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Monoclonal Antibody (M2) to Glial and Neuronal Cell Surfaces

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A monoclonal antibody designated M2 arose from the fusion of mouse myeloma cells with splenocytes from a rat immunized with particulate fraction from early postnatal mouse cerebellum. Expression of M2 antigen was examined by indirect immunofluorescence on frozen sections of developing and adult mouse cerebellum and on monolayer cultures of early postnatal mouse cerebellar cells. In adult cerebellum, M2 staining outlines the cell bodies of granule and Purkinje cells. A weaker, more diffuse staining is seen in the molecular layer and white matter. In sections of newborn cerebellum, M2 antigen is weakly detectable surrounding cells of the external granular layer and Purkinje cells. The expression of M2 antigen increases during development in both cell types, reaching adult levels by postnatal day 14. At all stages of postnatal cerebellar development, granule cells that have completed migration to the internal granule layer are more heavily stained by M2 antibodies than are those before and in process of migration. In monolayer cultures, M2 antigen is detected on the cell surface of all GFA protein-positive astrocytes and on more immature oligodendrocytes that express 04 antigen but not 01 antigen. After 3 days in culture, tetanus toxinpositive neurons begin to express M2 antigen. The same delayed expression of M2 antigen on neurons is observed in cultures derived from mice ranging in age from postnatal day 0 to 10.

Key words: cell surface antigen, cerebellum, development, mouse, indirect immunofluorescence

An understanding of normal and abnormal brain development requires the ability to distinguish interacting subsets of neural cells. At the same time, it is necessary to be able to detect the developmental stages or relative maturity of these interacting cellular subsets. In this context, the study of cell surface molecules, which may be directly concerned with cell-cell interactions, is of particular interest. Conventionally prepared, polyclonal antibodies to neural cell surface antigens have shown that the expression of surface antigens may change during development and that several antigens are restricted to subclasses of neural cells [1]. With the advent of techniques to produce monoclonal antibodies, immunological analysis of brain development has reached an unprecedented stage of refinement, which has been used in this laboratory to study the development of the nervous system [2–4].

The cerebellar cortex of the mouse is a particularly favorable region of the central nervous system to investigate cell–cell interactions of neural subclasses.

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It contains only five neuronal cell types which are organized in relatively simple geometric arrays repeated throughout the cerebellar cortex [5–7]. The connectivity of the neuronal cells is well understood and most transmitters have been identified. Some major events in neuron formation and migration as well as overt synaptogenesis take place postnatally so they become easily accessible to the investigator. In addition, a number of neurological mutants show selective neuronal death, perhaps based on developmental abnormalities in cell interaction [8,9].

To date, a limited number of antibodies have been described which detect cell surface molecules of the cerebellum. NS-4 antibody detects surface antigen(s) on neuronal cells and a minority of astrocytes and oligodendrocytes, in vitro [10]. The receptor for tetanus toxin, detected by sequential treatment with tetanus toxin and anti-tetanus toxin, is expressed on all neurons [10, 11]. Anti-bovine corpus callosum antiserum detects cell surface structures on cerebellar astrocytes and oligodendrocytes but not on neurons [12, 13]. At early postnatal ages, Thy-1 antigen is detectable on Purkinje cells and/or Golgi type II cells but not on smaller neurons, comprising granule, basket, and stellate cells. After several days in culture, some fibroblast-like cells and astrocytes also express Thy-1 antigen [10, 14]. Fibronectin expression is restricted to fibroblast and fibroblast-like cells [15]. Recently described monoclonal antibodies 01–04 are detected on the cell surface of oligodendrocytes. The 04 is expressed both on immature and more mature oligodendrocytes, while 01 antigen is found only on more mature oligodendrocytes [3, 4]. Galactocerebroside is detected on all 01 antigen-positive cells [3, 4]. Thus, with presently available antibodies, distinctions as to cell type and cell maturity are possible within cerebellar cell populations.

In this study, we describe the cellular localization and developmental expression of a new antigen designated M2 that is detected by a rat monoclonal antibody.

MATERIALS AND METHODS

Animals

C57BL/6J mice and Sprague-Dawley rats were obtained from and bred at the animal facilities of Theoretische Medizin of this university or of this department.

Cells and Cell Culture

Mouse myeloma P3 NS1/1Ag4-1 and hybridoma cell lines were maintained as previously described [2]. Primary monolayer cultures of early postnatal mouse cerebellar cells were maintained on polylysine-coated glass coverslips in Basal Medium Eagle's with Earle's salts, containing 10% horse serum [10]. All sera and media were obtained from Seromed, Munich, FRG.

Production of Monoclonal Antibody

M2 antibody producing hybridoma arose from the fusion of mouse myeloma cells with splenocytes from a Sprague-Dawley rat immunized with the particulate fraction from 6- to 8-day-old C57BL/6J mouse cerebellar homoge-

nates as previously described [2, 16]. Fusion was carried out also as previously described [2, 17].

Antibodies

Hybridoma supernatents containing monoclonal antibody M2 were concentrated 30-fold by precipitation with 45% saturated $(NH_4)_2SO_4$ and dialyzed against phosphate-buffered saline (PBS), pH 7.3. For immunolabeling, this concentrate was diluted 1:5. Monoclonal antibodies to 01 and 04 antigens have been described previously [3, 4]. Rabbit anti-GFA protein antibody was obtained from Dr. L. F. Eng. Rabbit anti-fibronectin antibody was obtained from Dr. R.O. Hynes. Tetanus toxin was obtained from Dr. E. Habermann. Rabbit anti-tetanus toxin antibody was obtained from Dr. V.R. Zurawsky. Sheep anti-rat immunoglobin (Ig), referred to in this text as SARat, rabbit anti-rat Ig (RARat), and rabbit anti-mouse Ig (RAM) were purified with DEAE cellulose [18] and coupled with either fluorescein isothiocyanate (FITC) or tetramethylrhodamine-isothiocyanate (TRITC) as described [18]. Goat anti-rabbit Ig coupled with TRITC (TRITC-GAR) was obtained from Nordic Immunology, Tilberg, The Netherlands. For double immunofluorescence utilizing antibodies to M2 (originating from rat) and antibodies to 01 and 04 (originating from mouse) it was necessary to prepare species-specific fluorescent second antibodies. For this purpose, Affi-Gel 10 (Bio-Rad, Munich, FRG) was coupled with either purified mouse or rat Ig (see below), at 5 mg protein/ml of Affi-Gel 10, following the protocol suggested by the manufacturer. To remove undesirable cross-reactive antibody, approximately 5 ml of FITC-RARat containing 5 mg protein/ml, were passed three times over a 0.5 ml column containing Affi-Gel coupled mouse Ig. TRITC-RAM was similarly absorbed with immobilized rat Ig. Resultant antibodies showed species-specific reactivity as shown by indirect immunofluorescence. Mouse and rat Ig was obtained by passing serum of mice or rats that had been immunized with hen ovalbumin (Sigma, Munich, FRG) over an Affi-Gel 10 column coupled with ovalbumin (10 mg protein/ml gel). After washing with 10-column volumes of PBS containing 0.1% NP 40 (Roth, Karlsruhe, FRG), Ig was eluted with 0.1 M diethylamine HC1, pH 11.5, and dialyzed against 0.2 M $NaHCO_3$, pH 8.0, for Affi-Gel 10 coupling.

Immunohistological Procedures

Indirect immunofluorescence on fresh frozen sagittal sections of early postnatal and adult mouse cerebellum and on cultured cerebellar cells was carried out as described previously [10, 19]. Cultures were routinely fixed with 4% paraformaldehyde at room temperature for 5 min after incubation with the first antibody. To facilitate access of antibodies to intracellular antigens, fixed cultures were dipped for 1 min into 100% ethanol at –20°C. To test for stability of M2 antigen, the above described treatments with paraformaldehyde or ethanol were performed prior to treatment with M2 antibody.

RESULTS

Localization of M2 Antigen in Sections of Mouse Cerebellum

Adult cerebellum. In sagittal sections of fresh frozen adult mouse cer-

ebellum, M2 antigen was detectable by indirect immunofluorescence around cell bodies of two neuronal cell types: granule cells and Purkinje cells (Fig. 1). Both the molecular layer and white matter showed a weaker, more diffuse staining of M2 antigen that was difficult to associate with specific structures in these areas. The relative intensity of fluorescent staining in the molecular layer was, however, much greater than that in the white matter. In the deep cerebellar nuclei, large neurons were strongly outlined by anti-M2 antibody. M2 antigen was also detectable in other brain regions, again apparently surrounding neuronal cell bodies.

Neonatal and early postnatal cerebellum. In newborn mice, M2 antigen was barely detectable by indirect immunofluorescence, both in cerebellum and in other brain regions. At this age, immunofluorescence staining could only weakly be detected around neurons. At postnatal day 5, M2 antigen was detectable on cells in the external granular layer. Its expression was more pronounced on granule cells in the internal granular layer (Fig. 2). M2 antigen seemed to be more strongly expressed on Purkinje cells than around granule cells during the second postnatal week. Adult levels of M2 antigen in cerebellum were attained by postnatal day 14, as detected by immunofluorescence.

Localization of M2 Antigen on Monolayer Cultures of Early Postnatal Mouse Cerebellum

To determine whether M2 antigen was expressed at the cell surface, monolayer cultures of postnatal day 6 mouse cerebellum were examined by indirect immunofluorescence after 2–7 days of maintenance in vitro. M2 antigen was present on live cultured cells, indicating that it was localized on the cell surface. M2 antigen remained detectable when cells were fixed with 4% paraformaldehyde prior to antibody incubation. Prior treatment of cultures with

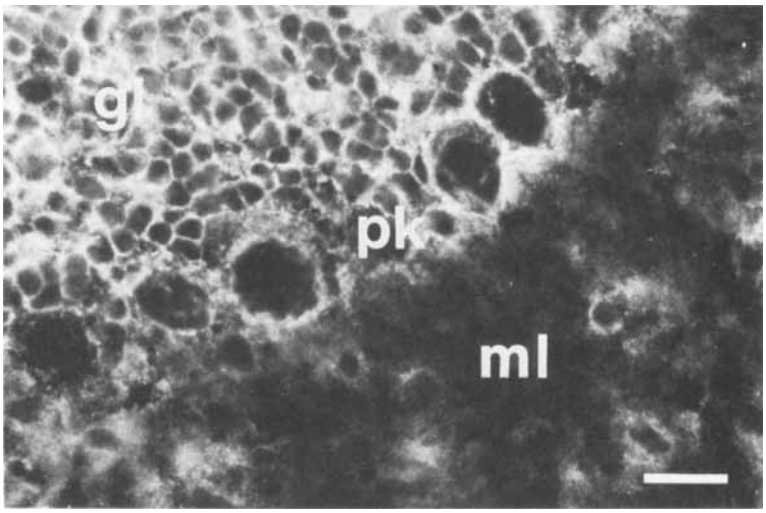

Fig 1 Immunofluorescent staining of M2 antigen in 20-day-old mouse cerebellum M2 antigen is visualized using FITC SARat (see Materials and Methods) gl, granule layer, ml, molecular layer, pk, Purkinje cell layer Bar=20 μm

ethanol (as described in Materials and Methods) prevented detection of M2 antigen.

To identify cell types that expressed M2 antigen, double immunolabeling experiments were carried out using antibodies directed against the following cell type-specific markers: GFA protein served as a marker for astrocytes [20, 21], fibronectin, for fibroblasts or fibroblast-like cells [15], tetanus toxin for neurons [10, 11], 01 antigen as a marker for more mature oligodendrocytes, and 04 antigen as a marker for immature and more mature oligodendrocytes [3, 4]. During the first 3 days in culture, M2 antigen was confined to non-neuronal cells. All GFA protein-positive astrocytes showed uniform cell surface staining with M2 antibody (Fig. 3). A subpopulation of oligodendrocytes that expressed 04 antigen was also M2 positive (Fig. 4). M2 was rarely found on more mature oligodendrocytes that expressed 01 antigen (Fig. 5).

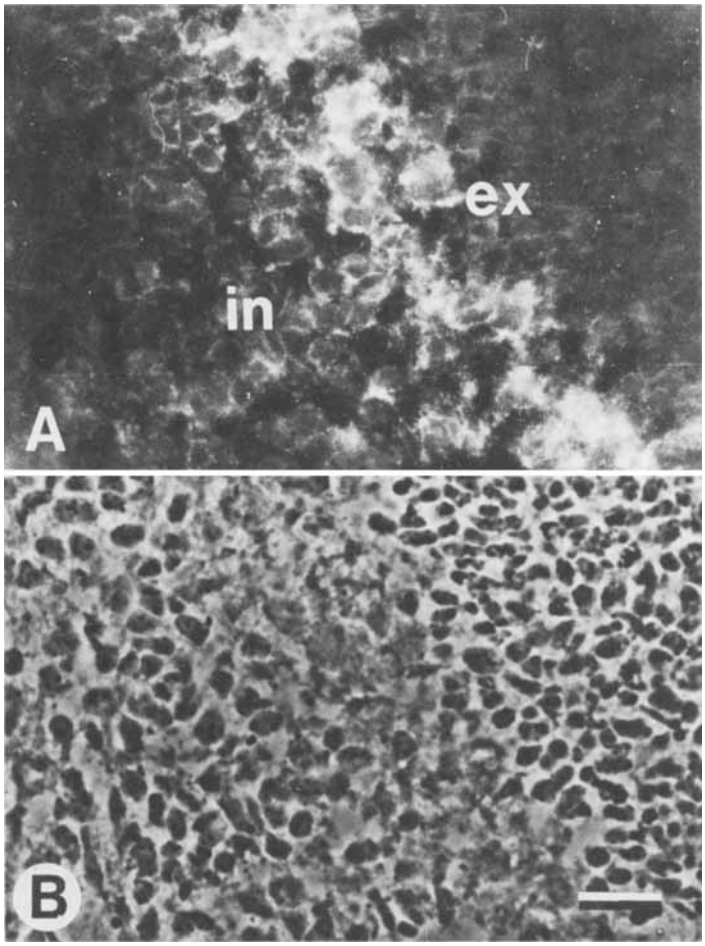

Fig. 2. Immunofluorescent staining of M2 antigen in 5-day-old mouse cerebellum. A) Immunofluorescence detection of M2 antigen. M2 antigen is visualized using FITC SARat: ex, external granular layer; in, internal granular layer. B) Phase contrast micrograph of identical visual field. Bar=20 μm.

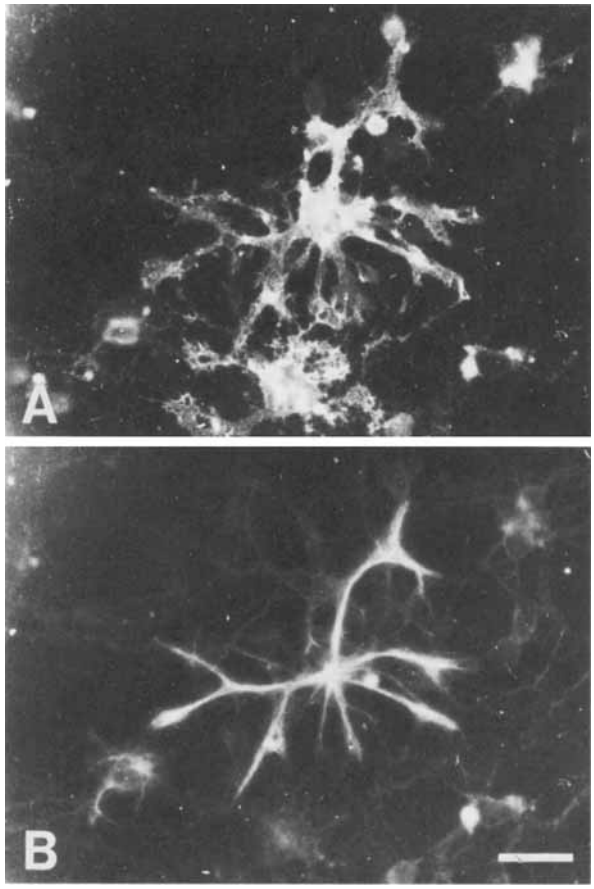

Fig. 3. Immunofluorescent staining of M2 antigen and GFA protein in monolayer cultures of cerebellar cells from 6-day-old C57BL/6J mice. A) M2 antigen is visualized using FITC SARat. B) GFA protein is visualized using TRITC GAR. Note the additional M2 antigen positive, GFA protein negative cell, possibly an immature oligodendrocyte (see Fig. 4). Bar = 20 μm.

M2 antigen was not expressed on tetanus toxin-positive neurons (ie, cells bearing the receptors for tetanus toxin) during the first 3 days of culture (Fig. 6). With 4 or more days in culture, M2 antigen was also detectable on tetanus toxin-positive neurons (Fig. 7). After 4 days in culture many small neurons, possibly mostly granule cell neurons, began to express M2 antigen, particularly where several neuronal cell bodies were contacting each other in clusters (Fig. 7). By 5 days in culture, most but not all tetanus toxin-positive neurons had become M2 positive. After 6 days in culture, only rare tetanus toxin-positive neurons could be found that were not M2 antigen-positive. After 5 days in culture, M2 antigen expression on neurons was much stronger than that on glial cells (for comparison, see Figs. 6 and 7 which show M2 antigen-positive and

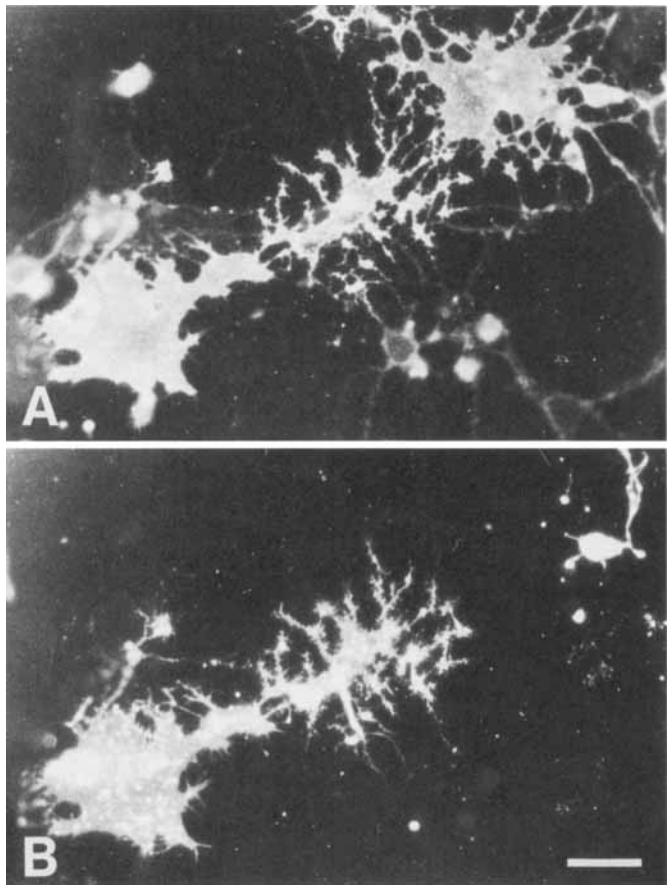

Fig. 4. Immunofluorescent staining of M2 and 04 antigens in monolayer cultures of cerebellar cells from 6-day-old C57BL/6J mice. A) The M2 antigen is visualized using FITC RARat. B) The 04 antigen is visualized using TRITC RAM. Note the additional M2 positive cell, possibly an astrocyte (see Fig. 3). Bar = 20 μm.

tetanus toxin-negative cells with epitheloid morphology). Relative to the distribution of tetanus toxin receptors on neuronal cell surfaces, M2 antigen was confined more to neuronal cell bodies and was less readily detected on neuronal processes (Fig. 7). Fibroblasts or fibroblast-like cells that expressed fibronectin did not express detectable levels of M2 antigen (results not shown).

Comparison of age of mice used for culture. To determine whether the expression of M2 antigen was dependent on the age of mice used to prepare cerebellar cultures, cultures from day 0 and 10 mice were compared to those derived from 6-day-old animals. Cultures from 0-, 6-, and 10-day-old mice showed essentially identical patterns of M2 antigen expression. In all cases, M2 antigen was detectable exclusively on glial cells during the first 3 days of cul-

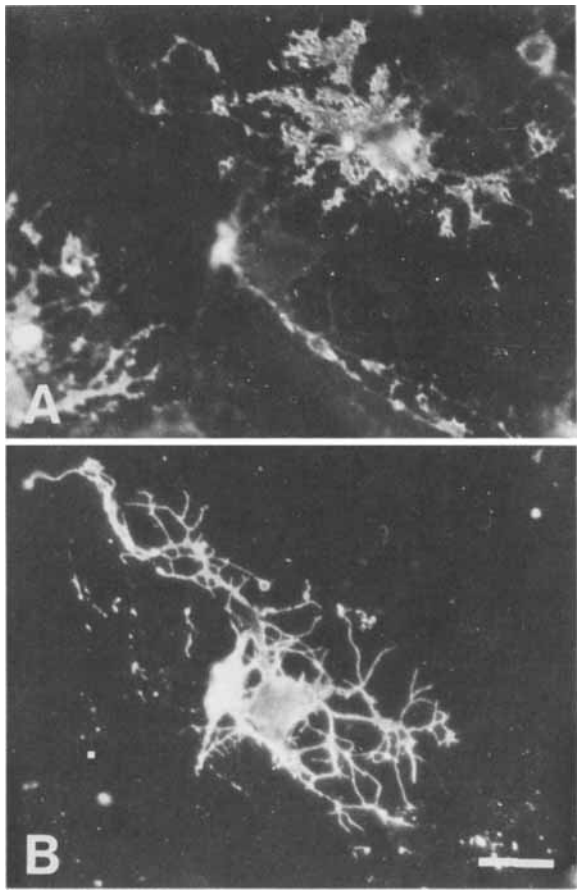

Fig. 5. Immunofluorescent staining of M2 and 01 antigens in monolayer cultures of cerebellar cells from 6-day-old C57BL/6J mice. A) The M2 antigen is visualized using FITC RARat. B) The 01 antigen is visualized using TRITC RAM. Bar=20 μm.

ture, whereas neuronal cells began to express M2 antigen after 4 days of maintenance in vitro.

DISCUSSION

In histological sections of developing and adult mouse cerebellum, M2 antigen is most prominently expressed in areas rich in neuronal cell bodies, such as granular and Purkinje cell layers. This observation is in agreement with the findings that on cultured cerebellar neurons, M2 antigen is predominantly present on cell bodies, and less on cellular processes. The presence of M2 antigen on astrocytes and the more immature oligodendrocytes is well detectable in culture, but is less easily documented in histological sections, where unequivocal staining of astrocytes or oligodendrocytes is not apparent. It is difficult to judge from immunofluorescence methods at the light-microscopic

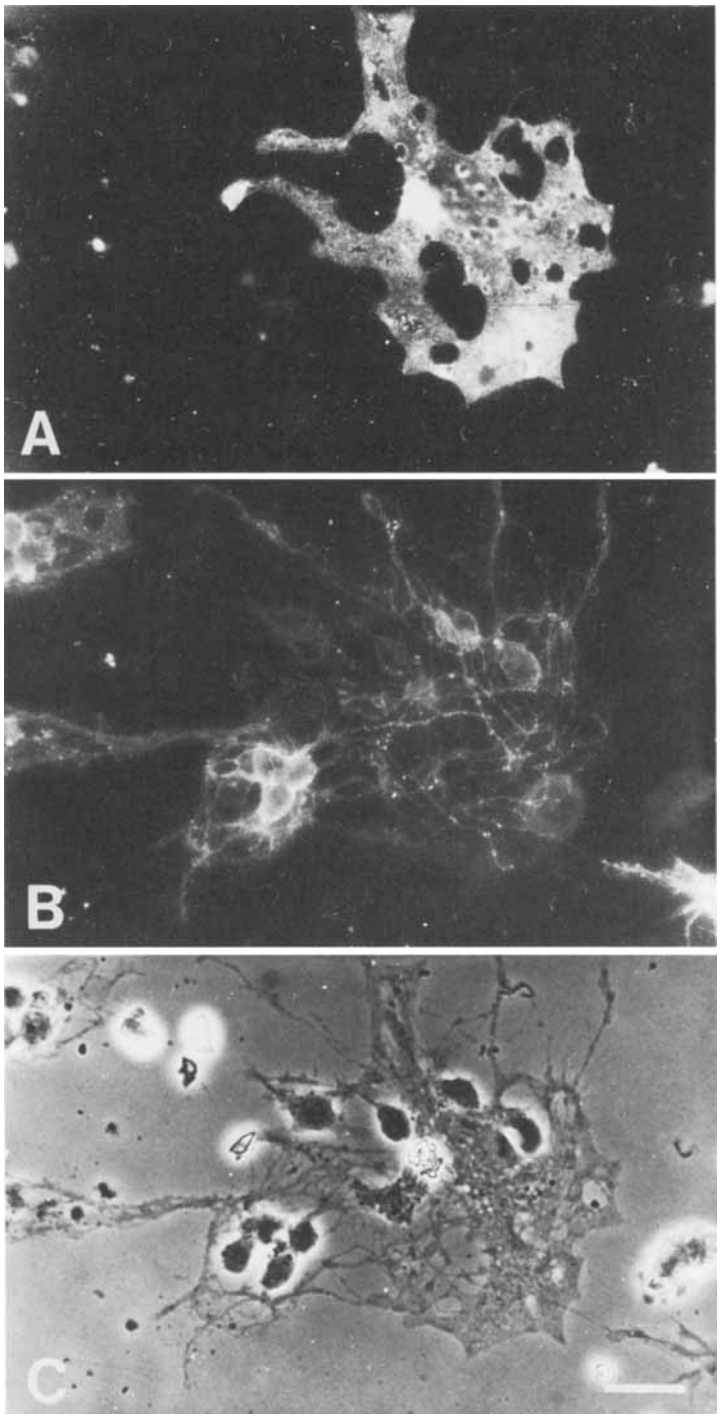

Fig 6 Immunofluorescent staining of M2 antigen and tetanus toxin receptors Cells from 6 day old C57BL/6J mouse cerebellum maintained for 3 days in culture A) M2 antigen is visualized using FITC SARat B) Tetanus toxin is visualized using TRITC GAR and rabbit anti tetanus toxin Bar=20 μm

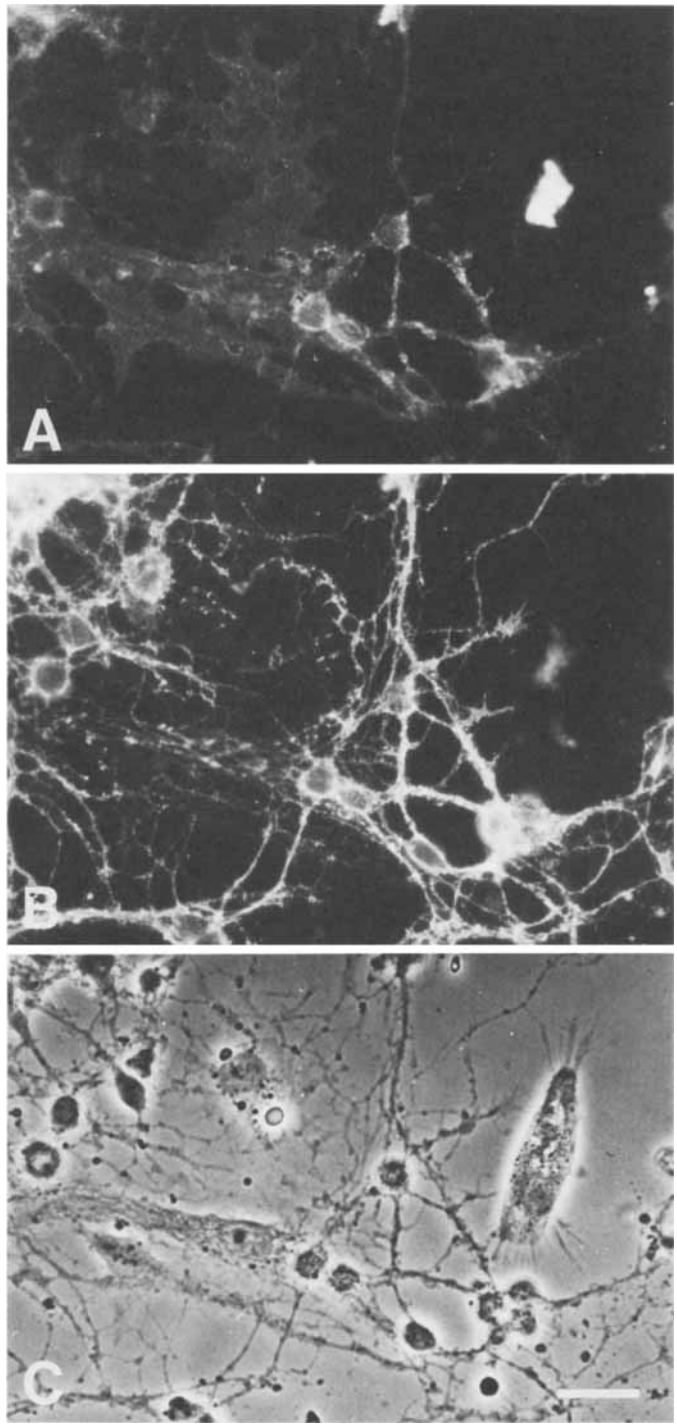

Fig 7 Immunofluorescent staining of M2 antigen and tetanus toxin receptors Cells from 6-day old C57BL/6J mouse cerebellum maintained for 6 days in culture stained for M2 antigen and tetanus toxin as indicated in Figure 6 A) M2 antigen B) Tetanus toxin Note that M2 antigen is more weakly expressed on neuronal processes than are tetanus toxin receptors Bar=20 μm

levels alone whether diffuse labeling of white matter and molecular layer represents antigen expression on glial cell surfaces.

The timing and levels of M2 antigen expression are not congruent when compared between cerebellar cells in situ and in culture. At 4 days of age, M2 antigen is only very weakly detectable in histological sections, whereas in cultures of neonatal cerebellum it is distinctly seen on small neurons by indirect immunofluorescence after 4 days in vitro. From day 6 onwards M2 antigen is more strongly expressed on increasing numbers of postmigratory granule cells as can be seen in histological sections. In vitro, however, the day and degree of onset of M2 antigen expression is the same in cultures of 6- or 10-day-old as of neonatal cerebella.

It could be speculated that neurons are induced to express M2 antigen once they have become firmly attached to a culture substrate or are in close contact with neighboring cells which is the case, for instance, with postmigratory granule cells when they take residence in the internal granular layer. The molecular mechanisms underlying differential timing of M2 antigen expression on neurons and on cultured glia which reveal detectable levels of M2 antigen within 24 hours of culture time remain at present obscure, but will be the topic of further investigations.

It is noteworthy, and equally unexplained at present, that M2 antigen is expressed on most if not all neurons and all more mature GFA protein-containing astrocytes, but not on all oligodendrocytes. Furthermore, among oligodendrocytes M2 antigen is predominantly present on the more immature oligodendrocytes, which are 04 antigen-positive, but 01 antigen-negative [3, 4]. Thus, M2 antigen serves as an additional marker for less differentiated oligodendrocytes within the 04 antigen-positive population of oligodendrocytes. It will be pertinent to determine if M2 antigen is also expressed by more immature, GFA protein-negative astroglia. Further characterization of M2 antigen concerning expression at embryonic ages, involvement in anchoring of neurons, definition of its molecular nature and ultrastructural localization may shed light on the antigen's functional significance during development.

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