Published in final edited form as:

Dev Neurosci. 2000 ; 22(0): 154–166.

Characterization and Distribution of a New Cell Surface Marker of Neuronal Precursors

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Abstract

In a screen for novel cell surface markers of neuronal progenitors, we recently identified mAb 2F7 that recognizes an epitope present on both progenitor cells and postmitotic neurons, in the developing CNS and PNS. In the embryonic rat telencephalon, the mAb 2F7 epitope is expressed by migratory and postmigratory neurons in the developing cerebral cortex, as well as by presumptive neuronal progenitor cells of the ventricular zone. In the neonatal forebrain mAb 2F7 labels postmitotic neurons, including those of the developing cerebral cortex and olfactory bulb, as well as the axons of the corpus callosum. While mAb 2F7 immunoreactivity is present on only a low density of the neuronal progenitor cells situated in the anterior part of the subventricular zone, a progressively higher proportion of cells forming the rostral migratory stream express this epitope. mAb 2F7 labels the surfaces of neurons and neuronal precursors, but not mature oligodendrocytes and astrocytes in primary cultures derived from the rat neural tube. In vivo, migrating neural crest cells, motor neurons, and axonal projections associated with the spinal cord express the mAb 2F7 epitope. Immunoblot analyses reveal that the mAb 2F7 epitope resides on several high-molecular-weight, membrane-associated proteins, and is likely to be composed of N-linked carbohydrate. These findings suggest that mAb 2F7 recognizes a novel epitope that is present on progenitor cells and postmitotic, differentiating neurons in the developing mammalian nervous system.

Keywords

Neuronal progenitor; Neuronal differentiation; Spinal cord; Forebrain; E-NCAM; HNK-1

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Introduction

The appropriate development of the nervous system requires not only the acquisition of positional information, but also the specification of cell types. Cell type specification is believed to be a process of sequential restriction in developmental potential. Indeed, cell types corresponding to several stages in the differentiation process, and pluripotent stem cells that can generate neurons, astrocytes and oligodendrocytes, have been identified [reviewed in Kalyani and Rao, 1999]. More restricted precursors that specifically generate neurons or glial cells have also been described [Raff et al., 1983; Kalyani et al., 1998; Luskin and McDermott, 1994; Luskin et al., 1997; Ray and Gage, 1994]. In the developing telencephalon, neuronal progenitor cells of the ventricular zone have been identified as early as the onset of cortical neurogenesis [Luskin et al., 1993]. Moreover, direct lineage relationships between stem cells and intermediate precursors have been established [Mayer-Proschel et al., 1997; Rao et al., 1997].

Elucidation of the mechanisms underlying cell type specification in the developing nervous system has been hampered by the absence of precursor-specific cell surface markers that can be used for the isolation of particular cell populations. For example, no cell surface markers for pluripotent cells exist, and only two monoclonal antibodies (mAbs), one specific for highly polysialated NCAM (or E-NCAM) and mAb A2B5, which distinguish neuronal and glial lineages, respectively, have been described as lineage-specific markers [see for example Mayer-Proschel et al., 1997; Rao et al., 1997]. While both of these mAbs can be used to isolate precursors at specific stages of development, neither is particularly specific at later developmental ages. In particular, E-NCAM immunoreactivity is present on neuronal precursors at early embryonic ages in the rat [see Mayer-Proschel et al., 1997], but also identifies glial precursors [Ben-Hur et al., 1998] and a pluripotent stem cell [Marmur et al., 1998], at later stages of development. Likewise, A2B5, a marker for glial precursors in the developing rat optic nerve [Raff, 1989], also labels specific neuronal cells in distinct regions of the developing brain [Eisenbarth et al., 1979].

In contrast to the paucity of neural markers, a large battery of lineage-specific cell surface epitopes (Thy1, Sca1, CD4, etc.) have been identified in the hematopoietic system [reviewed in Matthews et al., 1991; Spangrude et al., 1988]. These markers have proven to be invaluable reagents for identifying, isolating [Matthews et al., 1991; Spangrude et al., 1988] and elucidating the developmental potential [Geiger et al., 1998; Ikuta et al., 1990] of a variety of progenitor populations in the immune system. The documented differences in adhesive properties, cytokine dependence and receptor expression [Kalyani and Rao, 1999; Rao, 1999] between early neuronal precursors strongly suggest that these progenitors also express a number of unique markers on their surfaces.

A straightforward approach towards identifying novel, cell type-specific markers in the developing mammalian central nervous system (CNS) involves the production of mAbs. We recently used the cyclophosphamide immunosuppression method [Matthew and Patterson, 1983; Ou et al., 1991] to bias the immune response towards cell surface antigens present in NT2/D1 cells induced to express neuronal properties by retinoic acid (RA) treatment [Andrews, 1984; Pleasure and Lee, 1993]. Our studies demonstrate that one of the

antibodies generated in these screens, mAb 2F7, labels multiple neuronal/axonal populations in the embryonic rat and chick spinal cord and peripheral nervous system (PNS), as well as neurons and presumptive neuronal progenitor cells in the embryonic and neonatal forebrain.

Materials and Methods

mAb Production

The mAb production protocol was as previously described [Tole et al., 1995]. The hybridoma secreting mAb 2F7 [see Zhu et al., 1998] was subcloned by limiting dilution, and its isotype was determined to be an IgM by the use of the Mouse Monoclonal Antibody Isotyping kit (Sigma Immunochemicals). Undiluted hybridoma supernatant consisting of Iscove's modified Dulbecco's medium (Gibco BRL), 10% Fetal Clone I, bovine serum (Hyclone) and penicillin-streptomycin (Cellgro, Mediatech) was used as the source of mAb 2F7 for all experiments.

Cell Culture

NT2/D1 (clone D1 of the NTERA-2 teratocarcinoma) cells were obtained from P.W. Andrews, The Wistar Institute of Anatomy and Biology [Andrews, 1984; Andrews et al., 1984] and were cultured as previously described [Tole et al., 1995]. Pluripotent neuroepithelial (NEP) cells, neuron-restricted (NRP) and glial-restricted (GRP) precursor cells, and cell lines were cultured as previously described [Mayer-Proschel et al., 1997].

Immunohistochemistry

Cryosections—Inbred Sprague-Dawley rats were mated overnight and the females checked for vaginal plugs in the morning. The day of conception, embryonic day 0 (E0), was signified by the appearance of a positive vaginal plug. E22 was considered equivalent to postnatal day 0 (P0). E12–E22 embryos were removed from the uterus of deeply anesthetized pregnant females. Chick embryos were removed from properly staged eggs (Spafas, Inc.). The rat and chick embryos that were not used for the analysis of the forebrain were immersion fixed in cold 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.4, and cryoprotected in sucrose as previously described [Kaprielian et al., 1995]. Rat embryos (E15–E21) and P0–P2 pups (anesthetized by ether inhalation) to be used for the analysis of the forebrain, were perfused transcardially with the same fixative, and then the brains were dissected and postfixed at 4°C and equilibrated with 20% sucrose for 24 h. Whole embryo and brains were embedded in OCT (Miles Inc., Ind., USA), frozen with liquid nitrogen, and cut on a cryostat at various thicknesses.

The staining of the whole embryo-containing section (fig. 1) was performed exactly as previously described [Zhu et al., 1998]. The staining of the non-brain-containing chick and rat embryo sections was performed as previously described [Kaprielian and Patterson, 1993] with mAb 2F7 and a mAb specific for HNK-1 (mouse IgM; supernatant; ATCC), and a fluorescein-conjugated secondary antibody (Chemicon; anti-IgM; 1:200). Sections were coverslipped in the presence of Pro-Long Antifade (Molecular Probes). Micrographs were generated using a Nikon TE300 inverted research microscope equipped with a Nikon N70

camera back and Kodak Elite Chrome 400 film. Color slides were scanned on an Agfa Duoscan flatbed scanner.

The forebrain-containing sections were stained as previously described [Menezes and Luskin, 1994] using the following primary antibodies: neuron-specific polyclonal anti- β -tubulin class III (TuJ1 clone; mouse IgG 1:200, supplied by A. Frankfurter, University of Virginia, Charlottesville, Va., USA); anti-N-CAM (rabbit IgG 1:500, supplied by J. Sanes, Washington University, St. Louis, Mo., USA); mAb 2F7 (supernatant), mAb HNK-1 (supernatant), and secondary antibodies: FITC-conjugated goat anti-mouse IgG 1:200 (Jackson ImmunoResearch Labs, Pa., USA); rhodamine-conjugated goat anti-rabbit IgG 1:200 (Jackson ImmunoResearch Labs). For double labeling, the sections were incubated together in both primary antibodies and then in the corresponding secondary antibodies. Laminar boundaries were confirmed by staining adjacent sections with cresyl violet. The sections were examined by confocal microscopy (Zeiss Axiovert equipped with LSM 510) and the images captured. All images were processed using Adobe Photoshop (Adobe Systems, Mountain View, Calif., USA).

Cultured Neuronal and Glial Cells—Staining procedures were as previously described [Rao et al., 1997]. Staining for the cell surface markers mAb 2F7, HNK-1 and galactocerebroside (GalC) was carried out in living cells. To stain cells with antibodies against cytoplasmic antigens (GFAP and type III β -tubulin) cultures were first fixed with 2% formaldehyde for 20 min at room temperature. Double labeling experiments were performed by simultaneously incubating cells in appropriate combinations of primary antibodies followed by non cross-reactive secondary antibodies. Bisbenzimidazole (DAPI) histochemistry was performed after immunolabeling had been completed, and as previously described [Kalyani et al., 1997]. The following primary antibodies were used: anti-glial fibrillary acidic protein (GFAP; SMI.21; mouse IgG; 1:100 dilution of supernatant; Sternberger Monoclonal, Inc.), anti-GALC (mouse IgG; 1:1 dilution of supernatant; ATCC), and anti- β -tubulin class III (SDL.3D10; mouse IgG; 1:200 dilution of supernatant; Sigma Immunochemicals). Secondary antibodies were purchased from either Jackson Immunologicals or Southern Biotechnology.

Protein Preparations—Cytoplasmic (or soluble) and membrane/cytoskeletal fractions of embryonic and adult rat and embryonic chick tissues, and NT2/D1 cells were generated exactly as previously described [Tole et al., 1995]. Membrane/cytoskeletal fractions were further separated into detergent-soluble and detergent-insoluble fractions exactly as previously described [Zhu et al., 1998].

Electrophoresis and Immunoblotting—One-dimensional SDS-PAGE (12%) and subsequent immunoblot analyses were performed exactly as previously described [Zhu et al., 1998].

Enzymatic Deglycosylation—Membrane/cytoskeletal detergent-soluble NT2/D1 protein preparations (450 mg) were subjected to deglycosylation using Endoglycosidase-F (25 U; Boehringer Mannheim) as previously described [Chuang and McMahon, 1999].

Results

Generation of mAb 2F7

We used the cyclophosphamide immunosuppression method to generate mAbs that preferentially recognize cell surface antigens expressed in RA-treated NT2/D1 cells as previously described [Tol et al., 1995]. The resultant hybridoma supernatants were first screened for binding to sagittal cryosections containing large regions of the E16 rat CNS. One of the antibodies generated in this fusion, mAb 2F7, was chosen for further analysis since it strongly and rather specifically labeled the developing rat CNS along its entire rostrocaudal extent (fig. 1). Subsequent screening demonstrated that mAb 2F7 labels the surfaces of RA-treated NT2/D1 cells (data not shown).

Spatiotemporal Distribution of the mAb 2F7 Epitope in the Developing Nervous System

Embryonic Rat and Chick Neural Tube and Spinal Cord—In the early embryonic rat neural tube, specific mAb 2F7 immunoreactivity is first detectable at embryonic day (E)11.5 small cell bodies and their processes situated in the ventrolateral regions of the neural tube (likely to be young motor neurons). In addition, neural crest cells migrating adjacent to the neural tube at caudal, but not rostral, levels of the neuraxis, are labeled at E11.5 (data not shown). In a chick embryo at a similar stage of development (E2, HH 12) [Hamburger and Hamilton, 1951], mAb 2F7 intensely labels migrating neural crest cells at both rostral and caudal levels of the neuraxis and the outer surface of the notochord (fig. 2A). Interestingly, this labeling pattern overlaps considerably with the distribution of the HNK-1 epitope [see Jungalwala, 1994] (fig. 2C). In E12 rat embryos, both mAb 2F7 and HNK-1 intensely label cell bodies and processes in the ventral horn, the lateral exit points, and the ventral roots associated with the spinal cord at trunk levels (fig. 2B, D). During later stages of embryogenesis, E5 (HH 26) in the chick (fig. 2E) and E14 in the rat (fig. 2F), mAb 2F7 brightly labels the spinal cord at all rostrocaudal levels with a similar distribution to that observed in E12 rat embryos. In contrast to the overlapping patterns of labeling present within the spinal cord of chick and rat embryos, mAb 2F7 brightly labels chick (fig. 2E), but not rat (fig. 2F), dorsal root ganglia.

Embryonic Telencephalon—To determine whether the mAb 2F7 epitope is also expressed by young neurons in the mammalian brain, we analyzed its spatiotemporal pattern of expression in the laminae of the developing cerebral cortex during the period of rat cortical neurogenesis E14–E20 [Raedler and Raedler, 1978], and at two subsequent time points: E22 (or P0) and P2. The earliest generated neurons of the cerebral cortex migrate away from their site of origin in the ventricular zone and become subdivided into the marginal zone and subplate [Luskin and Shatz, 1985]. The later generated neurons, destined progressively for the more superficial layers of the cortical plate, similarly migrate away from the ventricular zone. At all embryonic ages examined a similar laminar pattern of expression of the mAb 2F7 epitope was observed. Although the highest levels of expression were always observed in the cells of the subplate and marginal zone (fig. 3A, C), containing the Cajal-Retzius neurons, the mAb 2F7 epitope was expressed in all layers. Even a majority of the cells in the ventricular zone, where cell division occurs, express this epitope. However, in the apical half of the ventricular zone, where the cells are in S-phase of the

mitotic cycle, there was less intense mAb 2F7 staining in comparison to cells of the basal half of the ventricular zone (fig. 3A). Overall, these findings suggest that at least a subset of the mAb 2F7-positive progenitor cells have become committed to the neuronal lineage. We compared the expression of the mAb 2F7 epitope with the labeling of TuJ1, an antibody that recognizes neuron-specific tubulin [Lee et al., 1990; Sullivan, 1988]. TuJ1 was previously shown to be expressed by the postmitotic neurons of the PNS and CNS, and is one of the earliest markers of a differentiated neuron [Easter et al., 1993; Menezes and Luskin, 1995]. The actively dividing cells of the cortical ventricular zone (i.e., those that incorporate the cell proliferation marker BrdU) do not express TuJ1 [Menezes and Luskin, 1994]. However, a small proportion of the cells in the ventricular zone do express TuJ1. Most likely they are neurons that have recently exited the cell cycle and are beginning their migration to the cortical plate. Similar to the staining pattern obtained with mAb 2F7, the cells of the marginal zone and subplate are the most intensely labeled by TuJ1. Our double labeling studies demonstrate that in the developing telencephalon virtually all of the cells that express the neuron-specific marker TuJ1 also express the mAb 2F7 epitope. However, many of the 2F7-positive ventricular zone cells are TuJ1-negative (fig. 3C). The presence of the mAb 2F7 epitope in the ventricular zone of the mammalian forebrain is in contrast to its relative absence from the ventricular zone of the developing spinal cord. Our findings suggest that mAb 2F7 is an earlier marker than TuJ1 of cells possessing a neuronal phenotype in the telencephalon.

Neonatal Rat Rostral Migratory Stream and Olfactory Bulb—We compared the expression of the 2F7 epitope in the embryonic telencephalic ventricular zone to its expression by the neuronal progenitor cells situated in the anterior part of the neonatal forebrain subventricular zone (SVZa). Unlike other CNS precursor cells, SVZa cells undergo division and also express a number of neuron-specific genes ordinarily found in postmitotic cells [Law et al., 1999, in press; Luskin et al., 1997; Menezes and Luskin, 1994]. In particular, the vast majority are MAP-2-positive and express neuron-specific type III β -tubulin recognized by the antibody TuJ1, but still incorporate the cell proliferation marker BrdU. Sections of the P0–P2 neonatal forebrain were double labeled with TuJ1 and mAb 2F7. In contrast to the nearly ubiquitous expression of TuJ1 by SVZa cells, mAb 2F7 labeled the SVZa more sparsely and predominantly in its dorsal half underlying the intensely TuJ1-positive/2F7-positive immunoreactive corpus callosum (fig. 4A, C). In order to determine when and if the SVZa-derived cells express 2F7, we examined the cells in the rostral migratory stream, the pathway SVZa-derived cells traverse to their final destination in the olfactory bulb. The level of expression of the mAb 2F7 epitope was considerably higher in the vertical and horizontal limbs of the rostral migratory stream than in the SVZa (fig. 4A, 5A, B). We observed a gradient of expression in the olfactory bulb; mAb 2F7 immunoreactivity was lower in the subependymal layer in the central part of the bulb and higher as the granule cell layer was approached (fig. 5C, D). We also compared the expression pattern of the mAb 2F7 epitope, in the pathway and olfactory bulb with the expression of neuron-specific tubulin and NCAM, a marker of differentiating neurons. There was prominent NCAM immunoreactivity in the SVZa, rostral migratory stream and in the middle of the bulb, as well as in the overlying neurons of the olfactory bulb (fig. 5C, D).

mAb 2F7 Labels Neurons and Neuronal Precursors, in vitro

The distribution of the mAb 2F7 epitope, in vivo, suggests that it is expressed on neuronal cell bodies early in embryonic development, and in axonal tracts at later developmental stages. To identify the specific cell types that express the mAb 2F7 epitope, we isolated precursor and differentiated cells from E13 rat neural tubes and compared the labeling of mAb 2F7 with that of cell type-specific markers. Virtually all type III β -tubulin-immunoreactive neuronal cells co-expressed the mAb 2F7 epitope (cf. fig. 6A and A'). In contrast, Gal-C-immunoreactive oligodendrocytes (fig. 6B) and GFAP-immunoreactive astrocytes (fig. 6C) do not express the mAb 2F7 epitope. mAb 2F7 immunoreactivity persisted on neurons maintained in culture for several weeks, and was not induced on cultured oligodendrocytes or astrocytes (data not shown).

mAb 2F7 Does Not Label Glial Cells or Their Precursors, in vitro

NEP, NRP and GRP cells have been isolated based on their differentiation potential and their expression of cell type-specific markers [Mayer-Proschel et al., 1997; Rao et al., 1997, 1998; Kalyani et al., 1998; Kalyani and Rao, 1999]. To determine whether mAb 2F7 could be used to distinguish between NEP, NRP and GRP cells, we examined the expression of the mAb 2F7 epitope on each of these populations, in vitro. mAb 2F7 labels many E-NCAM-selected NRP cells (fig. 6E), but only very few NEP cells (fig. 6D). Thus, it is possible that mAb 2F7 labeling identifies an NRP cell. Consistent with this notion, mAb 2F7 labels only a small number of A2B5-selected GRP cells (fig. 6F). To rule out the possibility that the immunoselected A2B5-positive population contains a population of contaminating neuronal precursors, we examined the expression of the mAb 2F7 epitope within an immortalized glial precursor cell line that we had previously generated [Mujtaba et al., 1998]. Consistent with the lack of significant labeling in the cultures containing GRP cells, mAb 2F7 recognizes only a very limited number of these immortalized cells (fig. 6G). Thus, mAb 2F7 may be a predominantly neuronal marker.

Biochemical Characterization of the mAb 2F7 Epitope

To identify the antigen(s) recognized by mAb 2F7, E14 rat whole spinal cord was first homogenized and separated into cytoplasmic and detergent-soluble and -insoluble membrane/cytoskeletal fractions (see Materials and Methods). By immunoblot analysis mAb 2F7 labels several high-molecular-weight proteins that are highly enriched in the detergent-soluble membrane/cytoskeletal preparation (fig. 7A, left). None of these proteins are labeled when mAb 2F7 is omitted from the analysis, or by other mAbs generated in the same fusion (data not shown). mAb 2F7 labels a similar pattern of high-molecular-weight proteins in both the E14 and adult rat spinal cord fractions (fig. 7B, left). While HNK-1 also labels several high-molecular-weight proteins in both the E14 and the adult fractions (fig. 7B, right), this antibody recognizes at least three lower-molecular-weight proteins that are specifically present in the embryonic preparation (fig. 7B, right). mAb 2F7 does not label these proteins, even when the loading is increased 3- to 5-fold (data not shown). Both mAb 2F7 and HNK-1 also recognize similar antigen profiles in detergent-soluble membrane/cytoskeletal fractions derived from whole E5 chick heads and trunks (fig. 7C). The heterogeneity and molecular weight range of the proteins recognized by mAb 2F7 suggest

that the corresponding epitope may be composed of carbohydrate. To test this possibility, we treated detergent-soluble membrane/cyto-skeletal fractions of NT2/D1 cells with Endoglycosidase-F (see Materials and Methods), and subjected the corresponding proteins to an immunoblot analysis. mAb 2F7, as well as HNK-1 [see Naegele and Barnstable, 1991] immunoreactivity is virtually abolished by the enzyme treatment (fig. 7D, left).

Discussion

mAb 2F7 was identified in a screen for novel antigens that are expressed in RA-treated NT2/D1 cells, as well as in the developing rat CNS [Tole et al., 1995]. We present evidence here that mAb 2F7 is a marker for both postmitotic neurons and neuronal progenitors. In particular, the mAb 2F7 epitope is expressed by migratory and postmigratory neurons in the developing cerebral cortex, most presumptive neuronal progenitor cells of the proliferative ventricular zone in the embryonic rat forebrain, and by postmitotic neurons and axonal projections in the neonatal rat forebrain. mAb 2F7 immunoreactivity is also present on the surfaces of migrating neural crest cells, developing motor neurons, and axonal projections within both the PNS and CNS. Furthermore, a substantial proportion of the neuronal progenitor cells traversing the rostral migratory stream (RMS) express mAb 2F7. The mAb 2F7 epitope resides on several high-molecular-weight cell surface proteins, and is likely to be composed of carbohydrate. The results of comparative immunohistochemistry and immunoblot analyses suggest that the mAb 2F7 and HNK-1 epitopes are likely to be quite similar.

Spatiotemporal Distribution of the mAb 2F7 Epitope, in vivo

mAb 2F7 is an Early Neuronal Marker in the CNS and PNS—Several aspects of the distribution of the mAb 2F7 epitope in the developing CNS and PNS suggest that it is an early neuronal marker. For example, mAb 2F7 may be the earliest identified marker of neurons in the developing forebrain. Extensive co-localization with TuJ1 was evident in the intermediate zone, through which immature neurons migrate from the ventricular zone to the cortical plate. In addition, many cells in the subplate, marginal zone and cortical plate expressed the mAb 2F7 epitope and neuron-specific tubulin. However, the prominent expression of the mAb 2F7 epitope in the ventricular zone suggests that this epitope precedes the expression of other known markers of newly generated neurons, and may also be expressed by the progenitor cells themselves. In the early chick and rat spinal cord, mAb 2F7 immunoreactivity is associated with motor neurons in the ventral horn [see Chen and Chiu, 1992] and migrating neural crest cells.

Neuronal Expression of the mAb 2F7 Epitope Persists throughout

Development—At later stages of development, mAb 2F7 immunoreactivity remains associated with neurons and axons. In the E14 rat, for example, mAb 2F7 labels the ventral horns, ventral funiculi, and ventral roots associated with the spinal cord, the optic nerve (data not shown), and DRG. At E16, strong mAb 2F7 immunoreactivity is associated with the entire rostrocaudal extent of the developing rat CNS. Moreover, in the neonatal rat forebrain, the postmitotic, postmigratory cells of the developing cerebral cortex and olfactory bulb display mAb 2F7 immunoreactivity. Similar patterns of mAb 2F7

immunoreactivity in membrane extracts of E14 and adult rat spinal cord are consistent with neuronal expression of the mAb 2F7 epitope persisting in the mature CNS.

The Onset of Neuronal Gene Expression in Forebrain Progenitor Cells—The expression pattern of the mAb 2F7 epitope exhibited by the progenitor cells of the embryonic telencephalon is different from that shown by the neuronal progenitor cells of the neonatal SVZa. In the embryonic brain the mAb 2F7 epitope is expressed by all layers of the developing cortex, including the ventricular zone where the progenitor cells reside. The expression pattern of the mAb 2F7 epitope was similar, but not identical, to the distribution of neuron-specific type III β -tubulin, reported to be among the earliest neuron-specific genes to be expressed by postmitotic neurons in most regions of the PNS and CNS [Easter et al., 1993; Memberg and Hall, 1995; Menezes and Luskin, 1994]. The main difference is that the extent of mAb 2F7 staining by the embryonic ventricular zone greatly exceeds that of TuJ1. mAb 2F7 labeling of numerous ventricular zone cells suggests the possibility that the mAb 2F7 epitope is expressed earlier by committed postmitotic neurons than TuJ1. Among the explanations also consistent with the ventricular zone labeling is that those ventricular zone progenitor cells that express the mAb 2F7 epitope have made the decision to express characteristics of a neuronal phenotype. Alternatively, only progenitor cells at a particular phase of the cell cycle express the mAb 2F7 epitope. This is consistent with our previously reported findings that neuronal and glial lineages diverge prior to the onset of cortical neurogenesis [Luskin et al., 1988, 1993]. In the remainder of the developing cortex, the mAb 2F7 and TuJ1 epitopes were often co-localized in the same cell (see fig. 3A–C), indicating that mAb 2F7 is a selective marker of cells with a neuronal identity.

Although we predicted that the neuronal progenitor cells of the SVZa would express the mAb 2F7 epitope since they express other neuron-specific markers, mAb 2F7 weakly labeled this cell population. There was increased expression of the mAb 2F7 epitope by the TuJ1-immunoreactive, MAP-2-immunoreactive neurons of the rostral migratory stream compared to the SVZa. Even higher levels of expression were detected on SVZa-derived cells once they reached their final destination, suggesting that as neurons become more mature, they may express higher levels of the mAb 2F7 epitope. Nevertheless, it is still not clear why the embryonic ventricular zone cells express the mAb 2F7 epitope while the TuJ1-positive SVZa cells do not. One possible explanation is that not all cell populations exhibit the same sequence of gene expression during neuronal proliferation and differentiation. In some cases, such as for the SVZa cells, neuron-specific tubulin is turned on even as cells are proliferating, and only much later is the mAb 2F7 epitope expressed. In other cell populations (e.g., the ventricular zone progenitor cells), expression of the mAb 2F7 epitope precedes TuJ1 expression.

Cell Type-Specific Expression of the mAb 2F7 Epitope, in vitro

The distribution of the mAb 2F7 epitope in vivo suggests that it is a relatively specific marker of neuronal cells. Consistent with this conclusion, mAb 2F7 immuno-reactivity appears to be predominantly associated with neuronal cells in vitro, as no expression is seen on oligodendrocytes and only a very small number of glial precursors are positive, even after long-term culture. In particular, the mAb 2F7 epitope is expressed on most E-NCAM-

selected NRP cells, but only on very few NEP cells, in cultures derived from the developing rat neural tube. These findings suggest that mAb 2F7 may be used to distinguish neurons and neuronal precursors from NEP cells, oligodendrocytes and astrocytes, in vitro.

We have previously shown that E-NCAM expression is present on NRP, but not NEP cells, and that this marker can be used to select NRP cells at early stages of development [Mayer-Proschel et al., 1997]. However, E-NCAM expression is down-regulated on neuronal cells, and present on several different cell populations during later stages of development (see Introduction). The epitope recognized by mAb 2F7, on the other hand, is present on neurons and neuronal precursors even at late developmental ages, both in vivo and in vitro. Thus, mAb 2F7 should represent a useful tool for the isolation of NRP cells during a broad window of development.

mAb 2F7 Epitope

Several lines of evidence suggest that the mAb 2F7 epitope is composed of carbohydrate. First, mAb 2F7 immunoreactivity is present on multiple, high-molecular-weight proteins contained within membrane extracts derived from a variety of tissues representing different stages of development and multiple species. Second, and most significantly, mAb 2F7 immunoreactivity is greatly reduced when membrane extracts are subjected to enzymatic deglycosylation prior to immunoblotting. The use of Endoglycosidase-F in these experiments further suggests that the mAb 2F7 epitope is likely to be composed, at least in part, of N-linked carbohydrate. The presence of mAb 2F7 immunoreactivity on the surfaces of living NT2/D1 cells is further consistent with the conclusion that this epitope is composed of carbohydrate.

Comparison of the Distribution of the mAb 2F7 and HNK-1 Epitopes

HNK-1 is a mouse mAb that was originally generated against cultured T cells and which recognizes an epitope that is expressed in a dynamic and spatially-restricted pattern in the developing vertebrate nervous system [for a review, see Jungalwala, 1994]. HNK-1 recognizes a sulfate- and glucuronic acid-containing N-linked carbohydrate [Chou et al., 1985, 1986; Naegele and Barnstable, 1991] epitope that is present on several glycoproteins which are thought to mediate cell-cell interactions in the developing nervous system [Schachner and Martini, 1995]. There is considerable overlap between the distributions of the mAb 2F7 and HNK-1 epitopes in the developing chick and rat neural tube and spinal cord (see fig. 2), and in the embryonic telencephalon (data not shown). These findings raise the possibility that the two epitopes are highly related or identical. However, despite these similarities, differences in the distribution patterns and expression levels of the mAb 2F7 and HNK-1 epitopes also exist. For example, HNK-1 (data not shown), but not mAb 2F7 (see fig. 6), labels GFAP-immunoreactive astrocytes, as has been previously described [Riopelle et al., 1986; Smith et al., 1993]. Furthermore, while both mAbs recognize multiple high-molecular-weight proteins in extracts of E14 and adult rat spinal cord, HNK-1, but not mAb 2F7, also recognizes relatively lower-molecular-weight species in the E14 preparations (see fig. 7). Since mAb 2F7 labels the high-molecular-weight proteins as strongly as HNK-1, but does not label the lower-molecular-weight species (even when 3–5 times the

amount of total protein is loaded; data not shown), the HNK-1 epitope must reside on at least some proteins that do not bear the mAb 2F7 epitope.

It is interesting to note that a relatively large number of epitopes, recognized by mAbs generated against a wide variety of antigens, are distributed in an HNK-1-like pattern in the developing embryo [see Jungalwala, 1994; Preobrazhensky et al., 1997 and references therein]. This has led to the suggestion that these mAbs be considered as members of the HNK-1 antibody family [Preobrazhensky et al., 1997]. The similarities between the distribution and composition of the mAb 2F7 and HNK-1 epitopes identified in this study suggest that mAb 2F7 may be a new member of this antibody family. The degree of similarity between the mAb 2F7 and HNK-1 epitopes may ultimately be clarified by immunodepletion experiments, the results of which will indicate whether or not both epitopes reside on a completely overlapping set of antigens.

Acknowledgments

We thank Dr. Paul Patterson for allowing one of us (Z.K.) to generate mAb 2F7 in his lab and Susan Ou for performing the fusion, Dr. Peter Andrews at the Wistar Institute for the NT2/D1 cells, Dr. Anthony Frankfurter at the University of Virginia, Charlottesville, Va. for the TuJ1 antibody, Erik Runko for assistance with chick embryo dissections and immunoblotting, and Christi Wideman for figure preparation. The hybridoma 5A5 was obtained from the Developmental Studies Hybridoma Bank. This work was supported by an NIH FIRST award and a MDA award to M.S.R., an R01 from NIDCD to M.B.L. and an NIH FIRST award to Z.K.

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mAb 2F7

Fig. 1.

mAb 2F7 labels the developing rat CNS. A sagittal cryosection of an E16 rat embryo was labeled with mAb 2F7. Strong immunoreactivity is present throughout the CNS, including the forebrain (fb), midbrain (mb), hindbrain (hb) and spinal cord (sc). mAb 2F7 also labels the developing vertebra (arrow). Primary antibody binding was detected with a biotinylated secondary antibody as previously described [Zhu et al., 1998]. The scale bar represents 1 mm.

Fig. 2.

Spatiotemporal distribution of the mAb 2F7 and HNK-1 epitopes in the embryonic rat and chick nervous system. **A–F** Photomicrographs of transverse cryosections representing various age chick or rat embryos labeled with either mAb 2F7 or HNK-1. **A, C** In semi-serial sections derived from the forelimb region of an E2 chick embryo, both mAb 2F7 and HNK-1 brightly label migrating neural crest cells (nc) adjacent to the neural tube, and the surface of the notochord (n), and weakly label the ventricular zone. **B, D** In semi-serial sections derived from the rostral trunk region of an E12 rat embryo, both mAb 2F7 and HNK-1 strongly label the ventral horns (vh), ventral roots (vr) and lateral exit zones (le), and weakly label the ventricular zone associated with the spinal cord. **E** At the trunk level of an E5 (HH 26) chick embryo, mAb 2F7 brightly labels the ventral horns (vh) of the spinal cord, axons associated with the ventral funiculus (vf), the ventricular zone, and the dorsal root ganglia (drg). **F** At the trunk level of an E14 rat embryo, axons associated with the ventral funiculus (vf), the ventral horns (vh), and the exiting ventral roots (vr), all exhibit mAb 2F7 immunoreactivity. mAb 2F7 labeling is barely detectable on dorsal root ganglia (drg) and in the ventricular zone. In all cases dorsal is up and ventral is down. Primary antibody binding was detected with a fluorescein-conjugated anti-IgM secondary antibody. No specific labeling was detected with the secondary antibody alone. Scale bar in **C** applies to **A–D** and represents 50 μ m. The scale bar in **F** applies to **E** and **F** and represents 300 μ m.

Fig. 3.

The expression pattern of the mAb 2F7 epitope compared to that of TuJ1 in the embryonic telencephalon. **A–C** Fluorescent photomicrographs of the identical 10- μ m parasagittal section representing the E16 dorsal part of the telencephalon stained with mAb 2F7 (green, **A**, **C**) and the neuron-specific antibody TuJ1 (red, **B**, **C**). **A** and **B** respectively are viewed with a fluorescein and rhodamine filter and **C** is their superimposed confocal image. **A** shows that mAb 2F7 immunoreactivity is associated with cells in all layers of the telencephalon. The postmitotic, postmigratory cells of the subplate and marginal zone exhibit the highest level of 2F7 expression. The expression level in the intermediate zone, traversed by the immature neurons, is at an intermediate level, while the neurons of the cortical plate showed comparatively less expression of the mAb 2F7 epitope. The lowest level of staining, although still readily apparent, is present in the ventricular zone (VZ). Note that there is less 2F7 staining in the apical half of the VZ, where the cells in S-phase undergo DNA synthesis, in comparison to the basal half of the VZ. **B** shows that the majority of VZ cells are TuJ1-negative, in agreement with the findings of Menezes and Luskin [1994]. The TuJ1-labeled VZ cells represent the newly postmitotic neurons destined for the cortical plate. The cells of the subplate and marginal zone are intensely labeled by TuJ1. The postmitotic neurons of the intermediate zone and cortical plate are also prominently labeled by TuJ1. **C** demonstrates that all the neurons which express TuJ1 also express the mAb 2F7 epitope, but that mAb 2F7 immunoreactivity is more extensive in the VZ. Representative double-labeled cells in the VZ and subplate are designated by an arrow and arrowhead, respectively. CP = Cortical plate; IZ = intermediate zone; MZ = marginal zone; SP = subplate. The scale bar in **C** applies to **A** and **B** and represents 40 μ m.

Fig. 4.

Distribution of mAb 2F7 immunoreactivity in the neonatal subventricular zone and rostral migratory stream (RMS). **A** Fluorescent photomicrograph of a 10- μ m sagittal section of the P2 rat fore-brain demonstrating the distribution of the mAb 2F7 epitope. The intensity of mAb 2F7 immunoreactivity in the subventricular zone varies along the anterior-posterior axis. The progenitor cells of the SVZa (asterisk) exhibit low levels of expression in comparison to the heavier staining by SVZp cells (also compare **B** and **C**). The arrow signifies the approximate transition between the neurogenic SVZa and gliogenic SVZp. The cells of the vertical and horizontal limb of the RMS express higher levels of mAb 2F7 immunoreactivity relative to the SVZa, while the postmitotic neurons in the cellular layers of the olfactory bulb were even more heavily stained by 2F7 than those in the subependymal zone. **B** Fluorescent photomicrograph of a 10- μ m parasagittal section of the posterior extent of the SVZp double labeled with mAb 2F7 (green) and TuJ1 (red). The SVZp cells express low levels of the mAb 2F7 epitope and TuJ1 compared to the overlying corpus callosum and cerebral cortex. **C–E** Fluorescent photomicrographs of the identical 10- μ m parasagittal section of the P0 SVZa and overlying corpus callosum showing the distribution of mAb 2F7 immunoreactivity (green, **C**), TuJ1 immunoreactivity (red, **D**) and superimposed confocal images of 2F7/TuJ1 immunoreactivity (**E**). The entire thickness of the SVZa, except for the ependymal cell lining, is TuJ1-positive (**D**), while the SVZa progenitor cells express low levels of 2F7 immunoreactivity (**E**), concentrated in the basal half underlying the corpus callosum. Note that the axons of the corpus callosum are intensely labeled by both mAb 2F7 and TuJ1. CC = Corpus callosum; CTX = cerebral cortex; hl = horizontal limb of the RMS; HP = hippocampus; LV = lateral ventricle; OB = olfactory bulb; SVZa = anterior part of the neonatal subventricular zone; SVZp = posterior part of the neonatal subventricular zone; vl = vertical limb of the RMS. The scale bar in **E** applies to **C** and **D** and represents 100 μ m. The scale bar represents 375 μ m in **A** and 100 μ m in **B**.

Fig. 5.

The distribution of the mAb 2F7 epitope in the rostral migratory stream and olfactory bulb compared to the expression of NCAM. **A–D** Fluorescent photomicrographs of 10- μ m parasagittal sections of the rostral forebrain double labeled with 2F7 (green) and an antibody to NCAM (red). **A** and **B** show the distribution of the 2F7 epitope and anti-NCAM in the vertical (**A**) and horizontal (**B**) limb of the rostral migratory stream. Note that the mAb 2F7 epitope is predominantly expressed at the periphery of the RMS while NCAM is expressed throughout the pathway. **C** and **D** show the distribution of the 2F7 epitope and anti-NCAM in the subependymal zone and overlying cellular layers of the OB. mAb 2F7 immunoreactivity is low in the middle of the SEZ whereas there is prominent staining of anti-NCAM. The periphery of the pathway and the layers of the OB are mAb 2F7-positive. **D** illustrates the boxed area in **C** at higher magnification. The typical punctate cell surface mAb 2F7 staining is evident surrounding many of the cells of the olfactory bulb. CC = Corpus callosum; CTX = cerebral cortex; epl = external plexiform layer; gcl = granular cell layer; gl = glomerular layer; hl = horizontal limb of the RMS; ml = mitral cell layer; onl = olfactory nerve layer; sez = subependymal zone; vl = vertical limb of the RMS. The 175- μ m scale bar in **B** also applies to **A**. The scale bar represents 200 μ m in **C** and 75 μ m in **D**.

Fig. 6.

mAb 2F7 labels cultured neurons from the embryonic rat neural tube and only a very small population of multipotent neuroepithelial cells, in vitro. **A–C** E13.5 rat neural tubes were dissociated and the cells grown in culture for a period of seven days. The distribution of the mAb 2F7 epitope and neuronal and glial markers were then assessed as described in the Materials and Methods. Double labeling with β -111 tubulin (**A'**, green) and mAb 2F7 (**A**, red) shows that virtually all neurons express the mAb 2F7 epitope (compare **A** and **A'**). In contrast, the mAb 2F7 epitope (**B** and **C**, green) is not present on GFAP-positive astrocytes (**B**, red), or GalC-positive oligodendrocytes (**C**, red). The cultures were established and the antibody labeling performed as described in Materials and Methods. **D–G** E10.5 and E13.5 rat neural tubes were harvested and precursor cells isolated as described in Materials and Methods. Neuroepithelial stem cells (NEP cells) [Mayer-Proschel et al., 1997] derived from the E10.5 neural tube (**D**), E-NCAM-immunoreactive, restricted neuronal precursors (**E**) and A2B5-immunoreactive glial-restricted precursors (**F**), isolated from the E13.5 neural tube, as well as an immortalized A2B5-immunoreactive glial precursor cell line (**G**), were all stained with mAb 2F7, and processed for DAPI histo-chemistry to visualize nuclei (**D–G**, blue). While mAb 2F7 labels only a very few NEP cells (**D**, green), it labels virtually all E13.5, E-NCAM-immunoreactive restricted neuronal precursors (**E**, red), as well as a small subset of

A2B5-immunoselected cells (**F**, red). mAb 2F7 also labels a small proportion of cells present within an immortalized A2B5-immunoreactive glial cell line (**G**, green).

Fig. 7.

Biochemical characterization of the mAb 2F7 epitope. **A** mAb 2F7 labels several high-molecular-weight (between 116 and 205 kD) proteins, and to a lesser extent two proteins centered at about 75 kD in a detergent-soluble membrane/cytoskeletal fraction derived from E14 rat spinal cord (lane 3). Some mAb 2F7 immunoreactivity is present in the detergent-insoluble membrane/cytoskeletal fraction (lane 2), but essentially none is present in the cytoplasmic fraction (lane 1). **B** mAbs 2F7 and HNK-1 label similar profiles of high-molecular-weight proteins in detergent-soluble membrane/cytoskeletal fractions derived from both E14 and adult (postnatal day 140) whole rat spinal cords. HNK-1, but not mAb 2F7, also labels several lower-molecular-weight (between 47 and 74 kD) proteins that are selectively present in the fractions derived from E14 spinal cords. **C** mAbs 2F7 and HNK-1 label similar profiles of high-molecular-weight proteins in detergent-soluble membrane/cytoskeletal preparations derived from E5 chick head and trunk. **D** Detergent-soluble fractions of membrane/cytoskeletal preparations derived from NT2/D1 cells were treated with Endoglycosidase-F (Boehringer Mannheim; see Materials and Methods; + lanes) or with buffer alone (– lanes) for 24 h at 37°C, and then subjected to an immunoblot analysis with mAb 2F7 and HNK-1. Both mAb 2F7 and HNK-1 immunoreactivity is essentially abolished by the enzyme treatment. In **A–D**, 150 µg of protein (concentration determined by the BCA protein Assay System; Pierce) was loaded per lane, and primary antibody binding was detected with an HRP-conjugated secondary antibody using ECL reagents (Amersham).