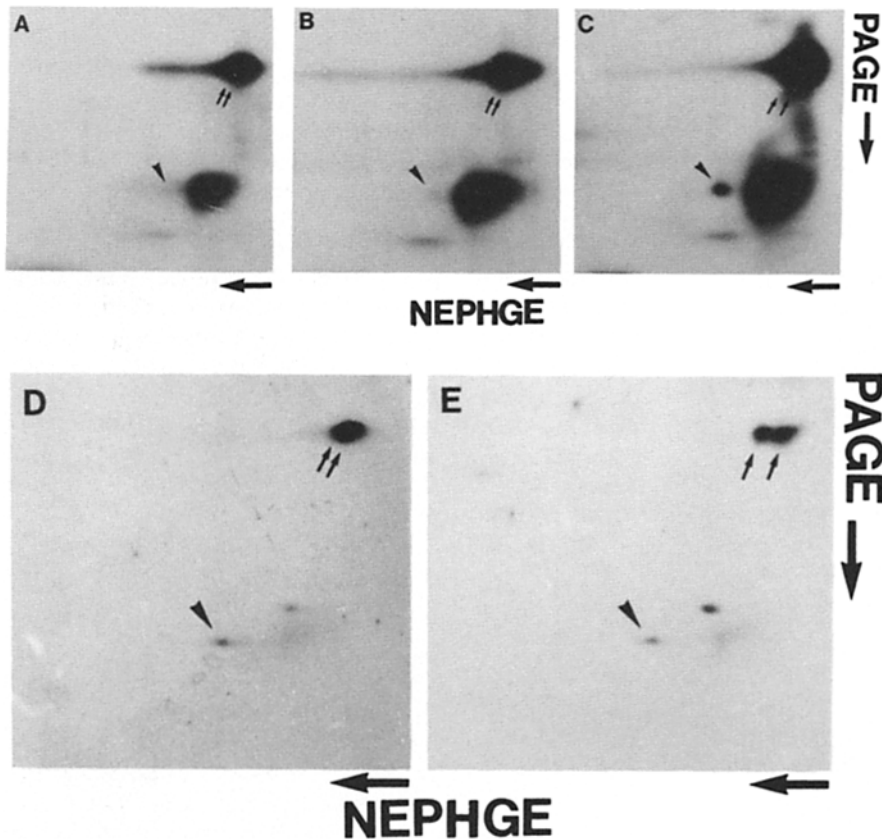
noblots analysis of the protein after transfer to an Immobilon blotting membrane using NM antibodies.

OL and type 1 As extracts have several proteins in common that are soluble in 2.5% perchloric acid and which are phosphoproteins. However, only OL expressed detectable amounts of NM. The NM antigen in OL was heavily stained with silver and migrated apparently as a single protein spot with a pI around 4.5 and a molecular mass of 46 kD. There was no significant difference in the silver staining pattern of NM from PMA-treated cells and nontreated controls (data not shown, see also below). The other heavily silver stained phosphoprotein present in both OL and type 1 As that migrated with a pI of  $\sim 4.2$  and a molecular mass of 85 kD (arrowheads) has been identified as the MARCKS protein (also named p87) (Graff et al., 1989). The other phosphoproteins have not been yet identified. Note the major phosphoprotein in OL and type 1 As that migrated in front of NM in the IEF gel, with a molecular mass of 43 kD and that is not stained with silver (small arrows). This protein has been characterized as a PKC substrate in both *in situ* and *in vitro* phosphorylation experiments (unpublished data).

In Fig. 6, *a-c*, two-dimensional gels of the 2.5% perchloric acid-soluble proteins of neuronal extracts are shown. Fig. 6 *a* shows the autoradiogram of the second dimensional gel where the perchloric acid-soluble proteins from cells that were not stimulated with PMA are run in tandem with their homologs from cells stimulated with PMA before the extraction procedure. The neuronal NM can be resolved into three distinct immunoreactive spots (Fig. 6 *c*, arrow and arrowheads) which can also be visualized on the silver-stained gel (Fig. 6 *b*, arrow and arrowheads). The two more acidic forms of NM were phosphorylated when cells were incubated with  $^{32}\text{P}$ i (Fig. 6 *a*, arrowheads). When one compares the silver staining intensity of the different NM spots with the corresponding phosphorylated forms on the autoradiogram it is clear that there is an apparent preferential  $^{32}\text{P}$ i incorporation into the more acidic NM forms. PMA stimulation of the neuronal cells increased the  $^{32}\text{P}$ i incorporation mainly into the more acidic NM species. The apparent stim-



**Figure 7.** Comparison of NM in OL and neuronal cells. Secondary cultures of OL, after 48 h in culture, were incubated with  $^{32}\text{P}$ i (0.5 mCi/ml) for 2 h and stimulated for 15 min with 1  $\mu\text{M}$  PMA before protein extraction. Proteins of 6-d-old primary neuronal cultures were extracted without  $^{32}\text{P}$ i labeling. The perchloric acid-soluble OL and neuronal cell extracts resuspended in lysis buffer (2.5% ampholine pH 3.5–9.5 plus 2.5% ampholine pH 3.5–10) were mixed together before separation on two-dimensional gel electrophoresis (0.1% LDS-12% polyacrylamide). After transfer of the proteins to an Immobilon blotting membrane, the NM protein spots were visualized using NM antibodies (1:1,000) and alkaline phosphatase-conjugated second antibodies (*a*). The OL NM spot (arrowhead) was specifically visualized on the autoradiogram after autoradiography of the blotting membrane (*b*). Only the areas where NM migrated are shown. Small arrowhead indicates the position of a minor phosphorylated protein in OL. In *a* arrows indicate the position of the neuronal NM spots.



**Figure 8.** NM in OLs is phosphorylated by PKC. Secondary cultures of OL after 6 d in culture were incubated with <sup>32</sup>Pi (0.5 mCi/ml) for 2 h before protein extraction. The perchloric acid-soluble proteins were then separated by two-dimensional gel electrophoresis. The first dimensional separation was done using NEPHGE and the second dimension by using 0.1% LDS-12.5% PAGE. Only the acidic end of the gels are shown and correspond to the regions where NM (arrowhead) and the MARCKS protein (double arrows) migrated. NM was identified from its specific interaction with NM antibodies by Western blot analysis. In the margins the arrows indicate the direction of the protein migration. *a* and *c* represent the autoradiograms of the second dimensional gels. *d* and *e* represent the silver staining of the gels that correspond to the autoradiograms shown in *a* and *c*, respectively. In *a* and *d*, OLs were preincubated with 5 μM PMA for 20 h before <sup>32</sup>Pi addition. *b* shows the control phosphorylation experiment. In *c* and *e*, OLs were stimulated with 5 μM PMA for 20 min before protein extraction.

ulation of PKC activity by PMA was much more evident for the MARCKS protein which was found to incorporate about four times more radioactivity compared to the control as deduced by Cerenkov counting of protein bands cut out of the gel. Less than a twofold increase in NM phosphorylation was found in cells treated with PMA. This rather low stimulation in the phosphorylation of NM may explain why the silver staining pattern of the different NM forms did not significantly change when cells were treated with PMA (data not shown).

To compare the NM species in OL with the three major forms in neurons, we mixed the OL and neuronal cell extracts and separated the proteins by two-dimensional gel electrophoresis. The OL were preincubated with <sup>32</sup>Pi to label the glial NM form and they were treated with 1 μM PMA before protein extraction. After transferring the proteins to an Immobilon blotting membrane, the neuronal and OL NM spots were identified by immunostaining using NM antibodies and the OL NM spot was further characterized by autoradiography (Fig. 7). Differences were observed between the electrophoretic mobilities in LDS-PAGE of the neuronal and OL NM antigens. The NM form in OL showed a lower electrophoretic mobility as compared to the neuronal NM forms (Fig. 7, large arrowhead). This NM form of OL undergoes phosphorylation and has a pI in the range of the two basic forms found in neurons. In this experiment we also noticed the presence of a minor <sup>32</sup>P-labeled protein spot in the OL extract that migrated with a slightly more acidic pI than the phosphorylated NM protein spot (Fig. 7, small arrowhead). Its nature remains to be determined. The better resolution of the NM protein spots compared to Fig. 5 could be ex-

plained by a change in the batch of ampholine used (in this last experiment we used LKB ampholine pH 3.5–9.5 instead of pH 3.5–10). The difference in the electrophoretic mobility between the NM spots in Figs. 7 and 8 results from changes in the polyacrylamide concentration used for the second dimension PAGE (12% polyacrylamide instead of 12.5%).

#### *NM in OLs Is a Substrate for Endogenous PKC*

Because NM is specifically phosphorylated by PKC in vitro (Alexander et al., 1987; Baudier et al., 1989) and is a major PKC substrate in neuronal cells (Nelson et al., 1989) we extended the characterization of NM in OL by studying its phosphorylation in intact cells and the stimulatory effect of the phorbol ester PMA, known as a PKC activator. Phorbol esters stimulate PKC activity by increasing the translocation of the enzyme to the membrane and this results in an eventual proteolytic degradation or "downregulation" of the enzyme after long-term exposure of the cells to phorbol esters (Huang et al., 1989).

NM phosphorylation in OL incubated with <sup>32</sup>Pi was, therefore, studied in three different ways (Fig. 8, *a–c*). (*a*) Cells were incubated 20 h with 5 μM PMA, in order to "downregulate" endogenous PKC, before incubation with <sup>32</sup>Pi (Fig. 8 *a*); (*b*) the control experiment employed cells that had never been in contact with PMA (Fig. 8 *b*); (*c*) activation of endogenous PKC was achieved by stimulating cells with 5 μM PMA for 20 min (Fig. 8 *c*). To improve the resolution of the NM spot in the two-dimensional gel analysis of the 2.5% perchloric acid-soluble proteins from OL we applied a NEPHGE for the first dimensional IEF as described



under Materials and Methods. In this electrophoretic system the separation of the NM protein spot (Fig. 8) from the other phosphoprotein spots is improved (compare with Fig. 5). Phosphorylation of NM was greatly enhanced in OL when PMA was added to the cultures for 20 min (Fig. 8 c) as compared to controls (Fig. 8 b). NM phosphorylation was totally inhibited in OL preincubated with PMA for 20 h (Fig. 8 a). These results strongly support the notion that NM is probably a specific substrate for PKC in OL. Note also the stimulatory effect of PMA on the phosphorylation of the MARCKS protein (fivefold stimulation as deduced after scintillation counting of the proteins cut out of the gel). In Fig. 8, d and e, the silver staining of the gels corresponding to the autoradiograms in Fig. 8, a and c, respectively, are shown. They confirm that phosphorylation of NM in OL by PKC does not change significantly the electrophoretic mobility of the protein in the two-dimensional gel analysis. On the other hand, silver staining of the NEPHGE gels revealed differences in the electrophoretic mobility of the MARCKS protein when cells were stimulated with PMA suggesting that its phosphorylation by PKC shifts the pI of the protein to a more acidic value.

## Discussion

### *NM Is Present in O-2A Glial Cell Lineage*

In serum-containing cultures of cells dissociated from different areas of the postnatal rat central nervous system, two populations of astrocytes (GFAP<sup>+</sup>) can be distinguished on the basis of morphological, antigenic, and functional criteria (Raff et al., 1983, 1984; Ffrench-Constant and Raff, 1986; Levi et al., 1986; Aloisi et al., 1988a,b; Behar et al., 1988; Sontheimer et al., 1989): the epithelioid flat type 1 As and the stellate type 2 As with many radial processes. As the cultures become more confluent, the number of cells with a stellate shape decreases, and at confluency such cells are no longer seen. These two astrocyte populations belong to two different cell lineages. Type 2 As is derived from the bipotential glial O-2A progenitor cells, which express on their surface specific neuronal gangliosides binding the mAb A2B5. O-2A progenitor cells may also differentiate into OL in serum-free medium (Raff et al., 1983; Aloisi et al., 1988b; Behar et al., 1988). The timing of the differentiation of cultured O-2A precursors from the optic nerve is now thought to be regulated by growth factors secreted by the type 1 As and neurons (Noble et al., 1988; Richardson et al., 1988; Levine, 1989).

Our immunocytochemical and biochemical data demonstrate the presence of NM in O-2A progenitor cells and their derived type 2 As and OL, but not in type 1 As. These results suggest that the previously reported transient expression of NM in high density rat astrocyte cultures (Vitkovic et al., 1988), was probably due to a contamination of the main type 1 As population with type 2 As during the first 2 wk of culturing. This possibility is further substantiated by the stellate aspect of the astrocytes labeled with the anti-GAP-43 and anti-GFAP antibodies after 3 d in culture (Vitkovic et al., 1988), their shape is typical of type 2 As.

The identification of a new neuronal phenotype, NM, in O-2A progenitor cells and their derived type 2 As and OL and its absence in the type 1 As, strongly support the hypoth-

esis that neurons and bipotential glial cells are of a common cell lineage (for a review of glial cell lineages, see Raff and Miller, 1984; McKay, 1989). Thus, NM appears to be an interesting protein to study the relationship between cell lineages during development of the nervous system. In the same line it should be mentioned that chromaffin cells and ciliary ganglionic neurons which are both of neural crest origin also express significant amounts of NM (unpublished data).

NM was previously considered to be specific for neurons and was believed to be integral to neuronal growth and plasticity (Kalil and Skene, 1986; Meiri et al., 1986; Nelson et al., 1989). In agreement with a role for NM in neuronal plasticity, it has recently been reported that the protein, which is expressed in the olfactory system of neonatal rat in the entire population of developing olfactory receptor neurons, becomes progressively restricted in the adult rat to a subpopulation of precursor cells from the olfactory neuroepithelium that maintain their capacity to migrate, divide, and differentiate (Verhaagen et al., 1989). The accumulation of NM in O-2A cells may be also associated with the capacity of these cells to divide and differentiate in embryonic as well as in adult brain (Ffrench-Constant and Raff, 1986; Norton and Farooq, 1989) and suggests a more general function of this protein in the development of the central nervous system. Direct immunolocalization of NM in adult rat brain failed to detect this protein in nonneuronal cells (McGuire et al., 1988). The probable dispersion of O-2A progenitor cells through the brain would make them difficult to detect. In the case of type 2 As and OL, the expression of NM in the cultured cells is possibly associated with the properties of these cells to form processes during their development and differentiation. Indeed, when secondary cultures of OL were maintained for 2 wk in culture we noticed a significant decrease in the percentage of NM-positive cells associated with the morphological maturation of these cells. A systematic study on the expression of NM in OL through their development and maturation is currently under investigation in our laboratory.

### *Comparison between NM in OLs and Neuronal Cells*

The labeling of OL with NM antibodies is very similar to that observed for neuronal cells. The reaction product shows intense, discontinuous, punctate staining of the processes which has been attributed to the membrane attachment of the protein (Meiri et al., 1988). Biochemical characterization of the NM protein revealed, however, striking differences between the OL and neuronal cells. OL NM apparently migrates as a single protein spot in two-dimensional electrophoresis, whereas three distinct protein spots are resolved for neurons. Differences in the electrophoretic mobility of the neuronal and OL NM in the second dimensional LDS-PAGE were also observed.

We do not have an explanation for the slight variation in the migration of the neuronal and OL NM protein in LDS-PAGE. It is well known that the apparent molecular weight of NM in SDS-PAGE (43,000–57,000) is dependent on the concentration of polyacrylamide used in the gel and does not reflect the true molecular mass of the protein (24 kD) (Cimler et al., 1986). The identical pI of the neuronal and OL NM protein suggest that the differences in their apparent molecular weights arise from variations in protein conformation

and/or posttranslational modifications. Further studies are required to resolve this point.

The heterogeneity of neuronal NM has been previously mentioned (Skene and Virag, 1989). The two more acidic spots correspond to posttranslational modifications of the primary translation product and have been called "mature forms" of the protein. In OL, NM phosphorylation is strongly stimulated by the phorbol ester (PMA) but this does not result in a significant change in NM pI. In neurons, only the two "mature forms" of NM are phosphorylated, with a preferential <sup>32</sup>Pi incorporation into the more acidic form which is only slightly enhanced by PMA. This raises the possibility that other posttranslational modifications of the neuronal protein may regulate neuromodulin phosphorylation by PKC.

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