# A Molecular View of Vertebrate Retinal Development

*Colin J. Barnstable*

*Laboratory of Neurobiology*
*The Rockefeller University*
*1230 York Avenue*
*New York, NY 10021*



## Abstract

Immunological probes have begun to identify molecules that delineate cell layers and cell types during the formation of the retina and other parts of the optic cup. Within the developing retina, cell-type-specific mono-

clonal antibodies have been used to show that differentiation occurs before cells reach their final laminar position. Cell surface molecules have been found expressed in position-dependent gradients across the retina. These molecules may convey positional information to the retinal cells and their topographic connections. One such molecule is a modified carbohydrate group on a ganglioside, suggesting that such groups may play a role in neural development. A variety of molecules that are expressed by rod photoreceptors at defined stages of their differentiation have been characterized. These molecules have been used to show the development of subcellular compartments within rods. In vitro studies have suggested that photoreceptor molecules expressed at different times are under different forms of regulation. Some of these cell-specific molecules have been shown to be under transcriptional control and thus defined cell interactions seem to be linked to changes in gene expression during retinal development.

**Index Entries:** Vertebrate retinal development; retinal development, in vertebrates; development, of the vertebrate retina; embryonic retina; optic cup formation; optic stalk; retinal lamination; positional information within the retina; axon outgrowth cues, retinal; cell adhesion molecules, retinal; photoreceptor development; proto-oncogenes and growth factors, retinal.

## Introduction

The establishment of pattern in developing organisms remains one of the least understood aspects of developmental biology. In the nervous system, some important aspects of pattern formation manifest themselves as the temporally and spatially ordered generation of distinct neuronal cell classes that migrate so as to take up characteristic positions and then acquire characteristic morphologies. The link between the events leading to specification of cell types and the later processes of axonal elongation and synapse formation is at present unknown.

Many of the efforts to understand the forces leading to the production of specific cell types have used simple organisms, such as the nematode *Caenorhabditis elegans*, or insects, such as the grasshopper or *Drosophila*, in which stereotypic cell lineages, ease of observation of the embryo, and small numbers of cells provide many practical advantages (*see* Sulston, 1983; Horvitz et al., 1983; Bastiani et al., 1985). In addition, the rapid life cycle of some of these organisms allows genetic approaches to the analysis of neural development to be employed.

In vertebrates, similar analyses are made more difficult by the inaccessability of the embryo, the number of cells within the nervous system, and the existence of long-range interactions that make it difficult to study any given area of the CNS in isolation. The retina has long been used as a model system to study neural development because it has features that can overcome some of these limitations. These features include a limited number of cell types that are arranged in a laminar structure, as shown in Fig. 1. The retina has also been investigated extensively, both anatomically and physiologically; it develops in the absence of any significant neural input and has a relatively late generation (final mitosis) of many of the cell types, which allows accessability of the developing tissue in neonatal animals.

Development of the retina has historically been described in terms of anatomical and physiological parameters. Because these probably involve many factors, we have taken a more reductionist approach and have sought to describe retinal development in terms of the expression of individual molecules. To identify useful molecular markers, we have produced a panel of monoclonal antibodies against retinal

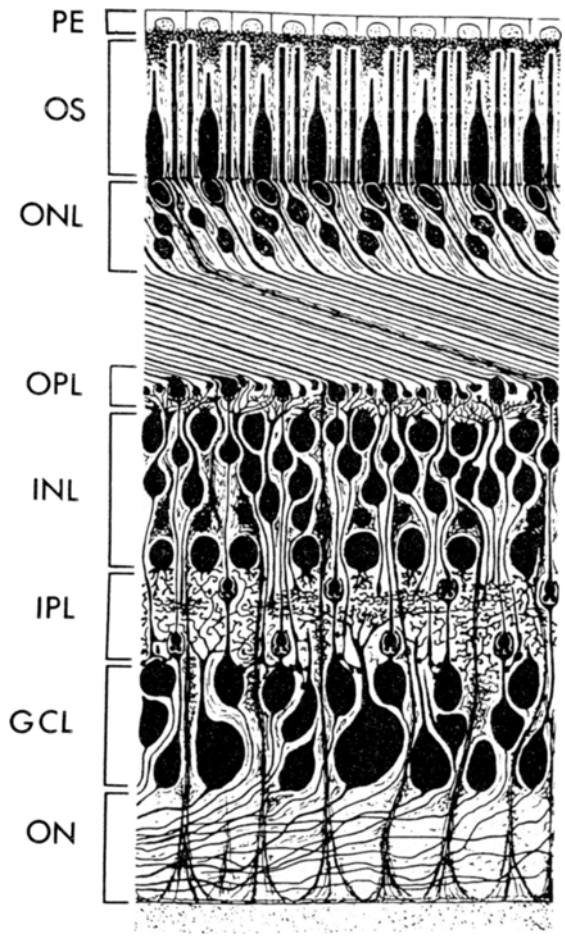


Fig. 1. Diagrammatic representation of a human retina showing the cell types, layered structure, and synaptic connections. PE, pigment epithelium; OS, photoreceptor outer segments; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; ON, optic nerve fiber layer. Taken from Barnstable (1985).

antigens. In the adult tissue we now have immunological markers of each of the major retinal cell types (reviewed in Barnstable, 1982, 1985). Many of these molecules are expressed at discrete times during development, and for some retinal cell types multiple developmental events can be described in terms of antigen appearance. In addition, the availability of these cell-type markers has allowed us to study retinal cells both in the intact tissue and in a variety of culture environments (Barnstable et al., 1983; Akagawa and Barnstable, 1986, 1987).

These initial studies in retina have shown that functional classes of cells can be identified by the expression of particular molecular markers. For example, a number of antigens have been localized to rod photoreceptors that are not found on any other neuronal cell (Barnstable, 1980). Some of these antigens are only on portions of the cell, indicating the degree of subcellular compartmentalization that can be detected in neurons. All retinal amacrine cells express a 35-kdalton membrane protein defined by the antibody HPC-1 (Barnstable et al., 1985). Such a grouping is clearly incomplete since it is known that further subdivisions of amacrine cells can be made on the basis of transmitter phenotype or morphology in the adult animal. Similarly, all

retinal ganglion cells express the surface marker Thy-1, but no good markers of known functional and morphological subclasses of ganglion cells have yet been described (Barnstable and Drager, 1984; Drager et al., 1984). Thus, it is still an open question as to how precisely the functional diversity of retinal cells can be mapped in terms of their detectable molecular diversity.

A further conclusion that comes from studies from a number of laboratories is that functionally related cells of different cell types or even in different regions can also express a common antigen. For example, an antibody raised against rat hippocampus seems to preferentially react with cells that can be classified as part of the limbic system (Levitt, 1984). A further antibody raised against cat spinal cord seems to react with cells in the visual system which have the common property of being involved in motion detection (Hockfield et al., 1983).

Given the range of available molecular markers that have already been described, it is clear that many of the questions of neural development can now be reformulated in terms of questions analyzing the mechanisms controlling the expression of individual molecules. In this article I would like to review some of the available information on molecules expressed during retinal development and describe some of the aspects of development of the mammalian retina that have been of interest within my own laboratory over the last few years. In particular, I want to discuss our results in the context of the series of genetic and epigenetic events that may be of importance both in the specification of position within the developing retinal epithelium and in the formation of mature retinal cell types.

## Embryonic Development of the Optic Cup

### *Formation of the Optic Cup*

The optic vesicle, from which the retina is eventually derived, starts as a bulging out from the ventrolateral aspect of each side of the forebrain. In rats the optic vesicles become visible at 11 d of gestation (E11). At this time the anterior of the neural tube has not fully closed so that the optic vesicles are forming before there is even an

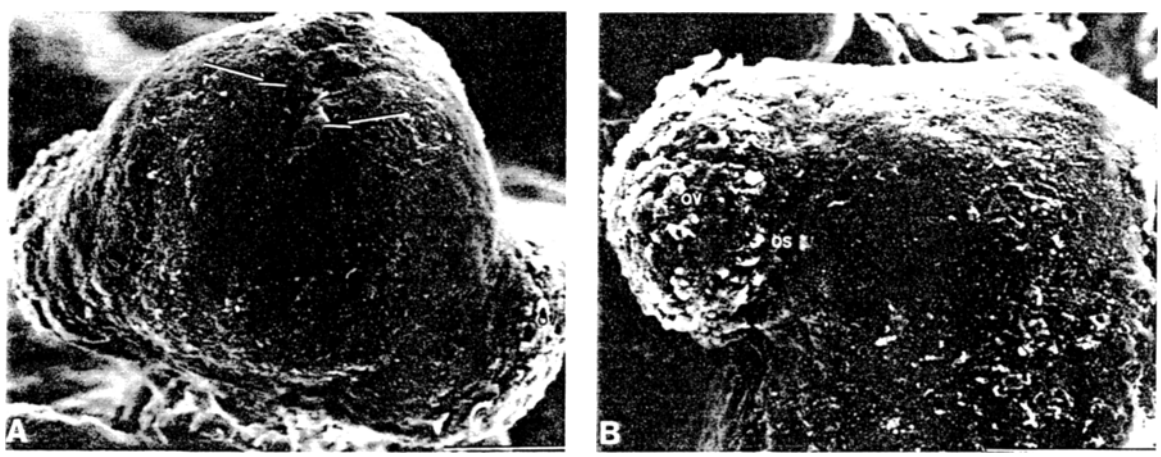


Fig. 2. (A) Scanning electronmicrograph of E11 anterior rat neural tube. The two early optical vesicles (OV) have a broad attachment to the forebrain. At this stage the midline of the neural groove has not completed closed (arows). (B) Posterior view of the same specimen showing the delineation of the optic vesicle (OV) from the narrower optic stalk (OS). Figure adapted from Morse and McCann (1984).

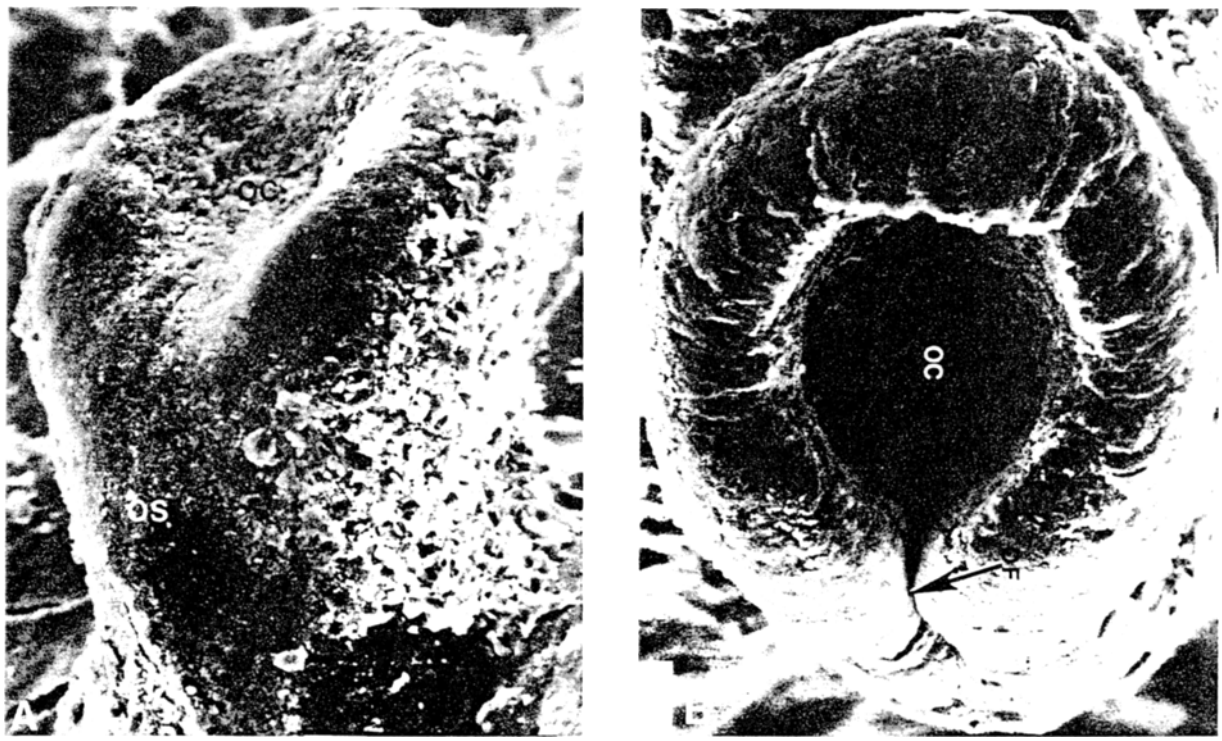


Fig. 3. (A) Scanning electron micrograph of an E12 rat optic (OC) and ventral aspect of the optic stalk (OS). The optic fissure is wide and shallow, and its border is formed by the lips of the cup (asterisks). (B) By E13 the optic cup (OC) is deep and the optic fissure (OF) has narrowed such that its lips appear to be in contact at some points. Figure adapted from Morse and McCann (1984).

isolated forebrain vesicle [Fig 2 (a)] (Morse and McCann, 1984).

Over the next day (E12) the optic vesicle grows out further, so that a definable optic stalk is present and becomes more closely apposed to the overlying ectoderm [Fig. 2 (b)]. As it grows, the optic vesicle begins to invaginate to create the optic cup [Fig 3. (a)]. A shallow furrow, the optic fissure, forms on the ventral suface of the optic cup, although this closes by E13 [Fig 3 (b)].

The molecular signals involved in these early events have not been fully explored, although there is evidence that both movements of calcium and new extracellular matrix material are important at some stages. Invagination of chick optic vesicles in vitro was promoted by the calcium ionophore A23187 in the presence of external calcium and was inhibited by the calcium antagonists verapamil and papaverine (Brady and Hilfer, 1982). The process was also inhibited by the drugs trifluoperazine and chlorpromazine, both of which interact with calmodulin. Calmodulin can confer calcium sensitivity upon nonmuscle actin, and the retinal cells contain actin filaments that show changes in organization during optic cup invagination (Hifler et al., 1981). Thus, it is possible that a calcium–calmodulin–actomyosin mechanism is involved in optic cup morphogenesis. It has not been excluded, however, that other calcium–calmodulin-dependent molecules, such as protein kinases, are also important links between calcium influx and optic vesicle invagination.

Other correlates of optic vesicle invagination have also been found. Anatomical studies have suggested that the developing lens may play a

role in the invagination process. The extracellular matrices of the optic vesicle and the lens epithelium are closely apposed, and the basal laminae of the two layers may actually fuse (Stroeva, 1960; Johnston et al., 1979). Both 6-diazo-5-oxo-L-norleucine and tunicamycin, which block the synthesis of N-linked carbohydrate chains, inhibited optic vesicle invagination and blocked the attachment of the retinal disk and the lens epithelium (Wang and Hilfer, 1982). Although the results support the idea of a role for components of the extracellular matrix, such as glycosaminoglycans, the use of such wide-spectrum inhibitors cannot exclude the possibility that other glycoconjugates are of importance. Immunofluorescence studies have shown that the extracellular matrix glycoproteins fibronectin and laminin are both present around the developing optic vesicle and cup and between the optic cup and the lens (Krotoski et al., 1986). Fibronectin staining was much weaker than that for laminin around parts of the optic cup and often appeared as a patchy distribution. Adjacent sections labeled with antibodies against CSAT, a membrane glycoprotein that binds to both fibronectin and laminin, showed fluorescence both around the basal surface of the developing optic cup and around individual cells within the optic cup and the lens. No obvious differences in the distribution of any of these molecules was observed at the time of optic vesicle invagination, but extracellular matrix components may provide an important scaffolding upon which other molecular changes can exert their actions.

## Determination of Cell Layers Within the Optic Cup

In the rat, optic vesicle invagination begins at E12, and the first postmitotic retinal neurons are formed by E13. Thus, the processes of invagination and retinal differentiation show a considerable temporal overlap. Whether the processes are linked or not has not yet been studied. Whatever the relationship, invagination leads to the formation of two cell layers, one of which goes on to form the pigment epithelium and the other of which gives rise to the retina. If the initial optic vesicle epithelium was equipotential, an assumption that has yet to be verified, then the different environments in which the cell layers find themselves after invagination may lead to their very different cellular and molecular differentiation. In a series of experiments in which E11.5-E12.5 rat optic vesicles were transplanted to the anterior eye chamber, it was found that in the absence of all mesenchymal tissue the entire vesicle developed into retina. In the presence of mesenchyme, usually iris, the cell layer that would normally become pigment epithelium carried out its normal development, but presumptive retina never become pigmented (Stroeva, 1960.) These results suggest a hierarchy of developmental potential whereby presumptive PE can become retina, but presumptive retina cannot become PE, and also stress the potential role of interactions with mesenchymal tissue in determining cell fates. These studies used primarily anatomical criteria to define development, and it was not possible to describe the development of either cell layer in molecular terms.

To try and analyze the cells' fates and potentials in more controlled conditions, we have begun to analyze the differentiation of optic cups explanted into tissue culture at various embryonic stages. Explants taken from E13 embryos were found to form all the major retinal cell types in the correct laminar pattern (Sparrow and Barnstable, 1986). In particular, photoreceptors were detected with an opsin antibody even though the tissue was put into culture before any photoreceptors had become postmitotic. These experiments suggest that either the dividing neuroblasts that give rise to photoreceptors (and the other cell types detected) are already determined within the retinal epithe-

lium or, more likely, that all the signals necessary for specific differentiation events are present in the groups of cells explanted.

## The Optic Stalk

The cells connecting the retina and PE to the forebrain comprise the optic stalk. These cells eventually differentiate into the supporting cells of the optic nerve, namely astrocytes and oligodendrocytes. It has been suggested, however, that before retinal ganglion cell axons pass through the stalk, the stalk cells are multipotential and can become neurons if placed in tissue culture (Juurlink and Federoff, 1980). Although it is tempting to suggest that contact with the axons can alter the developmental potential of these cells, there are complicating factors. In *Xenopus* it has been shown that ventral retinal cells migrate in through the optic stalk (Holt, 1980). There is some evidence that the same is true for rats (Morse and McCann, 1984). Thus, stalk cells may not change their developmental potential, but, rather, the constituent cell populations within the optic stalk may vary with time.

Thymidine birthdating studies have been used to try and establish the times at which the glial cell types become postmitotic (Skoff et al., 1976). This approach is less satisfactory for glia than neurons since recognizable glial cell types can still divide a number of times and thus create a greater difference between the time of formation and the time of last mitosis than is generally assumed to occur for neurons. The results of these studies have suggested that the first astrocytes became postmitotic in rat optic stalk at E15.5. The number of postmitotic astrocytes increased with time and peaked at PN5. The first postmitotic oligodendrocytes were found at PN5, and the peak formation was at about PN10. No gradient of formation was found along the length of the developing optic nerve.

At least two cell lineages arise from the optic stalk (Fig. 4). These were initially defined after it was found that cultures of rat optic nerve contained two types of GFAP$^+$ astrocytes (Raff et al., 1983). One type (Type 1) exhibited a fibroblast-like morphology and did not bind tetanus toxin or the antibody A2B5. The other type (Type 2) had a process-bearing morphology and bound both tetanus toxin and A2B5. Subsequent studies have suggested that Type 2 astrocytes derive from a precursor cell type that could be identified in culture as an A2B5-positive cell that has the potential to form both Type 2 astrocytes and oligodendrocytes (Raff et al., 1983; Abney et al., 1983). For this reason it has been termed the O2A precursor cell type.

Type 1 astrocytes form the protoplasmic astrocytes, which are found primarily at the periphery of the optic nerve and form the glial-limiting membrane. These cells differentiate first and probably account for the postmitotic cells thymidine-labeled in the embryonic optic nerve (Skoff et al., 1976; David et al., 1984). Many of the Type 2 astrocytes differentiate at the same time as the oligodendrocytes, suggesting that the O2A precursor cells are differentiating along the two pathways at the same time.

It has been suggested that contact with ganglion cell axons might trigger astrocytes to become postmitotic and that axon size might be a trigger for oligodendrocytes to stop dividing and begin myelination (Skoff et al., 1976). This idea is supported by studies that show a decrease in oligodendrocyte formation in optic nerves transected neonatally (Fulcrand and Privat, 1977). Neonatal transaction of the optic nerve appears to lead to a decrease in the O2A precursors and both the oligodendrocyte and astrocyte progeny (David et al., 1984). The mechanism of this has not been established, but it is thought that the effect is upon cell survival rather than proliferation or differentiation.

In culture the differentiation pathway chosen by O2A progenitor cells can be influenced by such factors as serum and contact with other cells. Growth of single cells in serum-free medium resulted in their differentiation into

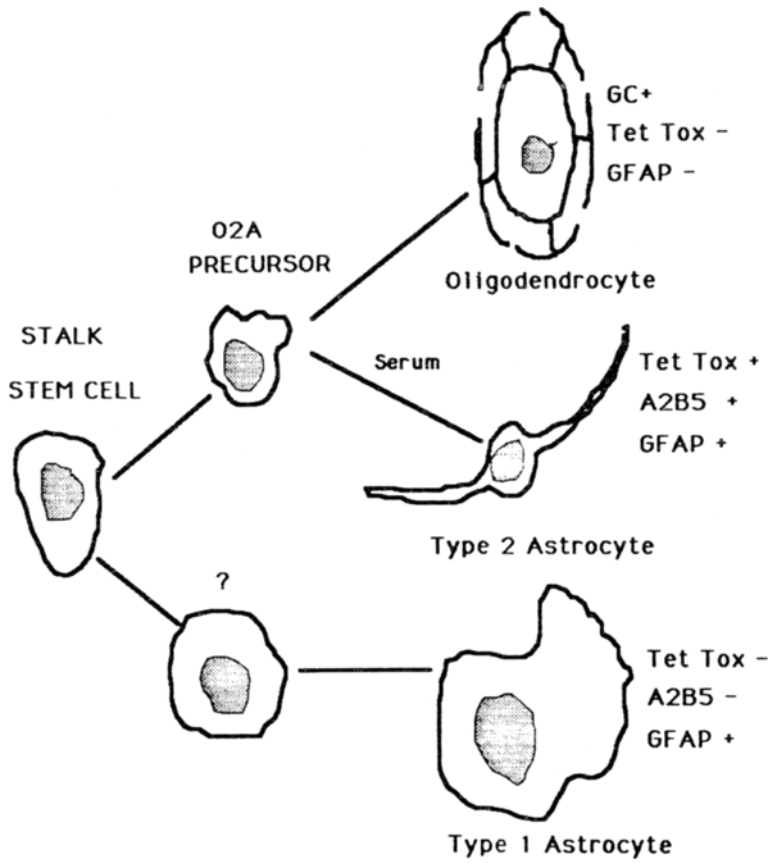


Fig. 4. A schematic representation of the development of optic stalk cells. The optic stalk stem cells give rise to two lineages. One of these leads to the formation of the O2A precursor cell type, which is bipotential and can form either oligodendrocytes or Type 2 astrocytes. In culture the pathway can be influenced by serum factors or conditioned medium. The other lineages eventually give rise to type 1 astrocytes. The cell labeled ? represents possible intermediate stages along the type 1 astrocyte development pathway.

oligodendrocytes, whereas growth in medium containing 20% fetal calf serum resulted in 62% of the cells differentiating into Type 2 astrocytes (Temple and Raff, 1985). Almost all (>90%) of O2A precursor cells differentiated into Type 2 astrocytes in bulk cultures of optic nerve (Raff et al., 1984). This effect could be mimicked by conditioned medium from the bulk cultures, suggesting that the cell–cell interactions might be mediated by soluble factors. The source of such factors and their relationship to the serum factors is not yet known. In addition, it is not yet clear whether these factors act in a permissive or an instructive way to influence different patterns of gene expression in the O2A cells.

## Specification of Positional Information Within the Retina

### *Lamination Within the Retina*

As described in the previous section, the embryological movements of the neuroepithelial sheet of the optic vesicle lead to a distinct

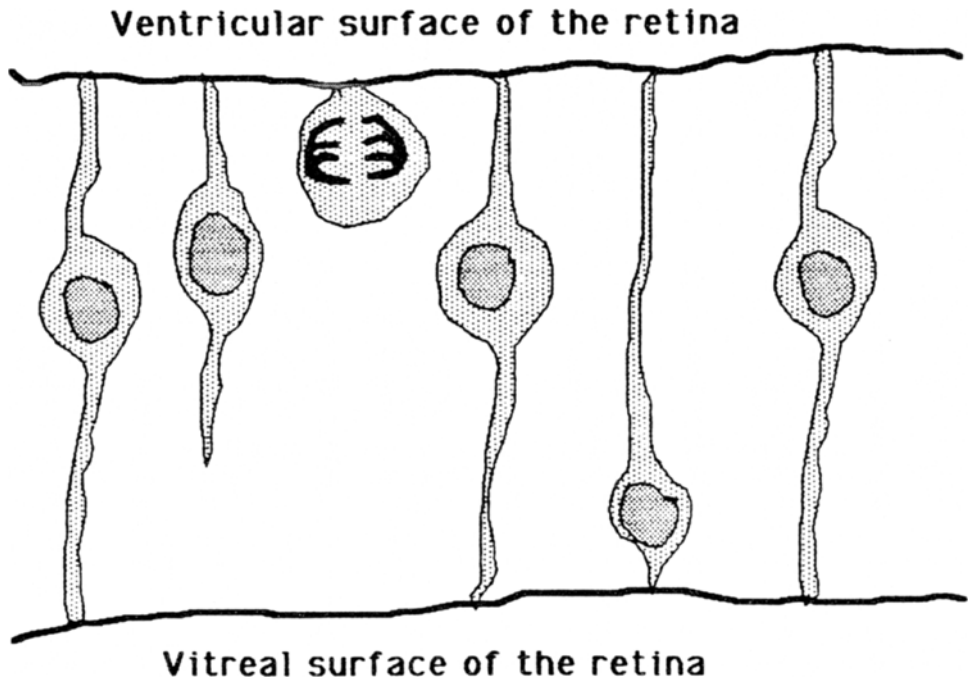


Fig. 5. A schematic representation of retinal cell position during the mitotic cycle. Undifferentiated neuroblasts span the thickness of the retina and have attachments at both the ventricular surface and the vitreal surface. As the cells move through the mitotic cycle the attachment at the vitreal surface may be lost. Division of the cells occurs exclusively at the ventricular surface. After division, the cells reestablish their connections, and the nuclei migrate down toward the vitreal surface before returning for another round of cell division. As the retinal layers differentiate, the extent of this interkinetic nuclear migration becomes less, such that undifferentiated nuclei do not migrate through differentiated cell layers.

invaginated structure that subsequently gives rise to all the cell types of the retina. This subsequent developmental phase involves extensive cell division to give a structure that is many cells thick. Initally, this proliferation results in the close packing of elongated cells that still have attachments at both edges of the retina. With continued development, some of these attachments disappear, and in the adult retina the only cells to span the full thickness of the tissue are the Müller glial cells.

All mitoses of the neuronal precursors take place at the ventricular surface of the tissue (Fig. 5). Between mitotic cycles the cell nuclei migrate down into the retina, returning for a further mitotic division (Denham, 1967; Fujita, 1962; Braekfelt and Hollenberg, 1970). After their final mitosis the cells migrate to their final position. This migration is much shorter than migrations of neurons in the developing cerebral cortex, and it is not clear whether the retinal neurons need any cells homologous to the radial glia found in many other areas of the CNS upon which to migrate (Rakic, 1981). The formation of laminae in the retina also differs from that of cerebral cortex in another way. In general, cortical layers are formed from the deeper layers to the more superficial layers, that is, from the layers closest to the ventricle to the layers furthest away. The retinal laminae, on the other hand, are formed from the layers furthest from the ventricle to the layers closest, that is, from ganglion cell layer first to photoreceptor layer last. Thus, retinal cells do not have to pass through

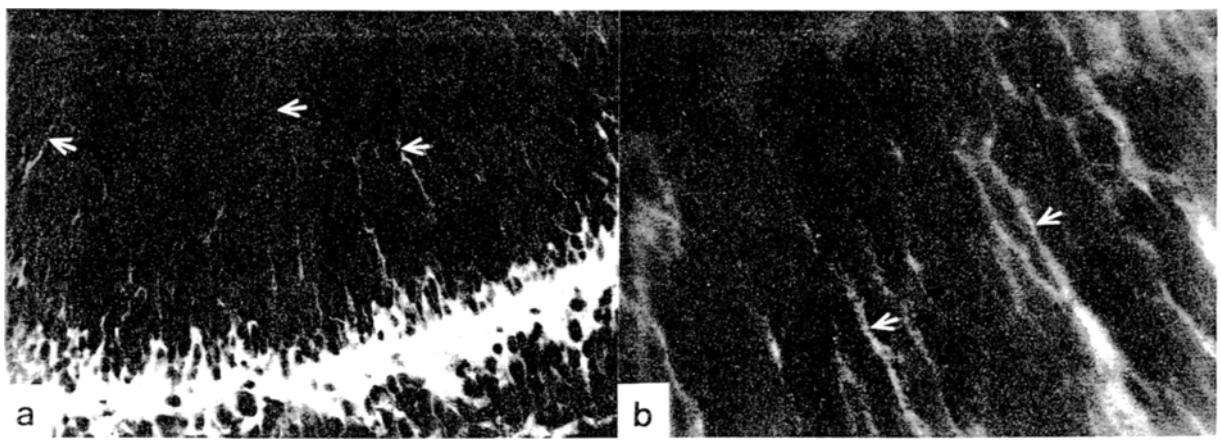


Fig. 6. Immunofluorescent labeling (b) of E17–18 rat embryo retina by amacrine cell antibody HPC-1. Tissue was dissected and fixed by imersion in 4% paraformaldehyde. Fifteen-micrometer sections were cut on a cryostat and labeled with monoclonal antibody followed by a fluorescent secondary antibody. Labeled cell bodies and radial processes could be found extending up to the ventricular surface (arrows). The bright band of label represents the forming IPL. Scale bar in (a) 50 μm. Taken from Barnstable et al. (1985).

layers of cells already formed, but only need to migrate until other differentiated cells are encountered. It is not clear whether such a simple mechanical model of retinal layer formation is appropriate, however, since several cell layers are forming simultaneously. To understand the formation of retinal laminae more fully, it is necessary to know whether the cell migrations involve undifferentiated neuroblasts or the homing of already differentiated cells. To distinguish these possibilities we examined the formation of amacrine cells in embryonic rat retina. The antigen HPC-1 is detectable at E17 in the retina (Barnstable et al., 1985). At this stage the ganglion cell and inner plexiform layers are not fully formed. HPC-1-labeled cells were detected not only in the forming amacrine cell layer, but also at the ventricular surface and positions in between (Fig. 6). This suggests that the cells begin to differentiate before they migrate and that layer formation involves the homing of differentiated cells. The migrating cells retain a radial process attached to the ventricular surface as they migrate. It is possible that such processes could be used by the cells to sense the depth to which they have migrated. Different layers would be formed if different cell types maintained radial attachments of different lengths.

There is some evidence that other factors can influence lamination in the retina. In reaggregate cultures consisting of all retinal cell types except ganglion cells, the outermost layer of cells formed consists of amacrine cells (Barnstable et al., 1983; Akagawa and Barnstable, 1987). In addition, these cells direct their processes in toward other retinal cell types, that is, in the opposite orientation to most nomal amacrine cells. One interpretation of this observation is that in the normal retina the developing dendrites of the ganglion cells play an important role in organizing the amacrine cell layer and the inner plexiform layer. If this is the case, it will be interesting to determine the nature of the interactions between these cell types in the embryo since synaptic interactions are not thought to be present in the inner plexiform layer until several days after birth.

One other cell type that may play an organizing role in retinal lamination is the axonless class of horizontal cells. Although the general order of formation of the retinal layers is as described above, namely ganglion cells to photoreceptors, thymidine birthdating studies have shown one exception to this (Sidman, 1961; Hinds and Hinds, 1978; Young, 1985). In the mouse, the peak of horizontal cell birth occurred at E13-13.5, before the peak of amacrine cell birth and many days before the peak of photoreceptor and bipolar cell birth. Histological examination of late embryonic retinas has shown that well-differentiated horizontal cells can be found in the middle of a broad neuroblastic cell layer (Barnstable and Constantine-Paton, 1984). This raises the question of what cues are used by these cells to form such a layer and whether the placement of these cells is critical to the formation of the outer plexiform layer, the division between photoreceptors, and the inner nuclear layer. The early-appearing horizontal cells at least include the axonless class of horizontal cells because we have been able to label these cells in embryonic rats with antibodies against neurofilament subunits. In the adult retina, neurofilaments are found only in axonless horizontal cells and ganglion cells (Drager et al., 1984). As was the case for the amacrine cells mentioned above, the horizontal cells maintained a radial connection until they completed their movement and began to extend horizontal processes. Once again the information provided by such a scaffolding for subsequent retinal development is unclear, as are the molecular mechanisms by which such information can be transferred.

Even though the detailed mechanisms of retinal lamination are not yet understood, one point to be emphasised is that there appear to be many instances of cell interactions that are important for correct cell positioning. As mentioned above, this cellular communication would seem to be separate from synaptic interactions that occur only after the cells are in their correct laminae.

## Gradients Along the Dorsoventral Retinal Axis

In addition to the laminar development of retinal cell types, each cell forms a characteristic network of processes and connections. The distribution of some of these cells and their processes is not uniform across the retina, but rather varies with position. Some of this variation is manifested as differences in the dendritic spread of otherwise similar cell types or in the packing density of many of the retinal cell types (Wässle and Rieman, 1978; Tauchi and Masland, 1984). These differences suggest that there is a mechanism for encoding position within the retinal sheet. As discussed in several sections below, there is also good evidence that retinal ganglion cells use positional cues for the direction of their initial axon outgrowth, and that such cues may be important in some aspects of setting up the topography of the retinal projection to other parts of the visual system.

It is a reasonable hypothesis that some correlates of the specification of position can be found in the differential expression of molecules by cells at different retinal coordinates. The search for such correlates has been going on for many years. Early studies measured the ability of retinal cells from dorsal or ventral positions to adhere to each other or to tectal cells from areas corresponding to the target sites of the retinal populations. Such experiments found that cells from dorsal retina adhered preferentially to ventral tectum and cells from ventral retina adhered preferentially to dorsal tectum (Barbara et al., 1973). This adhesive selectivity mimicked the normal pattern of innervation of the tectum by the retina. The adhesive assays measured the behavior of cell bodies rather than the ganglion cell axon tips, as occurs in vivo, thus suggesting that the molecules governing adhesive specificity might be more generally distributed within the retina. More detailed biochemical investigations of the molecules responsible

for this adhesive specificity suggested that two complementary gradients existed. One gradient within the retina was of a molecule that required a terminal β-*N*-acetylgalactosamine residue and appeared to be more concentrated in dorsal retina (Marchase, 1977). A complementary gradient, which is of a higher concentration in the ventral retina, of a molecule that was sensitive to trypsin digestion and could bind β–*N*-acetylgalactosamine was also proposed. Similar gradients within the tectum could then account for the binding specificity observed. Complete identification of these molecules was not possible. One candidate for the dorsal-enriched molecule was the ganglioside $GM_2$, but no difference in concentration of this molecule could be observed between dorsal and ventral portions of the retina. One enzyme, UDP-galactose $GM_2$ galactosyltransferase, was found to be 30% more concentrated in ventral retina than dorsal retina (Marchase, 1977).

With the introduction of monoclonal antibody methods it has become easier to search for molecules that are distributed in a graded manner across the retina. Using such methods there have been two molecular gradients described in the vertebrate retina. One is a gradient of a protein in the chick retina (Trisler et al., 1981a) and the other is a gradient of a modified ganglioside in rat retina (Constatine-Paton et al., 1986).

In the former study, monoclonal antibodies were raised against chick retina by immunizing mice with dissociated cells from whole retina and then boosting them with cells from either dorsoposterior or ventral retina. After fusion, hybridoma colonies were screened for the production of antibodies that bound preferentially to either dorsoposterior or ventral retina. One antibody, named TOP, showed 11-fold more binding to dorsal retina than ventral retina at E14, a ratio that declined to 2.85 in the adult (Trisler et al., 1981a,b). In more detailed studies in the embryonic retina, the gradient was found to be aligned with the dorsoposterior-to-ventroanterior axis of the eye, which is the axis defined by the optic fissure. At its extremes the gradient showed 35-fold more binding in dorsoposterior segments over ventroanterior segments (Trisler et al., 1981a). The gradient was first detected in the E4 embryo, although the antigen could be detected at E2. The results suggest that the gradient is maintained during the whole period of neuroblast proliferation and retinal histogenesis.

Biochemical studies of the molecule recognized by the TOP antibody have provided preliminary evidence that it is a protein. Trypsinization of retinal cells led to a loss of TOP antibody binding, as did heating to 100°C for 30 min. Incubation of retinal cells with wheat germ agglutinin lowered the binding of TOP antibody, whereas the lectins Concanavalin A and *Ulex europaeus* agglutinin I had no effect or enhanced binding.

One important feature of the TOP gradient is that it appears to be a variation in the amount of the molecule expressed by each cell within the retina. This conclusion was based upon immunofluorescence studies carried out on dissociated cells from different retinal regions grown as monolayer cultures. Cells from dorsal retina gave brighter fluorescence than those from middle or ventral retina, as judged visually (Trisler et al., 1981a). The experiment also suggests that the maintenance of the gradient is not under continuous regulation. Once dorsal cells have been programmed to express higher levels of TOP they can maintain this even in the isolated tissue culture environment.

To try and elucidate the function of the molecule recognized by TOP, the antibody was applied to the developing chick eye (Trisler et al., 1986). To maintain a constant supply of antibody over a period of several days, hybridoma cells secreting TOP antibody were injected into the vitreous. The gradient of TOP antigen was maintained in the presence of antibody. Injection of antibody altered the rate of synaptogensis in the retina. In control eyes, injected with other antibodies, the density of synapses rose to

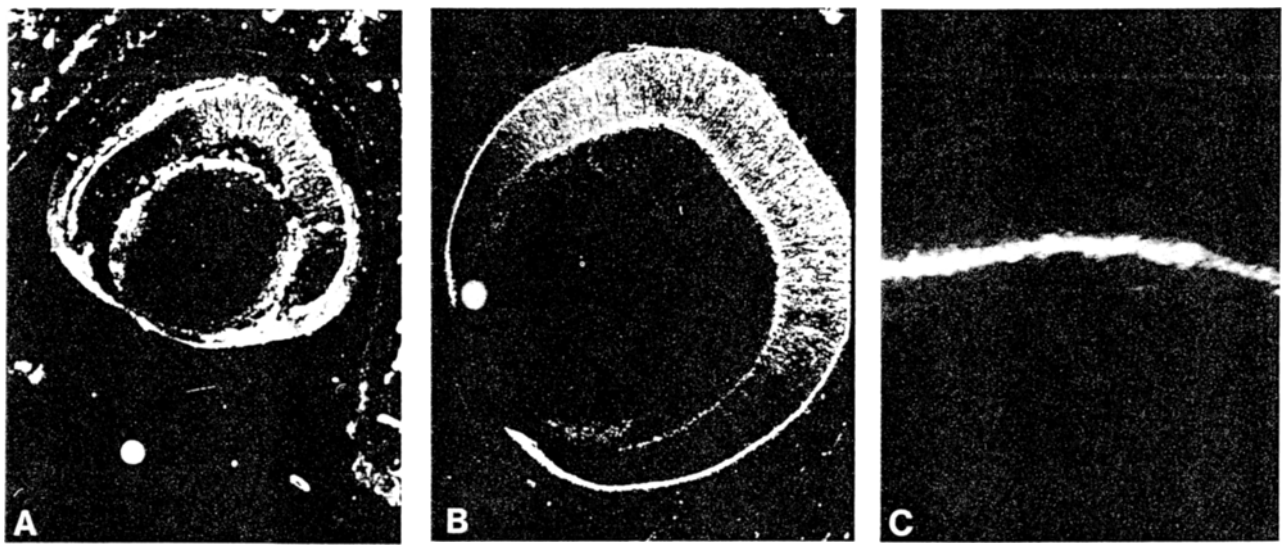


Fig. 7. Immunocytochemical distribution of JONES antibody labeling in the rat retina. (A) Labeling of central retina in the E13 eye. (B) Labeling of the E17 eye. The dorsoventral gradient can be easily seen. In the dorsal retina [upper in (B)] label is absent from the region close to the ciliary margin that contains only immature proliferating cells. In the cellular regions of the ventral retina little labeling can be detected. Labeling is seen in these regions along the forming ON. (C) Adult retina showing label confined to the OPL. Both the photoreceptor layer (upper) and the whole of the inner retina (lower) show no labeling above background. In (A) and (B) labeling was carried out using a silver-intensified gold labeling that was viewed and photographed with dark-field illumination. In (C) a fluorescent secondary antibody was used. (A) and (B) taken from Constatine-Paton et al. (1986).

a plateau value at E18. In eyes injected with TOP antibody, the rate of synapse formation was much slower, such that the density at E18 was only 50% of controls. By E19, however, the density of synapses was approximately the same in the two samples, suggesting that the TOP-treated retinas were capable of forming a normal number of synapses. There was also a striking difference in the density of growth cones during this period. In the TOP-treated animals there was a higher density of growth cones maintained for a longer period. One interpretation of the results was that the major effect of the TOP antibody was that growing neurites could not detect the TOP gradient and, hence, grew far longer before eventually making synapses. If this interpretation is correct, then it is possible that the dendritic fields of processes are increased and that the effects of TOP antibody injection may be detectable by changes in the physiological responses of cells, such as increases in receptive field size.

We have also been using an immunological approach to identify molecules that show unequal distribution in the developing rat retina. One of these, JONES, was first detected in the central portions of E12–13 retinas [Fig. 7(A)]. By E17-18, binding was observed along regions of the vitreal surface next to where an optic fiber layer had formed [Fig. 7(B)]. In cellular regions of the retina the antigen was clearly distributed in a gradient. JONES binding was invariably high in the dorsal retina, decreasing gradually in more ventral retina regions. The dorsal-to-ventral gradient was still detectable histologically at P0 and present though less pronounced at P3. In the adult, JONES binding was present only on the Outer Plexiform Layer [Fig. 7(C)].

These histological results probably underestimate the developmental period over which the gradient is present. To measure the gradient more accurately we fluorescently labeled dissociated cells from dorsal and ventral portions of retinas or whole retinas of various ages with JONES antibody and passed the cells through a Fluorescence Activated Cell Sorter (FACS). The results of these experiments allowed three conclusions to be drawn (Blum and Barnstable, 1986). The first was that, in agreement with the histological analysis, the percentage of JONES-labeled cells declined with time from a maximum of almost 40% at PN 2 to less than 5% in the adult. The second conclusion was that the observed gradient was at least in part related to differences in the percentage of cells labeled rather than to the amount of antigen expressed per cell since dorsal retina consistently gave a higher percentage of labeled cells than ventral retina. The third conclusion was that from E17 to about PN 5 the ratio of dorsal-to-ventral labeled cells remained constant at approximately 1.5. After this time the percentage of labeled cells was so low that any estimates of the gradient became unreliable.

Binding of JONES antibody to aldehyde-fixed sections of late fetal and early postnatal retinas was removed by pretreatment of the fixed tissue with organic solvents and detergents, and no binding could be found to immunoblots of retinal proteins separated by SDS polyacrylamide electrophoresis. These results suggested that the JONES antibody might be reacting with a lipid. Immunoreactivity was found in Folch extracts of retinal tissue, and when this was partitioned into aqueous and organic phases, all the immunoreactivity was found in the aqueous phase [Fig. 8(A)]. This indicated that the JONES epitope was carried on a polar lipid or ganglioside. Upon TLC analysis of the lipid extracts of retina, the JONES immunoreactivity migrated as a single major band. By comparing the migration with that of known markers, the major JONES band was found to migrate slightly below the ganglioside $GM_2$. The immunoreactive band was eliminated by treatment of the retinal extracts with neuraminidase [Fig. 8(B)]. It was also eliminated by treatment with base, such as 0.1*M* sodium carbonate, ammonia vapor, or methanolic sodium hydroxide.

These results suggested that the JONES epitope was carried on a ganglioside that was modified by the addition of a base-labile group. To determine the nature of the parent ganglioside, retinal lipid extracts were separated by TLC, blotted onto nitrocellulose, and treated with ammonia vapor. As shown in Fig. 9, this treatment removed the JONES reactivity, but revealed a new band that could be labeled by antibody R24, which is specific for the ganglioside $GD_3$. One possible modification of $GD_3$ was examined by chemically acetylating the sialic acids of $GD_3$ using the acetyl donor *N*-acetylimidazole. This was sufficient to create the JONES epitope, as judged by both binding assays and TLC/immunoblots (Blum and Barnstable, 1986). A minor, faster-migrating band was also observed in this experiment. This probably corresponded to the diacetylated form of $GD_3$. Since this was not seen in tissue extracts, the JONES epitope was primarily carried on a monoacetylated form of $GD_3$. The JONES epitope was insensitive to treatment with sodium periodate under conditions in which the reactivity of antibody R24 to $GD_3$ was totally obliterated. This strongly suggests that the acetylation occurs upon the 9-position of the sialic acid, since this is the only stable substitution that can protect the residue from periodate oxidation.

The probable structure of the major retinal form of the JONES molecule is shown in Fig. 10. The nature of the minor, slower-migrating band has yet to be elucidated. There are differences in the developmental profile of $GD_3$ and JONES in a number of CNS regions, suggesting that the acetylation that creates the unique JONES epitope is separable from the biosynthetic control of $GD_3$. The nature of this acetylating activity is at present unclear.

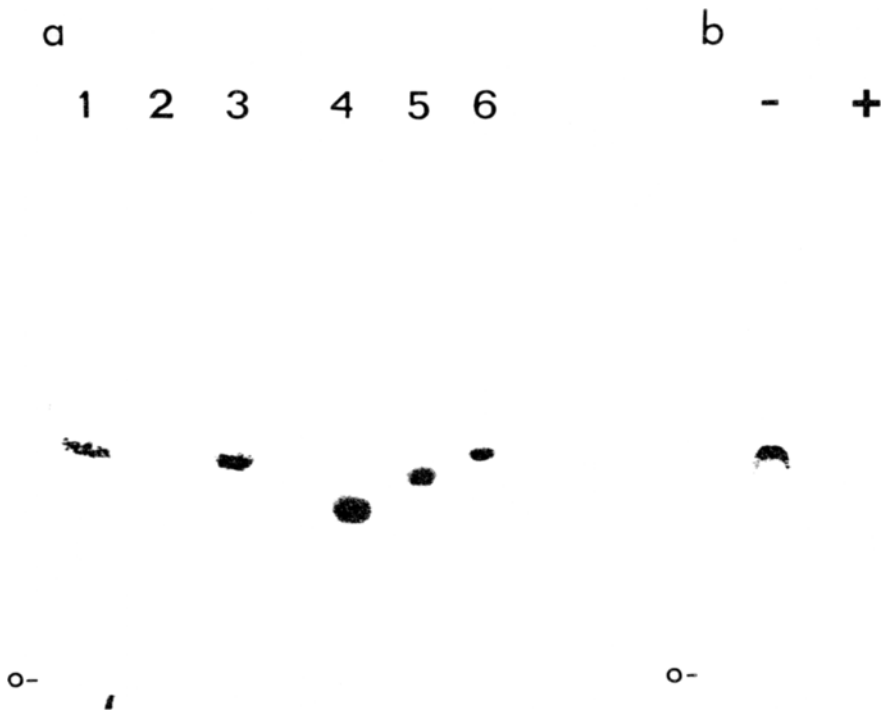


Fig. 8. Biochemical characterization of the JONES antigen. (a) The JONES antigen partitions into the aqueous phase of a Folch extract and is detected as a single band on immunoblots of lipids resolved by TLC. The band migrates to a position between that of $GM_2$ and $GM_1$ standards run at the same time: (1) whole lipid extract, (2) organic phase, (3) aqueous phase, (4) $GD_1$, (5) $GM_1$, (6) $GM_2$. (b) The epitope recognized by the JONES antibody contains sialic acid since immunoreactivity is removed by treatment with neuraminidase (+) but not buffer (-). Taken from Constantine-Paton et al. (1986).

The possibilities for synthesizing the JONES molecule are severalfold, and include the acetylation of $GD_3$, the addition of acetylated sialic acid to $GM_3$, or even removal of a terminal sugar from acetylated $GD_2$. There is tentative evidence for the existence of enzymes that can carry out the first two of these. In melanoma cell lines, which also express an acetylated $GD_3$, it has been suggested that there exists an enzymatic activity that can directly convert $GD_3$ into acetylated $GD_3$ (Cheresh et al., 1984). Other work has identified an acetylating activity in bovine submaxillary glands that can acetylate free sialic acid (Schauer, 1971). Determination of the pathway of biosynthesis of JONES, and particularly the rate-limiting step, will be of great importance for understanding the mechanism by which this cell surface molecule is expressed in a dorsoventral gradient.

The JONES determinant is one of a growing number of examples of carbohydrate antigens that shows a highly restricted localization. Such specificity suggests that they may play a far more significant role in patterning and cell interactions within the nervous system (and possibly other tissues) than had previously been realized. If mechanisms, such as acetylation, can impose even more detailed patterns of carbohydrate distribution, then sufficient diversity can be produced to account for a wide range of cell interactions and cell-patterning phenomena. Within the peripheral nervous system, the carbohydrate groups of the lacto- and globo-series of cerebrosides show discrete localizations that

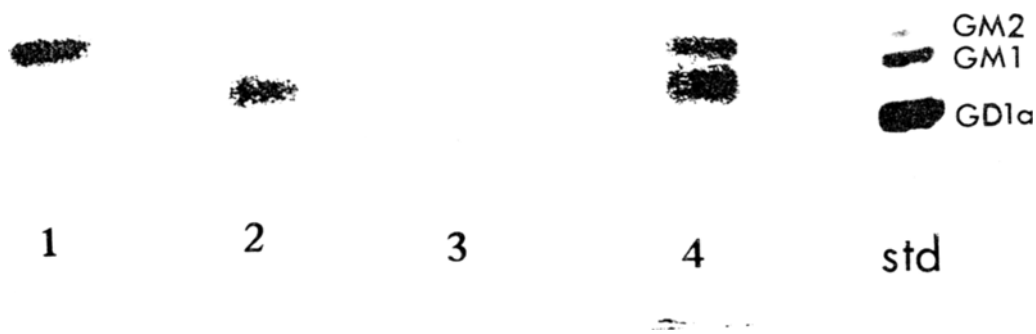


Fig. 9. The JONES antigen is a modified form of a $GD_3$ ganglioside. Retinal gangliosides were analyzed on a TLC immunoblot using either JONES antibody (1) or an anti-$GD_1$ antibody R24 (2). Each antibody shows a single band in nonoverlapping positions. Nitrocellulose blots of the same preparation were exposed to ammonia vapor and then reacted with the same antibodies. The immunoreactivity with JONES has disappeared (3). R24 now reacts with both the $GD_3$ band and a new band in the position of the former JONES band, showing that JONES is a modified form of $GD_3$ (4).

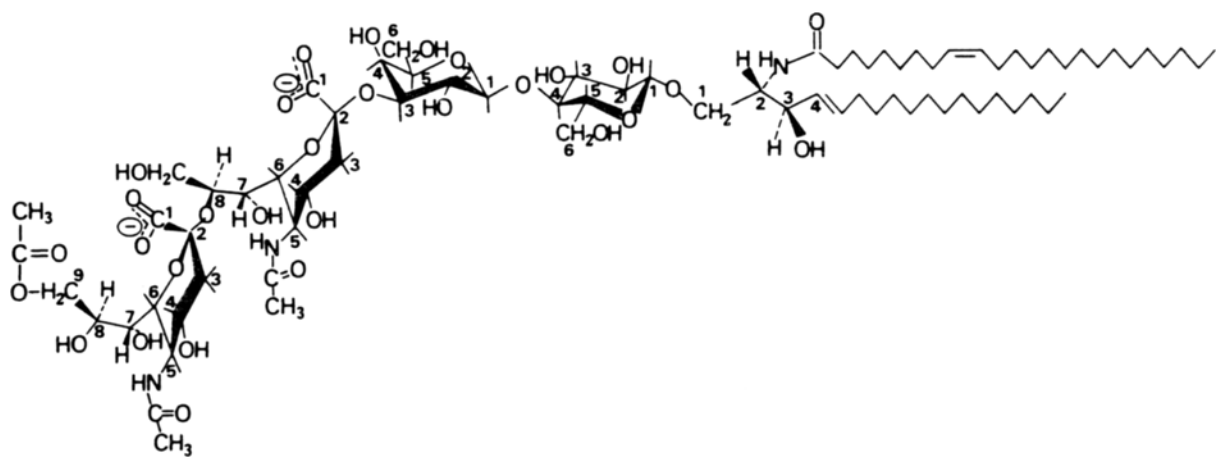

Fig. 10. A schematic diagram of the JONES molecule. The four sugar residues of $GD_3$ are shown attached to the cerebroside moiety. JONES is formed by the addition of an acetyl group to the 9-position of $GD_3$. This is shown on the terminal sialic acid, although modification of the inner sialic acid cannot be totally excluded. The nature of the fatty acid residues in JONES has not yet been determined.

appear to correlate with transmitter phenotype in rat dorsal-root ganglion cells (DRG) (Dodd et al., 1984; Jessell and Dodd, 1985). The different populations of DRG neurons terminate in different laminae of the spinal-cord dorsal horn, and the surface carbohydrate groups may act as molecular markers for the correct positioning of the synaptic terminals of these cells. Two endogenous, lactose-binding lectins have also been described in DRG neurons and the dorsal horn of the developing spinal cord (Regan et al., 1986). Thus, one role for cell-surface carbohydrates may be as part of a recognition system involving lectins that might be responsible for the organization of projections of an area to its target.

The carbohydrate determinants need not be part of a recognition system thenselves. Some, particularly charged carbohydrates such as gangliosides, may function by modifying other cell-surface molecules. There is evidence that gangliosides may play a role in cell adhesion by modifying the efficacy of adhesion molecules, such as fibronectin (Thomson et al., 1986). Disialogangliosides, including $GD_3$, have been shown to be preferentialy localized to the adhesive contacts of human melanoma cells (Cheresh et al., 1986). Thus, the combination of a uniformly distributed adhesion molecule with a graded concentration of a modifier, such as a ganglioside, could set up an adhesive hierarchy that might be of importance in determining positional information.

## Cues for Retinal Ganglion Cell Axon Outgrowth

As mentioned above, positional specification within the retina is probably important for the ordered growth of ganglion cell axons. That such an ordered growth does occur has been shown by studies of the early chick retina. These studies have shown that even the initial ganglion cell axon outgrowth is oriented toward the optic fissure or optic nerve head (Halfter et al., 1985). This has led to the suggestion that there are positional cues in the substrate over which these initial axons grow. The substrate is formed by the endfeet of retinal epithelial cells and the extracellular matrix secreted by them, which makes up the inner limiting membrane. The nature of such cues is not known, but it may well be related to the types of molecules dicussed in the previous section. There is another precedent for the existence of directional cues in axon outgrowth. In the developing insect nervous system, initial axon outgrowth of pioneer fibers is always in the correct direction (Bentley and Caudy, 1983a; Bastiani et al., 1985). This has been interpreted on the basis of the existence of directional cues present in the surface ectoderm or, more likely, in the extracellular matrix covering the cells (Bentley and Caudy, 1983b).

The patterning of ganglion cell axon growth may also be influenced by the selective interaction of axons from different regions of the retina. Axons from explants of either temporal or nasal regions of chick retina were allowed to make a choice and grow along either temporal or nasal axons (Bonhoeffer and Huf, 1985). The results of these experiments were that axons from nasal retina were able to grow along both the temporal and nasal axon substrates. The temporal axons, however, were unable to grow on nasal axons and grew exclusively along the temporal axons. The observed growth of temporal axons only on temporal axons is in agreement with in vivo observations in the frog, which indicate that temporal axons tend to fasciculate with each other. The data suggest a positive attraction that is graded across the nasotemporal axis of the retina, with the highest levels in the temporal region. An alternative explanation for the results has been offered following the finding of negative interactions between central and peripheral axons (Kampfhammer et al., 1986). Instead of a positive adhesive interaction between temporal axons, it has been suggested that nasal axons express molecules that prevent interac-

tion with temporal axons (Raper and Kapfhammer, 1986). Axon growth experiments that compared the behavior of dorsal and ventral portions of retina found no differences in growth selectivity (Bonhoeffer and Huf, 1985).

Thus, the early experiments measuring cell binding of retinal to tectum suggested dorsoventral gradient, whereas the axon growth experiments have suggested nasotemporal differences, but no dorsoventral differences. So far we have molecular candidates for the dorsoventral differences, but not for the nasotemporal differences.

## Cell Adhesion and Retinal Development

### *Cell Adhesion Molecules*

Adhesion of retinal cells has been shown to consist of at least two mechanisms, a calcium-independent and a calcium-dependent mechanism (Steinberg and Granger, 1966; Edwards et al., 1975; Takeichi, 1977; Takeichi et al., 1979; Magnani et al., 1981; Rutishauser, 1983; Edelman et al., 1983). These mechanisms can be distinguished because the molecules involved show differential sensitivity to proteolytic cleavage from the cell surface in the presence or absence of calcium. Cells trypsinized in the presence of EDTA lose both adhesion mechanisms. Cells trypsinized in the presence of calcium lose their calcium-independent adhesion mechanism, but not their calcium-dependent adhesion mechanism. The two adhesion mechanisms also differ in that only the calcium-dependent mechanism is temperature sensitive.

There are three molecules that are candidates for components of the calcium-independent mechanism of retinal cell adhesion. The reaggregation of dissociated chick retina cells was used as an assay for the purification of *N*eural Cell *A*dhesion *M*olecule (N-CAM), the best characterized of these. Fab fragments of an antiserum against chick retinal cells were shown to block the reaggregation of dissociated retinal cells, and this blocking activity could be removed by preincubation with a specific membrane protein (Brackenbury et al., 1977). N-CAM has been characterized extensively (Rutishauser, 1983; Edelman et al., 1983), and much of its amino acid sequence determined from its cDNA sequence (Murray et al., 1984; Goridis et al., 1985). N-CAM exists in the adult nervous system primarily as glycoproteins of apparent mol wt 180,000, 140,000, and 120,000. The two larger forms are both transmembrane proteins, but differ in the amount of polypeptide chain in the cytoplasmic portion of the molecule. This difference is related to differential splicing of the N-CAM mRNA that involves one exon (Murray et at., 1986). Although the functional consequences of changes in the ratio of the two forms are not yet understood, there is some evidence that the 180,000 form of N-CAM can interact with the cytoskeleton (Pollerberg et al., 1986). The 120,000 form of N-CAM is also formed by differential splicing of the mRNA, but it has no transmembrane region and no cytoplasmic domains. Instead, it has a novel C-terminal amino acid sequence and is linked to the plasma membrane through a phosphatidylinositol linkage (He et al., 1986).

In embryonic neural tissues a higher mol wt form of N-CAM is the predominant species and has an apparent mol wt of 220,000. The higher mol wt is related to a larger amount of polysialic acid attached to the polypeptide chain. The differences in amount of sialic acid have been shown to influence the affinity of N-CAM adhesion of artificial lipid vesicles (Hoffman and Edelman, 1983). The conversion of the high-sialic-acid, embryonic form of N-CAM to the lower sialic acid, adult form of the molecule occurs at different times and different rates in different parts of the nervous system. In most areas it occurs after histogenesis is complete and, thus, is probably not of major importance

during development. The retina is unusual in that it always expresses the adult, lower-sialic-acid form of N-CAM (Schlossauer et al., 1984). Along the optic nerve, however, the embryonic, higher-sialic-acid form can be found at the optic chiasm. This has been interpreted as representing a mechanism for decreasing axon adhesion at a point in the optic pathway at which it is known that fibers undergo a large amount of spatial rearrangement. What has yet to be explained is whether the modification is carried out by compartmentalization of molecules synthesized at different times in ganglion cell development or whether it can occur by extracellular neuraminidase activity, possibly secreted by other cell types.

The second identified component of the calcium-independent adhesion mechanism is the molecule called L1 or NILE in rodents or Ng-CAM in the chick (Schachner et al., 1983; Salton et al., 1983; Grumet et al., 1984). Ng-CAM appears as a single sharp band on gel electrophoresis and has an apparent mol wt of 135,000 (Grumet et al., 1984). Unlike N-CAM, which appears to mediate homophilic binding, Ng-CAM does not bind to itself, although its receptor has yet to be identified. In the developing retina, Ng-CAM is confined to the optic nerve fiber layer, although it later also appears in the inner plexiform layer (Daniloff et al., 1986). At no time in development was Ng-CAM observed on cell bodies or in the outer plexiform layer.

The third identified adhesion molecule, cognin, is a 50-kdalton integral membrane protein that was defined using an assay of chick retina membrane vesicle aggregation (Troccoli and Housman, 1985). It differs from the molecules described above in several ways. Cognin has not been detected in other neural or nonneural areas, making it one of the first region-specific cell adhesion molecules (Ben-Shaul et al., 1979). It also shows age-related decreases in the level of its expression and is not found at all on the last maturing photoreceptors and their outer segments (Ben-Shaul et al., 1980; Ophir et al., 1983).

There has only been one molecule described that appears to mediate calcium-dependent adhesion. A molecule of approximately 130 kdalton has been identified biochemically on the basis of proteolytic sensitivity and immunologically by antibody-mediated inhibition of cell aggreation (Grunewald et al., 1982; Hatta et al., 1985). Monoclonal antibodies produced against this molecule blocked aggregation of dissociated retinal cells under appropriate conditions (Hatta and Takeichi, 1986). The appearance of this adhesion molecule shows striking correlations with major morphogenetic events, such as gastrulation and neurulation, as well as events such as optic vesicle invagination and the accompanying lens formation.

The molecules described above are all thought to act by either self association or interaction with other membrane molecules. The alternative model, in which membrane molecules are linked by soluble bridging molecules, has also been described. Into this category would come those molecules that can bind to extracellular matrix molecules since the extracellular matrix can be thought of as an extended bridging molecule. In addition, other molecules and types of bridge may play a role. One candidate is the membrane molecule ligatin. This is a 10-kdalton plasma membrane protein that can bind peripheral proteins containing a terminal glucose-1-phosphate (Marchase et al., 1981, 1982). This protein has been found in many tissues and many species, including several mammals, chick, and even sea urchin. The reason for including the molecule in this section on cell adhesion is that the addition of ligatin was sufficient to block retinal cell aggregation, possibly by blocking the formation of appropriate bridges (Marchase et al., 1981).

## The Role of Cell Adhesion Molecules in Retinal Development

It seems unlikey that all of the different molecules described above exist simply to hold reti-

nal cells together. Thus, to understand their function more completely it is necessary to consider the ways in which patterns of different adhesivity could be set up or the effects upon other cellular functions transferred into the cytoplasm by some of these molecules when they interact with their appropriate partners.

Early experiments on differential adhesivity focused upon the ability of cells from one tissue to sort out from those of another tissue, for example liver and heart (Steinberg, 1964). More recent investigations of the reaggregation of a tissue of mixed cell types, such as the retina, has shown that retinal cell types can sort out into histiotypic layers (Moscona and Housman, 1977; Barnstable et al., 1983; Vollmer and Layer, 1986; Akagawa and Barnstable, 1987). There is also some evidence that this is related to active sorting out of cells after the reaggregates have formed rather than temporally separated addition of cell types to the reaggregates (Akagawa and Barnstable, 1987). By analyzing the distribution of GABA-ergic amacrine cells over several days in culture we have found sorting from an initial random array to a final external position (Fig. 11).

Addition of Fab fragments of antibodies against N-CAM to retinal reaggregates led to less complete histiotypic sorting, although the crtieria used did not measure the positions of identified cell types (Buskirk et al., 1980; Hoffman et al., 1986). In addition, the published experiments have not described the rates of cell division or cell death in antibody-treated explants. The comparision of treated and control retinas at the same time points may also hide information. As was found in the experiments described in Gradients Along the Dorsoventral Retinal Axis, where TOP antibody was injected into the chick eye, it is possible to slow developmental processes even though the same endpoint is eventually reached. It is unclear at present whether the effects of anti-N-CAM are on the rate of development. Interestingly, Fab fragments of antisera against Ng-CAM did not affect retinal histogenesis (Hoffman et al., 1986). This, combined with the preferential localization of this molecule to the optic nerve fiber layer suggests that Ng-CAM functions in optic nerve formation rather than in retinal histogenesis (Daniloff et al., 1986).

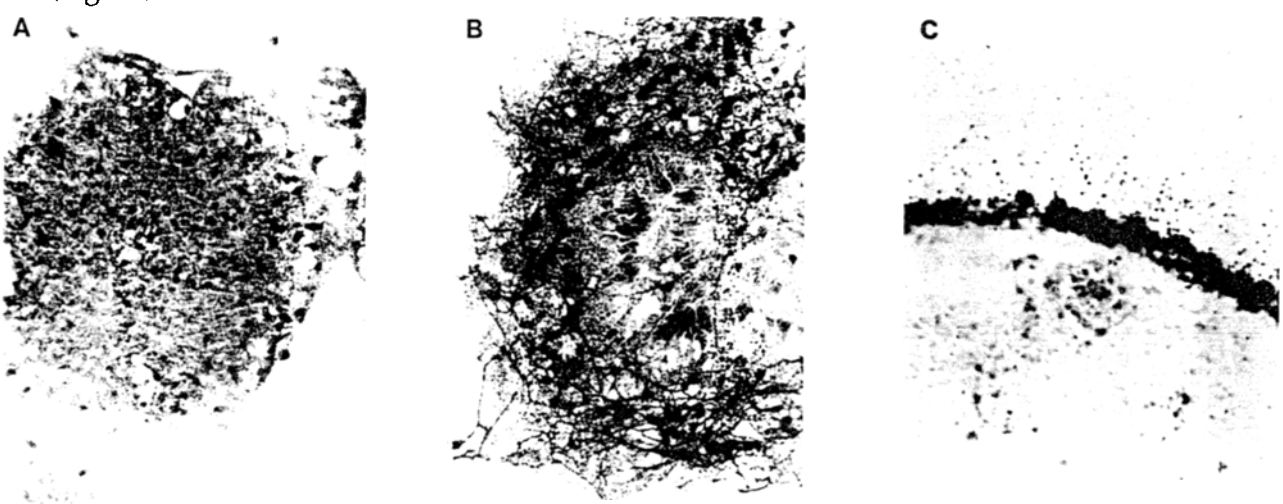


Fig. 11. Redistribution of GABA-ergic amacrine cells in retinal cultures. Cells were detected by uptake of radioactive GABA and subsequent autoradiography. At 20 h of culture (A) the labeled cells are scattered throughout the tissue. At 72 h (B) the cells are still broadly distributed, but are now excluded from a central zone of the culture. At 7 d (C) the GABA ergic cells are now confined to the outer layer of the aggregates. Taken form Akagawa and Barnstable (1987).

Thus, it would appear that generalized cell adhesion is a necessary prerequisite for retinal histogenesis. It is unclear, however, whether the observed lamination is mediated by these molecules, controlled in an appropriate spatial or temporal pattern, or by some differential mechanisms set up by an as yet unidentified set of cell-type-specific molecules. In addition, since sorting out in retinal reaggregates seems to involve active motility, it will be of interest to determine whether perturbation of sorting involves simply loss of adhesive strength or more complex transmembrane effects on the cytoskeleton or other components of cell motility.

## Development of Photoreceptors

### *Molecules Expressed During Photoreceptor Development*

Photoreceptors are among the last cells of the retina to be generated and to differentiate. In the rat, most photoreceptor differentiation occurs postnatally. One of the first detectable photoreceptor-specific molecules is the visual pigment protein opsin (Barnstable, 1981). In the neonatal rat retina, antibodies against N-terminal regions of the opsin molecule label a number of immature neuroblastic cells (Fig. 12a). These extend long radial processes across the tissue and have processes that reach the ventricular surface or outer limiting membrane. Labeling of dissociated living cells has confirmed that the labeling seen within the tissue is related to opsin inserted into the plasma membrane rather than to a cytoplasmic pool waiting to be inserted into the outer segment (Hicks and Barnstable, unpublished obervations). Over the first postnatal week the number of labeled cells in the outer part of the neuroblastic layer increases until essentially all appear to be labeled (Fig. 12b). As the developing photoreceptors form outer segments they take on a more mature morphology of closely packed spherical cell bodies connec-

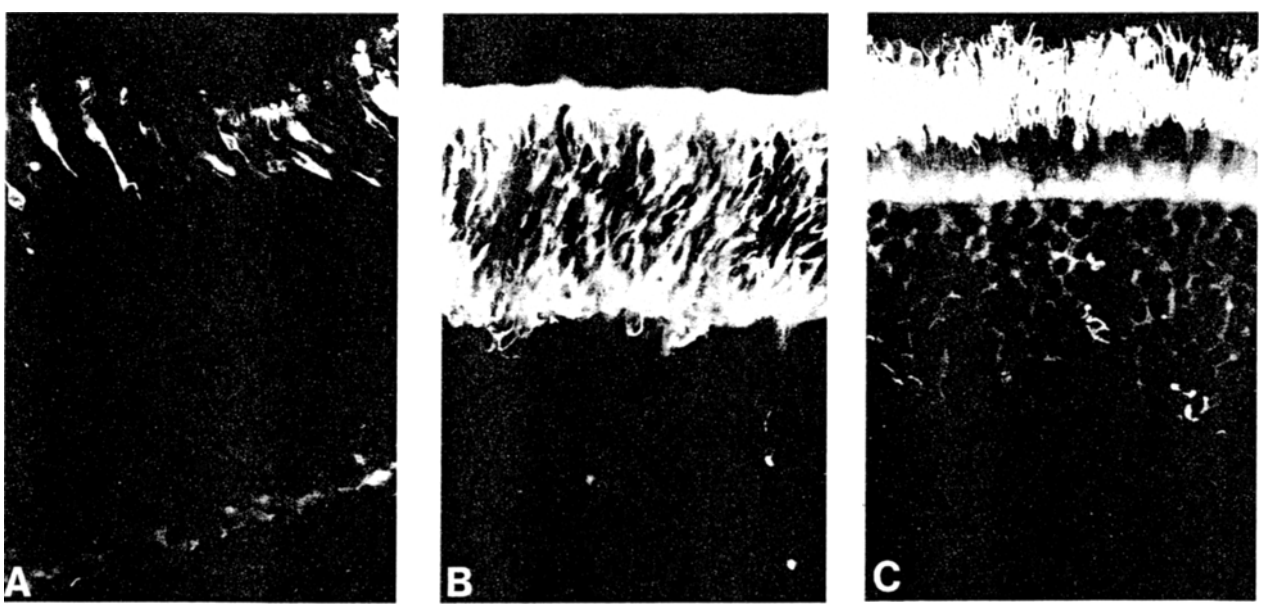


Fig. 12. Changing expression of photoreceptor antigen RET-P1, a determinant on the N-terminal portion opsin. (A) At PN2 a few immature cells are labeled at the ventricular surface of the tissue. (B) By PN8 most of the cells in the forming ONL are labeled, although they still have an immature morphology, and the outer segments have only just begun to extend. (C) In the adult the whole rod photoreceptor is labeled. Taken from Barnstable (1980,1981,1982).

ted to the inner and outer segments and the synaptic terminals by thin processes (Fig. 12c).

The developmental increase in the expression of opsin seems to be under transcriptional control. Using a dot-binding assay to total retinal RNA and an opsin cDNA probe, opsin mRNA was first detectable at PN3 and increased in amount until approximately PN28, at which stage adult levels were reached (Treisman et al., 1987). Given the very low number of cells labeled by opsin antibodies in the neonatal retina, it is perhaps not suprising that the amount of opsin mRNA was below the level of detection at these early times. Both methods give an early time of appearance for opsin than previous estimates. These other estimates have relied upon a spectrophotometric measurement of the loss of absorption of 498 nm upon bleaching. These estimates require not only the opsin polypeptide but also the retinal chromophore in the 11-*cis* configuration, such that it can respond to light by isomerization to the all-*trans* form. Using such measurements. the first detectable opsin was found at about PN7 (Dowling, 1964; Gonzalez-Fernandez et al., 1984a). The retinal chromophore is thought to be transported to the photoreceptors from the pigment epithelium as a complex with a soluble, interstitial retinol-binding protein (IRBP), which is thought to be secreted by photoreceptors (Gonzalez-Fernandez et al., 1984b). The developmental appearance of IRBP has been followed in the rat retina by measuring the amount of antibody bound to immunoblots of interphotoreceptor matrix from animals of different ages. Using this assay, the first detectable IRBP was found at PN5, and the amounts increased until adult levels were reached at about 4 wk of age (Gonzalez-Fernadez et al., 1984a). The curve of appearance of IRBP was almost identical to that for rhodopsin, using the spectroscopic assay, and so may also have missed the early expression by immature photoreceptors.

To gain information about both the onset of retinal transfer from the PE and the light sensitivity of photoreceptors during retinal development, we have carried out experiments using antibodies sensitive to light-dependent conformational changes in rhodopsin (Hicks and Barnstable, 1987). Such an analysis was possible because a number of antibodies have been studied with respect to the position of the epitopes on the opsin polypeptide. A summary of this information is given in Fig. 13.

At PN5 a number of opsin antibodies label the immature photoreceptors, and the labeling is not affected by bleaching the retina by exposure to bright light for 1 min prior to fixation. At PN8, on the other hand, two separate, light-dependent effects were found. The antibody RHO-C7 recognizes an epitope that includes an N-terminal region (Fig. 13), but also seems to require the native, three-dimensional structure of the molecule since it reacts poorly with opsin solubilized in many detergents and does not react well on immunoblots of retinal membranes or purified opsin. RHO-C7 labeling was decreased in the bleached PN8 retinas, whereas that of other N-terminal antibodies that are not obviously sensitive to conformation was unchanged. One consequence of light activation of rhodopsin is that it becomes susceptible to phosphorylation in the C-terminal region. This is thought to function physiologically as part of the mechanism that shuts off the phototransduction cascade. An antibody, 1C5, has been described that binds less well to phosphorylated rhodopsin (Molday and MacKenzie, 1983). In the PN8 retina, 1C5 reacts much less strongly to bleached tissue than to retinas fixed in the dark. Both of these findings suggest that the retinal chromophore is present in rhodopsin at PN8 and that the photoreceptors are capable of initiating a response to light. In phototransduction, the reception of photons by rhodopsin leads to the activation of a cGMP phosphodiesterase through a G-protein-coupling mechanism (Stryer, 1985). Thus, whether light can change the membrane potential of the immature photoreceptors will depend upon the presence of the light-dependent mem-

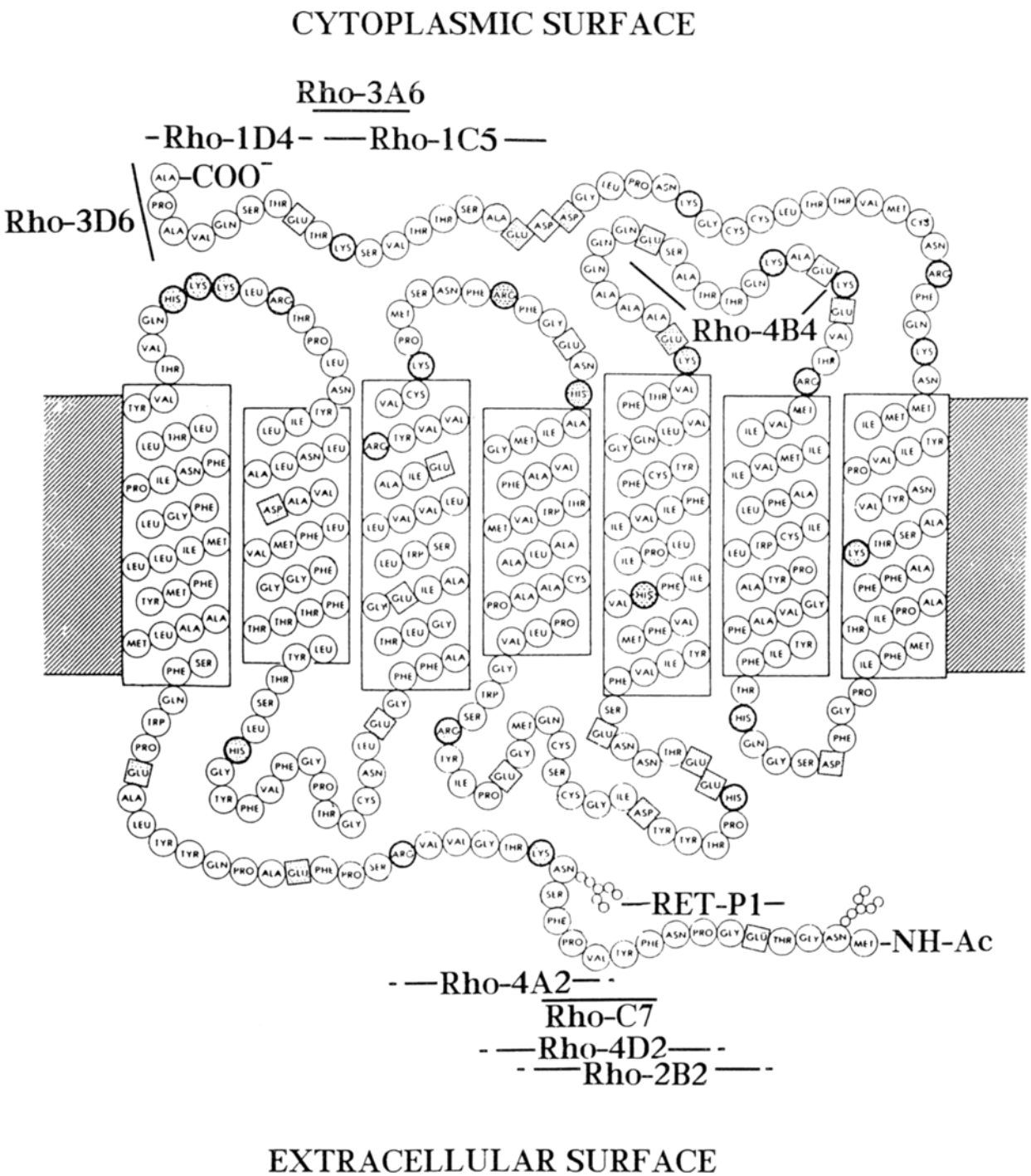


Fig. 13. Schematic representation of the opsin polypeptide. Onto this model have been drawn the known binding sites for a range of monoclonal antibodies, which were produced in several laboratories. The binding of some antibodies such as 1C5 to the C-terminal portion of the molecule is influenced by phosphorylation that accurs in this region after light-dependent conformational changes. Taken from Hicks and Barnstable (1987).

brane conductances and on the enzymatic machinery for the regulation of cGMP levels. Any effects upon other retinal cells will also depend upon the presence of synaptic machinery in the developing photoreceptor cells.

To examine the development of synapses by photoreceptors and other retinal cells, we have examined the expression of synaptic vesicle proteins. Photoreceptors are unusual in that they do not express synapsin I, a phosphoprotein thought to play a role in mobilizing synaptic vesicles for release (De Camilli et al., 1979). We have produced an antibody to a 38-kdalton vesicle membrane glycoprotein whose appearance does show a strong correlation with synaptogenesis throughout the nervous system (Devoto and Barnstable, 1987). This protein is detectable in a few vitreally directed processes of cells corresponding to differentiating photoreceptors in the PN 2 retina. Over the next 3–4 d the number of labeled cells in this layer increased and, on PN 5, labeling of both processes and presumptive synaptic terminals could be seen. Labeling in the inner plexiform layer lagged behind the outer plexiform layer by several days. In the electron microscope, at PN 5, accumulations of vesicles could be found at the sites of forming photoreceptor synapses, although these did not have the invaginated structure seen in the adult. In addition, synaptic ribbons were not observed until PN 6. Early studies indicated that an ERG, representing the existence of fully formed synaptic circuitry, could not be detected until the time of eye opening, at about PN 13 in the rat (Weidman and Kuwabara, 1968). The results mentioned above suggest that photoreceptors are capable of many light-dependent functions long before eye opening, and, thus, in our models of retina development it may be necessary to consider light input as a factor in the formation of retinal synaptic circuits.

The next phase of differentiation that we have defined in these cells takes place as outer segment formation begins, when a number of new molecules appear. Among them are RET-P2, a 38-kilodalton membrane protein that is restricted to the outer segments, and RET-B1, a molecule that is expressed by both photoreceptor inner segments and bipolar cells (Barnstable, 1980; Fekete and Barnstable, 1983; Akagawa and Barnstable, 1986). As discussed in the next sections, these two molecules are localized to different compartments of the cell and seem to be under different forms of developmental control.

A later phase of photoreceptor maturation has also been defined by the expression of the photoreceptor cell body marker RET-P3 (Barnstable, 1980). This can only be detected in fixed retinal sections at about PN10–12. How the expression of molecules such as this is controlled and how it changes the cell responses have yet to be determined.

## Cellular Compartments Within Photoreceptors

When opsin is first expressed it is found on the plasma membrane throughout the cell, suggesting that at these early stages the plasma membrane is homogeneous (Barnstable, 1981). In adult photoreceptors, on the other hand, many molecules are localized to distinct compartments of the cell. This finding has led us to investigate when such compartments are formed and to speculate how molecules end up in the right compartment (Hicks and Barnstable, 1986). Changes in the distribution of opsin can be used to show the formation of one of the compartments. As the inner and outer segments form, the expression of opsin on the cell bodies and inner segments declines and that on the outer segments is greatly enhanced [Fig. 14 (top)]. In some species it has been suggested that opsin is transported from the Golgi apparatus to a specialized region close to the base of the outer segment in specialized vesicles (Papermaster et al., 1985). If the onset of the utilization

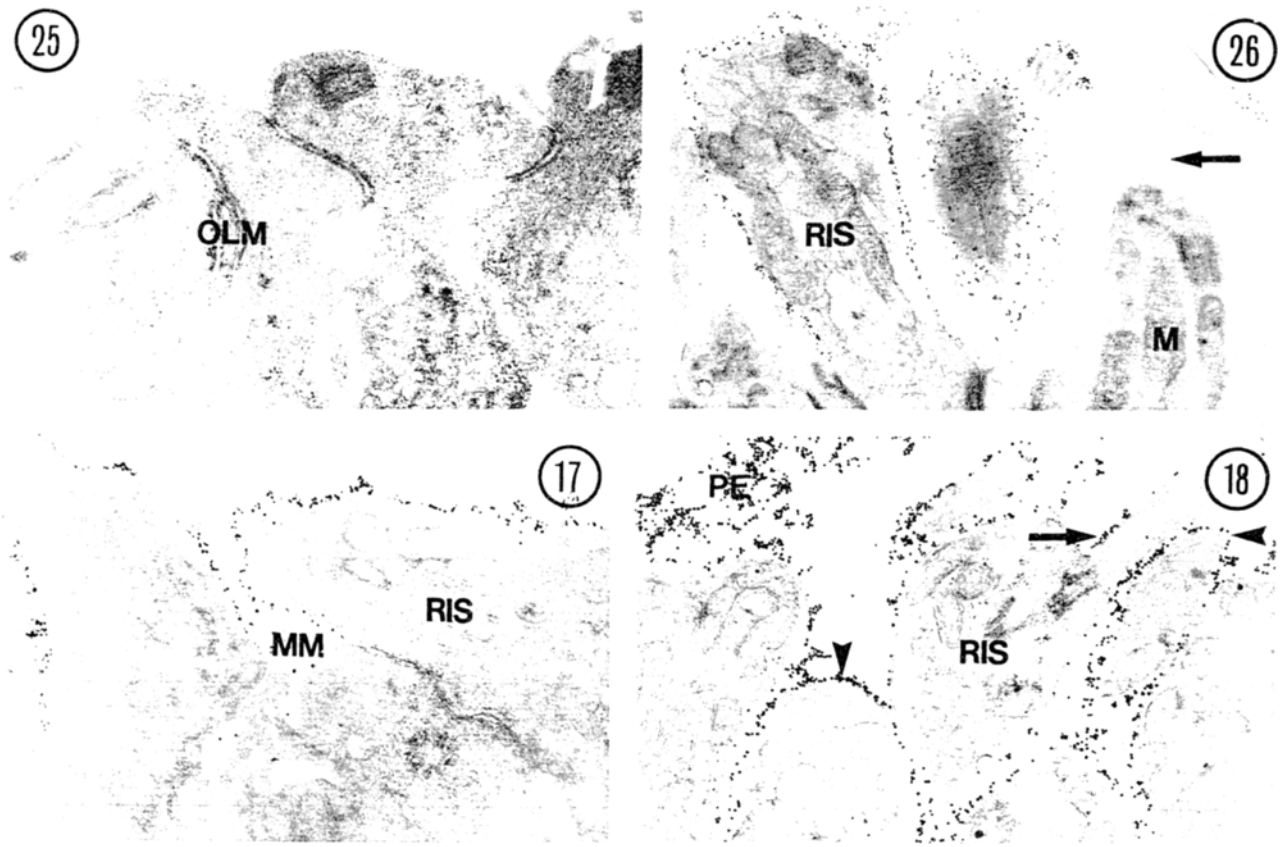


Fig. 14. (Top) The changing distribution of opsin on photoreceptors with development. At PN1 (top left) a rat retina incubated in antibody RET-P1 followed by a gam-$Au_5$-Dex complex shows very few gold particles on the cell membranes. By PN4 (top right) a retina incubated in a similar way shows some heavily labeled inner segments. As the cilia emerged the amount of label on the inner segment was sometimes found to decrease (arrow). (Bottom) Wheat germ agglutinin labels the surface of a PN1 rat retina uniformly (bottom left). At PN4 the proximal areas of cilia show a band of higher density of gold particles (arrow) than either the more distal cilium or the inner segment from which it arises (bottom right). Inner segments without cilia tend to show higher levels of labeling than those with cilia (arrowhead). Figure taken from Hicks and Barnstable (1986).

of these vesicles coincided with outer segment formation, then this might be sufficient to explain the change in opsin distribution. If other outer segment molecules also use these vesicles, it would also explain why molecules, such as RET-P2, are found only in outer segments. Such a mechanism also requires that once in the outer segment, molecules are prevented from diffusing back into the rest of the cell. We and others have noticed [Fig. 14 (bottom)] a ring of material at the base of the cilium connecting the rod outer segment to the rest of the cell, which can be detected by lectin labeling (Besharse et al., 1985; Hicks and Barnstable, 1986). This could act as a physical barrier to diffusion of membrane components, although such a function has not been formally demonstrated.

Within the membranes of the outer segment further compartmentalization seems to occur since it appears that the membrane components of the outer segment plasma membrane do not have the same composition as those of the free-

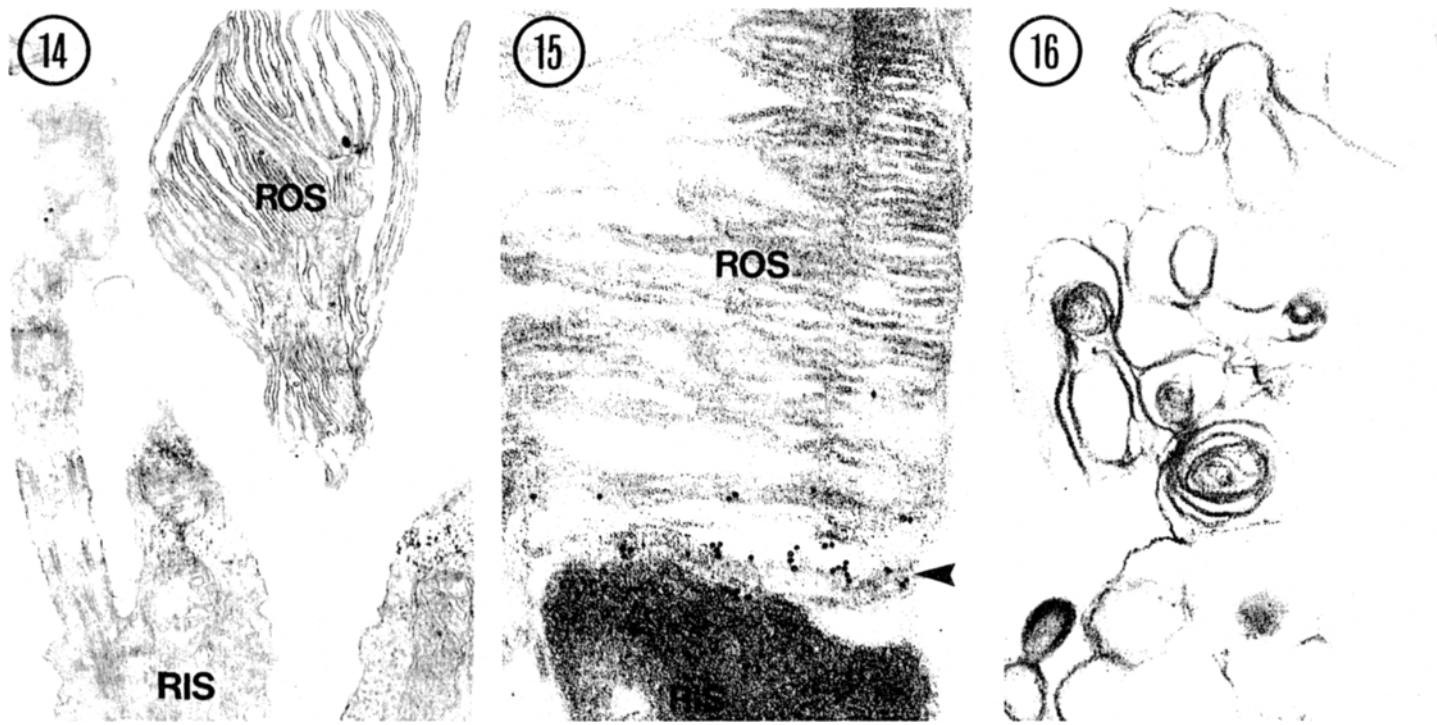


Fig. 15. Localization of RCAII lectin-binding sites in adult rat rod photoreceptors. (*Left*) Control section showing that in the presence of the competing sugar D(+)-galactose there is essentially no labeling. Pre-embedding labeling of photoreceptors showed that the plasma membrane did contain many RCAII binding sites. (*Middle*) Postembedding labeling shows that a region of increased RCAII binding is found at the base of the outer segment in the region where disks form, but no binding sites could be found over the mature disk membranes. (*Right*) Labeling of purified bovine disks incubated with RCAII-$Au_5$-Dex showing that the exposed disk membranes do not contain RCAII binding sites, which suggests that these binding sites become localized to the plasma membrane only. Figure taken from Hicks and Barnstable (1986).

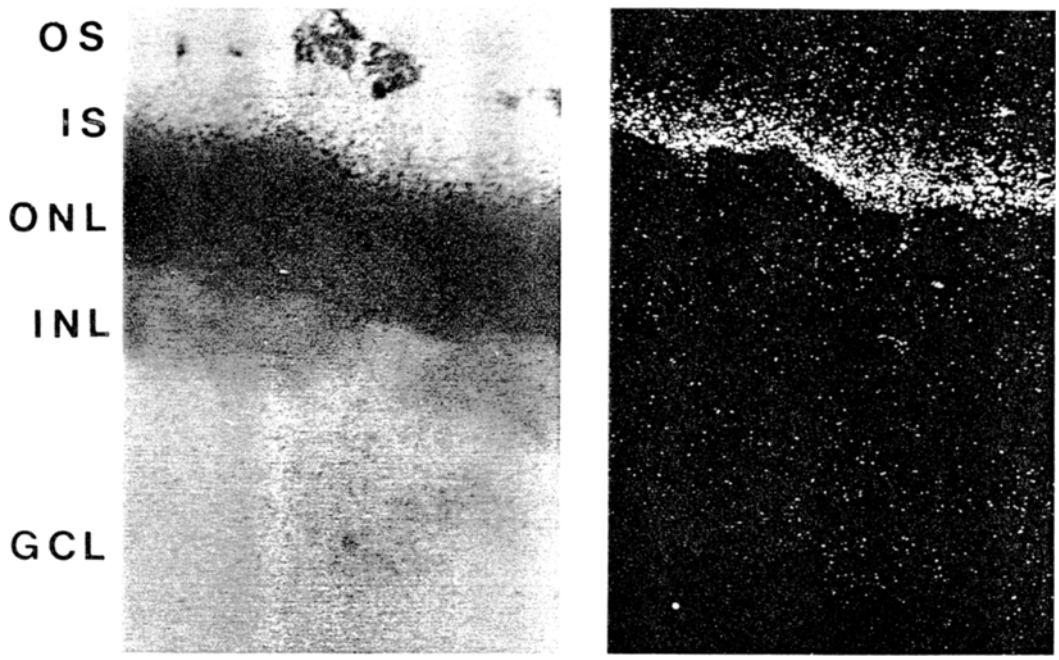


Fig. 16. *In situ* localization of opsin mRNA. Single-stranded, $^{35}$S-UTP-labeled RNA probes were prepared by transcription from a PT3/T7–18 plasmid containing a *Sma*I–*Pst*I fragment of opsin cDNA that spans the whole coding region. Probes were hybridized to crystat sections of adult rat retina and exposed to emulsion for 6–10 d. (*Left*) Toluidine-stained section showing the retinal layers. (*Right*) Dark-field illumination of the same section to show the silver grains. A band of grains is seen over the inner segments (IS), and some label is found over the photoreceptor cell bodies. The other retinal layers show no labeling above background. Hybridization to sense RNA gave no specific signal. Retinal layers are labeled as in Fig. 1.

floating disks. In our early studies of the RET-P2 antigen we suggested that it might be a component specifically localized to the outer segment plasma membrane (Fekete and Barnstable, 1983). Since then we have shown that other molecules detectable by binding of RCA II lectin are found only, or are greatly enriched, in the plasma membrane (Fig. 15). The disks are derived from the plasma membrane by evagination and pinching off (Steinberg et al., 1980). Thus, if molecules are to be excluded from the disks they must not enter this evaginating sheet of membrane. There is a concentration of cytoskeletal proteins in this region of the rod photoreceptor so that interactions between transmembrane proteins and these may lead to the separation of membrane domains (Drenckhahn and Wagner, 1985; Vaughan and Fisher, 1985).

The photoreceptor inner segment also seems to represent an isolated plasma membrane domain. The molecule RET-B1 is a cell-surface molecule that, in the adult, is found only, or is at least highly concentrated, in the inner segment (Akagawa and Barnstable, 1986). When it first appears, however, the molecule is found distributed throughout the photoreceptor (unpublished observations). Thus, like opsin, the distribution of this molecule changes as the photoreceptor matures. Whether this is by trapping of the molecule by interaction with other membrane or cytoskeletal components, or is by selective insertion in a domain that does not allow free diffusion is not yet clear.

Other cellular compartments in rod photoreceptors are found in the cell bodies and at the synaptic terminals. We have studied the cell-

body-specific marker defined by antibody RET-P3 less than other photoreceptor-specific molecules because we have not yet obtained a biochemical characterization of the molecule. When it is first expressed in the retina, outer and inner segments are forming. RET-P3 immunoreactivity seems to be confined to the immature cell body layer, which would be consistent with the results mentioned above, which indicate that the outer-segment compartment has been isolated by this time.

The synaptic vesicle protein SVP38 is confined to the synaptic terminals in the adult photoreceptor and is found only on the vitreally directed side of the cells, even when it is first detected. It is likely that, as in other neurons, synaptic vesicles are specifically transported down to the synaptic terminals. Once there, the mechanisms of exocytosis and membrane recycling are presumably efficient enough that these membrane components do not diffuse throughout the cell to any measurable extent.

One possibility for selective distribution of newly synthesized molecules within the photoreceptor is that they are produced in separate locations. It is known that the rod inner segments contain abundant rough endoplasmic reticulum. We have used *in situ* hybridization of $^{35}$S-labeled, single-stranded RNA probes for opsin mRNA to show that the inner segment is the major site of opsin biosynthesis (Fig. 16). Using a biotinylated cDNA probe, the site of synthesis of the secreted protein IRBP has been localized to the rod photoreceptor cell bodies in the outer nuclear layer (Van Veen et al., 1986). No concentration of mRNA was reported over the inner segments. Thus, it would appear that there is a difference in the localization of mRNA, and, presumably, of the whole protein synthetic apparatus, according to the cellular compartment for which the product is destined. It will be of great interest to determine the site of synthesis of molecules that are localized to other parts of the photoreceptor cell.

## Mosaic and Inductive Events in Photoreceptor Development

In the rat retina, rod photoreceptors become postmitotic in the late embryonic and early postnatal period. The photoreceptor antigens described above appear at different times over the first 10 d after birth. Two very different mechanisms can be proposed to account for the control of gene expression over such a long period in these nondividing cells. One mechanism would be for the cells to contain an internal clock, such that, once determined to be rod photoreceptors the cells would express appropriate gene products at the correct times in the differentiation pathway. The other mechanism would be that there is no single event of commitment and that at each stage particular genes are in duced by instructive environmental signals. These two mechanisms represent the extremes of mosaic and inductive mechanisms and are experimentally separable. The mosaic model predicts that an isolated photoreceptor, once committed, will be able to carry out its differentiation program in the absence of any cell interactions or specific factors. The inductive model predicts that such interactions or factors will be necessary at each developmental stage. We have been studying the differentiation of photoreceptors in culture to begin to distinguish these mechanisms. We have taken cells from PN 2 animals such that some of the photoreceptors can already be recognized by the expression of opsin. We have then placed these cells in various culture environments and measured the appearance of various photoreceptor molecules using specific antibodies. When maintained in low-density monolayer cultures the cells did not form outer segments or express outer-segment-specific molecules, even though the cells remained viable and healthy for many weeks (Akagawa and Barnstable, 1986). When the dissociated retinal cells were allowed to reassociate in reaggregate cultures, outer segments could be found both by immunocytochemical labeling

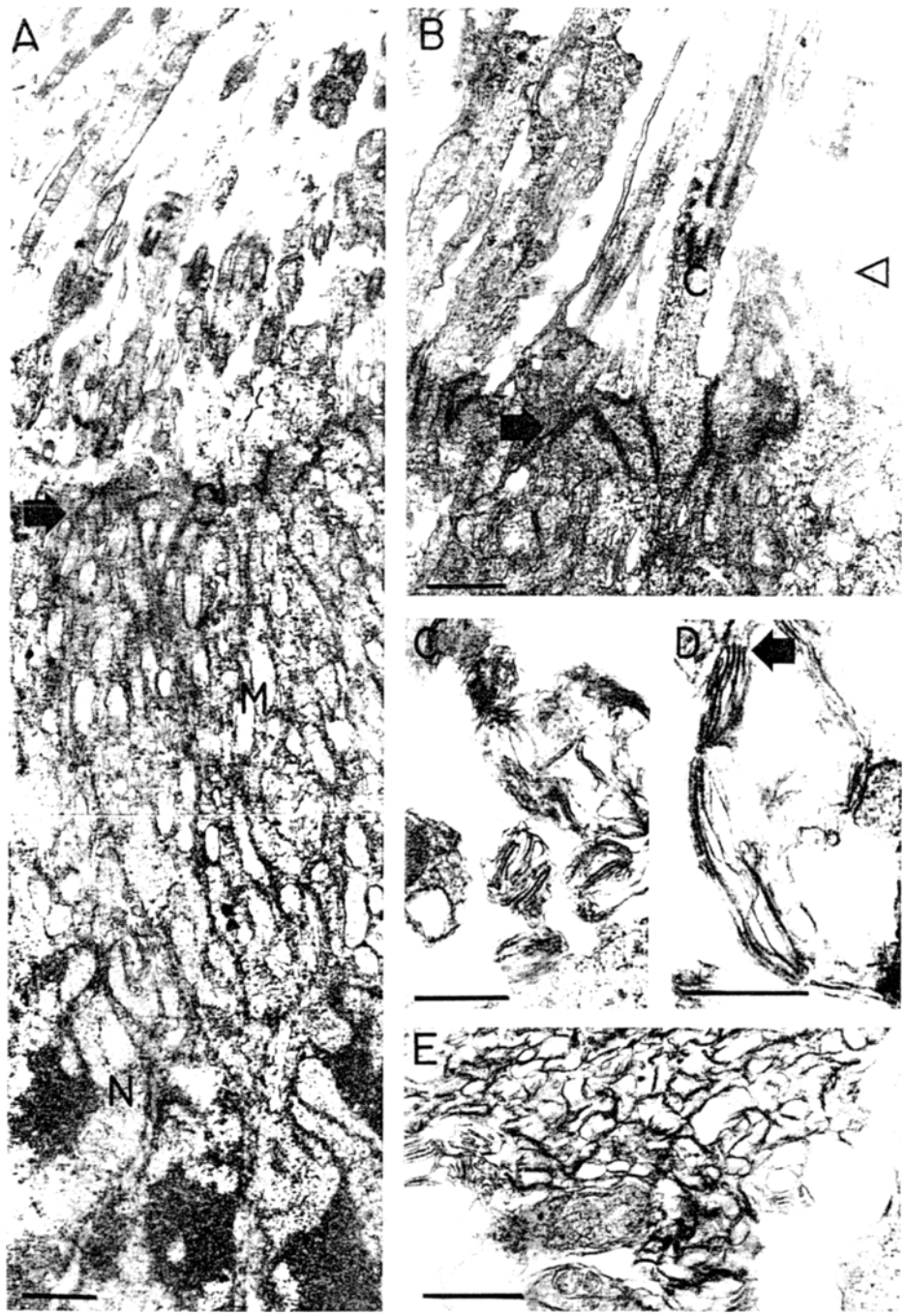


Fig. 17. Electron microscope view through the photoreceptor region of a rat retinal reaggregate culture. (A) The cell bodies contain irregular nuclei with clumped heterochromatin [N]. Mitochondria [M] collected luminally to the nuclei and the cell bodies were joined by desmosome-like junctions [arrow]. (B) Higher magnification of the inner segment region showing the same junctions [arrow]. Cellular protrusions into the rosette lumen often contained mitochondria, vesicles, and centrioles [C]. These structures often ended in a longitudinally oriented cilium. Between the photoreceptors, microvilli like those of Müller cells were observed (open arrowhead). (C and D) Within the lumen many photoreceptors ended in membranous expansions that contained small stacks of disks (arrow). (E) In some areas irregular accumulations of membranes could also be seen. Scale bar in (A) and (B) = 1 μm, in (C), (D), and (E) = 0.5 μm.

with outer-segment-specific antibodies and by morphological analysis in the electron microscope (Barnstable et al., 1983, and manuscript submitted). The photoreceptors in the reaggregates had also formed specific junctions with Müller glial cells analogous to those found at the outer limiting membrane of the intact retina (Fig. 17). Other photoreceptors in the reaggregates that had not formed these junctions did not make outer segments. This suggests that the specialized interactions between the Müller cells and the photoreceptors were necessary for the expression of the expression of outer-segment-specific gene products. Thus, at least one aspect of photoreceptor differentiation seems to require some inductive interaction. Morphological examination of the Müller cells in the reaggregate cultures showed that they had taken on a much more mature morphology than usually found in monolayer cultures, suggesting that the interaction between them and photoreceptors was reciprocal.

In the same set of experiments a very different result was found for the inner segment marker RET-B1. This molecule was expressed in low-density monolayer cultures about 5 d after plating, a time consistent with its time of exprssion in the intact tissue. Since the onset of expression did not seem to be affected by increased cell density, the result suggests that onset of expression was controlled by a mechanism intrinsic to the photoreceptors.

Thus, our results to date provide preliminary evidence for both mosaic and inductive mechanisms in photoreceptor differentiation. It is also important to emphasise that the different mechanisms can be operating at the same time, such as the period of outer segment formation. Among the things we do not yet know are whether any of the inductive events are dependent upon prior steps of differentiation; that is, would it be possible to express outer segments in cells that have not gone through the stage of opsin expression? The other major challenge is now to translate these results into an analysis of the events occurring at the level of the gene. Inductive events could act by either initiating transcription at uncommitted genes or by triggering transcription at genes that have already become activated by some prior stage of committment. Given that we have been able to describe photoreceptor differentiation in molecular terms, it is now possible to detect the genes for some of these molecules and to examine when their transcription is initiated and whether before this stage the genes are "activated," such as by the detection of 5'-DNAse-hypersensitive sites. Such studies will provide a solid molecular background with which to integrate results from studies examining the nature of the cell interactions or factors leading to the differentiation events.

## Oncogenes and Growth Factors in Retinal Development

Much of this review has dealt with cell-surface molecules and changing patterns of gene expression during retinal development. The mechanisms by which signals generated by interactions at the cell surface are transduced into changes in gene expression are unknown. Much of the current interest in the normal cellular homologs of oncogenes lies in the fact that some of them are related to growth factors or growth factor receptors and are strong candidates for carrying out this transduction.

So far only one proto-oncogene has been studied intensively in the retina. *C-src* is expressed in most if not all cells, but its expression in the nervous system has two interesting features. The first is that the specific activity of *c-src* is higher in neural tissue than most other tissues (Cotton and Brugge, 1983). The second is that neural *c-src* has a different molecular weight on SDS gels than *c-src* from other tissues (Brugge et al., 1985). This difference is localized to the N-terminal portion of the molecule and is found in neurons, but not glia. Immunocytochemical la-

beling of chick retina has shown that *c-src* can be found in both plexiform layers and cell bodies of at least amacrine and ganglion cells (Sorge et al., 1984). The levels of *c-src* mRNA and protein have been measured during chick retinal development (Sorge et al., 1984; Vardimon et al., 1986). Both show increases to a peak at d 11–13, after which the levels declined to a low level, which persisted after hatching. The declining phase observed by both groups was interpreted very differently. Measurements of declining protein were interpreted as relatively constant levels per retina, with increas es in structural proteins of the tissue giving an apparent decrease in *c-src* (Sorge et al., 1984). The decrease in mRNA, however, was interpreted as a true decrease because of previous studies that have suggested that total protein does not increase in the retina after E12 (Vardimon et al., 1986). *C-src* is similarly expressed in rat retina (unpublished observations). The availability of good antigenic markers for the differentiation state of various cell types will allow a closer correlation between changes in *c-src* and retinal cell differentiation.

The product of the *c-src* gene is a 60-kdalton phosphoprotein that contains a tyrosine kinase activity. The neuronal substrates for this enzyme have not yet been identified. It is interesting that the activity of *c-src* seems to have a different function to that of the viral form of *src*. *C-src* increases as neurons become postmitotic and, thus, appears to have a function in the maturation of these cells. V-*src*, on the other hand, is effective in blocking the expression of differentiated features in neuroectoderm cells, that is, it appears to prevent cells from differentiating (Keane et al., 1984).

Another tyrosine kinase with significant amino acid homology with *src* has been isolated as the transforming activity from the virus Y73 (Yoshida et al., 1980). The cellular homolog of this protein *c-yes* has also been detected in rat retina (Treisman et al., manuscript in preparation). It will be of interest to determine whether the developmental profiles of *c-src* and *c-yes* are similar. Since the two genes are thought to have diverged from a common precursor, it is, perhaps, more likely that they now carry out different functions that may be expressed at different times in development.

Both *c-src* and *c-yes* are attached to the cytoplasmic face of the plasma membrane and are thought to function by acting as a bridge between transmembrane receptors and cytoplasmic effector molecules. Although the identity of such transmembrane receptors has not been established, there have been a number of studies seeking factors responsible for retinal cell survival and differentiation.

Two soluble factors have been described that have an effect on the survival of retinal ganglion cells and outgrowth of their processes. One such factor has been purified from mammalian brain and has been called Brain-Derived Neurotrophic Factor or BDNF. This is a 12-kdalton protein that has an isoelectric point of about 10 and has been shown to support the survival of a variety of primary sensory neurons (Barde et al., 1982; Lindsay et al., 1985). This factor enhanced the survival of retinal ganglion cells in monolayer cultures (Johnson et al., 1986). Because ganglion cells do not normally survive well in monolayer cultures, it was possible to observe the effect of the factor. Since good markers for other retinal cell populations were not used in these experiments, no firm conclusions could be drawn about the effects of the factor upon other retinal populations.

A second class of factors has been isolated from the eye and has been shown to have effects upon the survival and process outgrowth of a number of neuronal cell types. It now seems clear that most of these factors are related to the class of acidic and basic fibroblast growth factors (FGFs) that have been isolated using a number of assays (Baird et al., 1985; Wagner and D'Amore, 1986). Although these factors seem to be mitogenic for many cell types, they have the property of enhancing process outgrowth by a number of neuronal cell types from retina, cere-

bral cortex, and the peripheral nervous system (Walicke et al., 1986; Morrisson et al., 1986; Wagner and D'Amore, 1986). Both basic FGF and acidic FGF can bind tightly to heparin. It has been suggested that such interactions could mediate both the adhesion of neurites to extracellular matrix and possibly even their growth (Wagner and D'Amore, 1986).

Thus far the growth factors studied have been assayed for their effects upon ganglion cell survival and axon elongation. Which of the two processes is the primary site of action has yet to be determined, as do the effects upon other retinal cells. In addition, it is unclear at present whether there are a further set of factors to be found that can influence the differentiation pathway of any retinal cell type.

## Conclusions

The retina has been a useful model for many studies of signal transduction, neurochemistry, neurophysiology, and general developmental biology. It is becoming clear that it is also useful for analyzing the molecular basis of many features of neural development. One of the principle advantages is that there now exist a wide range of molecular markers for many retinal cell types. The markers mentioned in this article are primarily those that have been generated in my own laboratory. Many other neurochemical and immunological markers have also been described by a number of other laboratories.

One of the other features that makes the retina such an attractive model system is that it now seems to be possible to get most if not all aspects of differentiation from a dividing neuroepithelium to occur in a culture dish. Thus, more precise experimental control can now be exerted over the differentiation process. As discussed in Development of Photoreceptors, we have been able to affect the differentiation of rod photoreceptors by altering the culture conditions to change the types of cell interactions observed. It is now possible to explore the molecular basis of these interactions and to learn how they can effect both cell morphology and the expression of new retinal molecules.

One aspect of neural differentiation that has been mentioned several times throughout this article is that many important events of patterning and cell interactions take place before the major times of synapse formation and are probably mediated by separate molecular systems. Thus, studies of activity-dependent patterning of synaptic contacts are frequently looking at later modulations or fine tuning of a pattern imposed by other mechanisms. In the retina, many of the events of cell differentiation and lamination have already occurred by the time of synapse formation.

Pattern, in the form of gradients across the retina, has been studied for many years. New approaches to this problem have yielded two molecules that show such a graded distribution (*see* Specification of Positional Information Within the Retina), both of which appear before synaptogenesis and are present during the major periods of retinal development. It is interesting to note that these two retinal gradients are the only two to have been described so far in vertebrate systems.

Even with the progress that has been made toward identifying molecules in the vertebrate retina, there is still a long way to go before we can understand how the initial sheet of neuroepithelium develops into the mature structure. We have reached the stage of formulation of specific hypotheses for aspects of retinal development and, more importantly, can propose ways in which these hypothese can be tested experimentally. With the tools now available, the next few years will almost certainly bring about a revolution in our understanding not only of individual cell types, but also of the mechanisms governing patterning in the nervous system and perhaps biology in general.

## Acknowledgments

Work from my laboratory described in this article was supported by NIH grants EY 05206 and NS 20483. Much of the work was carried out while the author was an Alfred P. Sloan Research Fellow. The contributions of K. Akagawa, A. S. Blum, S. H. Devoto, D. Hicks, J. R. Sparrow, and J. E. Treisman are gratefully acknowledged.

## References

Abney E. R., Williams B. P., and Raff M. C. (1983) Tracing the development of oligodendrocytes from precursor cells using monoclonal antibodies, fluorescence-activated cell sorting, and cell culture. *Dev. Biol.* **100**, 166–171.

Akagawa K. and Barnstable C. J. (1986) Identification and characterisation of cell types in monolayer cultures of rat retina using monoclonal antibodies. *Brain Res.* **383**, 110–120.

Akagawa K. and Barnstable C. J. (1987) Identification and characterisation of cell types accumulating GABA in rat retinal cultures using cell type specific monoclonal antibodies. *Brain Res.* **408**, 156–162.

Baird A., Esch F., Gospodarowicz D., and Guillemin R. (1985) Retina and eye derived endothelial cell growth factors: Partial molecular characterization and identity with acidic and basic fibroblast growth factors. *Biochemistry* **24**, 7855–7860.

Barbera A. J., Marchase R. B., and Roth S. (1973) Adhesive recognition and retinotectal specificity. *Proc. Nat. Acad. Sci. USA* **70**, 2482–2486.

Barde Y. A., Edgar D., and Thoenen H. (1982) Purification of new neurotrophic factor from mammalian brain. *EMBO J.* **1**, 549-553.

Barnstable C. J. (1980) Monoclonal antibodies which recognise different cell types in the rat retina. *Nature* **286**, 231–235.

Barnstable C. J. (1981) Developmental studies of rat retina cells using cell-type-specific monoclonal antibodies, in *Monoclonal Antibodies to Neural Antigens.* (McKay R., Raff M. C., and Reichardt L. F., eds.) pp. 219–230, Cold Spring Harbor, NY.

Barnstable C. J. (1982) Immunological studies of the retina, in *Neuroimmunology* (Brockes J., ed.) pp. 183–214, Plenum, New York, NY.

Barnstable C. J. (1985) Monoclonal antibodies as molecular probes of the nervous system, in *Hybridoma Technology in the Biosciences and Medicine* (Springer T., ed.) pp. 269–289, Plenum, New York, NY.

Barnstable C. J. and Constantine-Paton M. (1984) Initial events of lamination in the mammalian retina. *Soc. Neurosci. Abstr.* **10**, 787.

Barnstable C. J. and Dräger U. C. (1984) Thy-1: A ganglion cell specific marker of rodent retina. *Neuroscience* **11**, 847–855.

Barnstable C. J., Akagawa K., Hofstein R., and Horn J. P. (1983) Monoclonal antibodies that label discrete cell types in the mammalian nervous system. *Cold Spring Harbor Symp. Quant. Biol.* **48**, 863–876.

Barnstable C. J., Hofstein R., and Akagawa K. (1985) A marker of early amacrine cell development in rat retina. *Dev. Brain Res.* **20**, 286–290.

Bastiani M. J., Du Lac S., and Goodman C. S. (1985) The first neuronal growth cones in insect embryos. Model system for studying the development of neuronal specificity, in *Model Neural Networks and Behavior* (Selverston A. I., ed.) pp. 149–174. Plenum, New York, NY.

Ben-Shaul Y., Hausman R. E., and Moscona A. A. (1979) Visualization of a cell surface glycoprotein, the retina cognin on embryonic cells by immunolatex labeling and scanning electron microscopy. *Dev. Biol.* **72**, 89–101.

Ben-Shaul Y., Hausman R.E., and Moscona A. A. (1980) Age-dependent differences in cognin regeneration on embryonic retina cells: Immunolabeling and SEM studies. *Dev. Neurosci.* **3**, 66–74.

Bentley D. and Caudy M. (1983a) Pioneer axons lose directed growth after selective killing of guidepost cells. *Nature* **304**, 62–65.

Bentley D. and Caudy M. (1983b) Navigational substrates for peripheral pioneer growth cones: Limb-axis polarity cues, limb-segment boundaries, and guidepost neurons. *Cold Spring Harbor Symp. Quant. Biol.* **48**, 573–585.

Besharse J. C., Forestner D. M., and Defoe D. M. (1985) Membrane assembley in retinal photoreceptors. III. Distinct membrane domains of the connecting cilium of developing rods. *J. Neurosci.* **5**, 1035–1048.

Blum A. S. and Barnstable C. J. (1986) Structure and regulation of a developmentally restricted neuronal surface antigen. *J. Cell Biol.* **103**, 231a.

Bonhoeffer F. Huf J. (1985) Position-dependent properties of retinal axons and their growth cones. *Nature* **315**, 409–410.

Brackenbury R., Thiery J.-P., Rutishauser U., and Edelman G. M. (1977) Adhesion among neural cells of the chick embryo. I. An immunological assay for molecules involved in cell-cell binding. *J. Biol. Chem.* **252**, 6835–6840.

Brady R.C. and Hilfer S.R. (1982) Optic cup formation: A calcium regulated process. *Proc. Nat. Acad. Sci. USA* **79**, 5587–5591.

Braekvelt C. R. and Hollenberg M. J. (1970) The development of the retina of the albino rat. *Am. J. Anat.* **127**, 281–302.

Brugge J. S., Cotton P. C., Queral A. E., Barrett J. N., Nonner D., and Keane R. W. (1985) Neurones express high levels of a structurally modified activated form of pp60$^{c\text{-}src}$. *Nature* **316**, 554–557.

Buskirk D. R., Thiery J. -P., Rutishauser U., and Edelman G. M. (1980) Antibodies to a neural cell adhesion molecule disrupt histogenesis in cultured chick retinae. *Nature* **285**, 488–489.

Cheresh D. A., Reisfeld R. A., and Varki A. P. (1984) O-acetylation of disialoganglioside GD3 by human melanoma cells creates a unique antigenic determinant. *Science* **225**, 844–846.

Cheresh D. A., Pierschbacher M. D., Herzig M. A., and Mujoo K. (1986) Disialogangliosides GD2 and GD3 are involved in the attachment of human melanoma and neuroblastoma cells to extracellular matrix proteins. *J. Cell Biol.* **102**, 688–696.

Constantine-Paton M., Blum A. S., Mendez-Otero R., and Barnstable C. J. (1986) A cell surface molecule distributed in a dorsoventral gradient in the perinatal rat retina. *Nature* **324**, 459–462.

Cotton P. C. and Brugge J. S. (1983) Neural tissues express high levels of the cellular src gene product pp60*c-src*. *Mol. Cell. Biol.* **3**, 1157–1162.

Daniloff J. K., Chuong C.-M., Levi G., and Edelman G. M. (1986) Differential distribution of cell adhesion molecules during histogenesis of the chick nervous system. *J. Neurosci.* **6**, 739– 758.

David S., Miller R. H., Patel R., and Raff M. C. (1984) Effects of neonatal transection on glial cell development in the rat optic nerve: Evidence that the oligodendrocyte-type 2 astrocyte lineage depends on axons for its survival. *J. Neurocytol.* **13**, 961–974.

De Camilli P., Ueda T., E., Bloom F. E., Battenberg E., and Greengard P. (1979) Widespread distribution of protein I in the central and peripheral nervous systems. *Proc. Nat. Acad. Sci. USA* **76**, 5977–5981.

Denham S. (1967) A cell proliferation study of the neural retina in the two day rat. *J. Embryol. Exp. Morphol.* **18**, 53–66.

Devoto S. and Barnstable C. J. (1987) SVP38: A synaptic vesicle protein whose appearance correlates closely with synaptogenesis in the rat nervous system *Ann. NY Acad. Sci.* (in press).

Dodd J., Solter D., and Jessell T. M. (1984) Monoclonal antibodies against carbohydrate differentiation antigens identify subsets of primary sensory neurons. *Nature* **311**, 469–472.

Dowling J. E. (1964) Nutritional and inherited blindness in the rat. *Exp. Eye Res.* **3**, 348–356.

Dräger U. C., Edwards D. L., and Barnstable C. J. (1984) Antibodies against filamentous components in discrete cell types of the mouse retina. *J. Neurosci.* **4**, 2025–2042.

Drenckhahn D. and Wagner H. -J. (1985) Relation of retinomotor responses and contractile proteins in vertebrate retinas. *Eur. J. Cell. Biol.* **37**, 156–168.

Edelman G. M., Hoffman S., Chuong C.-M., Thiery J. -P., Brackenbury R., Gallin W. J., Grumet M., Greenberg M. E., Hemperly J. J., Cohen C., and Cunningham B. A. (1983) Structure and modulation of neural cell adhesion molecules in early and late embryogenesis. *Cold Spring Harbor Symp. Quant. Biol.* **48**, 515–526.

Edwards J. G., Campbell J. A., Robson R. T., and Vicker M. G. (1975) Trypsinized BHK 21 cells aggregate in the presence of divalent cations. *J. Cell Sci.* **19**, 653–667.

Fekete D. M. and Barnstable C. J. (1983) The subcellular localisation of rat photoreceptor specific antigens. *J. Neurocytol.* **12**, 785–803.

Fujita S. (1962) Kinetica of cellular proliferation. *Exp. Cell Res.* **28**, 52–60.

Fulcrand J. and Privat A. (1977) Neuroglial reactions secondary to Wallerian degeneration in the optic nerve of the postnatal rat: Ultrastructural and quantitative study. *J. Comp. Neurol.* **176**, 189–224.

Gonzalez-Fernandez F., Landers R. A., Glazebrook P. A., Fong S. -L., Liou G. I., Lam D. M. K., and Bridges C. D. B. (1984a) An extracellular retinol-binding glycoprotein in the eyes of mutant rats with retinal dystrophy: Development, localization, and biosynthesis. *J. Cell Biol.* **99**, 2092–2098.

Gonzalez-Fernandez F., Landers R. A., Fong S.-L., Liou F. I., and Bridges C. D. B. (1984b) Interstitial retinal retinol-binding protein in the interphotoreceptor matrix of normal and dystrophic rats, in *The Inter Photoreceptor in Matrix in Health tonreceptor in Matrix in Health and disease* (Bridges C. D. and Adler A. J., eds.) pp 213–229. *Alan R. Liss, New York, NY.*

Goridis C., Hirn M., Santoni M.-J., Genarini G., Deagostini-Bazin H., Jordan B. R., Kiefer M., and Steinmetz M. (1985) Isolation of mouse N-CAM-related cDNA: Detection and cloning using monoclonal antibodies. *EMBO J.* **4**, 631–635.

Grumet M., Hoffman S. and Edelman G. M. (1984) Two antigenically related neuronal cell adhesion molecules of different specificities mediate neuron–neuron and neuron–glia adhesion. *Proc. Nat. Acad. Sci. USA* **81**, 267–271.

Grunewald G. B., Pratt R. S., and Lilien J. (1982) Enzymatic dissection of embryonic cell adhesive mechanisms. III. Immunological identification of a component of the calcium-dependent adhesive system of embryonic chick neural retina cells. *J. Cell Sci.* **55**, 69–83.

Halfter W., Deiss S., and Schwarz U. (1985) The formation of the axonal pattern in the embryonic avian retina. *J. Comp. Neurol.* **232**, 466–480.

Hatta K. and Takeichi M. (1986) Expression of N-adherin adhesion molecules associated with early morphogenetic events in chicken development. *Nature* **320**, 447–449.

Hatta K., Okada T. S., and Takeichi M. (1985) A monoclonal antibody disrupting calcium-dependent cell–cell adhesion of brain tissues: Possible role of its target antigen in animal pattern formation. *Proc. Nat. Acad. Sci. USA* **82**, 2789–2793.

He H. T., Barbet J., Chaix J. C., and Goridis C. (1986) Phosphatidylinositol is involved in the membrane attachment of N-CAM-120, the smallest component of the cell adhesion molecule. *EMBO J.* **5**, 2489–2494.

Hicks D. and Barnstable C. J. (1986) Lectin and antibody labelling of developing rat photoreceptor cells: An electron microscope immunocytochemical study. *J. Neurocytol.* **15**, 219–230.

Hicks D. and Barnstable C. T. (1987) Different monoclonal antibodies reveal different binding patterns on developing and adult retina. *J. Histochem. Cytochem* (In Press).

Hilfer S. R., Brady R. C., and Wang J- J. W. (1981) Intracellular and extracellular changes during early ocular development in the chick embryo, in *Ocular Size and Shape: Regulation During Development* (Hilfer S. R. and Sheffield J. B., eds.) pp. 47–78, Springer-Verlag, New York, NY.

Hinds J. W. and Hinds P. L. (1978) Early development of amacrine cells in the mouse retina: An electron microscope, serial section analysis. *J. Comp. Neurol.* **179**, 277–300.

Hockfield S., McKay R. D., Hendry S. H. C., and Jones E. G. (1983) A surface antigen that identifies ocular dominance columns in the visual cortex and laminar features of the lateral geniculate nucleus. *Cold Spring Harbor Symp. Quant. Biol.* **48**, 877–890.

Hoffman S. and Edelman G. M. (1983) Kinetics of homophilic binding by E and A forms of the neural cell adhesion molecule. *Proc. Nat. Acad. Sci. USA* **80**, 5762–5766.

Hoffman S., Friedlander D. R., Chuong C.-M., Grumet M., and Edelman G. M. (1986) Differential contributions of Ng-CAM and N-CAM to cell adhesion in different neural regions. *J. Cell Biol.* **103**, 145–158.

Holt C. (1980) Cell Movements in *Xenopus* development. *Nature* **287**, 850–852.

Horvitz H.R., Sternberg P.W., Greenwald I. S., Fixsen W., and Ellis H. M. (1983) Mutations that affect neural cell lineages and cell fates during the development of the nematode *Caenorhabditis elegans. Cold Spring Harbor Symp. Quant. Biol.* **48**, 453–463.

Jessell T. M. and Dodd J. (1985) Structure and expression of differentiation antigens on functional subclasses of primary sensory neurons. *Phil. Trans. R. Soc. (Lond)* **B308**, 271–281.

Johnson J. E., Barde Y.-A., Schwab M., and Thoenen H. (1986) Brain-derived neurotrophic factor supports the survival of cultured rat retinal ganglion cells. *J. Neurosci.* **6**, 3031–3038.

Johnston M. C., Nodoen M. D., Hazelton R. D., Coulombre J. L., and Coulombre A. J. (1979) Origins of avian ocular and periocular tissues. *Exp. Eye Res.* **29**, 27–43.

Juurlink B. H. J. and Federoff S. (1980) Differentiation capabilities of mouse optic stalk in isolation of its immediate *in vivo* environment. *Dev. Biol.* **78**, 215–221.

Kapfhammer J. P., Grunewald B. E., and Raper J. A. (1986) The selective inhibition of growth cone

extension by specific neurites in culture. *J. Neurosci.* **6**, 2527–2534.

Keane R. W., Lipsich L. A., and Brugge J. S. (1984) Differentiation and transformation of neural plate cells. *Dev. Biol.* **103**, 38–52.

Krotoski D. M., Domingo C., and Bronner-Fraser M. (1986) Distribution of a putative cell surface receptor for fibronectin and laminin in the avian embryo. *J. Cell Biol.* **103**, 1061–1071.

Levitt P. (1984) A monoclonal antibody to limbic system neurons. *Science* **223**, 299–301.

Lindsay R. M., Thoenen H., and Barde Y. A. (1985) Placode and neural crest derived sensory neurons are responsive at early developmental stages to brain-derived neurotrophic factor. *Dev. Biol.* **112**, 319–328.

Magnani J. L., Thomas W. A., and Steinberg M. S. (1981) Two distinct adhesion mechanisms in embryonic neural retina cells. I. A kinetic analysis. *Dev. Biol.* **81**, 96–105.

Marchase R. B. (1977) Biochemical investigations of retinotectal adhesive specificity. *J. Cell Biol.* **75**, 237–257.

Marchase R. B., Harges P., and Jakoi E. R. (1981) Ligatin from embryonic chick neural retina inhibits retinal cell adhesion. *Dev. Biol* **86**, 250–255.

Marchase R. B., Koro L. A., Kelly C. M., and McClay D. R. (1982) Retinal ligatin recognizes glycoproteins bearing oligosaccharides terminating in phosphodiester linked glucose. *Cell* **28**, 813–820.

Molday R. S. and Mac Kenzie D. (1983) Monoclonal antibodies to rhodopsin: Characterization, cross-reactivity and application as structural probes. *Biochemistry* **22**, 653–660.

Morrison R. S., Sharma A., De Vellis J., and Bradshaw R. A. (1986) Basic fibroblast growth factor supports the survival of cerebral cortical neurons in primary culture. *Proc. Nat. Acad. Sci. USA* **83**, 7537–7541.

Morse D. E. and McCann P. S. (1984) Neuroectoderm of the early embryonic rat eye. Scanning electron microscopy. *Invest. Ophthalmol. Vis. Sci.* **25**, 899–907.

Moscona A. A. and Housman R. E. (1977) Biological and biochemical studies on embryonic cell–cell recognition, in *Cell and Tissues Interactions*. (Lash J. W. and Burger M. M., eds.) pp. 173–185. Raven, New York, NY.

Murray B. A., Hemperly J. J., Gallin W. J., MacGregor J. S., Edelman G. M., and Cunningham B. A. (1984) Isolation of cDNA clones for the chick neural cell adhesion molecule (N-CAM). *Proc. Nat. Acad. Sci: USA* **81**, 5584–5588.

Murray B. A., Hemperly J. J., Prediger E. A., Edelman G. M., and Cunningham B. A. (1986) Alternatively spliced mRNAs code for different polypeptide chains of the chicken neural cell adhesion molecule (N-CAM). *J. Cell Biol.* **102**, 189–193.

Ophir I., Moscona A. A., and Ben-Shaul Y. (1983) Localization of retina cognin in embryonic neural retina tissue by immuno-scanning electron microscopy. *Cell Differ.* **13**, 133–141.

Papermaster D. S., Schneider B. G., and Besharse J. (1985) Vesicular transport of newly synthesized opsin from the golgi apparatus toward the rod outer segment. *Invest. Ophthalmol. Vis. Sci.* **26**, 1386–1404.

Pollerberg G. E., Schachner M., and Davoust J. (1986) Differentiation state-dependent surface mobilities of two forms of the neural cell adhesion molecule. *Nature* **324**, 462–465.

Raff M. C., Miller R. H., and Noble M. (1983) A glial progenitor cell that develops *in vitro* into an astrocyte or an oligodendrocyte depending on culture medium. *Nature* **303**, 390–396.

Raff M. C., Williams B., and Miller R. H. (1984) The *in vitro* differentiation of a bipotential glial progenitor cell. *EMBO J.* **3**, 1857–1864.

Rakic P. (1981) Developmental events leading to laminar and areal organization of the neocortex, in *The Organization of the Cerebral Cortex* (Schmitt F. O., Worden F. G., Adelman G., and Dennis S. G., eds.) pp. 7–28. MIT, Cambridge, MA.

Raper J. A. and Kapfhammer J. P. (1986) The tissue specificity of contact mediated avoidance between growth cones and axons. *Soc. Neurosci. Abstr.* **12**, 1335.

Regan L. J., Dodd J., Barondes S. H., and Jessell T. M. (1986) Selective expression of endogenous lactose-binding lectins and lactoseries glycoconjugates in subsets of rat sensory neurons. *Proc. Nat. Acad. Sci. USA* **83**, 2248–2252.

Rutishauser U. (1983) Molecular and Biological properties of a neural cell adhesion molecule. *Cold Spring Harbor Symp. Quant. Biol.* **48**, 501–514.

Salton S. R. J., Richter-Landsberg C., Greene L. A., and Shelanski M. L. (1983) Nerve growth factor inducible large external (NILE) glycoprotein:

Studies of a central and peripheral neuronal marker. *J. Neurosci.* **3**, 441–454.

Schachner M., Faissner A., Kruse J., Lindner J., Meier D. H., Rathjen F. G., and Wernecke H. (1983) Cell-type specificity and developmental expression of neural cell-surface components involved in cell interactions and of structurally related molecules. *Cold Spring Harbor Symp. Quant. Biol.* **48**, 557–568.

Schauer R. (1971) Hydroxylation and O-acetylation of *N*-acetylneuraminic acid bound to glycoproteins of isolated subcellular membranes from porcine and bovine submaxillary glands. *Hoppe-Seyler's Z. Physiol. Chem.* **352**, 1282–1290.

Schlosshauer B., Schwartz U., and Rutishauser U. (1984) Topological distribution of different forms of neural cell adhesion molecule in the developing chick visual system. *Nature* **310**, 141–143.

Sidman R. L. (1961) Histogenesis of mouse retina studied with thymidine-$H^3$, in *Structure of the Eye* (Smelser G. K., ed.) pp. 487–506. Academic, London, UK.

Skoff R. P., Price D. L., and Stocks A. (1976) Electron microscopic autoradiographic studies of gliogenesis in rat optic nerve. I. Cell proliferation. *J. Comp. Neurol.* **169**, 291–312.

Sorge L. K., Levy B. T., and Maness P. F. (1984) $pp60^{c\text{-}src}$ is developmentally regulated in the neural retina. *Cell* **36**, 249–257.

Sparrow J. R. and Barnstable C. J. (1986) Expression of cell and synapse specific antigens in rat retinal explant culture. *Soc. Neurosci. Abstr.* **12**, 643.

Steinberg M. S. (1964) The problem of adhesive selectivity in cellular interactions, in *Cellular Membranes in Development.* (Locke M. ed.) pp. 321–366. Academic, New York, NY.

Steinberg M. S. and Granger R. E. (1966) The re-acquisition of adhesiveness by trypsinized chick embryonic cells *in vitro. Am. Zool.* **6**, 337a.

Steinberg R. H., Fisher S. K., and Anderson D. H. (1980) Disc morphogenesis in vertebrate photoreceptors. *J. Comp. Neurol.* **190**, 501–518.

Stroeva O. G. (1960) Experimental analysis of the eye morphogenesis in mammals. *J. Embryol. Exp. Morphol.* **8**, 349–368.

Stryer L. (1985) Molecular design of an amplification cascade in vision. *Biopolymers* **24**, 29–47.

Sulston J. E. (1983) Neuronal cell lineages in the nematode *Caenorhabditis elegans. Cold Spring Harbor symp. Quant. Biol.* **48**, 443–452.

Takeichi M. (1977) Functional correlation between cell adhesive properties and some cell surface proteins. *J. Cell Biol.* **75**, 464–474.

Takeichi M., Ozaki H. S., Tokumaga K., and Okada T. S. (1979) Experimantal manipulation of cell surface to affect cellular recognition mechanisms. *Dev. Biol.* **70**, 195–205.

Tauchi M. and Masland R. H. (1984) The shape and arrangement of the cholinergic neurons in the rabbit retina. *Proc. R. Soc. Lond. B* **223**, 101–119.

Temple S. and Raff M. C. (1985) Differentiation of a bipotential glial progenitor cell in single cell microculture. *Nature* **313**, 223–225.

Thomas J. B., Bastiani M. J., Bate C. M., and Goodman C. S. (1984) From grasshopper to *Drosophila*: A common plan for neruonal development. *Nature* **310**, 203–207.

Thomson L. K., Horowitz P. M., Bentley K. L., Thomas D. D., Alderete J. F., and Klebe R. J. (1986) Localization of the ganglioside-binding site of fibronectin. *J. Biol. Chem.* **261**, 5209–5214.

Treisman J. E., Morabito M., and Barnstable C. J. (1987) Developmental expression of opsin, a photoreceptor-specific gene. *Proc. Miami Winter Symp.* (in press).

Trisler D., Bekenstein J., and Daniels M. P. (1986) Antibody to a molecular marker of cell position inhibits synapse formation in retina. *Proc. Nat. Acad. Sci. USA* **83**, 4194–4198.

Trisler G. D., Schneider M. D., and Nirenberg M. (1981a) A topographic gradient of molecules in retina can be used to identify neuron position. *Proc. Nat. Acad. Sci. USA* **78**, 2145–2149.

Trisler G. D., Schneider M. D., Moskal J. R., and Nirenberg M. (1981b) A gradient of molecules in avian retina with dorsoventral polarity, in *Monoclonal Antibodies to Neural Antigens* (McKay R., Raff M. C., and Reichardt L. F., eds.) pp. 231–245. *Cold-Spring Harbor*, New York.

Troccoli N. M. and Housman R. E. (1985) Vesicle interactions as a model for the retinal cell–cell recognition mediated by R-cognin. *Cell Differ.* **16**, 43–49.

Van Veen T., Katial A., Shinohara T., Barrett D. J., Wiggert B., Chader G. J., and Nickerson J. M. (1986) Retinal photoreceptor neurons and pinealocyutes accumulate mRNA for interphotoreceptor retinoid binding protein (IRBP). *FEBS Lett.* **208**, 133–137.

Vardimon L., Fox L. E., and Moscona A. A. (1986) Accumulation of c-src mRNA is developmentally regulated in embryonic neural retina. *Mol. Cell. Biol.* **6**, 4109–4111.

Vaughan D. K. and Fisher S. K. (1985) Distribution of F-actin in retinal cells. *Invest. Ophthalmol. Vis. Sci.* **26 (suppl.)**, 248.

Vollmer G. and Layer P. G. (1986) An *in vitro* model of proliferation and differentiation of the chick retina: Coaggregtes of retinal and pigment epithelial cells. J. Neurosci. 6, 1885–1896.

Wagner J. A. and D'Amore P. A. (1986) Neurite outgrowth induced by an endothelial cell mitogen isolated from retina. *J. Cell biol.* **103**, 1363–1367.

Walicke P., Cowan W. M., Ueno N., Baird A., and Guillemin R. (1986) Fibroblast growth factor promotes survival of dissociated hippocampal neurons and enhances neurite extension. *Proc. Nat. acad. Sci. USA* **83**, 3012–3016.

Wang J. W. and Hilfer S. R. (1982) The effect of inhibitors of glycoconjugate synthesis on optic cup formation in the chick embryo. Dev. Biol. 92, 41–53.

Wässle H. and Riemann H. J. (1978) The mosaic of nerve cells in the mammalian retina. *Proc. R. Soc. Lond. B* **200**, 441–461.

Weidman T. A. and Kuwabara T. (1968) Postnatal development of the rat retina. An electron microscopic study. *Arch. Ophthalmol.* **79**, 470–484.

Yoshida M., Kawai S., and Toyoshima K. (1980) Uninfected avian cells contain structurally unrelated progenitors of viral sarcoma genes. *Nature* **287**, 653–654.

Young R. W. (1985) Cell differentiation in the retina of the mouse. *Anat. Rec.* **21**, 199–205.