

# Immunolocalization of a Neuronal Growth-dependent Membrane Glycoprotein

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**ABSTRACT** Monoclonal antibody (mAb) 5B4 recognizes in the rat a large, developmentally regulated membrane glycoprotein. The larger form of this antigen (185–255 kD) occurs in the developing nervous system and is present in membranes of nerve growth cones, as determined by analysis of a growth cone particle fraction. An immunochemical characterization of this antigen and of a smaller form (140 kD), sparsely present in the mature nervous system, has been described (Ellis, L., I. Wallis, E. Abreu, and K. H. Pfenninger, 1985, *J. Cell. Biol.*, 101:1977–1989). The present paper reports on the localization by immunofluorescence of 5B4 antigen in cultured cortical neurons, developing spinal cord, and the mature olfactory system. In culture, mAb 5B4 stains only neurons; it is sparsely present in neurons at the onset of sprouting while, during sprouting, it appears to be concentrated at the growth cone and in regions of the perikaryon. In the developing spinal cord, 5B4 labeling is faintly detectable on embryonic day 11 but is intense on fetal day 13. At this stage, the fluorescence is observed in regions of the cord where axonal growth is occurring, while areas composed of dividing or migrating neural cells are nonfluorescent. With maturation of the spinal cord, this basic pattern of fluorescence persists initially, but the staining intensity decreases dramatically. In the adult, faint fluorescence is detectable only in gray matter, presumably indicating the presence of the 140 kD rather than the fetal antigen. The only known structure of the adult mammalian nervous system where axonal growth normally occurs is the olfactory nerve. mAb 5B4 intensely stains a variable proportion of olfactory axons in the mucosa as well as in the olfactory bulb. Based on both immunochemical and immunofluorescence data, the 5B4 antigen of 185–255 kD is associated specifically with growing neurons, i.e., neurons that are generating neurites.

In the previous article (3), the generation of a monoclonal antibody (mAb)<sup>1</sup> to a neuronal membrane antigen that is developmentally regulated and concentrated in growth cone membranes was reported. In the brain of the fetal rat, this antibody (mAb 5B4) recognizes a 185–255-kD membrane glycoprotein that is rich in sialic acid residues and resembles in its biochemical properties the previously described neuronal antigen(s) designated D2 (8, 10), N-CAM (for neural cell adhesion molecule) (e.g., 2, 7, 12), or BSP-2 (4, 6, 13). In the mature brain, mAb 5B4 does not seem to bind to synaptosomal membranes but recognizes a very small amount of a

140-kD protein in crude membrane preparations.

In the present paper, we present results obtained by immunofluorescence in order to address the following questions. (a) Is the localization of the 5B4 antigen in the developing brain consistent with the data obtained by subcellular fractionation? (b) How is the expression of the 5B4 antigen related to neurogenesis? (c) How is the expression of the 5B4 antigen related to axonal growth and synaptogenesis? To study these issues, we have examined the labeling of neurons sprouting in culture, during spinal cord development, and in the mature olfactory system. The culture system enables one to look at individual neurons at different times after seeding in vitro, whereas the developing neural tube enables one to monitor

<sup>1</sup>Abbreviations used in this paper: mAb, monoclonal antibody; N-CAM, neural cell adhesion molecule.

in vivo neuronal differentiation from the mitosing neuroblast to the synapsing neuron. The olfactory nerve is of particular interest because it is the only known mammalian system in which new neurons are generated and axons grow out *de novo* in the adult (5).

## MATERIALS AND METHODS

**Immunoreagents:** The techniques used for the generation and screening of monoclonal antibody (mAb) 5B4 have been described in the previous paper (3). Hybridoma culture supernatants and different batches of ascites fluid were used for the present studies. Ascites fluid, diluted in phosphate-buffered saline (pH 7.3) containing 1% bovine serum albumin (fraction V) (PBS-BSA) (Sigma Chemical Co., St. Louis, MO), was used for all experiments illustrated here. The fluorescent secondary antibody was an affinity-purified goat anti-mouse IgG conjugated to fluorescein isothiocyanate (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, and Boehringer Mannheim Biochemicals, Indianapolis, IN).

**Cell Culture:** Fetal rats (day 18 of gestation) were used for the preparation of neural cultures. The brains were dissected and the cortices dissociated as described in the previous paper (3). The dissociated cells were pelleted from solution, resuspended in medium, and plated into 35-mm poly-D-lysine-coated plastic dishes. The cells were grown in F-12 medium (Gibco Laboratories, Grand Island, NY) containing 10% fetal calf serum, insulin, transferrin, progesterone, putrescine, selenous acid, and estradiol (all from Sigma Chemical Co.) at 37°C, with ~3% CO<sub>2</sub> in humidified air (1). Cultures were maintained for 3 d to 1 wk. They were fixed with 2% formaldehyde in phosphate buffer (0.1 M, pH 7.3) as described in reference 3.

**Cryotomy:** All tissue used for cryotomy was fixed with 2% formaldehyde (prepared fresh from paraformaldehyde powder) (Fisher Chemical Co., Springfield, NJ) in phosphate buffer (0.1 M, pH 7.3). Fetuses between days 11 and 18 of gestation were removed from the anesthetized mother by cesarian section and placed into fixative. Adult tissues (spinal cord, olfactory mucosa, and bulb) were obtained after perfusing the rat with the fixative. The dissected tissues were then immersed in the same fixative for 1 to 2 h at room temperature. For sectioning of intact olfactory mucosa and bulb, the surrounding cartilaginous and bony tissues were softened by demineralization. The area of the skull containing these olfactory structures was incubated in 10% EDTA in 0.1 M Tris/0.15 M NaCl, pH 7.4, at 4°C while rocking for 5 d. The demineralizing solution was changed daily. The tissues were then transferred into 30% sucrose in 0.1 M phosphate buffer, pH 7.3, and kept at 4°C until the samples sank (usually overnight). After equilibration with 30% sucrose, whole fetuses (11 and 13 d) or tissue blocks from the 15- and 18-d-old fetuses and adult animals were embedded in OCT (Lab-TEK, Miles Laboratories Inc., Naperville, IL), and frozen by slow submersion in liquid nitrogen. The frozen blocks were kept at -80°C until used for sectioning. Sections were cut in a cryostat to a thickness of ~10 μm.

**Immunostaining:** For indirect immunofluorescence, fixed cultures and frozen sections were treated with identical immunoreagents and wash buffers except that 0.02% saponin was added to all solutions used to prepare the cultured material. Samples for labeling were first incubated in PBS-BSA for 30 min to quench any residual aldehyde groups. mAb 5B4 (ascites) and, as a control, myeloma ascites were used for the primary antibody incubations. These immunoreagents were diluted in PBS-BSA 250- or 500-fold for labeling of frozen sections and 100-fold for cultures. Incubation in primary antibody was 2 h for cultures and 1 h for cryostat sections, both at room temperature. Unbound antibody was removed by washing with PBS-BSA six times for 5 min each time for cultures, three times for 5 min each time for sections. Incubation with secondary antibody (goat anti-mouse IgG conjugated to fluorescein isothiocyanate, diluted 50-fold in PBS-BSA) was for 2 h (cultures) or 1 h (sections) at room temperature. Excess secondary antibody was washed out as described for the primary antibody. Cultures were mounted under round coverslips after breaking away the wall of the 35-mm dish and adding a drop of 50% glycerol in PBS (pH 7.3). Coverslips were sealed to the surface of the dish with nail polish. Frozen sections were sealed between glass coverslips containing 50% glycerol in PBS and then cemented to slides with Permount (Fisher Chemical Co., Springfield, NJ). Immunofluorescence microscopy was done using a Leitz Ortholux microscope equipped with phase-contrast and epifluorescence optics. Photomicrographs were taken with a Leitz automated camera system using Tri-X film (Eastman Kodak Co., Rochester, NY). However, for comparison of fluorescence intensities in different sections and anatomical regions, exposure times were held constant, i.e., the automatic exposure system was not used. Likewise, negatives were printed using equal exposure times and conditions of paper development.

## RESULTS

### Cultures of Cerebral Cortex

In the previous paper (3), the use of indirect immunofluorescence demonstrated that mAb 5B4 labels the sprouting neuron, and that the staining requires pretreatment with saponin. In these cell cultures, prepared from fetal rat cortex, the 5B4 antigen is observed only on cells with the morphological appearance of neurons (Fig. 1). Fig. 1A is a phase-contrast micrograph of a 2-3-d-old culture. A small cluster of rounded cells of high contrast, typical of neurons in culture, is attached to less prominent, flat, and polygonal cells. The flat elements are supporting cells of fibroblastic or glial nature (Fig. 1A). After saponin treatment and labeling with mAb 5B4 and the FITC-conjugated second antibody, fluorescence microscopy reveals weak staining in the cluster of rounded cells only. Not all the rounded cells are labeled, however. Where present, the staining is ring-like, but a small region in the rounded cells appears to exhibit a more intense fluorescence. None of the flat cells exhibit any fluorescence. Control cultures incubated with myeloma-conditioned medium are totally devoid of fluorescence (data not shown; see reference 3). Culturing these cells for ~5 d results in neuronal sprouting. Such neurons are 5B4-positive as seen in Fig. 1, C and D. These cells have round or ellipsoid perikarya and each exhibit a single large neurite. The entire neuron seen in Fig. 1C is outlined by fluorescence, and it has a brightly stained, protruding region at its perikaryon opposite to the origin of the major neurite. The neuron shown in Fig. 1D has a major neurite that appears to bifurcate (small arrowhead). At the tip of one of the branches is a large amoeboid structure, a growth cone, whose veil-like processes are outlined by fluorescence (Fig. 1D, large arrowhead). The proximal portion of this growth cone and the distal shaft of the axon immediately contiguous with it are brightly fluorescent. Intense fluorescence is also seen in an amorphous patch and in discrete structures near the nucleus. The discrete structures may be circular or ring-shaped in appearance. The perinuclear fluorescence is the result of superimposition of cytoplasmic elements with those of the tip of a neurite branch projecting back onto the cell. A second amoeboid structure originating from the perikaryon is seen at the lower left of the perikaryon.

### Developing Spinal Cord

Having established that the 5B4 antigen is present on sprouting neurons and not detectable by immunofluorescence in non-neuronal cells of the brain in culture, the appearance and distribution of the 5B4 antigen was investigated during neural development in vivo. The developing spinal cord is particularly suitable for this study because it exhibits clearly defined morphological regions whose structure and development have been well characterized in independent studies (e.g., reference 11).

Fig. 2 shows a series of photographs from frozen sections of fetal spinal cord, labeled for indirect immunofluorescence with mAb 5B4. At day 11 of gestation, a cross section through the embryo shows faint labeling of parts of the neural tube and the ectoderm (Fig. 2, E11). By fetal day 13, certain regions of the developing cord are intensely fluorescent (Fig. 2, F13). The most fluorescent area is the marginal layer, the periphery on the ventrolateral sides of the spinal cord (Fig. 2, F13, *mr*). This region contains developing axon bundles that are as-

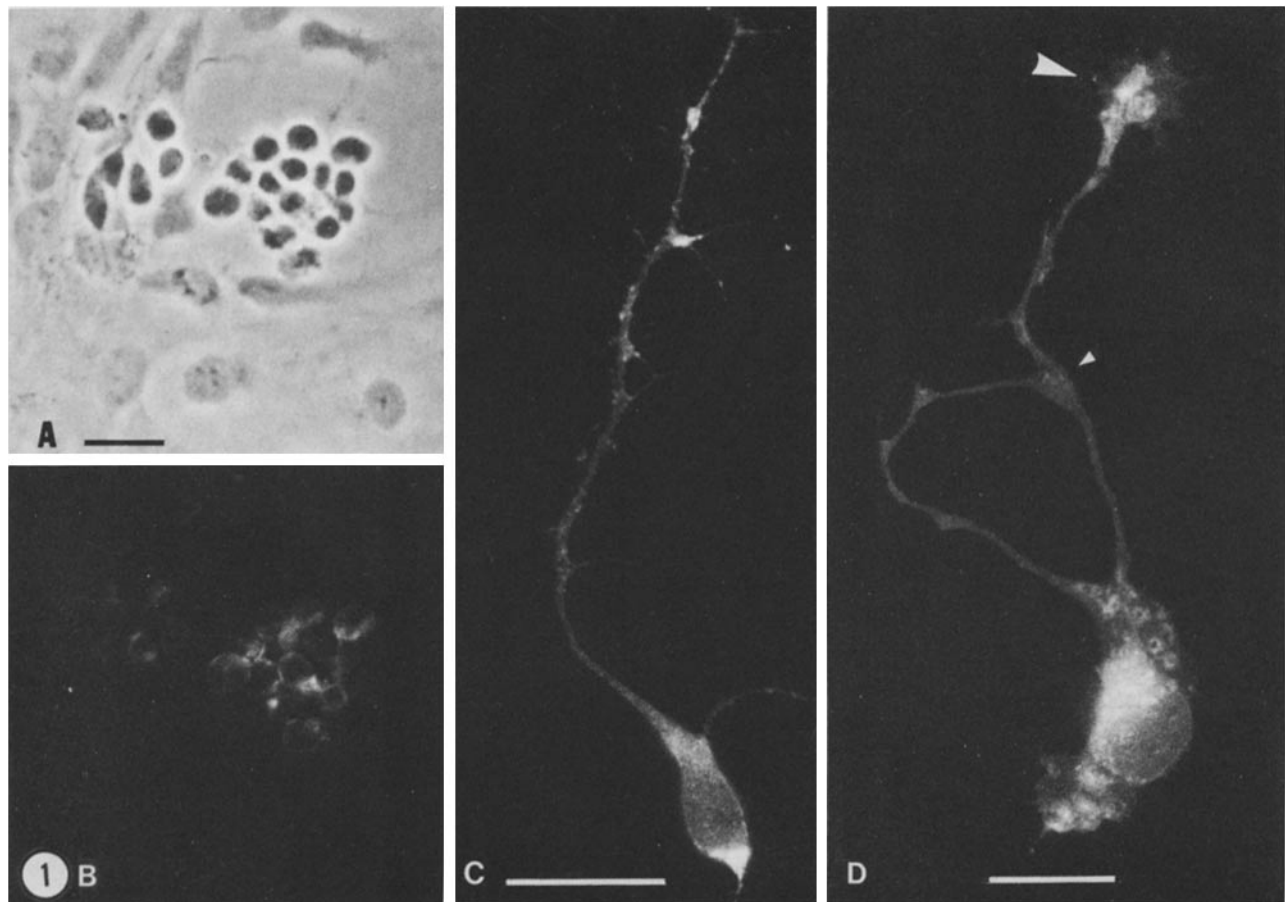


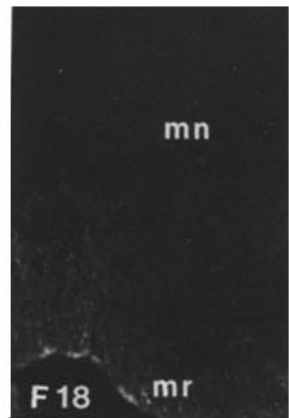
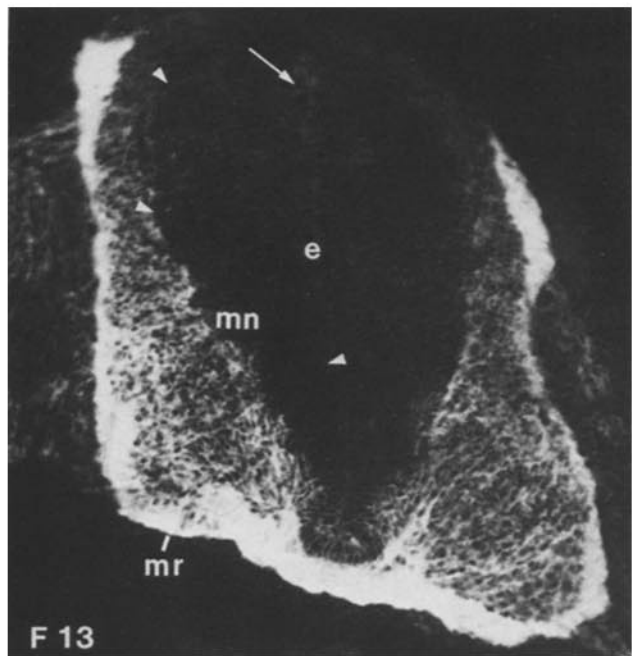
FIGURE 1 Neural cell culture. (A) Phase-contrast micrograph of young neurons growing on a carpet of non-neuronal cells. (B) Fluorescence micrograph of field seen in A. Only neuronal cells bind mAb 5B4. (C and D) Neurons maintained in culture for 5 d. Intense fluorescent labeling with mAb 5B4 is seen on the perikarya, at the growth cone (D, large arrowhead) and in areas along the neurite's shaft (C). Small arrowhead in D shows the point of bifurcation of the neurite. Bars, 20  $\mu\text{m}$ .

ending, descending, entering, and leaving the spinal cord. In appropriate sections, the developing ventral roots can also be seen to be fluorescent at this stage (not shown). In addition, substantial amounts of the 5B4 antigen are also found in parts of the mantle layer, especially in the areas of the developing anterior horns (Fig. 2, *F13*, *mn*). These areas contain the cell bodies of the spinal cord neurons that have gone through terminal mitosis and have begun to elaborate their axons and dendrites. In contrast to the intense continuous labeling of the marginal zone, the mantle layer exhibits more patchy fluorescence outlining the perikarya. The most central regions of the cord, the ependyma and subependymal areas of the mantle zone, reveal little if any specific labeling (Fig. 2; *F13*).

This nonfluorescent zone contains neurons that are still undergoing mitosis and migration. Part of a control section of 13-d fetal spinal cord, from the same block as that shown in Fig. 2, *F13*, is illustrated in Fig. 2, *F13C*. This section was incubated with myeloma-conditioned medium instead of mAb 5B4 and then treated identically to the other sections. No fluorescence is detectable.

In the 15-d fetus (Fig. 2, *F15*) the basic pattern of fluorescence is similar to that of the 13-d gestation spinal cord. The relative intensity of the fluorescence is generally decreased, but the area of mAb 5B4 binding is increased due, in part, to the dramatic growth of the spinal cord. The marginal zone is still the most fluorescent region. The fluorescent portion of

FIGURE 2 Indirect immunofluorescence of frozen sections of developing spinal cord stained with mAb 5B4. (*E11*) Cross section of rat embryo at day 11 of gestation. Faint fluorescence is visible in the ectoderm and the neural tube (arrowheads point out the outline of the neural tube). (*F13*) Fetal spinal cord, day 13 of gestation. The marginal layer (*mr*) containing growing axons is strongly fluorescent. The developing ventral horn areas and intermediolateral regions of the mantle layer (*mn*) also exhibit bright, patchy fluorescence. A more central area of the mantle zone (between arrowheads), which is broader dorsally and extends to the ependymal layer (*e*), is not labeled. The arrow points to the slit-like spinal canal. (*F15*) 15-d fetal spinal cord. The cord has increased greatly in size. The marginal layer now forms a fluorescent zone that encircles the entire cord. The staining in the mantle layer (*mn*) has extended dorsally and medially. Only a narrow nonfluorescent region remains just underneath the unstained ependymal cells (*e*). (*F18*) Cross section of the ventro-medial area of an 18-d fetal spinal cord. Fluorescence in both *mr* and *mn* has decreased dramatically relative to *F13*. (*F13-C*) Control: region of the *mr* and *mn* of a 13-d fetal spinal cord labeled with conditioned medium. No fluorescence is seen in this control section. All photographs were taken and printed under identical conditions of exposure. Bar, 200  $\mu\text{m}$ .



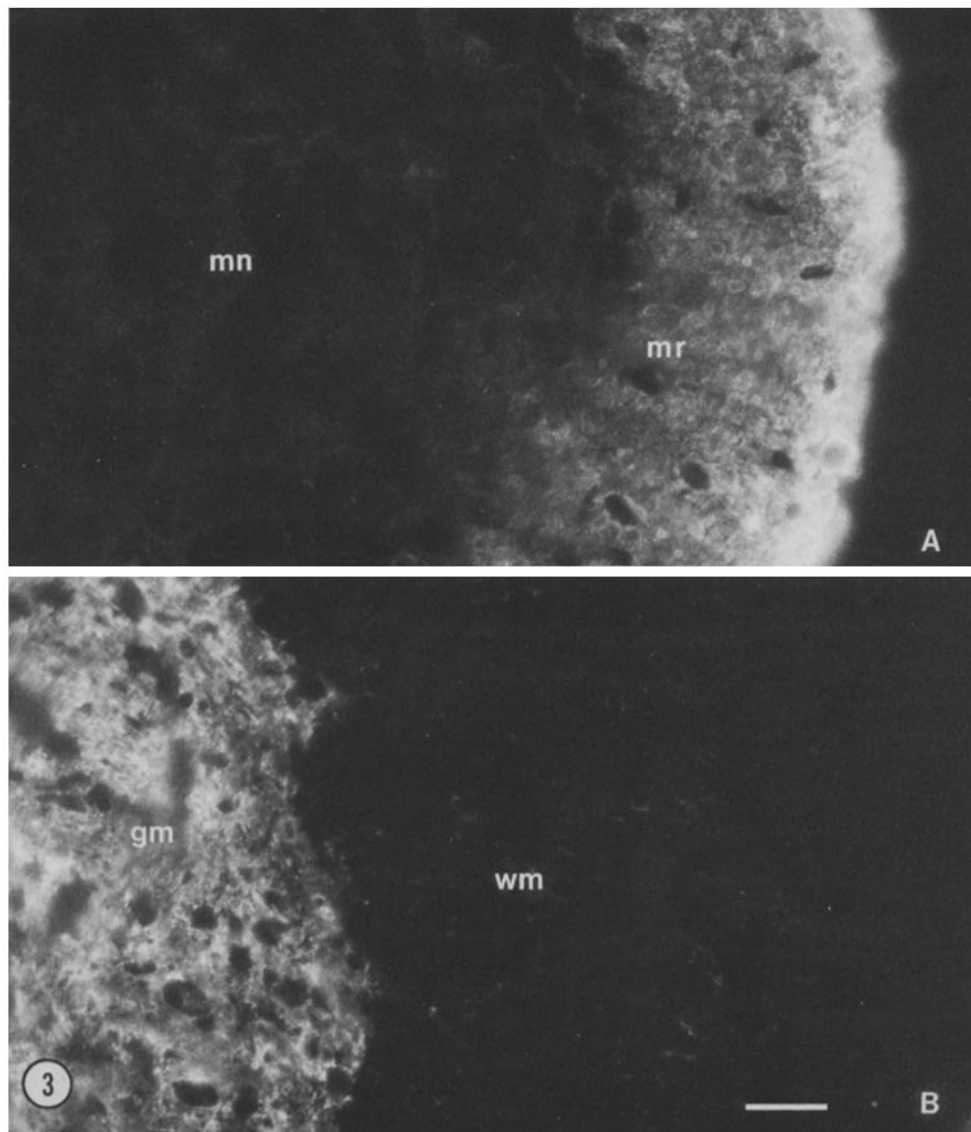


FIGURE 3 High magnification fluorescence micrographs of spinal cord, frozen sectioned and labeled with mAb 5B4. These photographs have been exposed longer than those in Fig. 2 so as to enhance the weak fluorescence that is present (cf. Fig. 2). (A) 18-d fetal spinal cord; almost all the labeling is restricted to the marginal layer (*mr*). Fluorescence in the mantle layer (*mn*) is barely detectable. (B) Adult spinal cord; labeling, still very weak, is now confined to the gray matter (*gm*). White matter (*wm*) does not stain with mAb 5B4. Bar, 20  $\mu$ m.

the mantle layer (*mn*), still patchy in appearance, has grown at the expense of the more central, nonfluorescent area. The ependymal layer lining the spinal canal and immediately subjacent cells are unlabeled as before (Fig. 2, *F15*).

By fetal day 18, the intensity of staining with mAb 5B4 is greatly diminished in the cord. This decrease is particularly dramatic in the marginal layer (Fig. 2, *F18*, *mr*), which is now barely visible in fluorescence conditions used for the other panels of Fig. 2. Fig. 3 shows micrographs taken at higher magnification of 18-d fetal (*A*) and adult (*B*) spinal cord. The negatives of these micrographs have been over-exposed and then less darkly printed in order to enhance the actual low level of fluorescence. The 18-d cord (Fig. 3*A*) exhibits relatively more labeling in the developing marginal layer (*mr*) as compared to the mantle layer (*mn*). In the mantle layer, the diffuse fluorescence appears to outline cell perikarya, which are unstained. For the adult spinal cord, the fluorescence

pattern appears reversed (Fig. 3*B*): the marginal zone (white matter [*wm*]), which now contains the myelinated nerve fibers of the spinal cord, is essentially devoid of fluorescence, whereas faint but significant staining is observed in the mantle zone (gray matter [*gm*]). The pattern of fluorescence in the gray matter spares cell perikarya.

#### Adult Olfactory Mucosa and Bulb

Different regions of olfactory axons of the adult rat were analyzed by indirect immunofluorescence with mAb 5B4. Fig. 4 consists of a series of micrographs from frozen sections of the olfactory mucosa. In the phase-contrast micrographs in Fig. 4, *A* and *C*, the mucosal epithelium (*e*), connective tissue septa and nerve bundles (fila olfactoria, arrows, and arrowheads) are quite apparent. Staining with mAb 5B4 results in the fluorescent patterns seen in Fig. 4, *B* and *D*. The epithelial cells (*e*) are faintly stained, but much more dramatic is the

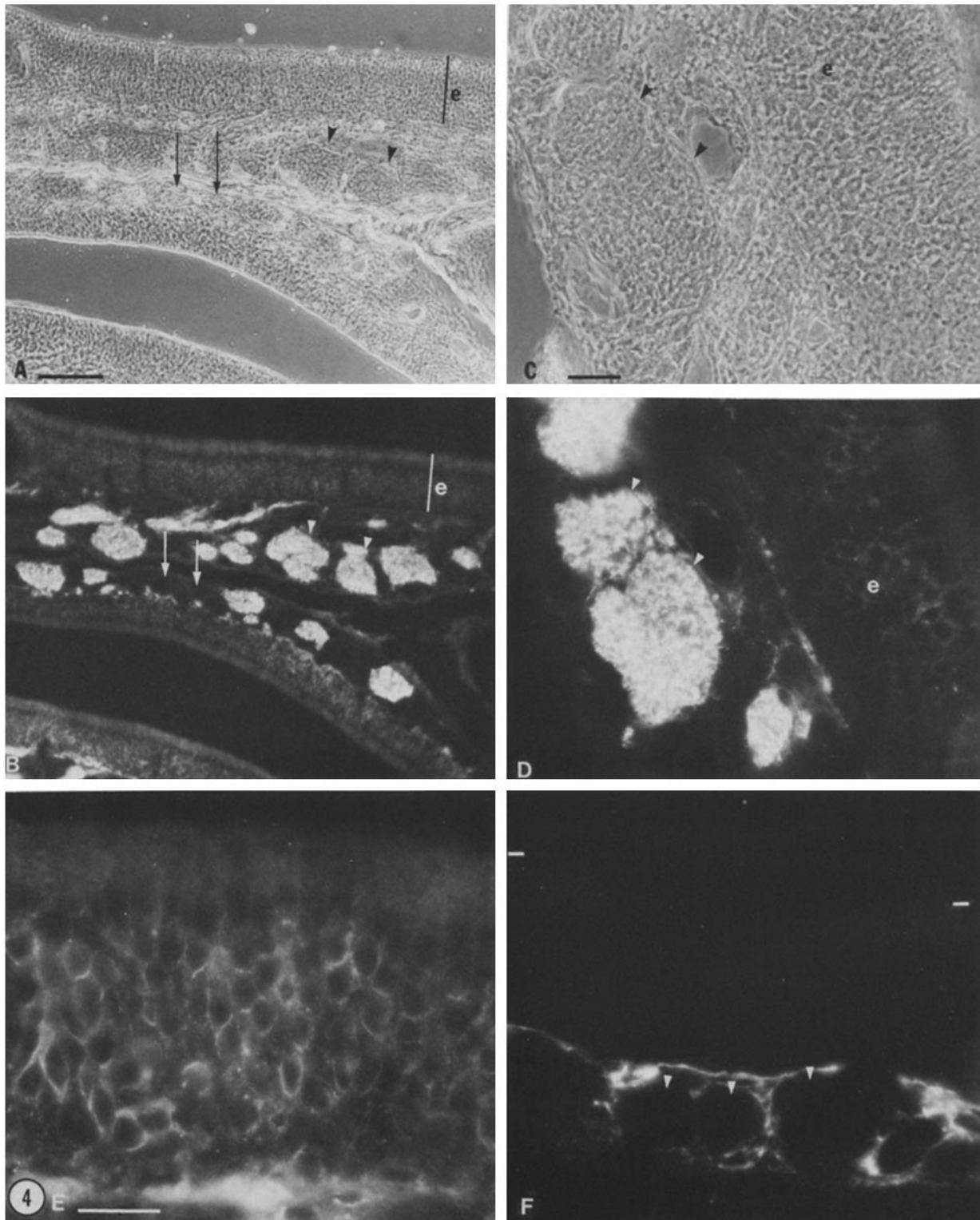


FIGURE 4 Frozen sections of adult olfactory mucosa, immunostained with mAb 5B4. (A) Phase-contrast photomicrograph of the olfactory mucosa. The extent of the epithelium containing the olfactory receptor cells is indicated by a black vertical line (e). Axons from these receptors form variously sized bundles termed fila olfactoria (arrows and arrowheads). (B) Fluorescent micrograph of olfactory mucosa seen in A. Intensely fluorescent regions corresponding to the fila olfactoria are evident (arrowheads). However, not all axon bundles are labeled (arrows). Faint but specific labeling is seen in the epithelium (e). (C and D) Phase-contrast and fluorescent micrographs, respectively, of a region of mucosa taken at higher magnification. The fila olfactoria are heavily stained; however, note the differences in fluorescence intensity within and between individual axon bundles (arrowheads). Faint, ring-like fluorescence is seen in the epithelium (e). (E) In this high magnification micrograph of the epithelium, fluorescent labeling around the somata of some cells is seen. Cell bodies near the surface of the epithelium appear to be unstained. The fluorescence pattern seen is consistent with surface labeling of the neuronal cell population of the epithelium. The brightly fluorescent region near the bottom of the micrograph may be a fascicle of olfactory receptor axons exiting the epithelium. (F) Control section of the olfactory mucosa, incubated with myeloma ascites instead of mAb 5B4. The fila olfactoria are unstained (arrowheads); however, blood vessels and connective tissue surrounding the axon bundles (perineurium) stain nonspecifically. No staining is seen in the epithelium, whose surface is indicated by the bars. Bars (A and B) 100  $\mu\text{m}$ ; (C-F) 20  $\mu\text{m}$ .

fluorescence of the axon bundles below the epithelium (arrowheads). The smaller fluorescent patches and a narrow band of fluorescence are probably fascicles of axons exiting the epithelium to form the fila olfactoria. Note that not all nerve fiber bundles are fluorescent (see arrows in Fig. 4, *A* and *B*). Furthermore, cross sections through nerve fiber bundles exhibit nonfluorescent patches, thus producing an uneven staining pattern (Fig. 4*D*, arrowheads). The control picture (Fig. 4*F*) shows that the fine network of connective tissue surrounding the axon bundles is nonspecifically fluorescent. At high magnification, one can see relatively weak fluorescence in the pseudostratified epithelium forming the olfactory mucosa. It is variable in intensity and of ring-like character, surrounding the somata of some epithelial cells (Fig. 4*E*). The faint fluorescence on the surface of the mucosa is nonspecific (note that Fig. 4*E* is overexposed relative to the control shown in Fig. 4*F*).

The axons of olfactory receptor cells travel in the olfactory nerves to terminate ipsilaterally in the paired olfactory bulbs. Fig. 5 illustrates the morphology and staining pattern of the olfactory bulbs. A low-power phase-contrast micrograph is shown in Fig. 5*A*. This section is from the medial region of the bulbs, and parts of both bulbs are evident. Small circular areas of higher contrast, olfactory glomeruli (arrowheads) are visible. The space between the bulbs is partially filled with connective tissue and blood vessels. The fluorescence micrograph of Fig. 5*B* shows the pattern of mAb 5B4 staining. A superficial zone of the bulbs, the area containing the afferent fila olfactoria, exhibits labeling, and the staining is patchy. However, the more central regions of the bulbs are completely unstained. Round, variably fluorescent areas corresponding to the circular areas in the phase-contrast micrograph (cf. Fig. 5*A*) are also evident (arrowheads). Fig. 5*C* is a control section incubated with myeloma-conditioned medium instead of mAb 5B4. This fluorescent micrograph was overexposed to reveal a small amount of nonspecific fluorescence associated with the connective tissue between the bulbs and the surface covering of the bulb. The outer edge as well as the rest of the bulb are without any fluorescence. To obtain more detailed information on the fluorescent labeling of the bulb, photomicrographs were taken at higher magnification (Fig. 5, *D* and *E*). The phase-contrast micrograph (Fig. 5*D*) is from a lateral region of the bulb. Again, a few olfactory glomeruli are seen below the surface (arrowheads). The corresponding fluorescence pattern (of the same section) is shown in Fig. 5*E*. Round (or ellipsoid) fluorescent structures can now be seen to correspond to the glomeruli (arrowheads). The fluorescence intensity seems to vary among them and within an individual glomerulus.

## DISCUSSION

Two general, basic observations with mAb 5B4 are that (*a*) the patterns of immunofluorescence are quite distinctive for different brain regions of the central nervous system at specific times of development, and (*b*), in the undifferentiated neural tube and the adult brain (except for areas containing olfactory nerve), specific fluorescence is very low, just barely detectable. These conclusions are also based on immunofluorescence studies of the cerebellum and cerebral cortex, which are not illustrated in this paper. The bright fluorescence observed in fetal brain and its almost complete disappearance during maturation are consistent with the decrease in 5B4 binding

to brain membranes observed biochemically during maturation (3). These findings indicate that immunofluorescence studies with mAb 5B4 monitor primarily the presence or absence of the 185–255 kD, polysialylated fetal antigen. Therefore, the following discussion will focus on the expression and distribution of this antigen.

### *Mitotic Neural Cells and Sprouting Neurons*

During the transformation of the neural tube into the spinal cord, dividing neuroblasts are found in a central region around the spinal canal, whereas the first postmitotic and sprouting neurons can be seen in ventrolateral domains corresponding to the future anterior horns. The poorly differentiated neural tube of embryos at day 11 of gestation, as well as the more central areas of the mantle zone of the maturing spinal cord (where neurites are not yet present), exhibit only faint 5B4 immunofluorescence. This is consistent with our observations on dissociated neurons in culture: neurons that have not yet formed neurites are either nonfluorescent or may have just a small fluorescent “pole.” Therefore, nonsprouting neurons which have not proceeded through terminal mitosis do not express the 5B4 antigen at detectable levels. This observation is in clear contrast to the staining of developing spinal cord with anti-N-CAM antibodies, which results in striking immunolabeling of the undifferentiated neural tube and ectoderm (14).

Neurons sprouting in culture are brightly fluorescent, especially in somata and proximal growth cone regions. In the neuronal perikarya one occasionally sees irregularly shaped, fluorescent structures that may represent the Golgi complex and/or aggregates of membrane sacs ready to be exported into the growing neurite. These observations raise questions as to the biochemical synthesis of the 5B4 antigen and its export to the growth cone. Quantitative comparison of the various plasmalemmal regions is not possible because the signal strength of fluorescence is dependent not only on the density of antigen per unit area of membrane but also upon the amount of membrane superimposed in a particular optical cross section. Thus, the label density on the very thin lamellipodia (~0.1  $\mu\text{m}$  thick) is underestimated relative to the contours of the perikaryon, which is at least several micrometers thick. The question of enrichment of the 5B4 antigen in growth cone plasmalemma will have to be answered by quantitative mapping at the electron microscopic level.

In vivo, bright fluorescence appears in the developing spinal cord as growing neurites emerge. Again, this observation is consistent with the data from isolated growth cone particles and cultured neurons (3). mAb 5B4 does not appear to discriminate among different types of neuron. Immunofluorescence of developing fiber tracts has been observed in various parts of the central and peripheral nervous systems. The labeling patterns are consistent with the expression of the 5B4 antigen in axons, whereas no conclusive data are available on its presence in, or absence from, dendrites. Based on the sum of observations made to date, the expression of the 185–255-kD 5B4 antigen is closely correlated with the sprouting phase of neuronal differentiation in development.

### *Neuronal Maturation and Synaptogenesis*

With increasing age of the spinal cord, immunofluorescence is observed in greater and greater areas of the developing

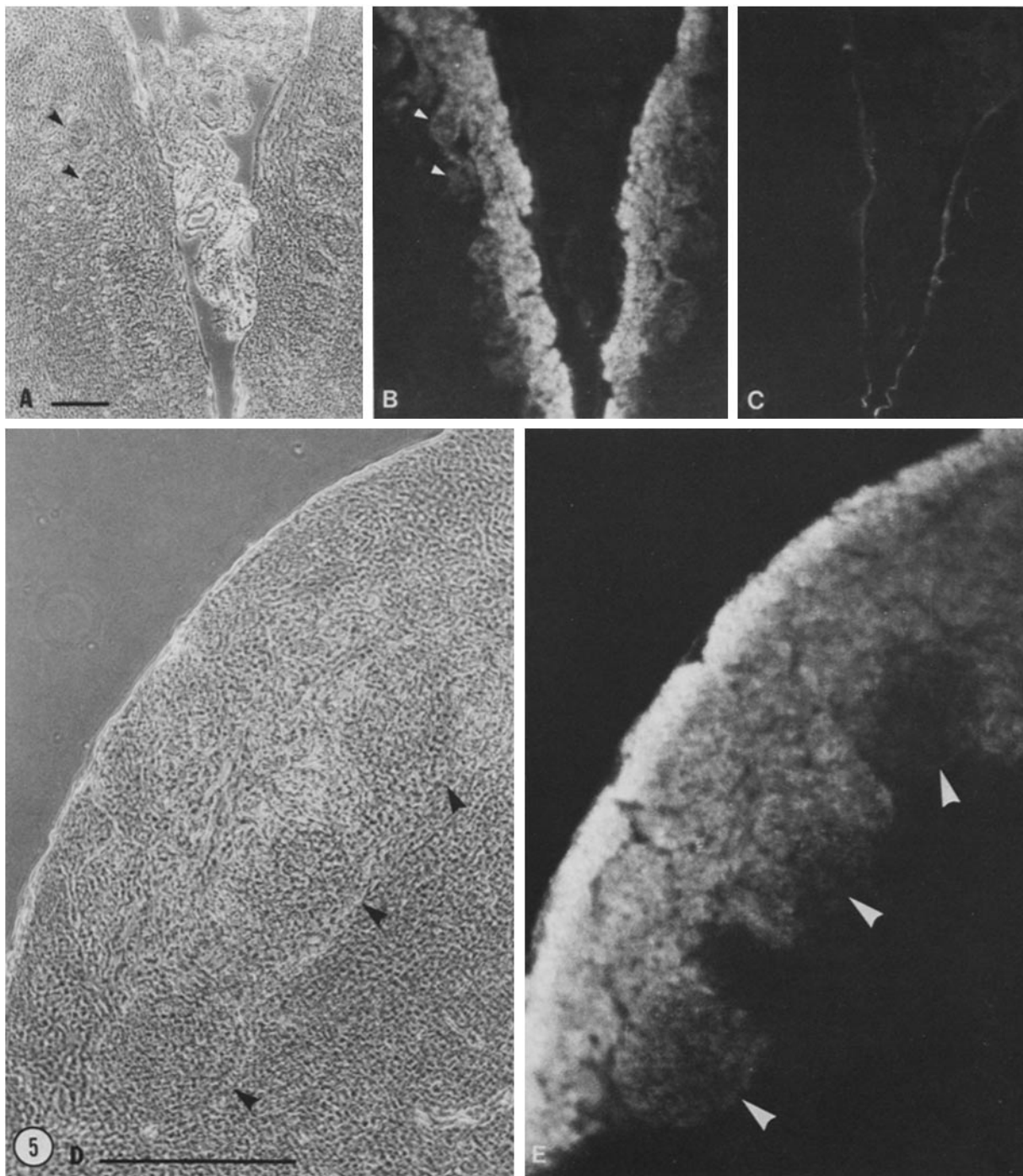


FIGURE 5 Immunostaining with mAb 5B4 of adult olfactory bulb. (A) Phase-contrast micrograph of a medial region of a pair of olfactory bulbs. Two olfactory glomeruli are indicated by the arrowheads. (B) Fluorescence micrograph of the same section as shown in A. Fluorescence is seen around the periphery of the bulbs and in some of the glomeruli (arrowheads). A control section using myeloma ascites as the primary antibody is seen in C. No staining is seen within the bulbs. Some slight nonspecific labeling can be detected in the meninges. (D and E) Phase-contrast and fluorescence micrographs of the same section of lateral olfactory bulb viewed at higher magnification. Fluorescence is limited to the periphery of the bulb and to the more deeply located glomeruli (arrowheads). Note the patchy fluorescence, varying in intensity within and between glomeruli. This pattern is consistent with that of the fila olfactoria, the axon bundles that project to the glomeruli (cf. Fig. 4, B and D). No immunolabeling is detected in the medial area of the bulb, which is not reached by olfactory axons. Bars, (A–C) 100  $\mu$ m; (D and E) 50  $\mu$ m.

spinal cord, but the fluorescence intensity decreases. This is consistent with the biochemical observation (3) that the expression of the 5B4 antigen, especially its fetal form, decreases during the maturation of newly formed neurites. In the adult spinal cord, virtually no fluorescence is detectable

after staining with mAb 5B4. In particular, the peripheral fiber tracts of the developing spinal cord, which were most brightly fluorescent in the fetus, are devoid of fluorescence in the adult, whereas low levels of staining are detectable in the spinal cord's gray matter. On the basis of these observations



and our biochemical results (3), one may tentatively conclude that the immunofluorescence in adult neuropil is the 140-kD antigen. Therefore, our results obtained by immunofluorescence corroborate the biochemical data and demonstrate that expression of the fetal 5B4 antigen decreases when axonal growth ceases, most likely in conjunction with synaptogenesis.

### Neuronal Growth in the Adult

Our observations on 5B4 expression in the developing spinal cord per se leave open the possibility that this antigen is present during a particular phase of nervous system development rather than being dependent upon axonal growth. Therefore, the analysis of an adult system in which neurons are constantly turned over is of particular interest.<sup>2</sup> The bright fluorescence of olfactory nerve bundles stained with mAb 5B4 indicates that the expression of this antigen is not restricted to the developing animal.<sup>3</sup> Bundles of fluorescent fibers can be followed from the mucosa to the glomerular layer of the olfactory bulb, where they will eventually form synaptic terminals. Interestingly, fila olfactoria or glomeruli include a variable proportion of non- or poorly fluorescent structures.<sup>4</sup> This suggests that mAb 5B4 discriminates between growing and, perhaps, immature (fluorescing) and mature (nonfluorescing) axons. In other words, the presence of the 5B4 antigen is not simply a function of chronologic age; rather, 5B4 expression is consistent with that of a marker of neurite growth.

### Conclusions

Immunofluorescence studies with antibodies to N-CAM and BSP-2 have shown several phases of expression of cross-reactive material during nervous system development (6, 14), and D2 has been localized in synaptic endings (9). Labeling with mAb 5B4 produces a quite different result: the antigen is present predominantly in postmitotic, sprouting neurons, regardless of the developmental stage of the animal. This phase of expression of the (185–255-kD) 5B4 antigen is followed by a massive decrease, most likely in conjunction with synaptogenesis. In contrast to antigen D2, which is related or

<sup>2</sup> In the mouse, the olfactory neuron's life span after terminal mitosis is ~30 d, about 8 d of which are taken up by differentiation and outgrowth. The growing neurons are distributed throughout the mucosa, scattered among the mature neurons (Graziadei, P. P. C., and G. A. Monti Graziadei, 1978, in *Handbook of Sensory Physiology*, Vol. IX, M. Jacobson, editor, Springer-Verlag, New York, 131–153).

<sup>3</sup> This is in striking contrast to another peripheral nerve in the adult, the sciatic nerve, which is normally nonfluorescent. However, preliminary data suggest that fluorescence is increased in sciatic nerve regeneration after crush.

<sup>4</sup> A monoclonal antibody, R4B12, has recently been reported to stain only a fraction of olfactory axons, grouped into bundles, and only selected olfactory glomeruli. In contrast to 5B4 labeling, these glomeruli are stained in toto and confined mostly to the bulb's lateral regions (Fujita, S. C., K. Mori, K. Imamura, and K. Obata, 1985, *Brain Res.*, 326:192–196).

identical to N-CAM, the 5B4 antigen is virtually not detectable in synaptosomes based on dot immunobinding assays and immunoblots (3). The biochemical similarities (see reference 3) and the differences in immunofluorescence patterns between 5B4 and BSP-2/N-CAM/D2 suggest the following interpretation. The latter antibodies may recognize a small number of similar but distinct antigens which are expressed sequentially, at different stages of neuronal differentiation, whereas mAb 5B4 may recognize one of these antigens, a molecule expressed predominantly during axonal growth. In conclusion, we have identified a large membrane glycoprotein (185–255 kD) whose expression is correlated with neurite growth.

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