

## Temporally Correlated Expression of nAChR Genes During Development of the Mammalian Retina

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In situ hybridizations and RNase protection assays have been used to characterize nicotinic acetylcholine receptor (nAChR) gene expression in the developing and adult rat retina. At the earliest time examined (embryonic day 13) a low level of  $\alpha$ -3 and  $\beta$ -4 gene expression could be detected. During the next 48 hr there was a dramatic induction of the  $\alpha$ -3,  $\alpha$ -4,  $\beta$ -2,  $\beta$ -3 and  $\beta$ -4 genes in the recently differentiated retinal ganglion cells. By post-natal day 4 we detected nAChR gene expression in the inner nuclear layer. In the adult retina, in situ hybridizations showed these genes are expressed by cells residing in the ganglion and inner nuclear layers. These results suggest a common regulatory mechanism for the induction of nAChR expression in retinal ganglion cells during development. In addition, the variety of nAChR genes expressed in the retina imply a relatively large number of different types of nAChRs can be expressed by these cells.

*Key words:* retina; development; nAChR; gene expression; ganglion cell.

### 1. Introduction

A central issue in neurobiology is determining the molecular mechanisms by which specific cell types arise in the developing nervous system. One approach to this question is to identify genes whose expression is unique to a particular cell type. Characterizing the mechanisms by which these genes are developmentally regulated should shed light on the mechanisms involved in cell differentiation. We have decided to take this approach using the retina as a model system. The retina provides an excellent system for studying central nervous systems (CNS) development and cell determination. The reasons for this include: the well defined stages of retinal cell differentiation; the continued post-natal mitosis of retinal precursors; the laminar organization of the various classes of retinal neurons; and its accessibility to experimental manipulations. In addition, most of the retinal neurotransmitters have been identified and the six major classes of retinal neurons have been well described (reviewed in Massey and Redburn, 1987). Each class of neuron has several subtypes, which brings the total number of different retinal neurons to around 60.

The retina develops from the anterior aspect of the neural tube. Neuroepithelial precursors proliferate to eventually give rise to the entire retina (reviewed by Barnstable, 1987). After undergoing terminal mitosis at the ventricular zone, these cells (retinal neuroblasts) migrate to their final position. Developmental studies indicate that these neuroblasts are multipotential

(Turner and Cepko, 1987; Turner, Snyder and Cepko, 1990; Wetts and Fraser, 1988; Holt et al., 1988) and become committed to their phenotype about the time they withdraw from the cell cycle (Adler and Hatlee, 1989). The molecular mechanisms by which uncommitted cells differentiate into various retinal neurons are not known, although both genetic and epigenetic events are likely involved. It is clear, however, that cell-type specific genes must be induced in these cells as they differentiate. Understanding the mechanisms responsible for regulating the expression of these genes during development should further our understanding of the differentiation process.

One set of such genes are those encoding nAChRs. These receptors are encoded by a family of related genes whose membership includes a variety of ligand-binding  $\alpha$ -subunits and structural  $\beta$ -subunits (reviewed by Leutje, Patrick and Seguela, 1990). Expression studies indicate that a functional neural nAChR can often be formed by co-expressing a single  $\alpha$ -subunit with a particular  $\beta$ -subunit.

Electrophysiological assays indicate that rat retinal ganglion cells express nAChRs (Lipton, Aizenman and Loring, 1987). In the neuropil of the innerplexiform layer some of these receptors may mediate communication between cholinergic amacrine cells (Masland, Mills and Hayden, 1984) and directionally sensitive ganglion cells (Ariel and Daw, 1982a, b). In addition, immunohistochemical staining and in situ hybridizations have shown that nAChRs and the genes encoding them are expressed in the adult vertebrate retina (Swanson et al., 1987; Cauley, Agranoff and Goldman, 1989, 1990; Wada et al., 1989; Matter, Matter-Sadzinski and Ballivet, 1990). However, neither the number of different types of

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nAChRs, nor the variety of cells that express these molecules in the retina is known. In addition, little is known about when these genes begin to be expressed during retinal development. Here we characterize the expression of the  $\alpha$ -2,  $\alpha$ -3,  $\alpha$ -4,  $\beta$ -2,  $\beta$ -3 and  $\beta$ -4 nAChR subunit-encoding genes in the developing and adult rat retina and show their expression to be temporally correlated during retinal ganglion cell development.

## 2. Materials and Methods

### Probes

nAChR cDNA clones (Fig. 1) were kindly provided by Drs Stephen Heinemann, Jim Patrick and associates (The Salk Institute, La Jolla, CA). A 1.1-kb *Bam*H I/*Acc* I fragment of the  $\alpha$ -2 cDNA (Wada et al., 1988) was subcloned into the Bluescript SK(+) vector (Stratagene, La Jolla, CA) and linearized with *Bam*H I. The  $\alpha$ -3 cDNA (Boulter et al., 1986) was subcloned into the pSP73 vector (Promega, Madison, WI) and linearized with either *Acc* I or *Xho* I. The  $\alpha$ -4 cDNA (Boulter et al., 1987; Goldman et al., 1987) was subcloned into the pSP73 vector and linearized with *Cla* I or *Eco*R I. A 1.4-kb *Eco*R I/*Pvu* II fragment of the  $\beta$ -2 cDNA (Deneris et al., 1988) was subcloned into pSP73 and linearized with *Bgl* II. The 3' 1.85-kb *Eco*R I fragment of the  $\beta$ -3 cDNA clone (Deneris et al., 1989) was subcloned into the Bluescript SK(-) vector and was linearized with *Cla* I. The  $\beta$ -4 cDNA (Duvoisin et al., 1989) was linearized with *Eco*R I. A pSP65 plasmid harboring the human U1 gene, encoding a small nuclear RNA, was kindly provided by Dr J. Gregory Howe (Howe and Steitz, 1986). This plasmid was linearized with *Hind* III. Antisense [<sup>35</sup>S]RNA probes were generated from these linearized constructs by run-off transcription using either SP6 ( $\alpha$ -3, U1), T3 ( $\beta$ -3) or T7 ( $\alpha$ -2,  $\alpha$ -4,  $\beta$ -2,  $\beta$ -4) promoters. Probes were hydrolyzed with 100 mM Na<sub>2</sub>CO<sub>3</sub>/NaHCO<sub>3</sub> (pH 10.2) to an average size of 200 nucleotides prior to in situ hybridization (Cox et al., 1984).

### In Situ Hybridization

Adult male, post-natal and fetal Sprague-Dawley rats were used for this study. Animals were anesthetized with ether before death, and the retinas were removed and placed into a 4% paraformaldehyde/PBS [10 mM NaPO<sub>4</sub> (pH 7.2), 145 mM NaCl] solution at 4°C. After fixing the tissue for 2 hr, the retinas were removed and cryoprotected in a 30% sucrose/PBS solution at 4°C for approximately 2–3 hr. The retinas were then mounted into a mold filled with OCT (Tissue Tek, Elkhardt, IN), and frozen with dry ice. Frozen sections were cut 15  $\mu$ m thick on a cryostat and collected on poly-L-lysine subbed slides. The sections were then dried in a 80°C vacuum oven for 20 min

and stored at -20°C. Prior to hybridization the sections were hydrated through graded alcohols, rinsed in 2X SSC (0.3 M NaCl, 0.03 M Na-citrate, pH 7.4), and digested with Proteinase K (10  $\mu$ g ml<sup>-1</sup>) in 0.1 M Tris (pH 7.2) and 0.05 M EDTA for 15 min at 37°C. The sections were then acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0) for 10 min at room temperature, before dehydrating through graded alcohols. Approximately 25  $\mu$ l of hybridization solution [10 mM Tris (pH 7.4), 0.3 M NaCl, 1 mM EDTA, 10% Dextran sulfate, 0.1 mM DTT] containing 50 000 cpm  $\mu$ l<sup>-1</sup> of radio-labeled [<sup>35</sup>S]cRNA probe was placed onto each section. The sections were coverslipped, and the edges of the coverslips sealed with glue. The slides were then placed into a humid chamber and incubated for 12–18 hr at 65°C. Following hybridization, the glue was removed, the slides were placed in a rack and the coverslips were floated off by rinsing three times for 10 min in 2XSSC. Slides were then immersed in 50% formamide, 2XSSC (pH 7.4), and 10 mM DTT at 55°C for 30 min. Single-stranded RNA still binding to the section was removed by digestion with RNase A (50  $\mu$ g ml<sup>-1</sup>) in 10 mM Tris (pH 7.5), 0.5 M NaCl, and 1 mM EDTA. Sections were then rinsed in 10 mM Tris (pH 7.5), 0.5 M NaCl, and 1 mM EDTA, for 30 min at 65°C and dehydrated through graded alcohols containing 0.4 M ammonium acetate. After air drying, the sections were dipped in photographic emulsion [NTB-2 (Kodak), 1:1 dilution with water], and dried in a light-tight container. The sections were exposed for 4 days before developing. The sections were then stained with Hematoxylin and Eosin B, dehydrated and differentiated through graded alcohols and xylenes. The sections were then coverslipped and viewed with a Zeiss Axiophot microscope using bright and dark field optics. Retinal cells were considered to express nAChR mRNA above background if they possessed greater than five silver grains clustered over their nuclei. The percentage of cells expressing nAChR genes in the ganglion cell layer were quantitated by counting the number of positively hybridizing cells and dividing by the total number of cells in this layer. Reported values are averages from four to five different retinal sections.

### RNase Protection Assays

Total RNA was isolated from retinas as previously described (Chirgwin et al., 1979; Goldman et al., 1985). Radiolabeled antisense RNA probes were prepared by run-off transcription of linearized vectors harboring the nAChR  $\alpha$ -2 and  $\beta$ -4 cDNAs. The  $\alpha$ -2 cDNA was restricted with *Alu* I, which resulted in a 350-nucleotide probe (excluding vector sequences). These nucleotides encode amino acids spanning the cytoplasmic domain. The  $\beta$ -4 cDNA was linearized with *Xba* I. This yielded a 409-nucleotide probe (excluding vector sequences) containing 3' untrans-

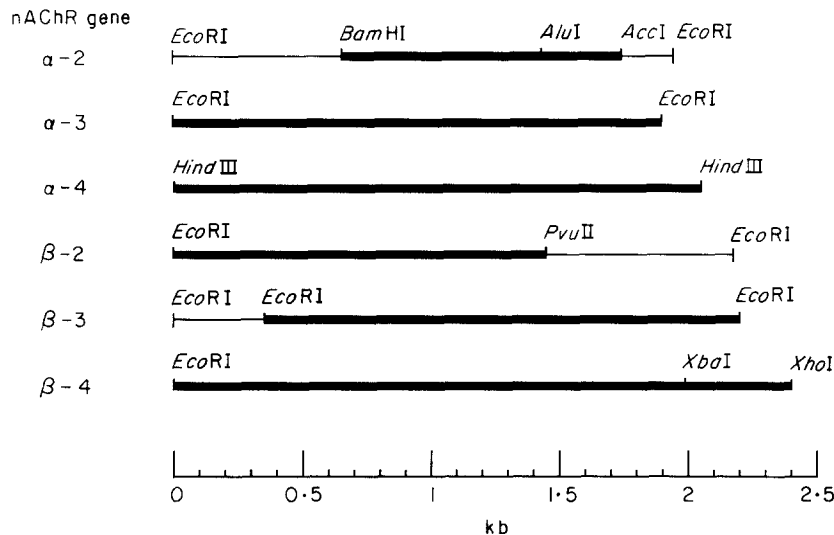


FIG. 1. Line diagram of nAChR cDNAs and probes used for in situ hybridizations. The thin horizontal line represents the nAChR cDNA portion of the recombinant plasmids described in Materials and Methods. The closed boxes represent portions of the cDNA used to generate probes for in situ hybridizations.

lated sequence. The hybridization reaction contained 25–78  $\mu$ g total retinal RNA, approximately 500 000 cpm of radiolabeled [ $^{32}$ P]cRNA probe, 50% formamide, 40 mM PIPES (pH 6.7), 750 mM NaCl, and 1 mM EDTA. After annealing for 20 hr at 55°C, 300  $\mu$ l of RNase digestion buffer [10 mM Tris (pH 7.5), 5 mM EDTA, 300 mM NaCl, RNase A (40  $\mu$ g ml $^{-1}$ ), RNase T1 (1000 U ml $^{-1}$ )] was added to the hybridizations. The samples were incubated at room temperature for 1 hr. RNase digestion was terminated by the addition of 20  $\mu$ l of 10% SDS and 50  $\mu$ g of proteinase K for 45 min at 37°C. The samples were then extracted with phenol:chloroform and the RNA precipitated with ethanol. RNase resistant hybrids were fractionated on a denaturing 8% acrylamide, 8 M urea gel. Gels were dried and exposed to X-ray film at room temperature for 8 days.

#### Retrograde Labeling of Retinal Ganglion Cells

Retinal ganglion cells were identified by retrograde transport of Fluoro-Gold (Fluorochrome Inc., Englewood, CO) or DAPI (4,6-diamidino-2-phenylindole, Sigma) injected into the brain (Perry, 1981). For identifying retinal ganglion cells in the 15-day-old embryo, retrograde labeling was performed in utero. Time-mated Sprague–Dawley rats were used for these experiments. Embryonic day 1 (E1) is approximately 24 hr after the vaginal plug was released. At E14, pregnant females were anesthetized with ether and the uterus surgically exposed. One microliter of a 1% Fluoro-Gold or 10 mg ml $^{-1}$  DAPI solution in H $_2$ O was slowly injected through the uterus into the embryonic brain. Following the injection, incisions were sutured and the animals were allowed to survive an additional 24 hr before being killed. Embryos were then isolated from the uterus, heads were removed, placed into fixative and processed as described above for in situ hybridization. Cells retrogradely labeled with tracers

were visualized using a Zeiss Axiophot microscope equipped with fluorescent optics. Ganglion cells were identified as fluorescent cells residing in the appropriate anatomical laminae. Photomicrographs were taken and the coordinates of the slide recorded.

### 3. Results

#### Nicotinic AChR Gene Expression in the Adult Retina

In situ hybridization experiments and RNase protection assays were used to determine which members of the nAChR gene family were expressed in the adult retina, and the variety of retinal cells expressing these genes. Probes used to identify nAChR RNAs are shown in Fig. 1. In situ hybridizations showed that the  $\alpha$ -3,  $\alpha$ -4,  $\beta$ -2,  $\beta$ -3 and  $\beta$ -4 nAChR genes are expressed in the ganglion and inner nuclear cell layers of the adult rat retina (Fig. 2). The level of  $\beta$ -4 expression was consistently lower than the other nAChR genes studied. We were unable to obtain a reliable in situ hybridization signal with the  $\alpha$ -2 probe. Therefore, we used an RNase protection protocol to assay for the expression of the  $\alpha$ -2 gene (Fig. 3). The experiment showed that RNA isolated from adult retinas completely protected the  $\alpha$ -2 probe (excluding vector sequences), confirming its expression in the adult rat retina.

The rat's retinal ganglion cell layer is composed of both displaced amacrine and ganglion cells in about equal proportions (Perry, 1981). In this layer the  $\alpha$ -3 and  $\beta$ -3 genes were expressed by about 50% of the cells. In contrast, the other nAChR genes studied were expressed in over 70% of these cells and were therefore likely expressed by both ganglion and displaced amacrine cells.

The inner nuclear layer (INL) of the rat contains horizontal, bipolar, interplexiform, amacrine and displaced ganglion cells (Massey and Redburn, 1987;

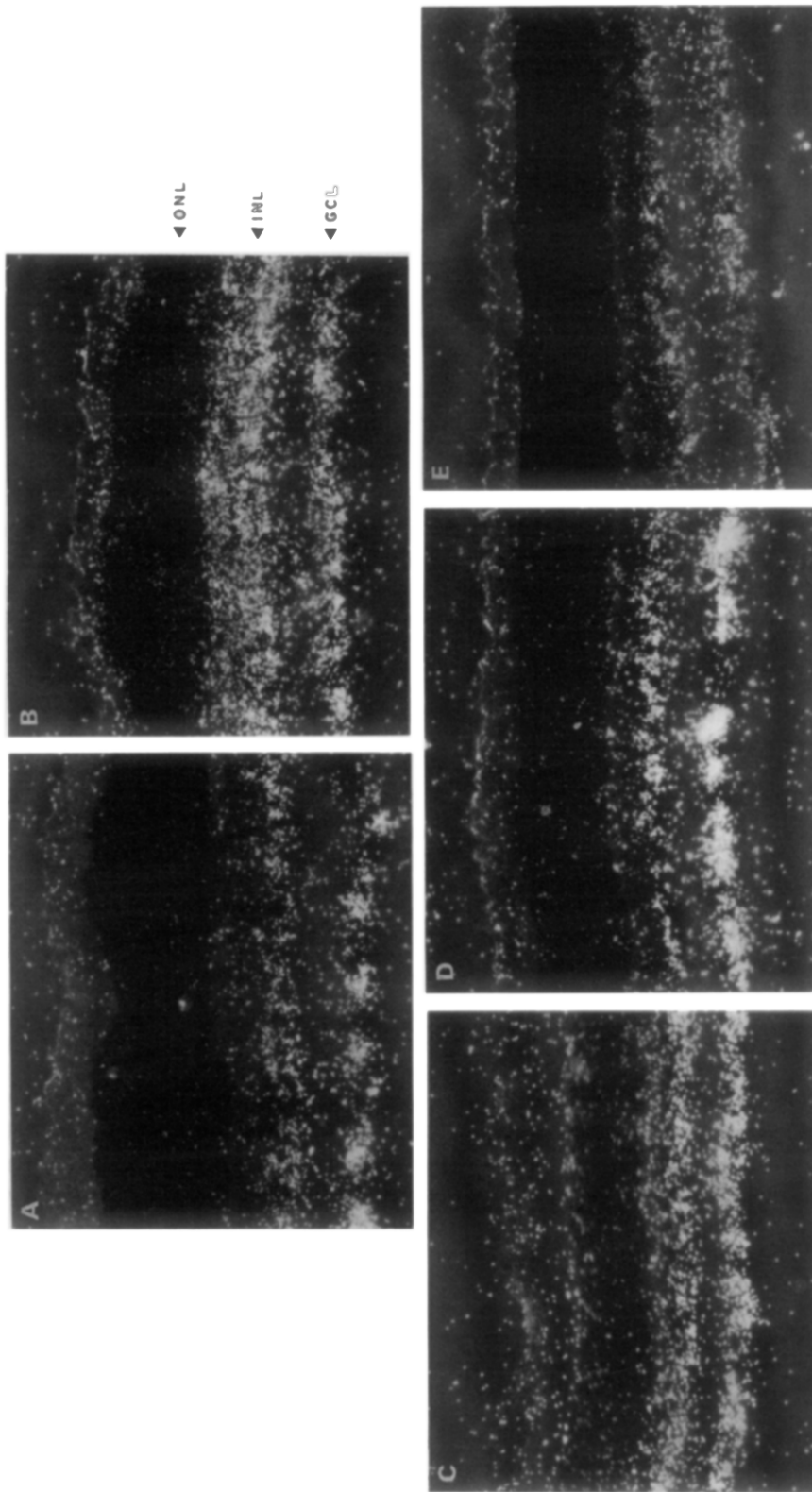


FIG. 2. Multiple nAChR genes are expressed in the adult rat retina. In situ hybridizations of the  $\alpha$ -3 (A),  $\alpha$ -4 (B),  $\beta$ -2 (C),  $\beta$ -3 (D), and  $\beta$ -4 (E) nAChR antisense probes to adult retinal sections. White grains represent regions of the retina expressing the nAChR genes. Photomicrographs were taken using dark field optics and the  $\times 20$  objective. ONL, Outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer.

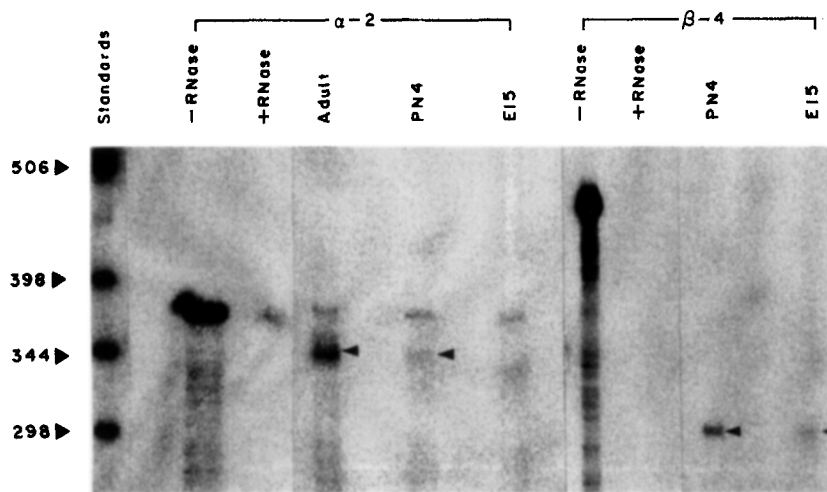


FIG. 3. Expression of the  $\alpha$ -2 and  $\beta$ -4 genes in the adult and developing retina. A 385 nucleotide radiolabeled  $\alpha$ -2 antisense RNA probe (-RNase) was hybridized alone (+RNase) or used to hybridize with total RNA isolated from either, adult retinas (40  $\mu$ g), post-natal day 4 retinas (78  $\mu$ g) or E 15 retinas (50  $\mu$ g). This probe contained 35 nucleotides of vector sequence. A 438-nucleotide radiolabeled  $\beta$ -4 antisense RNA probe (-RNase) was hybridized alone (+RNase) or with total RNA isolated from post-natal day 4 retinas (78  $\mu$ g) and embryonic day 15 retinas (25  $\mu$ g). This probe contained 29 nucleotides of vector sequence. Following hybridization, the samples were digested with RNase (except those labelled -RNase) and those duplexes remaining were fractionated in a denaturing acrylamide gel. The dried gel was exposed to X-ray film without an intensifying screen for 8 days. The  $\alpha$ -2 probe is completely protected (excluding vector sequences), while the  $\beta$ -4 probe is only partially protected from RNase digestion by hybridization with retinal RNA. The partially protected  $\beta$ -4 probe is consistent with the  $\beta$ -4 transcript using a poly (A) addition signal sequence about 100 nucleotides upstream of the reported poly (A) addition signal sequence (see Results). Arrowheads point to the protected regions of the probes. The left lane is a radiolabeled standard DNA ladder. The ladder was generated by restricting  $\lambda$  DNA with *Eco*R I and *Hind* III, and subsequently end-labeling with [ $^{32}$ P]dATP.

Liu and Jen, 1986). In this layer, *in situ* hybridizations consistently showed higher levels of  $\alpha$ -4 and  $\beta$ -2 RNA compared to  $\alpha$ -3,  $\beta$ -3 and  $\beta$ -4 RNA (Fig. 2). Except for  $\alpha$ -4 and perhaps  $\beta$ -2, the expression of these genes appears to be concentrated at the inner edge of the INL where amacrine cells reside (Masland et al., 1984; Vaney, 1985). Alpha-4 and  $\beta$ -2 expression occurs throughout the INL suggesting that, in addition to amacrine cells, other INL neurons express these genes (Fig. 2).

#### Expression of nAChR Genes in the Developing Retina

In order to gain insight into possible mechanisms by which retinal nAChR genes were induced, we assayed for nAChR gene expression in the developing retina. These experiments showed that nAChR genes were expressed in the embryonic retina at very early stages of development (Figs 4 and 5). At the earliest time examined (E13), we detected a low level of  $\alpha$ -3 and  $\beta$ -4 gene expression throughout the neuroblast layer (data shown for  $\alpha$ -3, Fig. 4). During the next 2 days of development (E14 and 15) we found a large induction in the level of expression of the  $\alpha$ -3,  $\alpha$ -4,  $\beta$ -2,  $\beta$ -3 and  $\beta$ -4 genes adjacent to the inner limiting membrane (data shown for  $\alpha$ -3 and  $\beta$ -2 in Figs 4 and 5). There was a central-to-peripheral gradient of expression, which correlates with the maturation gradient of retinal ganglion cells (Sidman, 1960; Morest, 1970).

In order to determine if these positive hybridizing ganglion cells had already sent their axons to the brain, we attempted to retrogradely label them by

injecting DAPI into the embryonic brain. For this experiment DAPI was injected into the E14 brain. Twenty-four hours later retinas were isolated, sectioned and examined microscopically with fluorescent optics. This experiment showed that those ganglion cells residing at the inner limiting membrane of E15-16 day retinas have already sent their axons to the brain (Fig. 6).

By post-natal day 4 (PN4), the nAChR genes assumed a pattern of expression more like that seen in the adult retina. At this stage of development an inner plexiform layer has formed, separating the ganglion cell and inner nuclear layers. Nicotinic AChR gene expression in the inner nuclear layer was now obvious (Figs 4 and 5).

The presence of  $\alpha$ -2 and  $\beta$ -4 RNA in the developing retina was verified with RNase protection assays (Fig. 3). This experiment showed that RNA isolated from PN4 retinas completely protected the  $\alpha$ -2 probe (excluding vector sequences), while this RNA only partially protected the  $\beta$ -4 probe (i.e. approximately 300 nucleotides were resistant to RNase digestion out of 409 nucleotides contained in the probe, excluding vector sequences), confirming that these genes are expressed in the retina. Since the  $\beta$ -4 probe was generated from 3' untranslated sequence, we thought it was likely that the partially protected probe resulted from the retinal  $\beta$ -4 transcript using a poly (A) addition signal sequence about 100 nucleotides upstream from the reported poly (A) addition signal sequence (Duvoisin et al., 1989). Inspection of the  $\beta$ -4 3' untranslated sequence shows that another

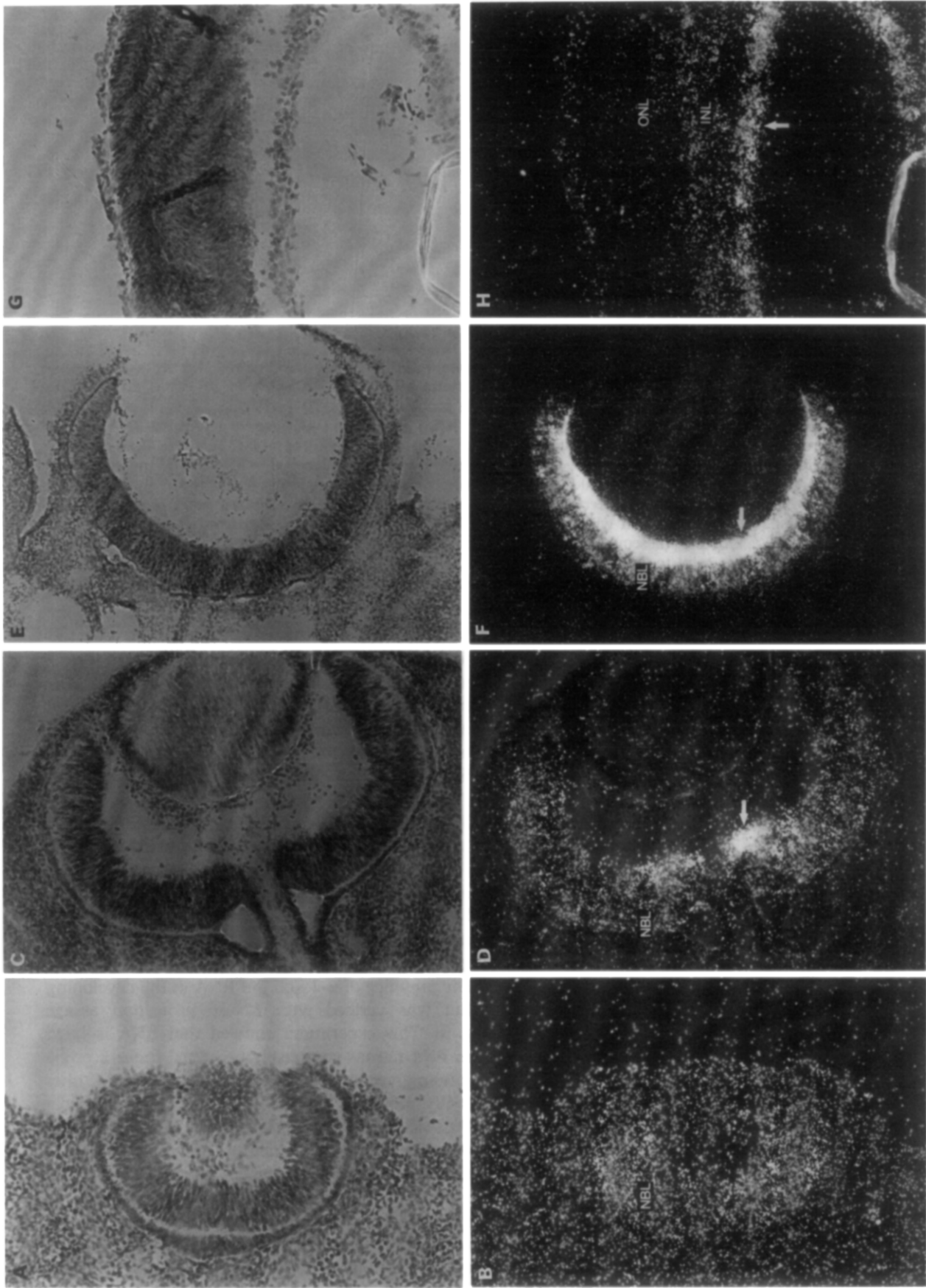


FIG. 4. Alpha 3 gene expression in the developing retina. In situ hybridization of a radiolabeled antisense  $\alpha$ -3 RNA probe hybridized to retinal sections taken from rats at various developmental stages: embryonic day 13 (E13) (A, B); post-natal day 4 (PN4) (G, H). Top row (A, C, E and G) shows retinal sections using bright field optics, while the lower row (B, D, F and H) is the same section photographed with dark field optics. The white grains correspond to regions of retina expressing the  $\alpha$ -3 gene. Although not shown, an identical pattern of hybridization was seen with the  $\beta$ -4 gene probe. Photomicrographs were taken with either a  $\times 10$  (E13, E14, E15) or a  $\times 20$  objective (PN4). NBL., Neuroblast layer; INL., inner nuclear layer; ONL., outer nuclear layer; arrows point to ganglion cell layer hybridization.

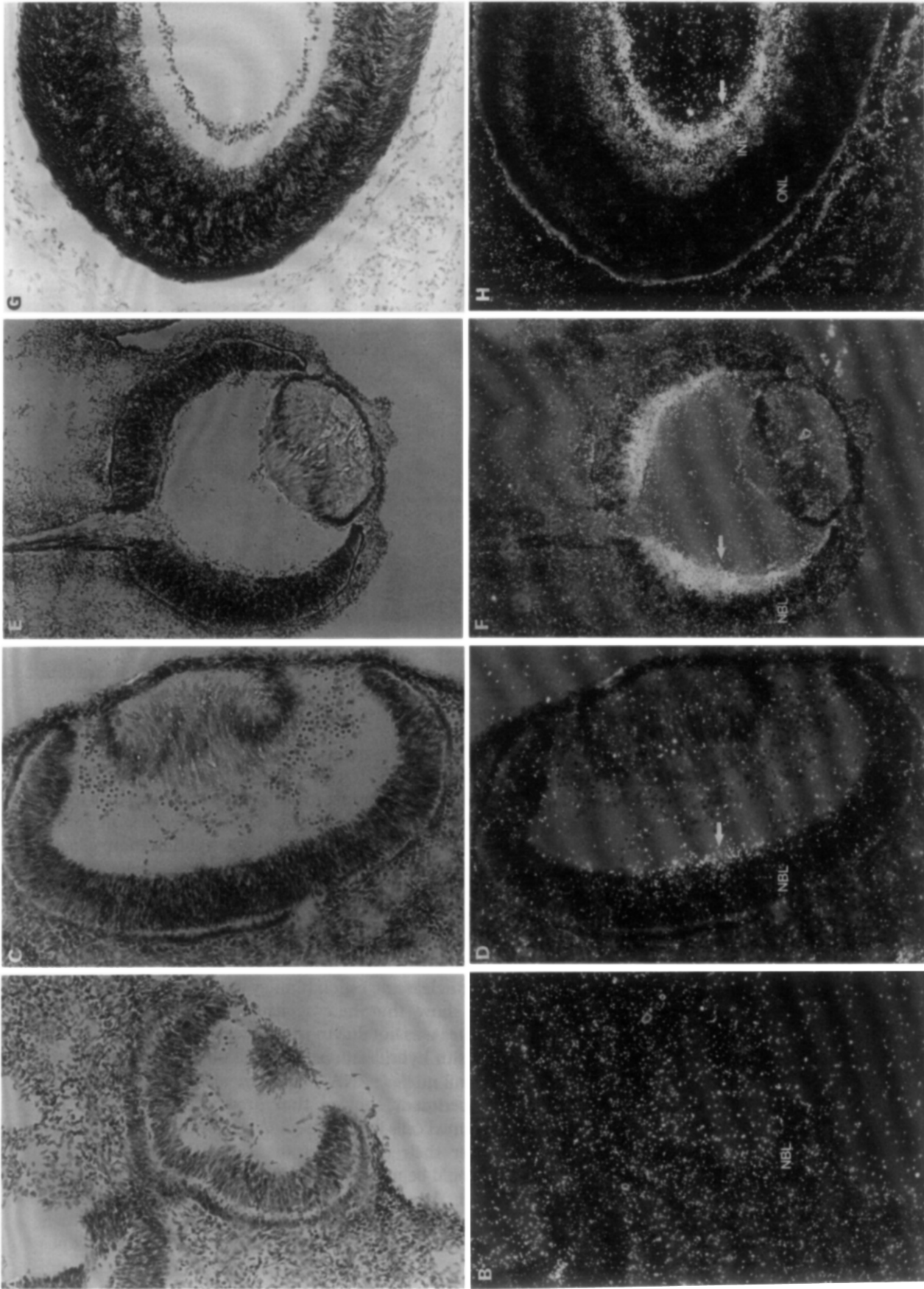


FIG. 5. Beta 2 gene expression in the developing retina. In situ hybridization of a radiolabeled antisense  $\beta$ -2 RNA probe hybridized to retinal sections taken from rats at various developmental stages: embryonic day 13 (E13) (A, B); E14 (C, D); E15 (E, F) post-natal day 4 (PN4) (G, H). Top row (A, C, E and G) shows retinal sections using bright field optics, while the lower row (B, D, F and H) is the same section photographed with dark field optics. The white grains correspond to regions of retina expressing the  $\beta$ -2 gene. Although not shown, identical patterns of hybridization were witnessed with the  $\alpha$ -4, and  $\beta$ -3 gene probes. Photomicrographs were taken with either a  $\times 10$  (E13, E14, E15) or a  $\times 20$  objective (PN4). Abbreviations as in Fig. 4. The arrows point to ganglion cell layer hybridization.

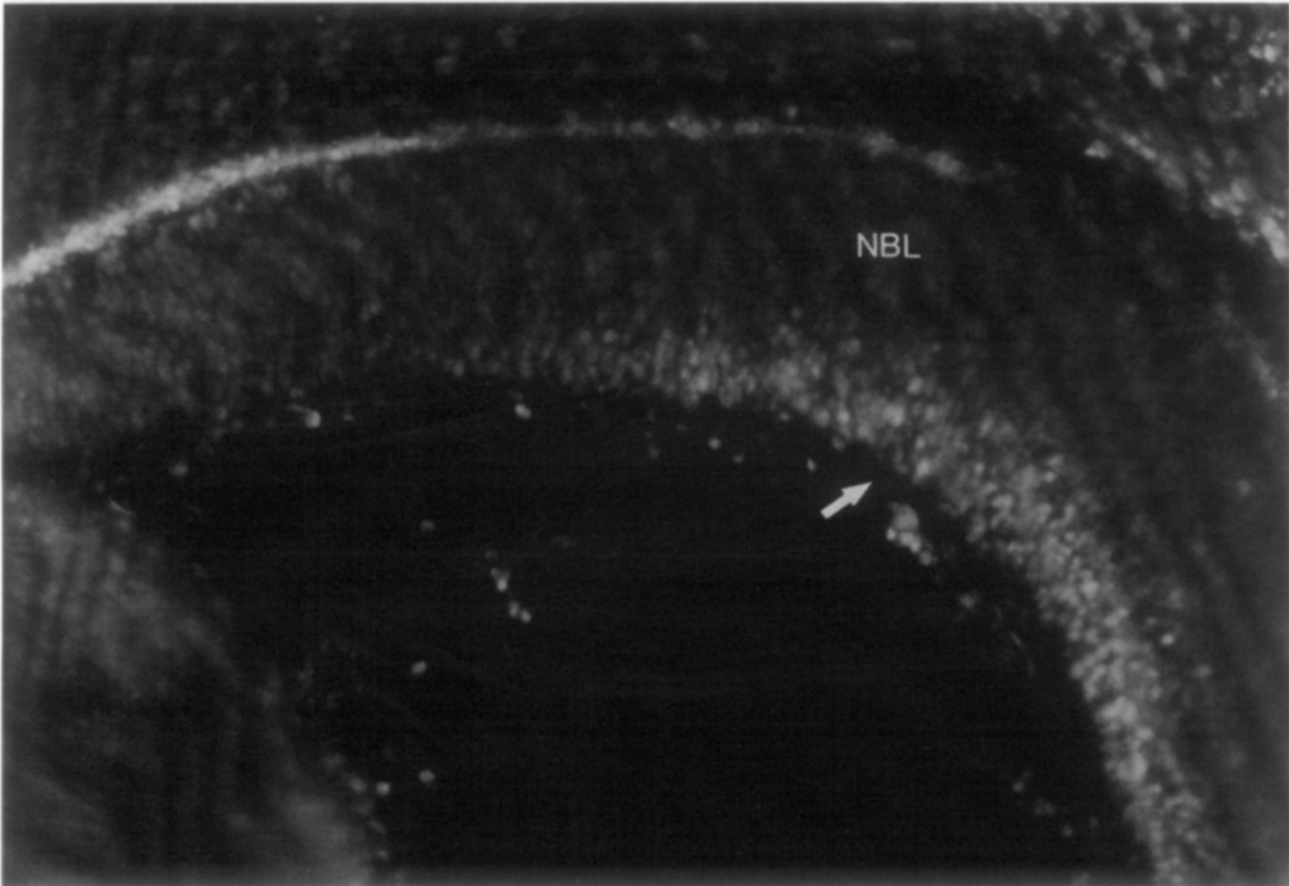


FIG. 6. Retrograde labeling identifies ganglion cells in the developing retina. The fluorescent tracer DAPI was injected into the brains of embryonic day 14 surgically exposed embryos. Twenty four hours later, retinas were fixed, sectioned and observed microscopically using fluorescent optics. Photomicrographs were taken using the  $\times 20$  objective. The arrow points to ganglion cells that were backlabeled with DAPI. Abbreviations as in Fig. 4.

poly (A) addition signal sequence (Wickens and Stephenson, 1984) does exist in this region (nucleotide no. 2347; Duvoisin et al., 1989).

#### 4. Discussion

In situ hybridization and RNase protection experiments showed that all the members of the nAChR gene family investigated were expressed in the adult rat retina (Figs 2 and 3). However, there were significant differences in the level and pattern of expression of these genes. These differences appeared to reflect the levels of retinal nAChR encoding RNAs and were not due to differences in probe, exposure times or regions of the retinas used. This is because all sections had a normalized amount of radioactivity, the probes were all of the same relative specific activity, and all the sections were exposed to emulsion for 4 days before developing. In addition, in situ hybridizations were performed many times on different retinal serial sections. Control hybridizations were done with sense orientation probes, which consistently showed only background levels of hybridization. Specificity of probes was confirmed by: (1) subunit specific hybridization to Southern blots of restricted

DNA corresponding to each of the nAChR clones using the same hybridization and post-hybridization conditions as were used for in situ hybridization experiments; (2) specific hybridization to brain regions previously shown to express these genes (Wada et al., 1989; Deneris et al., 1989; Duvoisin et al., 1989); and (3) nuclease protection assays showing even the most homologous nAChR RNA sequences are sensitive to nuclease digestion (Cauley et al., 1990). In addition, to show that heterogeneous expression of nAChR genes was not due to probe accessibility, we performed in situ hybridizations with the ubiquitously expressed small nuclear RNA, U1 (Howe and Steitz, 1986). This experiment showed that greater than 95% of the stained cells in the ganglion cell layer had silver grains over their cell bodies (data not shown). Finally, it should be noted that all the genes studied here, except  $\beta$ -3, have been shown to encode proteins that participate in functional ligand-gated ion channels (Deneris et al., 1989; Duvoisin et al., 1989; Papke et al., 1989). That the  $\beta$ -3 gene product does not participate with any of the known  $\alpha$ -subunits in forming a functional nAChR may indicate its requirement for other undiscovered subunits in order to form a functional receptor.



### *nAChR Gene Expression in the Adult Retina*

All the nAChR genes examined in this report were found to be expressed by cells in the ganglion and inner nuclear cell layers (Fig. 2). The ganglion cell layer is composed of both cholinergic amacrine cells and ganglion cells (Perry, 1981; Masland et al., 1984). Ganglion cells are known to express functional nAChRs that likely respond to input from cholinergic amacrine cells (Ariel and Daw, 1982a, b). In addition, ganglion cells express presynaptic nAChRs found on optic nerve terminals (Henley et al., 1986; Prusky and Cynader, 1988).

The expression of nAChR genes in the inner nuclear layer clearly shows a preference for those cells at the inner margin where amacrine cells reside (Fig. 2). There are many different types of amacrine cells, of which a small percentage are cholinergic (Massey and Redburn, 1987). Nicotinic acetylcholine receptor gene expression in the amacrine cell layer implies that besides providing input to ganglion cells, the cholinergic amacrine cells may also synapse with other amacrine cell types. In the goldfish and mouse retina such synapses have been documented (Pourcho, 1979; Zucker and Yazulla, 1982). In addition, it has been reported that cholinergic amacrine cells synapse with other cholinergic amacrine cells in rabbit, chick and goldfish retinas (Millar and Morgan, 1987), thus suggesting that these cells also express nAChRs.

This latter report may help explain why we observed a majority of cells (over 70%) in the ganglion cell layer expressing their  $\alpha$ -4,  $\beta$ -2 and  $\beta$ -4 nAChR genes. Since the ganglion cell layer is composed of about equal numbers of cholinergic amacrine and ganglion cells, one would predict a maximum of 50% of the cells in this layer to express their nAChR genes if confined to the ganglion cell type. However, if cholinergic amacrine cells can synapse with each other, one would expect them to contribute to the percentage of cells expressing nAChR genes in the ganglion cell layer. Confirmation of this possibility requires a double labeling protocol, in which nAChR RNA or protein is identified on a choline acetyltransferase positive amacrine cell.

Alpha-4 and perhaps  $\beta$ -2 gene expression in the adult retina are unique in that they appear to be expressed in a region of the inner nuclear layer that harbors bipolar cells (Fig. 2). Although it is not clear that these cells synthesize functional nAChRs, there are reports of  $\alpha$ -bungarotoxin binding on bipolar cells at amacrine-bipolar cell synapses (Pourcho, 1979; Zucker and Yazulla, 1982; James and Klein, 1985).

These data are consistent with immunohistochemical studies of nAChR expression in chick retina (Keyser et al., 1988) and indicate the ganglion cell and inner nuclear layers have the potential to synthesize a wide variety of different nAChR subtypes. This may reflect a differential subcellular distribution of these receptors, such as pre-, post- or extra-synaptic and/or it may reflect the functional heterogeneity of the cells

expressing them. Although the potential number of different types of nAChRs expressed in the retina is large, we have not been able to determine which combinations of nAChR subunit-encoding genes are expressed in any one particular cell.

### *nAChR Gene Expression During Retinal Development*

At the earliest time investigated (E13), we detected a low level of  $\alpha$ -3 and  $\beta$ -4 gene expression throughout the developing retina (Fig. 4). At this time of development the majority of cells are undifferentiated (Sidman, 1960; Braekevelt and Hollenberg, 1970). The significance of expressing nAChR genes at this early stage of development is not known, however, like nAChRs expressed throughout embryonic muscle fibers (Jaramillo, Vicini and Schuetze, 1988), they may be playing a physiological role independent of synaptic communication. Around E14 there is a coordinate induction of nAChR gene expression along the inner limiting membrane (Figs 4 and 5). Retrograde labeling studies using dyes injected into the embryonic brain at E14 show the cells expressing these genes are retinal ganglion cells that have already sent their axons to the brain (Fig. 6). It is interesting to note that the high level of E15 nAChR gene expression is concentrated at the inner wall of the retina over ganglion cell bodies. Clearly, ganglion cells are being born at the ventricular surface, yet we failed to detect any cells which express their nAChR genes at a level comparable to those which have reached their final position.

Two likely mechanisms exist to explain the induction of the nAChR genes at E14–15. First, a target derived factor retrogradely transported back to the ganglion cell body may be responsible for inducing nAChR gene expression. It is important to emphasize that this does not suggest that a synaptic connection is required, but rather a factor released by the target is picked up by retinal axon terminals. Consistent with this hypothesis is our finding that those ganglion cells expressing their nAChR genes correspond to cells that can be retrogradely labeled by injection of dye into the embryonic brain (Fig. 6). Alternatively, it is conceivable that the induction of nAChR gene expression in these ganglion cells is in response to an environmental cue at the inner limiting membrane. An environmental effect appears to be important in determining differentiation of many retinal cells (Adler and Hatlee, 1989; Reh and Kljavin, 1989). Consistent with both hypotheses is the observation that only those ganglion cells at the inner limiting membrane in E14 or E15 embryos express their nAChR genes at a relatively high level (Figs 4 and 5). Regardless which of the above mechanisms is correct it is interesting that patch clamp studies of dissociated retinal ganglion cells in culture showed the majority of these cells to have a single conductance of approximately 48 pS in response to acetylcholine (Lipton et al., 1987). Yet we

show here that these cells have the potential to synthesize many types of nAChR, and others have demonstrated that different combinations of nAChR subunits can combine to form functional channels with different conductances (Papke et al., 1989). It is possible that ganglion cell-target interactions or the environment the cells reside in are crucial for maintaining nAChR gene expression and that dissociated retinal cells no longer express many of their nAChR genes.

Between E18 and PN4 the ganglion cell layer separates from the neuroblast layer and we begin to detect nAChR gene expression in what will become the inner nuclear layer (Figs 4 and 5). The apparent decrease in gene expression of the adult versus the embryonic ganglion cell layer is probably due, in a large part, to cell death which occurs throughout this time period (Potts, Dreher and Bennett, 1982).

In summary, these data show that: (1) the  $\alpha$ -2,  $\alpha$ -3,  $\alpha$ -4,  $\beta$ -3, and  $\beta$ -4 nAChR subunit-encoding genes are expressed in the rat retina; (2) these genes are coordinately induced in ganglion cells beginning about embryonic day 14; and (3) multiple retinal cell types express these nAChR genes in both the ganglion and inner nuclear cell layers.

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