

# Olfactory Neurons Express a Unique Glycosylated Form Of the Neural Cell Adhesion Molecule (N-CAM)

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**Abstract.** mAb-based approaches were used to identify cell surface components involved in the development and function of the frog olfactory system. We describe here a 205-kD cell surface glycoprotein on olfactory receptor neurons that was detected with three mAbs: 9-OE, 5-OE, and 13-OE. mAb 9-OE immunoreactivity, unlike mAbs 5-OE and 13-OE, was restricted to only the axons and terminations of the primary sensory olfactory neurons in the frog nervous system. The 9-OE polypeptide(s) were immunoprecipitated and tested for cross-reactivity with known neural cell surface components including HNK-1, the cell adhesion molecule L1, and the neural cell adhesion molecule (N-CAM). These experiments revealed that

9-OE-reactive molecules were not L1 related but were a subset of the 200-kD isoforms of N-CAM. mAb 9-OE recognized epitopes associated with N-linked carbohydrate residues that were distinct from the polysialic acid chains present on the embryonic form of N-CAM. Moreover, 9-OE N-CAM was a heterogeneous population consisting of subsets both with and without the HNK-1 epitope.

Thus, combined immunohistochemical and immunoprecipitation experiments have revealed a new glycosylated form of N-CAM unique to the olfactory system. The restricted spatial expression pattern of this N-CAM glycoform suggests a possible role in the unusual regenerative properties of this sensory system.

**P**ERHAPS the most intriguing aspect of the developing nervous system is the formation of specific synaptic connections between neurons that are often considerable distances apart. The growth cones of pioneer neurons may be guided by cues in the form of chemotropic molecules, adhesion or recognition molecules, and even inhibitory molecules in their microenvironment (Jessell, 1988). Considerable attention has been devoted to identifying the molecular basis of axonal guidance (Goodman et al., 1984; Dodd and Jessell, 1988). Two main classes of cell surface molecules have been proposed to be involved in this process in the vertebrate central nervous system. There are those molecules, represented by neural cell adhesion molecule (N-CAM)<sup>1</sup> and N-cadherin, that are present on both cell bodies and axons of most neurons. Through both their temporal and spatial modulation, these molecules are probably involved in the histogenesis of many regions (Edelman, 1988; Matsunaga et al., 1988). The second class of molecules, whose distribution appears to be more limited to the surface of axons (e.g., L1, contactin, F11, and G4), appears to facilitate self-adhesion of fasciculating axons (Jessell, 1988).

Recently, several new forms of N-CAM have been identified, raising the possibility that certain members of this family of molecules may be involved in more selective aspects of neural development than general cell-cell adhesion

(Gower et al., 1988; Prediger et al., 1988; Small et al., 1987, 1988). This possibility is further highlighted by reports that fasciclin II, a molecule expressed selectively on specific subsets of axons in the developing invertebrate nervous system, is homologous to vertebrate N-CAM (Harrelson and Goodman, 1988). Although such restricted expression patterns have not yet been reported for individual N-CAM forms or N-CAM-related molecules in the vertebrate nervous system, it is possible that there are many, as yet, unidentified forms of these molecules whose distribution may be more restricted, like that of fasciclin in invertebrates.

The adult olfactory system is a particularly interesting model for investigating the molecular basis of axon guidance. The primary sensory olfactory neurons are the only known neurons in the vertebrate nervous system that undergo a continual cycle of proliferation from stem cells and subsequent cell death both during development and in mature animals (Graziadei and Metcalf, 1971). Furthermore, in mammals these neurons are the only neurons capable of regenerating and forming functional synaptic connections within the central nervous system (Monti Graziadei and Graziadei, 1979). The primary sensory olfactory neurons are located in the periphery, in the olfactory epithelium of the nose. The axons of these neurons synapse directly within the telencephalon in a specialized cortical formation called the olfactory bulb. The mechanisms responsible for the guidance of growth cones of differentiating olfactory neurons into the central nervous system of a mature animal are, as

1. *Abbreviations used in this paper:* E N-CAM, embryonic form of neural cell adhesion molecule; HRP, horseradish peroxidase; N-CAM, neural cell adhesion molecule; SBA, soybean agglutinin.

yet, undefined. Therefore, in the present study we sought to identify molecules that may be implicated in the unusual axonal growth and guidance occurring in the adult olfactory system.

We have generated a series of mAbs reactive with the frog and rat olfactory systems. We describe here the expression and biochemical characterization of the antigens recognized by three of these mAbs (9-OE, 13-OE, and 5-OE) in the frog nervous system. These antibodies bind to distinct molecular isoforms within the family of N-CAMs, each of which have different anatomical expression patterns in the frog nervous system. One of these mAbs (mAb 9-OE) binds to a unique glycosylated form of N-CAM that is present on only olfactory neurons. The existence of such N-CAM subtypes suggests that they may be selectively involved in adhesive mechanisms specific to different neuronal populations.

## Materials and Methods

### Tissue Preparation

The olfactory bulb with attached nerves, the brain (between the medulla and olfactory bulb), and the olfactory epithelium were dissected from 117 adult (15–23 cm) *Rana catesbeiana* bullfrogs (Lemberger Co. Inc., Oshkosh, WI). Before dissection, all animals were anaesthetized with methanesulfonate and drained of blood. Tissues were homogenized by hand in 100 mM Tris, pH 7.4, containing 0.6% NaCl, 2 mM EDTA, and 100 mM PMSF at 4°C. The crude membrane preparations were subsequently washed three times by centrifugation at 40,000 *g* for 30 min in the above buffer and then stored in aliquots at –70°C.

The olfactory epithelium was removed from five adult Sprague-Dawley rats anaesthetized with sodium pentobarbital and drained of blood. This tissue was prepared as above, except the Tris buffer contained 0.9% NaCl. The protein content of membrane preparations was determined by the Lowry method using BSA as the standard.

### mAb Production

Four mice (C57BL/6J; Jackson Laboratory, Bar Harbor, ME) were immunized with intraperitoneal injections of membrane proteins (prepared as above) together with 300 µg polyinosinic-cytidylic acid (Sigma Chemical Co., St. Louis, MO) every 2 wk. The first of three injections consisted of 1 mg frog olfactory epithelium, the second was 2 mg rat olfactory epithelium, and the third was 2 mg frog olfactory epithelium. Three of the four mice, however, died after the third injection of frog olfactory epithelium. Subsequent experiments revealed that repeated immunizations with milligram quantities of frog olfactory epithelium were toxic to mice due to the presence of endogenous bacteria. 2 wk subsequent to the third intraperitoneal injection and 3 d before fusion, the remaining mouse was boosted, both intravenously and intraperitoneally, with 125 µg frog olfactory epithelium. The spleen from this animal was fused to X63-Ag8.653 myeloma cells as previously described (Allen and Akeson, 1985a). After 14 d of growth, hybridoma media was assayed by ELISA for reactivity against frog and rat olfactory epithelium and frog liver. Hybridoma media with high reactivity to olfactory epithelium was then screened by immunohistochemistry on cryostat sections of fixed frog olfactory epithelium. A number of these hybridomas were diluted, cloned, three of which (mAbs 5-OE, 9-OE, and 13-OE) are described in the present study. mAbs 5-OE, 9-OE, and 13-OE were isotypized as IgG<sub>3</sub>/k, IgM/k, and IgG<sub>2a</sub>/k, respectively. For all immunohistochemical experiments, media from cell lines grown in DME containing 20% FCS, 100 mM hypoxanthine, and 16 mM thymidine was used.

### Other Antibodies

Rabbit polyclonal anti-rat N-CAM 161 sera (Akeson et al., 1988) and mAb 2B8 (Allen and Akeson, 1985a) were previously generated in our laboratory. Rabbit polyclonal anti-frog N-CAM RO14 sera (Jacobson and Rutishauser, 1986) was obtained from Dr. U. Rutishauser (Case Western Reserve University, Cleveland, OH). Immunoprecipitation and immunoblot analysis revealed that 161 and RO14 were binding to the frog isoforms of N-CAM with relative molecular masses of 200 and 140 kD (data not

shown). Monoclonal and rabbit polyclonal anti-mouse L1 antibodies (Rathjen and Schachner, 1984) were obtained from Dr. M. Schachner (University of Heidelberg, Heidelberg, FRG). Rabbit polyclonal antibodies against NILE-β-galactosidase fusion protein (Prince et al., 1989) were provided by Dr. W. B. Stallcup (La Jolla Cancer Research Foundation, La Jolla, CA). L1 and NILE are the same protein and will be referred to here as L1. mAb 5A5, which is reactive with the highly sialylated forms of N-CAM (Dodd et al., 1988) was provided by Dr. J. Dodd (Columbia University, New York) and Dr. U. Rutishauser (ascites). Dr. F. Margolis (Roche Institute of Molecular Biology, Nutley, NJ) provided goat polyclonal anti-rat olfactory marker protein antibodies (Keller and Margolis, 1975). Olfactory marker protein is widely used as a specific marker for olfactory receptor neurons in many species. We did not, however, detect any cross-reactivity of this polyclonal sera in *Rana catesbeiana* by immunohistochemical techniques.

### Immunohistochemistry

Nine adult *Rana catesbeiana* bullfrogs were anaesthetized in methanesulfonate and perfused transcardially with ice-cold 100 mM potassium phosphate buffer, pH 7.4, containing 0.6% NaCl and 1% sodium nitrate followed by 4% paraformaldehyde in this same buffer solution. The spinal cord, brain, and olfactory epithelium were dissected and fixed for a further 4 h at 4°C. This tissue was then washed in 30% sucrose in 100 mM potassium phosphate buffer, pH 7.4, for 24 h at 4°C before embedding in O.C.T. compound (Miles Scientific, Naperville, IL) and freezing with liquid nitrogen-cooled isopentane. Cryostat sections were cut (15–20 µm) and mounted on gelatin-coated microscope slides.

Tissue sections of brain and spinal cord were reacted using the avidin-biotin-horseradish peroxidase (HRP) Vectastain kit (Vector Laboratories, Inc., Burlington, CA) using standard methods. Control sections were treated similarly except the primary antibody was 1% normal mouse serum in X63-Ag8.653-conditioned media or mAb 3C5.59-conditioned media (an IgM, previously generated against rat brain membranes, that does not detectably cross react with frog tissues). Sections were developed with DAB (0.05%) and H<sub>2</sub>O<sub>2</sub> (0.01%) in 100 mM Tris, pH 7.4. TRITC-conjugated affinity-purified rabbit anti-mouse IgG (1:40; Cappel Laboratories, West Chester, PA) was used for immunofluorescent analysis of olfactory epithelium. For lectin labeling, sections were reacted with soybean agglutinin (SBA) conjugated to HRP (Sigma Chemical Co.) as previously described (Key and Giorgi, 1986a).

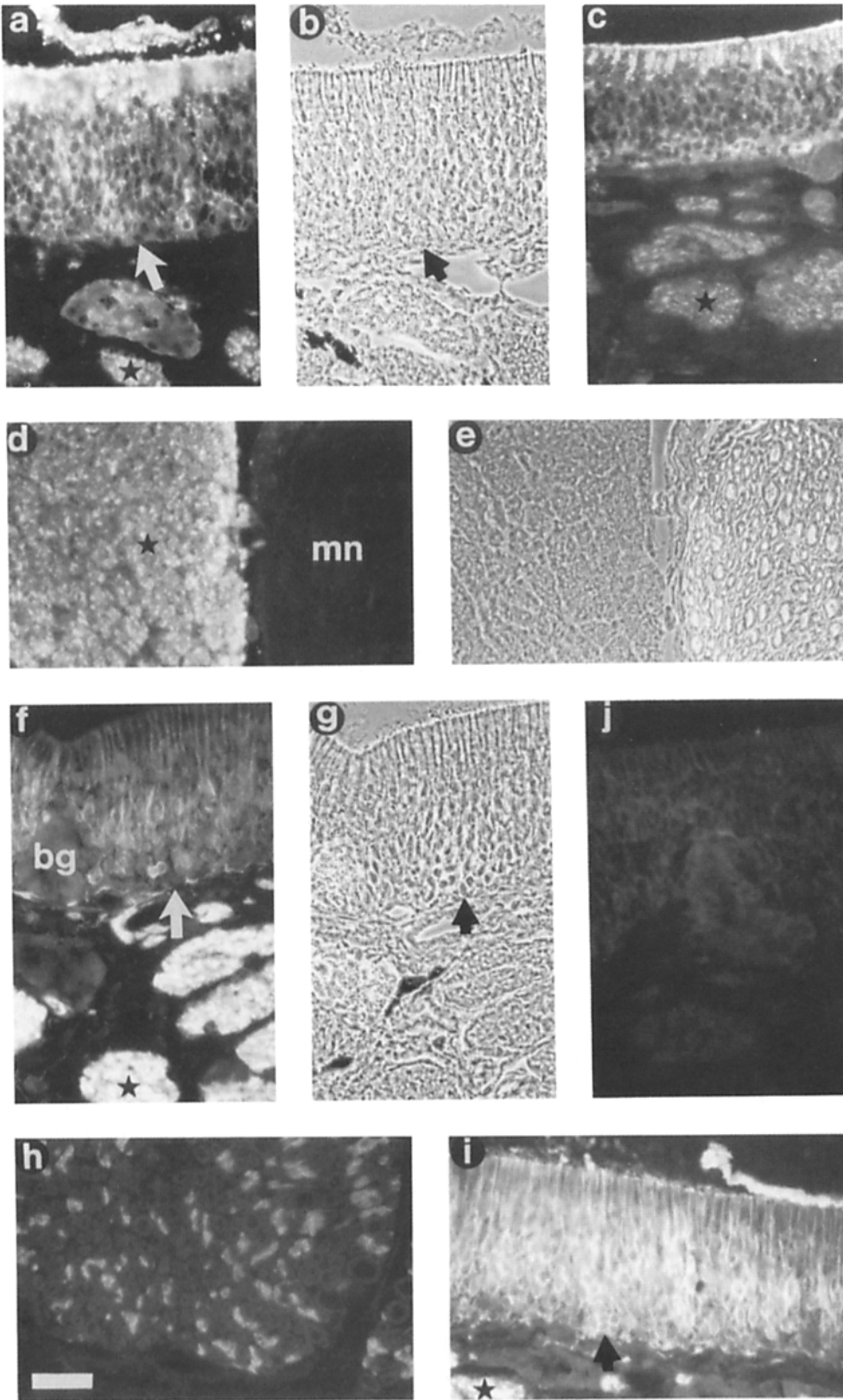
### Immunoblotting and Lectin Blotting

Membrane preparations, immunoprecipitated antigens, and affinity-purified glycoproteins were all solubilized in Laemmli's sample buffer, boiled for 5 min, and then electrophoresed on a discontinuous SDS gel system consisting of 6% polyacrylamide stacking and separating components. Electrophoresed proteins were electroblotted at 4°C onto Immobolin (Millipore Continental Water Systems, Bedford, MA) in 25 mM Tris-glycine buffer, pH 8.3, containing 20% methanol and 0.1% SDS. Blots were blocked in 100 mM Tris, pH 7.4, containing 0.9% NaCl, 0.2% Tween-20, and 10% nonfat dried milk (blocking buffer) and reacted using the avidin-biotin-HRP Vectastain kit (Vector Laboratories, Inc.) as described above. Controls were reacted similarly using either 1% normal mouse serum or mAb 3C5.59 as primary antibody. In most cases, reaction procedures between different experiments were standardized by simultaneously processing experimental blots and blots of frog olfactory bulb membranes with the same primary antibody. Relative molecular mass was determined using the SDS-6H standard molecular mass kit (Sigma Chemical Co.).

For lectin blotting, the Immobolin membranes were blocked in 100 mM Tris, pH 7.4, containing 0.9% NaCl, 0.2% Tween-20, and 4% BSA for 1 h. Blots were then incubated in SBA-HRP (50 µg/ml; Sigma Chemical Co.) in this buffer for 4 h at 25°C, washed in buffer (three times for 10 min each), and developed as above. Some blots were reacted with <sup>125</sup>I-SBA as previously described (Allen and Akeson, 1985a).

### Immunoprecipitations

Crude membrane preparations were solubilized in 1% NP-40 in 100 mM Tris, pH 7.4, containing 0.9% NaCl and protease inhibitors (aprotinin, 500 Kallikrein inhibitor units/ml; pepstatin, 1 µg/ml). In some cases, 0.4% SDS was added to this solubilization buffer. Pansorbin (fixed *Staphylococcus aureus*; Calbiochem-Behring Corp., San Diego, CA) was reacted with either 161, mAb 5-OE, or mAb 13-OE and then used to immunoprecipitate proteins from the membrane extracts as previously described (Williams et al., 1985). For immunoprecipitation with mAb 9-OE, a double antibody sand-



**Figure 1.** Immunofluorescence analysis of mAb binding to the olfactory epithelium. The antibodies used were: (a, c, and d) mAb 9-OE; (f and h) mAb 13-OE; (i) mAb 5-OE; and (j) normal mouse serum as a negative control. b, e, and g are phase micrographs of a, d, and f, respectively. The photographic and printing conditions were identical for all antibodies. Arrows point to basal cell layer. Stars label olfactory nerve bundles in the lamina propria. bg, Bowman's glands. Bar, 50  $\mu$ m.

wich technique was applied because this mAb bound very poorly to either Pansorbin or G-Sorbin (fixed group B *Streptococci*; Calbiochem-Behring Corp.). In this procedure, G-Sorbin was first coated with affinity-purified goat anti-mouse IgM antibodies (Vector Laboratories, Inc.) before reaction

with mAb 9-OE. In this way, the anti-mouse IgM antibodies acted as cross-linkers between G-Sorbin and mAb 9-OE.

To assess whether some of the antibodies were recognizing the same population of molecules in the frog olfactory bulb, it was necessary to com-

pletely deplete membrane extracts of particular antigens. This was achieved by sequential rounds of immunoprecipitation with either mAb 13-OE or polyclonal 161. At the completion of each immunoprecipitation, the supernatant was assayed for any remaining antigen by immunoblotting. When the membrane extracts were assessed to be depleted of a particular antigen, the supernatant was cleared of any contaminating unbound antibodies by incubation in uncoated Pansorbin. Controls consisted of parallel sequential immunoprecipitations with normal mouse serum for mAb 13-OE or normal rabbit serum for 161.

### Deglycosylation of Glycoproteins

N-CAM was immunoprecipitated from frog olfactory bulb with polyclonal 161 as described above and removed from *S. aureus* by boiling for 5 min in 0.5% SDS and 0.1 M beta-mercaptoethanol and then centrifuging. Aliquots of the supernatant were diluted to a final SDS concentration of 0.1% in 250 mM Tris buffer, pH 8.6, containing 10 mM 1,10-phenanthroline hydrate and 1.25% NP-40. The enzyme N-glycanase (5 U/ml; Genzyme Corp., Boston, MA) was added, and the incubation was allowed to proceed for 48 h at 37°C. In controls, N-glycanase was omitted from the reaction. Aliquots were boiled in Laemmli's sample buffer for 5 min, electrophoresed, and electroblotted.

Polysialic acid residues were removed from N-CAM by boiling in Laemmli's sample buffer for 30 min (Hoffman et al., 1982). The effectiveness of this procedure was verified by heating embryonic day-19.5 rat brain membranes for 3 min at 80°C or boiling for 30 min in sample buffer before electrophoresis and subsequently reacting blots with 161 (1:1,500) and mAb 5A5 (ascites, 1:200).

### Lectin Affinity Chromatography

Frog olfactory bulb membranes (24 mg) were solubilized in 2 ml of 1% NP-40 in 10 mM Tris, pH 7.4, containing 0.9% NaCl. This extract was loaded onto a 3-ml chromatography column of SBA conjugated to agarose (Sigma Chemical Co.) and allowed to stand at 25°C for 30 min. The column was then washed with 20 ml of 1% NP-40 in Tris buffer (flow rate 1 ml/3 min) that had been previously shown to clear the column of unbound protein. Bound glycoproteins were eluted with 2 ml of 200 mM *N*-acetyl-D-galactosamine in the above detergent buffer. The competing sugar was loaded and allowed to stand for 30 min before elution at a flow rate of 1 ml/15 min. Fractions (1 ml) were collected, and aliquots were electrophoresed on SDS-polyacrylamide gels and either silver stained or electroblotted onto nitrocellulose for lectin blotting with SBA.

## Results

A number of mAbs were generated against crude membrane fractions of olfactory epithelium using the protocol described. Some of these were selected for characterization on the basis of their immunohistochemical labeling patterns in the frog nervous system. We describe (a) the distribution of binding sites of mAbs 9-OE, 5-OE, and 13-OE and (b) the biochemical characterization of the antigens with which they react. The intent of our immunization protocol was to optimize the immunogenic response to antigenic determinants common to both frog and rat. Although such a procedure has been previously used for producing cross-reactive rabbit antisera (Akeson and Seeger, 1977), this is a novel approach for generation of mAbs. In the present study, our analysis has been restricted to reactivity of antibodies in frog. Data on species cross-reactivity of these mAbs will be described elsewhere.

### mAb 9-OE, 13-OE, and 5-OE Binding in the Olfactory Epithelium

The epithelium in the principal olfactory cavity is pseudostratified and consists of three basic cell types: a basal layer of proliferative stem cells, a middle layer of olfactory neurons, and an apical layer of supporting cells (Graziadei and

Metcalf, 1971). mAb 9-OE labeled cells throughout the whole depth of the olfactory epithelium (Fig. 1, a-c). This mAb appeared to be broadly reactive with the perikaryal membranes of both the basal cells which abut against the basement membrane and the middle layer of olfactory sensory neurons. The labeling of the basal cells, however, was considerably weaker than that observed on the other cell types in the epithelium (Fig. 1 a). Due to the intense fluorescence of the mucous granules in the apical cytoplasm of the supporting cells (Fig. 1 a), it was difficult to be certain whether either the dendrites of the olfactory neurons or the surface of the supporting cells were labeled by mAb 9-OE. The thick mucous layer lining the apical surface of the olfactory epithelium which contains the embedded olfactory cilia was also intensely immunoreactive. Bowman's glands, which secrete mucus onto the epithelial surface, protrude through the basement membrane into the epithelium proper from the lamina propria. These structures contained mAb 9-OE-immunoreactive antigens both on their perikaryal membranes and intracellularly on mucous granules. In the lamina propria, mAb 9-OE also stained other mucosal glands and the many small bundles of olfactory axons (Fig. 1, a and c, stars).

The thickness of the olfactory epithelium varies considerably in different regions of the nasal cavity. No difference was, however, observed in the labeling pattern obtained with mAb 9-OE between these regions (compare Fig. 1, a with c). In Fig. 1 c, the plane of focus was centered on the supporting cells which allowed for a clearer image of the immunofluorescent mucous granules than that observed in Fig. 1 a. Mixed nerves, most likely of trigeminal origin, were present within the lamina propria (Fig. 1, d and e, mn). No mAb 9-OE antigens were detected on either the myelinated or nonmyelinated axons within these nerve branches despite strong reactivity of the nonmyelinated olfactory axons in adjacent olfactory nerve bundles (Fig. 1 d). mAb 13-OE preferentially stained the perikaryal membranes of cells in the lower two-thirds of the epithelium (Fig. 1 f). These cells had labeled apical dendrites and basal axons and were identical, by both position and morphology, to primary olfactory sensory neurons. Due to the close juxtaposition of the plasma membranes of adjacent cells, it was difficult to be certain whether all of the olfactory neurons within a section were reactive with mAb 13-OE. mAb 13-OE also intensely labeled the fascicles of olfactory axons which exit the basement membrane of the epithelium and form small bundles in the lamina propria (Fig. 1 f, star). In contrast to mAb 9-OE, neither the basal stem cells, the apical supporting cells, the luminal surface of the epithelium, nor the Bowman's glands appeared to be stained by this mAb. The trigeminal nerve branches present in the lamina propria contained small patches of immunoreactivity that most likely was associated with fascicles of unmyelinated axons (Fig. 1 h). The axons of the myelinated axons in these nerves were unlabeled. mAb 5-OE appeared to react more strongly than mAb 13-OE with the perikaryal and dendritic membranes of the olfactory neurons (Fig. 1 i). This mAb also labeled basal cells and the apical mucous layer of the epithelium. Control sections of olfactory epithelium that were reacted with normal mouse serum exhibited minimal nonspecific fluorescence (Fig. 1 j).

In summary, all three antibodies showed distinct patterns

Table I. Summary of Immunohistochemical and Immunoblot Labeling with mAbs

Reagent	Histochemistry			Immunoblot		
	Olfactory epithelium	Olfactory bulb	Brain	Olfactory epithelium	Olfactory bulb	Brain
				<i>kD</i>	<i>kD</i>	<i>kD</i>
mAb 13-OE	Olfactory neuronal perikarya, dendrites, and axons	Most or all cells	Most or all cells	200	200, 140, and 115	200, 140, and 115
mAb 5-OE	Olfactory neurons, basal cells, mucus, and mucous glands	Olfactory nerve and glomeruli; weak in remaining layers	Weak in most or all cells; neuron subsets with strong label	205, 95, 55 and 50	205	205
mAb 9-OE	Most or all cells, mucus, and mucous glands	Olfactory nerve and glomeruli	Negative	205	205	Negative

of reactivity within the olfactory epithelium (Table I). Although they all stained primary olfactory sensory neurons, mAbs 5-OE and 9-OE also reacted with nonneuronal elements in the olfactory epithelium.

#### *mAb 9-OE, 13-OE, and 5-OE Binding in the Olfactory Bulb*

The frog main olfactory bulb consists of three principal layers (Hoffmann, 1973): (a) the outer nerve fiber layer and glomeruli (spherical structures consisting of the terminations of olfactory axons on the dendrites of the second order neurons—the mitral and periglomerular cells); (b) a dispersed mitral cell layer; and (c) the granule cell layer (Fig. 2, a and b). mAb 9-OE immunoreactivity was exclusively located on the outer nerve fiber layer and glomeruli in the olfactory bulb (Fig. 2, c and d). The inner mitral and granule cell layers were not stained by mAb 9-OE (Fig. 2, c and d). This distribution of label was very similar to that observed with the lectin SBA (Fig. 2, compare a and b with c and d, respectively). Note that the sections in Fig. 2, a and b, were counter-stained to show cell nuclei. SBA had previously been reported to label the surface of a subset of olfactory axons in *Xenopus* (Key and Giorgi, 1986a). In *Rana catesbeiana*, however, SBA bound to all olfactory axons (Fig. 2 a). In contrast to the discrete labeling pattern obtained with SBA and mAb 9-OE, mAb 13-OE reactivity was widespread and associated with all laminae of the olfactory bulb (Fig. 2 e). mAb 5-OE, on the other hand, more strongly stained the outer nerve fiber layer and glomeruli than the deeper olfactory bulb laminae (Fig. 2 f). In addition, mAb 5-OE also weakly labeled a subset of mitral cells and their dendrites (Fig. 2 f, open arrows, and i). This neuronal reactivity was relatively stronger than the diffuse background labeling of the surrounding neuropil and granule cell layer. No immunoreactivity was observed in control sections of olfactory bulb that were reacted with either normal mouse serum (Fig. 2 g) or control mAb 3C5.59 (not shown).

#### *mAb 9-OE, 13-OE, and 5-OE Binding in Brain and Spinal Cord*

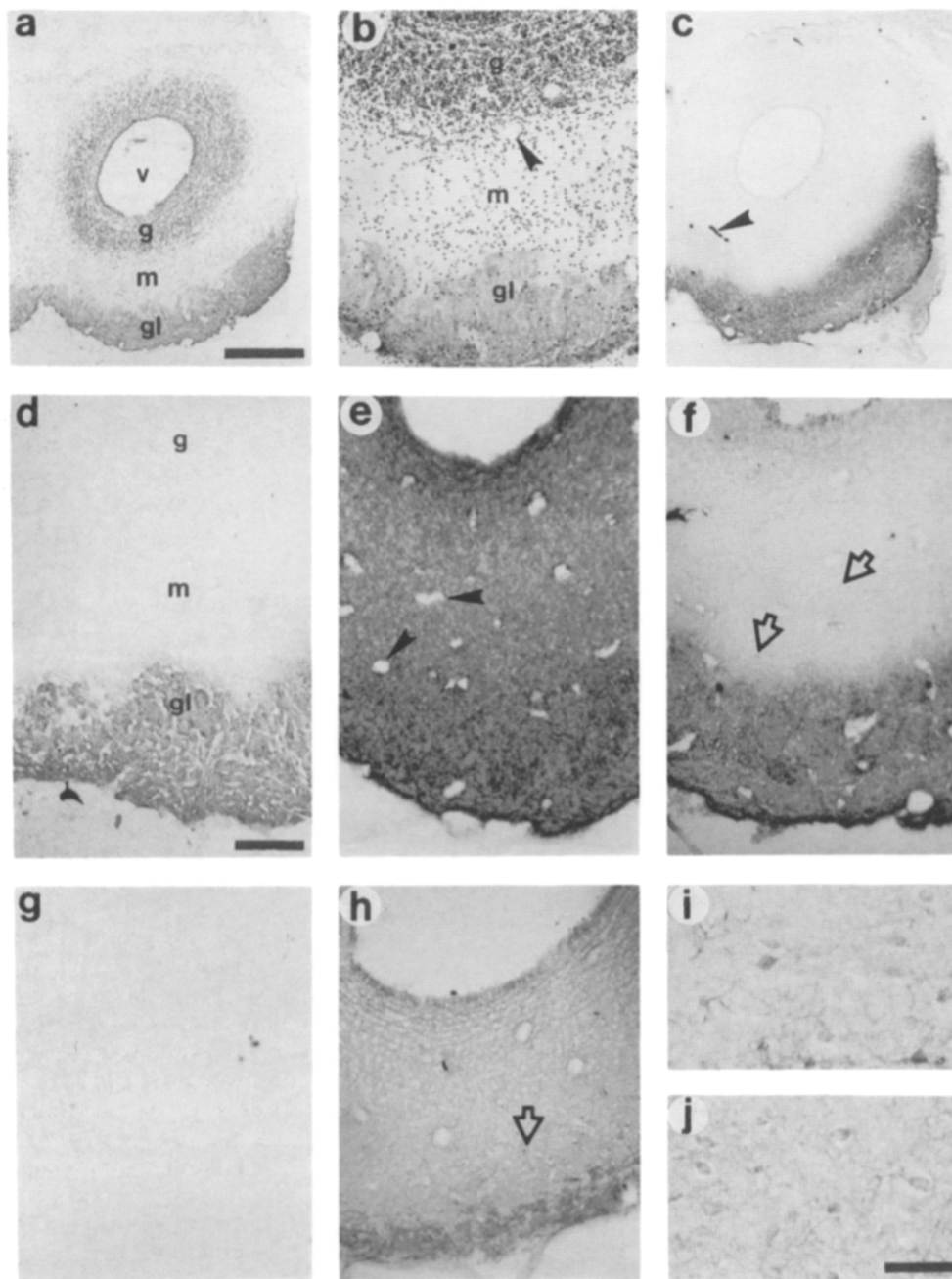
Antigen expression was assessed in other regions of the ner-

vous system to further examine the specificities of these antibodies. Primordial olfactory cortex and hippocampus, dorsal pallium, thalamus, tectum, mesencephalic tegmentum, medulla oblongata, and spinal cord were screened for mAb labeling. Some of these regions (see Hoffman, 1973) were chosen to include central higher order connections of the olfactory bulb (primordial olfactory cortex) and of other sensory systems (for example, the tectum and thalamus for the visual system).

mAb 9-OE binding could not be detected in any of these brain regions or in the spinal cord (data not shown). These results indicate the specificity of mAb 9-OE for primary sensory olfactory neurons among neuronal populations within the frog nervous system, at least at the level of sensitivity of this technique. In all brain regions tested, mAb 13-OE immunoreactivity reflected the broad reactivity observed in the olfactory bulb (Fig. 2 e). This mAb uniformly labeled many structures including the neuropil, axon tracts, and the cell surface of neuronal perikarya in a relatively nonspecific manner. In brain, mAb 5-OE preferentially stained small populations of neurons in the dorsal pallium, thalamus, tectum, and spinal cord (not shown). The distinct patterns of immunoreactivity obtained with these mAbs (Table I) suggest that they are each recognizing different antigenic determinants in the frog nervous system.

#### *Biochemical Characterization of the 9-OE, 13-OE, and 5-OE Antigens*

To characterize the antigens to which these mAbs bind, proteins from whole membrane fractions of three tissues (olfactory epithelium, olfactory bulb, and brain without olfactory bulb) were extracted in NP-40, separated by SDS-PAGE, electroblotted, and immunoblotted with mAbs 9-OE, 13-OE, and 5-OE. It should be pointed out here that the density of the labeled bands on immunoblots presented below can not be compared across different lanes when different mAbs are used. This is due to differences in the avidities and affinities of the various primary and secondary antibodies and also to the different development times of the color reactions. However, qualitative comparisons can be made between la-



**Figure 2.** Histochemical analysis of SBA and antibody reactivity in the olfactory bulb. All panels are coronal sections through the right olfactory bulb; lateral is to the right, dorsal is to the top, and ventral is to the bottom of each section. Sections were labeled with SBA-HRP (*a* and *b*), SBA-HRP and mAb 9-OE (*c* and *d*), mAb 13-OE (*e*), mAb 5-OE (*f* and *i*), normal mouse serum (*g*), and mAb 5A5 (*h* and *j*). *a* and *c* are micrographs of the entire right olfactory bulb. (*b* and *d-h*) are higher power micrographs of ventral olfactory bulb laminae. The SBA-HRP-labeled sections in *a* and *b* have been counter-stained with cresyl violet. *i* and *j* are enlargements of mitral cells and their dendrites, which are marked by arrows in *f* and *h*, respectively. Arrowheads mark blood vessels that in some cases contain blood cells not cleared by perfusion and therefore stained nonspecifically. Bars: (*a* and *c*) 500  $\mu$ m; (*b* and *d-f*) 100  $\mu$ m; (*j* and *i*) 50  $\mu$ m.

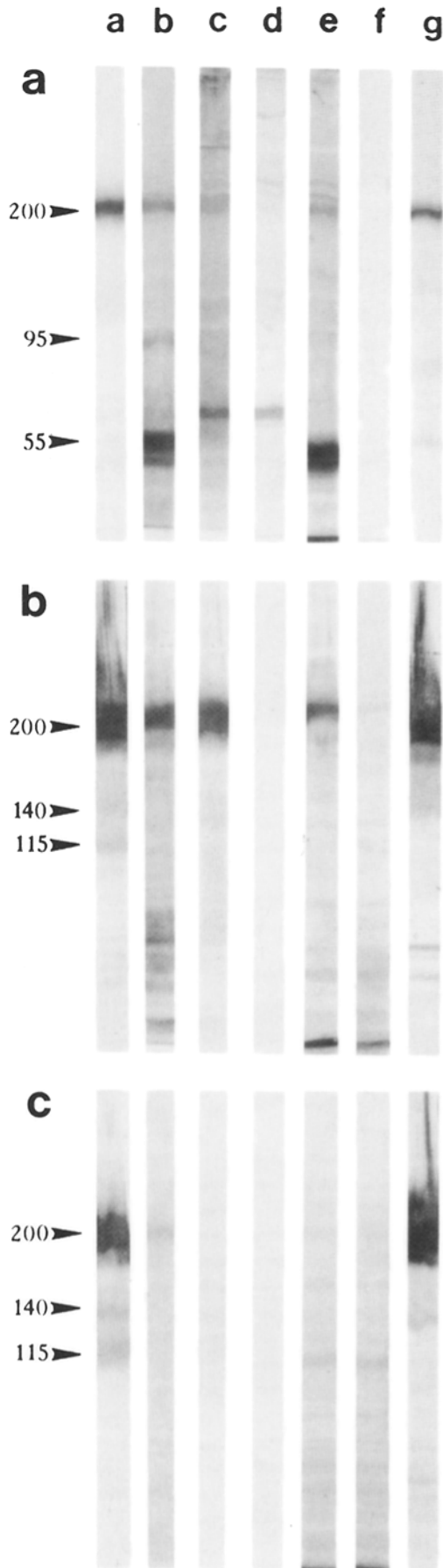
beled bands in different figures when the same primary antibody was used.

In olfactory epithelium, all three mAbs reacted strongly with a component(s) in the 190–210-kD range. mAb 13-OE bound only a single broad band with relative molecular mass centered around 200 kD (Fig. 3 *a*, lane *a*). In the same tissue, mAb 5-OE reacted with bands at 205, 95, 55, and 50 kD (Fig. 3 *a*, lane *b*). mAb 9-OE reacted with two bands at 205 and 65 kD (Fig. 3 *a*, lane *c*). In control blots, using normal mouse serum as the primary antibody and a goat anti-mouse IgM as the secondary antibody, the 65-kD band was found to be nonspecifically labeled (Fig. 3 *a*, lane *d*). Therefore, it appeared that mAb 9-OE specifically labeled only a 205-kD protein in olfactory epithelium.

In the olfactory bulb, again as in the olfactory epithelium, all three mAbs reacted strongly with component(s) in the 190–210-kD range. mAb 13-OE detected a broad band at 200 kD (Fig. 3 *b*, lane *a*) that was markedly smeared in the high molecular mass range of the blot. In addition, two lighter bands were present at 140 and 115 kD. Both mAbs 5-OE (Fig. 3 *b*, lane *b*) and 9-OE (Fig. 3 *b*, lane *c*) recognized a prominent band centered at  $\sim$ 205 kD that overlapped in size with the top of the broad band recognized by mAb 13-OE. mAb 9-OE did not bind to any other proteins in the olfactory bulb. mAb 5-OE, however, reacted faintly with numerous proteins with molecular masses <75 kD.

In extracts of brain without the olfactory bulb (subsequently referred to as brain), mAb 13-OE detected antigens





of similar size to those observed in the bulb (Fig. 3 c, lane a), with predominate reactivity at 200 kD. mAb 5-OE weakly labeled only a single band at 205 kD in brain (Fig. 3 c, lane b), while mAb 9-OE did not react with any proteins in this tissue (Fig. 3 c, lane c). Even when lanes were overloaded with brain protein (500  $\mu$ g), no binding was detected with mAb 9-OE, which was consistent with the lack of reactivity as observed with immunohistochemistry. Table I summarizes both the immunohistochemical and immunoblot data for all three mAbs.

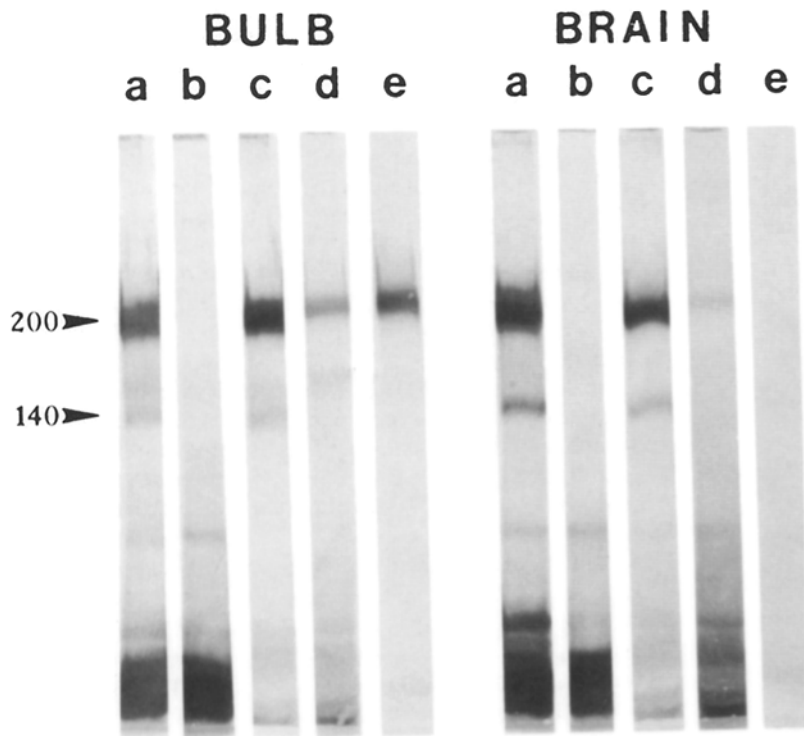
Since mAb 9-OE immunohistochemical labeling in the olfactory bulb was similar to that observed with SBA, we also probed blots of the three tissues with this lectin. In the olfactory epithelium, SBA detected three prominent bands at 205, 55, and 50 kD (Fig. 3 a, lane e). Several other weakly labeled bands were also detected. This lectin bound to proteins of similar size to those recognized by mAb 5-OE (Fig. 3 a, lane b). In the olfactory bulb, a single band at 205 kD was specifically stained (Fig. 3 b, compare lane e with f). In brain, while several bands were weakly labeled (Fig. 3 c, lane e), control incubations of SBA together with *N*-acetyl-D-galactosamine revealed these to be nonspecific (Fig. 3 c, lane f).

Interestingly, all three mAbs as well as SBA recognized a similar sized band centered at  $\sim$ 200–205 kD in both the olfactory epithelium and bulb.

#### ***mAb 9-OE Reacts Specifically with the 200-kD Isoform of N-CAM***

Since the antigen profile in immunoblots of mAb 13-OE resembled that previously reported for N-CAM in frog brain (Levi et al., 1987; Sunshine et al., 1987), we probed the three tissue extracts with a polyclonal anti-rat N-CAM antibody designated 161 (Akeson et al., 1988). In olfactory epithelium, 161 detected a single band at 200 kD (Fig. 3 a, lane g). A similar sized prominent band at 200 kD was also labeled in olfactory bulb preparations (Fig. 3 b, lane g). The low molecular mass bands observed in these blots represented nonspecific labeling. When the olfactory bulb blots were overdeveloped, a band at 140 kD became more apparent. In brain, 161 again detected a prominent band at 200 kD and a weak band at 140 kD (Fig. 3 c, lane g). In the olfactory bulb and brain, the 200-kD band recognized by 161 was very broad and smeared. This labeling was similar to that

**Figure 3.** Direct immunoblots of membrane extracts of olfactory epithelium (a), olfactory bulb (b), and brain (c). Individual lanes (150  $\mu$ g membrane protein) were labeled with (lanes a–g, respectively) mAb 13-OE, mAb 5-OE, mAb 9-OE, normal mouse serum, SBA-HRP, SBA-HRP plus 0.2 M *N*-acetyl-D-galactosamine, and anti-N-CAM 161 sera. In olfactory epithelium, both mAb 5-OE and 9-OE produced a diffuse background stain throughout the whole lane. No such labeling occurred in controls when normal mouse serum was used with either goat anti-mouse IgM (lane d) or IgG (data not shown) as the second antibody. In general, comparisons of the density of labeled bands between lanes are not indicative of relative protein levels due to differences in development time of the color reagents and to the use of antibodies of different affinities and avidities (see Results). Qualitative comparisons can only be made between lanes when the same primary antibody is used since attempts were made to standardize procedures in these cases (see Materials and Methods).



**Figure 4.** Immunoblots of N-CAM immunoprecipitated with 161 from olfactory bulb and brain (each lane represents antigen obtained from 150  $\mu$ g membrane protein). Antibodies used were (lanes *a-e*, respectively) 161, normal rabbit sera, mAb 13-OE, mAb 5-OE, and mAb 9-OE. 161 weakly labeled a 160-kD band in lane *a* in olfactory bulb. The same band was also labeled by mAbs 13-OE (lane *c*) and 5-OE (lane *d*). A similar weakly stained band was observed in direct immunoblots of olfactory bulb using 161 (Fig. 3 *b*, lane *g*) and mAbs 13-OE (Fig. 3 *b*, lane *a*) and 5-OE (Fig. 3 *b*, lane *b*). The nature of this band is unknown.

obtained with mAb 13-OE and resembled that previously reported for highly glycosylated forms of N-CAM (Hoffman et al., 1986).

Next the relationships between N-CAM and the molecules that react with mAbs 13-OE, 5-OE, and 9-OE were determined. N-CAM was immunoprecipitated from both olfactory bulb and brain with 161, separated by SDS-PAGE, blotted, and then reacted with each of the antibodies (Fig. 4). mAb 13-OE (Fig. 4, lanes *c*) clearly detected both the 200- and 140-kD N-CAMs labeled by 161 (Fig. 4, lanes *a*) from olfactory bulb and brain. mAb 5-OE reacted with the 200-kD N-CAM from both tissues (Fig. 4, lanes *d*). In contrast to the above two mAbs, mAb 9-OE reacted with N-CAM from only the olfactory bulb and not from the brain (Fig. 4, lanes *e*). Moreover, mAb 9-OE only recognized the 200-kD N-CAM isoform. Although the 200-kD band labeled by mAb 13-OE was exactly the same width and relative molecular mass as that labeled by 161, both mAbs 5-OE and 9-OE labeled only the top portion of the 161 band in olfactory bulb. In this tissue, both mAb 9-OE and 5-OE consistently labeled bands centered at 205 kD. In brain, mAb 5-OE also reacted with the 205-kD N-CAM band.

The lack of mAb 9-OE reactivity with brain immunoprecipitates confirmed results obtained from direct immunoblots of brain membranes (Fig. 3). The immunohistochemical data and the slightly higher relative molecular mass of the N-CAM labeled by mAb 9-OE suggest that this mAb is recognizing a unique subset of N-CAMs present only in the olfactory bulb and not in the rest of the brain.

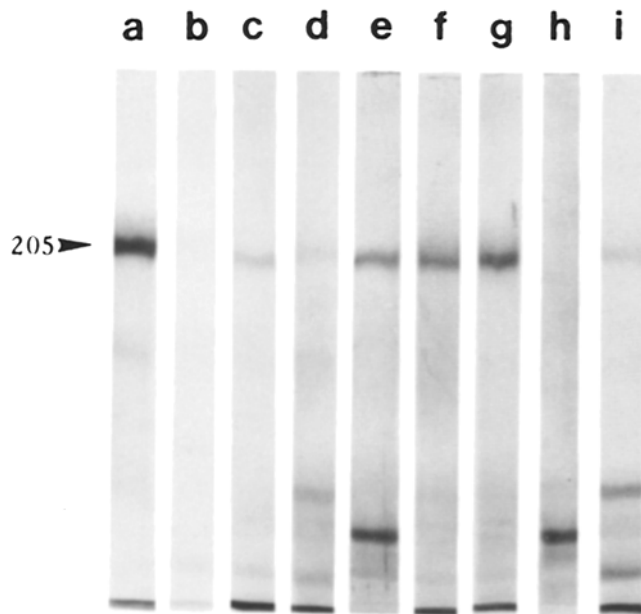
To further test whether mAb 9-OE was recognizing N-CAM, and not another molecule associated with N-CAM, we next immunoprecipitated olfactory bulb directly with mAb 9-OE and then immunoblotted the precipitates with other antibodies. Since both nonspecific protein interactions

and specific N-CAM-N-CAM binding can potentially occur in nonionic detergents, we tested immunoprecipitations in the presence or absence of 0.4% SDS, which disrupts many protein-protein interactions (Ezzell et al., 1988). Both mAb 13-OE and 161 reacted with the mAb 9-OE antigen that was immunoprecipitated in the presence of either 1% NP-40 (not shown) or 1% NP-40 and 0.4% SDS (see below). Therefore, mAb 9-OE must be specifically immunoprecipitating N-CAM from the olfactory bulb rather than another protein that nonspecifically binds to N-CAM. These experiments confirmed the 9-OE antigen as an N-CAM.

#### *Subsets of the Frog 200-kD N-CAM*

To assess whether mAbs 9-OE, 13-OE, and 5-OE were recognizing independent or overlapping subsets of N-CAMs, we tested the ability of each mAb to bind to the N-CAM isoforms immunoprecipitated by the other mAbs in the presence of 1% NP-40 and 0.4% SDS. Primary immunoprecipitates were made with mAb 9-OE which reacts specifically with a band at 205 kD in only the olfactory bulb (Fig. 5 *a*). Although controls revealed the absence of any nonspecifically labeled bands in this immunoprecipitate (Fig. 5 *b*), the other antibodies used (lanes *c-i*) reacted with the heavy and light immunoglobulin chains of IgM as well as with other proteins at the bottom of the blots. Both mAbs 13-OE (Fig. 5 *c*) and 5-OE (Fig. 5 *d*) bound weakly to the 205-kD protein immunoprecipitated by mAb 9-OE. These results suggested that at least some of the N-CAM forms recognized by mAb 9-OE in the olfactory bulb also possessed epitopes recognized by mAbs 5-OE and 13-OE. These experiments could not, however, distinguish whether mAb 9-OE was binding to a heterogeneous population of N-CAMs and perhaps even to other proteins of the same relative molecular mass as





**Figure 5.** Immunoblots of mAb 9-OE immunoprecipitated antigen from olfactory bulb (each lane represents antigen obtained from 150  $\mu$ g membrane protein). Lanes a-i, respectively, were reacted with mAb 9-OE, normal mouse sera, mAb 13-OE, mAb 5-OE, 161, anti-HNK-1, mAb 2B8, anti-L1, and SBA-HRP. The labeled bands at the bottom of the blots represent background immunoreactivity.

N-CAM—some possessing only the 9-OE epitope and others sharing different combinations of the three mAb epitopes.

Relatively weak labeling of the mAb 9-OE antigen was obtained with mAbs 13-OE (Fig. 5 c) and 5-OE (Fig. 5 d) and 161 (Fig. 5 e) in comparison with that obtained with these antibodies on direct immunoblots of olfactory bulb membranes (Fig. 3 b, lanes a, c, and g, respectively). These results suggested that the 9-OE protein made up only a small subset of all the 200-kD N-CAM isoforms and of the 5-OE and 13-OE proteins on olfactory neurons. However, because the mAb 9-OE antigens are expressed only on the axons and terminal arbors of primary sensory olfactory neurons, a weaker labeling of the immunoprecipitated mAb 9-OE antigen from olfactory bulb would be expected with 161 and

both mAbs 13-OE and 5-OE. No polypeptides were immunoprecipitated from brain with mAb 9-OE. This was consistent with the lack of binding of mAb 9-OE either to total brain proteins or to N-CAM immunoprecipitated from brain.

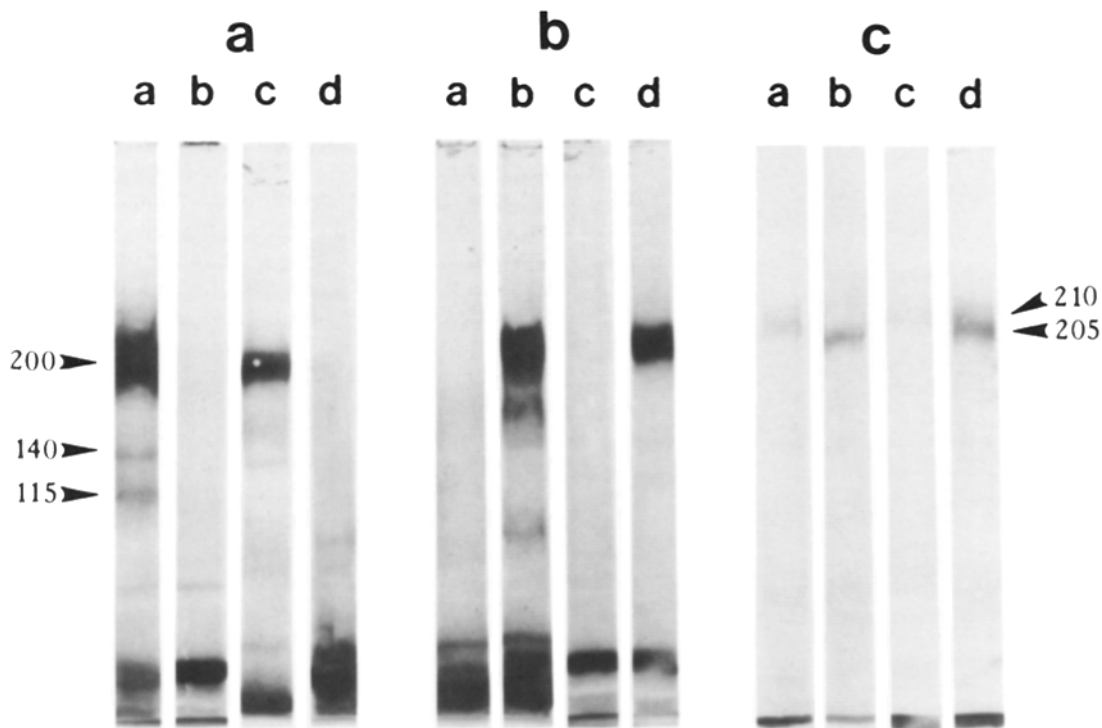
Next, primary immunoprecipitates were made with mAb 13-OE. mAb 13-OE immunoprecipitated the two major isoforms of N-CAM from olfactory bulb with relative molecular masses of 200 and 140 kD and also a band at 115 kD. Both mAbs 5-OE and 9-OE reacted with only a portion of the 200-kD N-CAM isoforms immunoprecipitated by mAb 13-OE from olfactory bulb (Table II). These mAbs labeled bands that migrated slightly slower in SDS gels at a relative molecular mass of 205 kD. These molecular mass differences were further confirmed by prolonged electrophoresis, which resulted in a more complete separation of the bands. As in olfactory bulb, mAb 13-OE immunoprecipitated all three isoforms of N-CAM from brain (Table II). mAb 5-OE bound weakly to the top portion of the 200-kD N-CAM immunoprecipitated from brain by mAb 13-OE. In olfactory bulb, mAb 9-OE recognized an N-CAM subset at 205 kD that possessed the mAb 13-OE epitope, however, in brain this mAb did not react with any N-CAM isoforms. These data are consistent with results obtained from mAb labeling of N-CAMs immunoprecipitated by 161 from both olfactory bulb and brain (Fig. 4). mAb 5-OE was subsequently used to immunoprecipitate antigens from the olfactory bulb and brain. mAbs 13-OE and 9-OE and polyclonal 161 all labeled the 205-kD band immunoprecipitated from olfactory bulb by mAb 5-OE (see Table II). mAb 5-OE did not appear to immunoprecipitate antigens as effectively as mAb 13-OE. In fact, we did not obtain sufficient yields from immunoprecipitations of brain to analyze binding of the different antibodies. This is also most likely due to the lower level of expression of the mAb 5-OE protein in brain.

The immunoprecipitation experiments revealed all three mAbs were recognizing overlapping sets of N-CAMs (Table II). It appeared that mAb 13-OE bound the largest set of N-CAMs, with epitopes present on N-CAMs immunoprecipitated by mAbs 5-OE and 9-OE and by 161. mAb 5-OE, however, detected epitopes present on only a subset of the N-CAMs recognized by mAb 13-OE and 161. The most discrete population of N-CAMs identified here was delineated by mAb 9-OE. This mAb detected unique epitopes present

**Table II. Summary of Cross-reactivity of Immunoprecipitated Antigens from Olfactory Bulb and Brain with Antibodies and SBA**

Antigen	Tissue	Subsequent immunoblot with antibody or lectin							
		mAb 9-OE	mAb 13-OE	mAb 5-OE	mAb HNK-1	mAb 2B8	Polyclonal anti-L1	Polyclonal anti-N-CAM	SBA
		kD	kD	kD	kD	kD	kD	kD	kD
N-CAM	Bulb	205	200 and 140	205	200 and 140	ND	—	200 and 140	ND
N-CAM	Brain	—	200 and 140	205	200 and 140	ND	—	200 and 140	ND
9-OE	Bulb	205	200	205	200	200	—	200	205
9-OE	Brain	—	ND	ND	ND	ND	ND	—	ND
13-OE	Bulb	205	200, 140, and 115	205	200, 140, and 115	200	—	200 and 140	205
13-OE	Brain	—	200, 140, and 115	205	200, 140, and 115	—	—	200 and 140	—
5-OE	Bulb	205	205	205	—	205	—	205	ND
SBA	Bulb	205	205	205	—	205	—	205	205

(—) No specific bands.



**Figure 6.** (a) Complete removal of antigens from olfactory bulb by immunoabsorption. Immunoblots of the initial (lane a) and final (lane b) sequential mAb 13-OE immunoprecipitation (each lane equivalent to antigen obtained from 150  $\mu$ g membrane protein) reacted with mAb 13-OE to show the complete removal of this antigen from olfactory bulb preparations. Similarly, the initial (lane c) and final (lane d) immunoprecipitation of olfactory bulb with 161 were reacted with 161 to show depletion of this antigen. (b) Cross immunoprecipitation of antigen depleted preparations. Next, the mAb 13-OE-negative olfactory bulb preparation was both immunoprecipitated and immunoblotted with 161 (lane a). Controls were sequentially immunoabsorbed with normal mouse sera and then subsequently both immunoprecipitated and immunoblotted with 161 (lane b). The 161-negative olfactory bulb preparation was both immunoprecipitated and immunoblotted with mAb 13-OE (lane c). In this case, controls were sequentially immunoabsorbed with normal rabbit sera and then both immunoprecipitated and immunoblotted with mAb 13-OE (lane d). The labeled N-CAM bands in control lanes revealed that antigens survive intact the rigorous sequential immunoprecipitation protocol. (c) mAb 9-OE immunoprecipitation of N-CAM-depleted preparations. mAb 9-OE was subsequently used to both immunoprecipitate and immunoblot the mAb 13-OE-depleted (lane a) and normal mouse sera-immunoabsorbed control olfactory bulb preparations (lane b). Similarly, the 161-depleted preparations (lane c) as well as the normal rabbit sera-immunoabsorbed preparations (lane d) were also both immunoprecipitated and immunoblotted with mAb 9-OE. In both controls (lanes b and d), mAb 9-OE bound to the 205-kD N-CAM subset. In the N-CAM-depleted preparations (lanes a and c), mAb 9-OE continued to bind, albeit very weakly, to a 210-kD band.

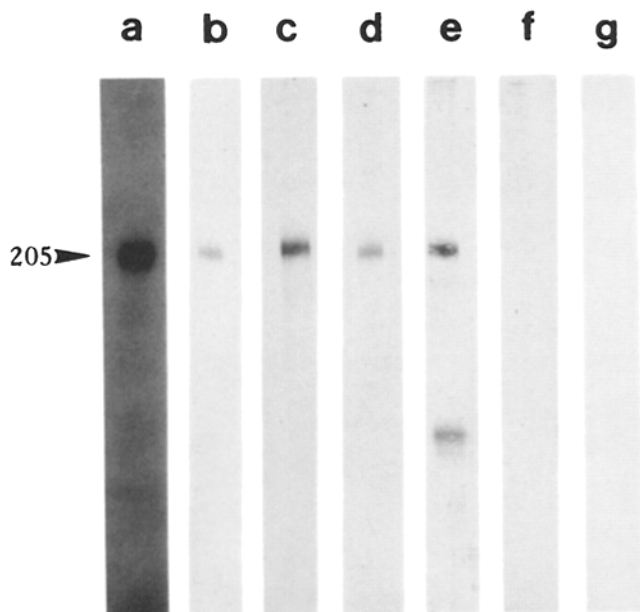
on only a small subset of the 200-kD N-CAMs that were restricted in their localization to the olfactory bulb and also had a distinct relative molecular mass of 205 kD.

#### **HNK-1, L1, mAb 2B8, and SBA Reactivity with 9-OE N-CAM**

We further assessed the nature of the different N-CAM subsets by testing their reactivity with SBA as well as with previously characterized antibodies known to react with cell surface components of the brain and olfactory system. mAb HNK-1, mAb 2B8, and anti-L1 polyclonal antibodies were used to probe blots of olfactory bulb and brain immunoprecipitated by mAbs 9-OE, 13-OE, and 5-OE. HNK-1 is a carbohydrate moiety containing 3-sulphated glucuronyl or similar polysaccharide chains (Chou et al., 1986) that are found on several different cell surface adhesion molecules in the nervous system including a subset of N-CAMs (Kruse et al., 1984). mAb 2B8 recognizes a developmentally regulated carbohydrate moiety present on several polypeptides in a subset of olfactory neurons in rat (Allen and Akesson, 1985a,

b). The epitope recognized by this mAb is distinct from the binding site detected by SBA (Key and Giorgi, 1986b). L1 is an adhesion molecule found predominantly on the surface of fasciculating axons in many neural structures during development (Stallcup et al., 1985). On direct immunoblots of NP-40 extracts of frog olfactory bulb membrane proteins, mAb HNK-1 recognized bands at 200, 140, and 115 kD, anti-L1 recognized bands at 205, 185, 60, and 55 kD, and 2B8 recognized bands at 200, 170, 160, and 140 kD.

Although HNK-1 epitopes were also found on the single band immunoprecipitated by 9-OE (Fig. 5, lane f), the proteins immunoprecipitated by mAb 5-OE lacked this carbohydrate epitope. HNK-1 epitopes were detected on the 200-, 140-, and 115-kD forms of N-CAM immunoprecipitated by mAb 13-OE in both olfactory bulb and brain. Since both mAbs 9-OE and 13-OE reacted with the mAb 5-OE antigen (see Table II), it is apparent that the molecules recognized by these two mAbs contain subpopulations without HNK-1 epitopes. Therefore, the 205-kD subset of N-CAMs recognized by mAb 9-OE consists of at least two subpopulations: one possessing the HNK-1 carbohydrate moiety but lacking



**Figure 7.** Affinity-purified SBA binding molecule from olfactory bulb. Lanes *a-g*, respectively, were reacted with  $^{125}\text{I}$ -SBA, mAb 13-OE, mAb 5-OE, mAb 9-OE, 161, anti-HNK-1, and rabbit polyclonal anti-L1. Lane *a* is an autoradiograph and appears darker against the white blotting membranes used in the other lanes due to photographic conditions.

the 5-OE epitope, and the other possessing the 5-OE epitope but lacking the HNK-1 epitope.

Both mAb 2B8 and SBA bound proteins immunoprecipitated by mAbs 9-OE, 13-OE, and 5-OE from the olfactory bulb (Table II). The very weak SBA staining of immunoprecipitated mAb 9-OE antigens (Fig. 5 *i*) in comparison with that observed on direct immunoblots (Fig. 3 *b*, lane *c*) suggested that mAb 9-OE reacted with only a subset of the SBA binding molecules. Because L1 has a similar molecular mass to the 200-kD N-CAM, we tested the proteins immunoprecipitated by mAbs 9-OE, 13-OE, and 5-OE for cross-reactivity with polyclonal anti-L1 antibodies (Fig. 5 *h* and Table II). No cross-reactivity was detected with these two polyclonal antibodies in either olfactory bulb or brain.

#### **Immunoabsorption of 9-OE N-CAM from Olfactory Bulb**

The relatively strong mAb 9-OE staining of immunoprecipitated 161 (in olfactory bulb; Fig. 4 *e*) and mAb 13-OE proteins in comparison with that obtained with this mAb on direct immunoblots of olfactory bulb (Fig. 3 *b*, lane *c*) suggested that most, if not all, of the mAb 9-OE antigen was N-CAM. To more rigorously test the degree of overlap between the N-CAM subsets, we sequentially depleted N-CAM (by immunoprecipitation) from olfactory bulb and subsequently probed the supernatant by immunoprecipitation for the presence of remaining antigens.

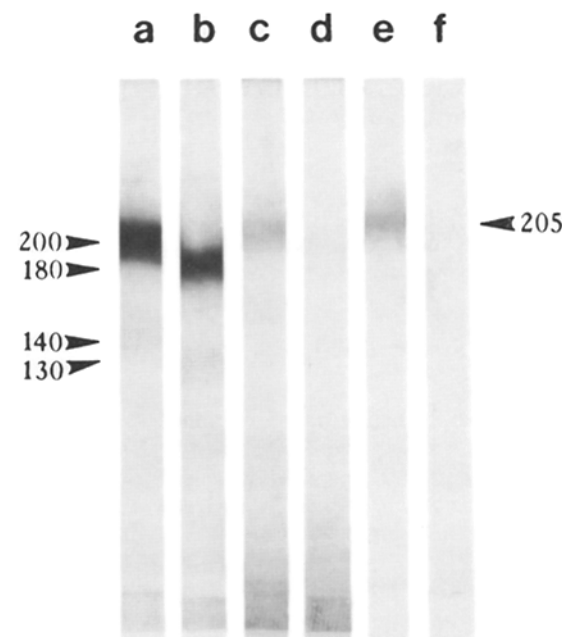
In parallel experiments, we depleted olfactory bulb membrane preparations of mAb 13-OE and 161 antigens (see Fig. 6 *a* and legend for experimental details). mAb 13-OE completely removed all N-CAM (as recognized by 161) from olfactory bulb (Fig. 6 *b*, lane *a*). Similarly, 161 completely removed all mAb 13-OE antigens from olfactory bulb (Fig. 6

*b*, lane *c*). These results indicated that both 161 and mAb 13-OE were binding to the same population of 200- and 140-kD N-CAMs present in olfactory bulb. We next tested these two N-CAM-depleted olfactory bulb extracts for the presence of mAb 9-OE antigens (Fig. 6 *c*). Most mAb 9-OE reactivity was removed by either 161 or mAb 13-OE. However, mAb 9-OE immunoprecipitated from both preparations a 210-kD protein barely detectable by immunoblotting (Fig. 6 *c*, lanes *a* and *b*). Therefore, despite having completely removed all N-CAM (as recognized by 161 and mAb 13-OE) from olfactory bulb, mAb 9-OE still bound to a small population of proteins. This subset of mAb 9-OE antigens migrated at the very top of the 205-kD bands labeled by mAb 9-OE in control preparations (Fig. 6 *c*, lanes *b* and *d*). These results indicate that most 9-OE antigenic sites are on N-CAM and only a small number of these epitopes were present on some proteins other than N-CAM in the olfactory bulb. The identity of this apparently small subset of mAb 9-OE proteins is unknown.

Although it appeared that the 9-OE antigens in olfactory bulb were predominantly N-CAM, we have not, as yet, directly tested the reverse of this; that is whether all the N-CAMs present on the primary sensory olfactory neurons possess the 9-OE epitope. However, the slightly higher and distinct relative molecular mass of the 9-OE antigens in comparison with the 161 and 13-OE N-CAMs in olfactory bulb indicated that the 9-OE epitope was present on only a subset of the high molecular mass N-CAM isoforms.

#### **The SBA Binding Molecule in Olfactory Bulb Is a Subset of 9-OE N-CAM**

We subsequently determined whether the glycoconjugates



**Figure 8.** N-CAM was immunoprecipitated from olfactory bulb with 161 and either deglycosylated with N-glycanase (lanes *b*, *d*, and *f*) or left intact (lanes *a*, *c*, and *e*) before electrophoresis and blotting. Blots were subsequently reacted with mAb 13-OE (lanes *a* and *b*), mAb 5-OE (lanes *c* and *d*), and mAb 9-OE (lanes *e* and *f*).

detected by SBA in the olfactory bulb were also recognized by mAb 9-OE and the anti-N-CAM antibodies. The SBA binding molecules were isolated from solubilized olfactory bulb membranes using affinity chromatography, separated on SDS-PAGE, and electroblotted. <sup>125</sup>I-SBA blotting of the material isolated on the SBA column revealed a single protein band at 205 kD (Fig. 7 a). mAbs 9-OE, 13-OE, and 5-OE and the polyclonal N-CAM antisera all reacted with a glycoprotein of the same relative molecular mass (Fig. 7, b-e, respectively). An additional band at 55 kD was labeled by 161 (Fig. 7 e). This protein was most likely a proteolytic degradation product that was detected by the polyclonal sera (Hoffmann et al., 1982). The 205-kD SBA binding molecule did not react with either the anti-HNK-1 (Fig. 7 f) or the anti-L1 (Fig. 7 g) antibodies. This lack of HNK-1 immunoreactivity further confirmed the presence of subpopulations of 9-OE N-CAM that either possess or lack the HNK-1 epitope as had been previously suggested by the absence of this epitope on the mAb 5-OE antigen.

#### Carbohydrate Nature of the 9-OE Epitope

To test whether SBA was binding to N-linked sugars on N-CAM, we enzymatically cleaved these carbohydrate chains from olfactory bulb membrane on dot blots with N-glycanase. These enzymatically cleaved preparations did not react with SBA (data not shown), which suggested that a portion of the diversity in N-CAM forms in the frog olfactory bulb was associated with N-linked carbohydrate chains. Therefore, we tested the possibility that mAbs 5-OE, 9-OE, and 13-OE were also binding to similar carbohydrate moieties.

N-CAM was immunoprecipitated from olfactory bulb by 161, enzymatically cleaved with N-glycanase to remove N-linked sugars, and subsequently electrophoresed and blotted. These deglycosylated N-CAM polypeptides reacted with mAb 13-OE (Fig. 8 b) but not with mAbs 5-OE (Fig. 8 c) and 9-OE (Fig. 8 e), indicating that the antigenic determinants recognized by the latter two mAbs were associated with carbohydrate moieties. The removal of N-linked sugars resulted in a shift in the size of the N-CAMs recognized by mAb 13-OE from 200 to 180 kD and from 140 to 130 kD. These data indicated that both 5-OE and 9-OE were recognizing either carbohydrate determinants on N-CAM associated with N-linked sugar chains, amino acid epitopes whose conformation depended on the presence of carbohydrates, or possibly a combination of the above. However, on these blots the tertiary structure of the immobilized and denatured N-CAMs is probably not maintained. Therefore, the most likely interpretation of these results is that both mAb 5-OE and mAb 9-OE recognize N-linked carbohydrate determinants on N-CAM.

#### Olfactory Bulb Embryonic N-CAM Is Distinct from 9-OE N-CAM

Recently, it was reported that the embryonic form of N-CAM (E N-CAM), which contains a high proportion of polysialic acid, is present on only a subset of olfactory axons while it is uniformly distributed throughout the deeper laminae of the mouse olfactory bulb (Miragall et al., 1988). Since it was possible that mAb 9-OE was recognizing the frog equivalent to E N-CAM, we analyzed the distribution of E N-CAM immunoreactivity in frog olfactory bulb using mAb 5A5 which

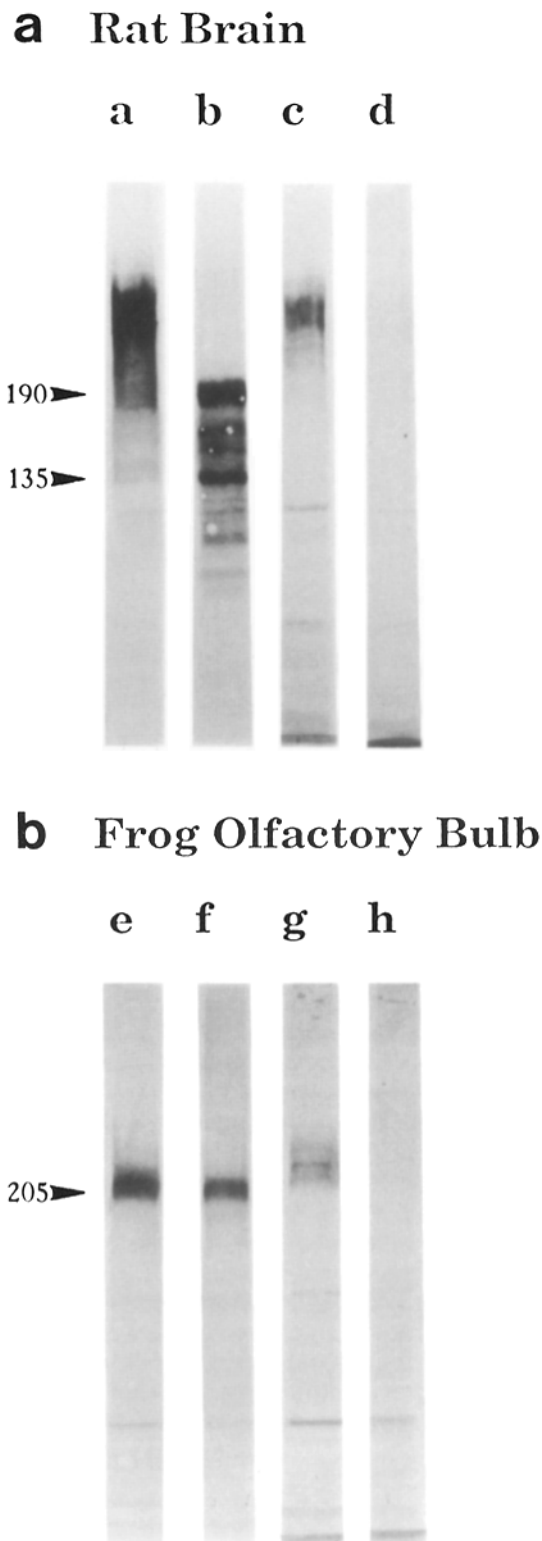


Figure 9. Embryonic day-19.5 rat brain (a) or frog olfactory bulb (b) membranes were heated at 80°C for 3 min (lanes a, b, e, and f) or boiled for 30 min (lanes c, d, g, and h) in Laemmli's sample buffer before electrophoresis. Blots were reacted with 161 (lanes a and b), mAb 5A5 (lanes c, d, g, and h), or mAb 9-OE (lanes e and f).

reacts with a determinant associated with the polysialic acid chains (Dodd et al., 1988). As in mouse, mAb 5A5 staining was found throughout the whole olfactory bulb with some olfactory glomeruli exhibiting elevated levels of immunoreactivity (Fig. 2 *h*). However, in addition we observed a subset of mitral cell perikarya and their dendrites that also preferentially expressed this form of N-CAM (Fig. 2, *h* and *j*). When compared with Fig. 2, *c* and *d*, these results indicated that mAb 9-OE was not specifically recognizing the polysialic acid residues present in E N-CAM expressed in frog brain. This was further confirmed in both the adult rat and mouse olfactory systems, where mAb 9-OE and mAb 5A5 again reacted with different populations of olfactory neurons (not shown).

To further test the immunohistochemical observations that 9-OE N-CAM was distinct from E N-CAM, frog olfactory bulb membranes were desialylated by boiling (Hoffman et al., 1982) and analyzed for their reactivity with mAb 9-OE (Fig. 9). Control experiments using embryonic rat brain membranes revealed that boiling for 30 min removed polysialic acid chains. This was determined by (a) the shift from diffuse high molecular mass sialylated N-CAM forms (Fig. 9, lane *a*) to two prominent desialylated N-CAM polypeptide bands at 190 and 135 kD (Fig. 9, lane *b*) as previously described (Hoffman et al., 1982) and (b) the concomitant loss of mAb 5A5 immunoreactivity (Fig. 9, lanes *c* and *d*). Desialylation of frog N-CAM, while removing mAb 5A5 epitopes (Fig. 9, lanes *g* and *h*), had no effect on mAb 9-OE immunoreactivity (Fig. 9, lanes *e* and *f*). Thus, the mAb 9-OE epitope was not associated with the polysialic acid chains of E N-CAM present in the adult olfactory system.

## Discussion

Unique glycosylated forms of N-CAM were identified in the present study with mAbs generated against olfactory epithelium. mAbs have been widely used to characterize molecules that may be of developmental significance in the nervous system. However, this is the first time that such an approach has identified a bonafide cell adhesion molecule with a region-specific glycosylation pattern in the nervous system. This report identifies a form of N-CAM that is expressed only on olfactory neurons, has a relative molecular mass of 205 kD, and contains unique N-linked carbohydrate(s), distinct from the polysialic acid chains, that are identified specifically by mAb 9-OE. Although other subsets of N-CAMs have been reported, this is the only set that has a very restricted distribution pattern in the vertebrate nervous system.

### Regional Expression of N-CAM

The frog N-CAMs recognized by our panel of antibodies were immunoprecipitated and tested for the presence of specific epitopes. This approach has enabled the characterization of several distinct subsets of the 200-kD N-CAMs. mAb 13-OE appears to bind the total population of N-CAMs present in the frog brain. On the other hand, mAbs 9-OE and 5-OE recognize only a subset of these N-CAMs. mAb 9-OE binds a subset of N-CAMs of 205 kD relative molecular mass that are expressed only by olfactory neurons. At the level of sensitivity of the immunohistochemical and immunoblot

techniques used in the present study, we could not detect expression of this N-CAM subset elsewhere in the nervous system. The mAb 9-OE-immunoreactive N-CAMs were shown to consist of, at least, two separate populations: those with HNK-1 epitopes and those with 5-OE epitopes and SBA binding sites. mAb 5-OE also binds to a subset of 205-kD N-CAMs that are weakly expressed throughout the brain. A higher level of expression, however, is detected on primary sensory olfactory neurons. Since this form of N-CAM lacks the HNK-1 epitope, two further subsets of N-CAM become apparent: those that have 13-OE and HNK-1 epitopes but lack 5-OE epitopes and those that have 13-OE and 5-OE epitopes but lack HNK-1 epitopes. mAb 5-OE also appears to intracellularly label subsets of neurons throughout the brain. However, this immunoreactivity may be due to cross-reactivity with molecules other than N-CAM since similar intracellular labeling was not observed in sensory olfactory neurons in olfactory epithelium. This may explain the additional labeled bands <75 kD in immunoblots of olfactory bulb reacted with mAb 5-OE.

Taken together, these results revealed the presence of the following four distinct subsets of N-CAMs in the frog nervous system which either possess (+) or lack (-) particular mAb epitopes or SBA binding sites: (a) 9-OE<sup>+</sup>, 5-OE<sup>+</sup>, 13-OE<sup>+</sup>, SBA<sup>+</sup>, and HNK-1<sup>-</sup> in olfactory bulb; (b) 9-OE<sup>+</sup>, 13-OE<sup>+</sup>, HNK-1<sup>+</sup>, SBA<sup>-</sup>, and 5-OE<sup>-</sup> in olfactory bulb; (c) 13-OE<sup>+</sup>, HNK-1<sup>+</sup>, 9-OE<sup>-</sup>, 5-OE<sup>-</sup>, and SBA<sup>-</sup> in olfactory bulb; and (d) 5-OE<sup>+</sup>, 13-OE<sup>+</sup>, SBA<sup>-</sup>, and 9-OE<sup>-</sup> in brain. Clearly, additional N-CAM glycosylation subsets may exist that are not detected by the reagents presently available. Interestingly, the diversity in N-CAM carbohydrate structure we have described is restricted to only the 200-kD isoform. The two smaller N-CAMs (140 and 120 kD) do not appear to possess binding sites for mAbs 9OE, 5OE, and 2B8 or for the lectin SBA. It is possible that a small subset of these lower molecular mass forms contain these epitopes at levels beneath the sensitivity of the immunochemical techniques. Both of the lower molecular mass isoforms do, however, possess mAb 13-OE and HNK-1 epitopes.

### Diversity in N-CAM Carbohydrates

The first evidence for heterogeneity within the extracellular domain of the two large forms of N-CAM (180 and 140 kD) came from immunochemical experiments showing that mAb 3G6 bound to only a subset of these isoforms (Williams et al., 1985). Interestingly, Chuong and Edelman (1984) also generated a mAb that appeared to be directed against a subset of the 180- and 140-kD N-CAMs. This mAb did not bind to either adult mouse olfactory epithelium or cerebellum N-CAMs whereas it reacted with N-CAMs from other regions of the nervous system. More recently, a cDNA encoding for the 140-kD N-CAM polypeptide in rat was isolated and shown to contain an additional 30-base alternatively spliced exon not present in other previously identified N-CAM cDNAs (Small et al., 1987). Additional alternative splicing patterns have subsequently been reported (Dickson et al., 1987; Gower et al., 1988; Prediger et al., 1988; Santoni et al., 1989).

Some diversity in N-CAMs are known to occur from post-translational modifications. Notably, as shown in the present study, differences in the glycosylation of the N-CAM poly-

peptide can generate distinct subsets. Although we have shown that mAb 9-OE is associated with N-linked carbohydrates distinct from the polysialic acid chains, the precise structures of the mAb epitopes and SBA binding sites in frog N-CAM remain uncharacterized. Recently, human N-CAMs from brain and skeletal muscle have been shown to be differentially glycosylated (Walsh et al., 1989). Skeletal muscle N-CAM contains a unique O-linked oligosaccharide recognized by the lectin peanut agglutinin. The presence of this carbohydrate was attributed to a novel attachment site for O-linked sugars contained within a putative muscle-specific alternatively spliced domain. Tissue-specific glycosylation has also been reported for other cell surface glycoproteins such as Thy-1 (Parekh et al., 1987). However, as opposed to skeletal muscle N-CAM, the carbohydrate heterogeneity observed between brain and thymus in Thy-1 is associated with tissue differences in the addition of oligosaccharides rather than primary differences at the level of the amino acid sequences. Therefore, the glycosylation patterns observed in olfactory N-CAMs in the present study could arise either from amino acid sequence differences as in human skeletal muscle N-CAMs (Walsh et al., 1989) or from differences in the regulatory mechanisms for glycosylation in different cell types (Rademacher, 1988).

### Significance of N-CAM Oligosaccharides

The spatial and temporal regulation of N-CAM expression has been proposed as one means for modulating the adhesiveness of cells during the histogenesis of the nervous system (Hoffman et al., 1986). In view of both the proposed role of oligosaccharides in N-CAM-mediated cell adhesion (Kunemund et al., 1988; Rutishauser et al., 1988) and the very restricted spatial expression pattern for 9-OE N-CAMs, it seems plausible that this N-CAM subset could be selectively involved in adhesive mechanisms mediating axonal fasciculation, olfactory glomerulus formation, or other events in the mature olfactory system. This possibility of course does not preclude a role for this N-CAM subset during early development. Furthermore, N-CAM subsets may also be present in the mammalian nervous system since the lectin SBA also binds to subsets of primary sensory neurons in the rodent olfactory system and spinal cord (Key and Giorgi, 1986b; Plenderleith et al., 1988, 1989).

### Conclusion

Evidence documenting that a large repertoire of N-CAMs arise from both alternative splicing and posttranslational events is increasing. Unique N-CAM glycoforms have now been identified in olfactory neurons and muscle. The full range of N-CAM diversity in amino acid sequence and posttranslational modifications remains to be determined. It has been hypothesized that individual N-CAM isoforms may, through their topographical localization, contribute to the modulation of specific cell-cell interactions in the vertebrate nervous system. The olfactory system in frog, an animal most amenable to experimental manipulation, is a powerful model to test this hypothesis during both development and regeneration.

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