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Colocalization of Choline Acetyltransferase and γ-Aminobutyric Acid in the Developing and Adult Turtle Retinas

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ABSTRACT

Acetylcholine and γ -aminobutyric acid (GABA) are putative neurotransmitters in the adult vertebrate retina. In this study, cells that coexpress choline acetyltransferase (ChAT) and GABA or glutamic acid decarboxylase (GAD) were investigated in turtle retinas from stage 14 (S14) to adulthood by using a double-labeling immunofluorescence technique. ChAT immunoreactivity was observed at S15 and included not only the presumptive starburst cholinergic amacrine cells but also a population in the ganglion cell layer (GCL) that expressed ChAT transiently during the embryonic stages (see the accompanying paper: Nguyen et al. [2000] J. Comp. Neurol. 420:512–526). J. Comp. Neurol. 420:527–538, 2000.

Indexing terms: amacrine cells; choline acetyltransferase; development; double labeling; fluorescence microscopy; γ-aminobutyric acid; glutamic acid decarboxylase; immunohistochemistry; retinal ganglion cells; starburst amacrine cells; transient population; turtle; type III cholinergic cells; vertebrates

Starburst amacrine cells in the adult vertebrate retina have been shown to contain not only acetylcholine but also γ-aminobutyric acid (GABA). GABA localization in these cells has been demonstrated by immunohistochemistry (Kosaka et al., 1988; Vaney and Young, 1988) and by the uptake of tritiated muscimol ([^{3}H]muscimol; Chun et al., 1988; Massey et al., 1991) and its release (Chun et al., 1988; Massey et al., 1991; O'Malley et al., 1992). In the adult retina, GABA has been demonstrated to be an important transmitter in several functions, such as lateral inhibition (Grzywacz et al., 1997), response transience (Werblin et al., 1988), and directional selectivity (Caldwell et al., 1978; Ariel and Adolph, 1985; Burgi and Grzywacz, 1997; Grzywacz et al., 1997). However, the role of GABA in cholinergic cells remains unclear. Some studies have suggested that GABA may influence the release of acetylcholine (Massey and Redburn, 1982; Ariel and Adolph, 1985; Neal et al., 1992). It is possible that GABA released by starburst amacrine cells influences the release of acetylcholine from these cells themselves through autoreceptors (Zucker et al., 1998). In the developing retina, cholinergic cells have been implicated in spontaneous activity (Masland, 1977; Meister et al., 1991; Sernagor and Grzywacz, 1995; Zucker et al., 1998) and expansion of receptive field size (Sernagor and Grzywacz, 1996). Irrespective of the precise role of the cholinergic amacrine cells, two questions arise: 1) How early does colocalization occur; and 2) is colocalization common across species?

In the accompanying paper (Nguyen et al., 2000), it is demonstrated that cells immunoreactive to choline acetyltransferase (ChAT), an enzyme that synthesizes acetylcholine, are present early in the developing turtle retina. They include not only the adult-like cholinergic amacrine cells but also a population of cells that expresses ChAT transiently. This transient population disappears around birth. Therefore, early in retinal development, it is not possible to discriminate between the two cholinergic populations that express ChAT. It is not until the disappearance of the transient population that the presumed starburst amacrine cells are distinguishable. Although several studies have demonstrated that the starburst amacrine cells in the adult vertebrate retinas coexpress ChAT and GABA and/or glutamic acid decarboxylase (GAD), with the exception of their colocalization in the chick retina

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(Reiss et al., 1997), little is known about their expressions during embryonic stages. Moreover, it is important to determine whether the population that transiently expresses ChAT also expresses GABA/GAD during development. If this transient cholinergic population was not labeled by GABA or GAD antibodies, then it would be possible to separate the cholinergic cells into two populations early in development: one that is stable and, thus, is the presumed starburst amacrine cells and one that expresses ChAT only transiently. In the current study, immunofluorescence colocalization of ChAT and GABA or GAD_{67} is observed in turtle retinas from stage 15 (S15) to adulthood. Findings indicate that only the adult-like cholinergic amacrine cells express GABA or GAD_{67} and that these cells are present in the retina early in development.

MATERIALS AND METHODS

Data in this study were derived from the same experiments from which data on the immunofluorescence localization of ChAT were obtained and are described in the accompanying paper (Nguyen et al., 2000). Two isoforms of GAD, GAD_{65} and GAD_{67}, have been found in the vertebrate retinas. Immunohistochemistry in this study was carried out to localize GAD_{67}, because antibodies against this isoform (but not the GAD_{65} isoform) labeled displaced amacrine cells in the rabbit retina (Brandon and Criswell, 1995). Furthermore, Vardi and Auerbach (1995) found that antibodies against GAD_{67} from Chemicon International Inc. labeled a higher percentage of GABA-IR cells in the cat retina than the GAD_{65} antibodies. In addition, GAD_{67} is localized in cells that are known to contain other neurotransmitters, notably acetylcholine (Vardi and Auerbach, 1995). For the demonstration of colocalization of ChAT and GABA or GAD_{67}, sections were reacted first for ChAT, blocked, and then reacted for GABA or GAD_{67}. For the localization of ChAT, briefly, retinal sections were blocked with 4.0% normal horse serum (NHS) in phosphate buffer (PB), pH 7.4, followed by incubation with affinity-purified goat anti-ChAT (Chemicon International Inc., Temecula, CA) in 1.0% NHS/PB, pH 7.4, at 1:800–1:1,600 in a humid chamber overnight at room temperature. The following day, sections were washed and incubated in horse anti-goat immunoglobulin G (IgG; Vector Laboratories, Burlingame, CA) for 1–2 hours. Next, sections were washed with PB and incubated in indocarbocyanine (Cy3)-conjugated streptavidin ($Cy^{TM}3$; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA).

After reacting for ChAT, sections were washed through several changes of buffer and blocked in an avidin-biotin blocking kit (Sigma, St. Louis, MO) for 1 hour. Sections were then rinsed with PB, and the immunohistochemistry procedure was repeated but for GABA or GAD. Sections were blocked with 10% normal goat serum (NGS) in PB, pH 7.4, for 1 hour at room temperature and incubated overnight in rabbit-anti-GABA (1:16,000–1:20,000; Sigma) or -GAD_{67} (1:1,000–1:1,600; Chemicon International Inc.) in 1% NGS/PB, pH 7.4, in a humid chamber. The following day, sections were washed in 1% NGS/PB and incubated in goat-anti-rabbit IgG (Vector Laboratories) at dilution 25 μl/5 ml in 1% NGS/PB, pH 7.4, for 1–2 hours. Sections were then washed in PB and labeled with Fluorescein avidin D (1:500; Vector Laboratories) for 30–40 minutes. After washing through several changes of PB, sections were coverslipped with Vectorshield (Vector Laboratories) and examined with a Leitz epifluorescence microscope (Leitz, Wetzlar, Germany) equipped with rhodamine and fluorescein isothiocyanate filters. Images were photographed at a magnification of ×25 using separate filters.

Control experiments

Several sections from each eyecup were used for control experiments to test the specificity of the primary and secondary antibodies. Negative controls were performed by excluding the primary or secondary antibody from incubation according to the experimental paradigm described above.

Quantitative data analysis

To produce images for analysis, 10.2 cm × 15.2 cm pairs of photomicrographs of the same retinal section but that contained different immunofluorescence labeling were scanned with an Agfa StudioScan IIsi scanner (Agfa-Gevaert NV, Montsel, Belgium) at 29.5 pixels/cm and 8-bit RGB. The images containing different dye labeling were imported into Adobe Photoshop software (Adobe Systems, Mountain View, CA), superimposed, cropped, and analyzed. Analysis was performed for the inner nuclear layer (INL) and the ganglion cell layer (GCL) separately. In Photoshop, the images were arranged into layers used to analyze the number of cells that separately labeled by ChAT and GABA or GAD_{67} antibodies. Data on the relative percentage of ChAT-immunoreactive (-IR) cells that also localized GABA or GAD_{67} were obtained by dividing the number of cells that contained the two labels by the total number of ChAT-IR cells. A cell was counted as ChAT-IR if it met criteria for intensity of labeling and size. Cells were counted for their immunoreactivity to GABA or GAD_{67} by using the same criteria. A detailed analyses of ChAT-IR cells, including density and dendritic stratification in the IPL, are reported in the accompanying paper (Nguyen et al., 2000).

RESULTS

Sequential labeling for ChAT and GABA/GAD_{67} on the same retinal sections by using secondary antibodies generated in different species did not result in cross reactivity. Double labeling with antibodies against GABA and GAD_{67} resulted in similar populations and relative number of cells labeled. Therefore, a high percentage of cells that labeled for GABA also were GAD_{67}-IR. Like the accompanying paper (Nguyen et al., 2000), data are presented by developmental stages and under the subheadings "Descriptive findings" and "Quantitative analysis."

Fig. 1. **A–D:** Fluorescence photomicrographs of adult retinas demonstrating labeling for choline acetyltransferase (ChAT; A,D) and γ-aminobutyric acid (GABA; B,E) and colocalization of ChAT and GABA (C,F). In the adult stage, most ChAT-IR cells are positioned on both sides of the inner plexiform layer (ipl) and display a mirror-like symmetry. A type III cholinergic cell (D, arrowhead) is shown to be faintly labeled by antibodies against GABA (E, arrowhead). The arrowhead in F shows a double-labeled type III cell. GABA antibodies also discretely label fibers (B, arrow) in the nerve fiber layer (nfl). opl, outer plexiform layer; inl, inner nuclear layer; gcl; ganglion cell layer. Scale bars = 20 μm in C (also applies to A,B) and F (also applies to D,E).

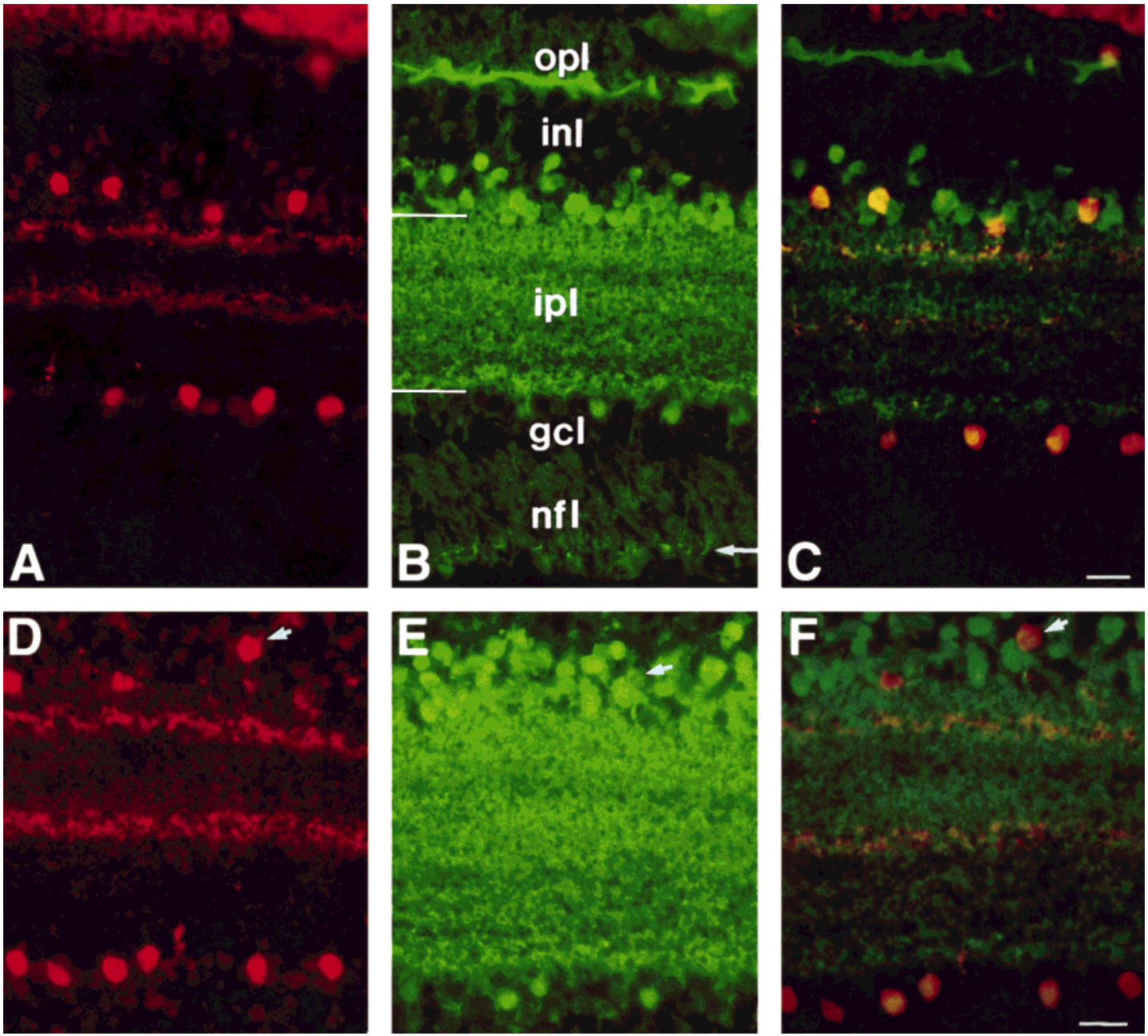

Figure 1

Data for the adult retina are reported first to establish a baseline with which the rest of the developmental stages are compared.

Descriptive findings

Adult. Almost all cells in the adult turtle retina that were ChAT-IR (Fig. 1A,D) also were GABA/GAD_{67}-IR (Fig.1B,E). The double-labeled cells that are located on both sides of the IPL (Fig. 1C,F) resembled the starburst amacrine cells that have been described in mammals (Famiglietti, 1983; Brecha et al., 1988; Vardi and Auerbach, 1995). They were round and homogeneous in appearance, were situated in the INL and the GCL, and displayed mirror-like symmetry (Famiglietti, 1983; Vaney, 1984) and regular spacing. In the GCL, the majority of GABA-/GAD_{67}-IR cells also were ChAT-IR. By contrast, in the INL, the ChAT-IR cells represented only a small subpopulation of the GABAergic amacrine cells. Compared with the rest of the GABAergic cells in the INL, those that also strongly expressed ChAT were rounder and more homogeneous and were situated close to the INL/IPL border. In the IPL, antibodies against GABA/GAD_{67} labeled densely, often obscuring the identification of individual dendritic stratification. Therefore, in the current study, characterization of individual GABA-/GAD_{67}-IR dendritic stratification was not explored. In other studies, approximately four to five GABA-IR dendritic stratifications (Sandmann et al., 1997) and five to seven GAD_{67}-IR dendritic stratifications (Hurd and Eldred, 1989; Criswell and Brandon, 1992) in the adult turtle IPL have been reported. By contrast, only two ChAT-IR dendritic strata clearly were present in the IPL, and they are analyzed in detail in the accompanying paper (Nguyen et al., 2000).

Type III cholinergic amacrine cells also were present in the INL, and, in this study, they were observed to contain GABA/GAD_{67} (Fig. 1E,F). These type III cells have been found to be inconsistently ChAT-IR (weak-to-strong immunoreactivity). Therefore, an attempt was not made to quantify these cells: It is only noted that they colocalized with GABA/GAD_{67}.

In the outer retina, ChAT immunoreactivity was observed, but it was not specific to cell bodies (for a discussion, see Nguyen et al., 2000). Antibodies against GABA/GAD_{67} labeled cells in the position of horizontal cells and their processes: These cells were not ChAT-IR.

Finally, GABA (but not GAD_{67}) labeled axons in the nerve fiber layer (Fig. 1B). These axons may belong to a small population of ganglion cells that are GABAergic (Blazynski, 1989; Caruso et al., 1989; Hurd and Eldred, 1989; Guiloff and Kolb, 1992a)

S14–S16 (approximately embryonic days 15–22). At S14, the earliest stage examined in this study, the retina was very thin and had no apparent subdivisions into nuclear or plexiform layers. Whereas ChAT immunoreactivity was not specific to cells (Fig. 2A; see Nguyen et al., 2000), cells in the same region of the retina already were GABA-/GAD_{67}-IR (Fig. 2B). This early expression of GABA/GAD_{67} by cells in the retina is supported elsewhere (Versaux-Botteri et al., 1994). In a less-developed region of the retina (in regions located farther from the optic nerve head), few cells showed GABA-/GAD_{67} immunoreactivity and appeared to be in the process of migration (Fig. 2B). In cases in which the retina was more developed, in regions near the optic nerve head, GABA-/GAD_{67}-IR cells were positioned in two regions of the retina, in the proximal and distal two-thirds (not shown). In the proximal one-third, antibodies to GABA/GAD_{67} labeled several rows of cells, whereas, in the distal one-third, labeled cells were distributed sparsely and formed a single row. At this and all subsequent stages, the GABA-/GAD_{67}-IR cells in the distal one-third of the retina resembled those in the adult stage and possibly belonged to the horizontal cells. An alternative explanation would be that, because this was an early stage in retinal development, instead, some or all of these cells may be in the process of migration from distal to proximal retina. The evidence against all of these cells undergoing migration is that GABAergic cells in the distal one-third of the retina appeared to line up uniformly in a single row, assuming the position of horizontal cells. However, at S14, the processes from the presumed horizontal cells were not yet observed. Therefore, at S14, approximately one-fourth of the way through development, the retina already had synthesized GABA/GAD_{67} slightly prior to acetylcholine.

At S15, whereas the retina still was immature and had no divisions into nuclear and plexiform layers, colocalization of ChAT and GABA/GAD_{67} already was observed in cells that were situated near the vitreous (Fig. 2E). Cells that were ChAT-IR, including the population that transiently expressed ChAT, were located next to the vitreal surface (Fig. 2C). A separate population of cells that were only GABA-/GAD_{67}-IR was located immediately distal to the ChAT-IR population (Fig. 2D). At S15, most ChAT-IR cells also were GABA-/GAD_{67}-IR.

With the emergence of a proto-IPL, indicated by a groove (Fig. 3A–C, arrows), the retina at S16 was beginning to be divided into two regions. Cells that expressed both ChAT and GABA/GAD_{67}, for the most part, were present on both sides of this proto-IPL, although more colocalized cells were observed distal to this proto-IPL, in the proto-INL. ChAT-IR cells, including the transient population, were located predominantly in the proto-GCL, whereas the majority GABA/GAD_{67}-IR cells were located in the proto-INL. The GABA-/GAD_{67}-IR cells that expressed ChAT were not uniform in size on the two sides of the proto-IPL at this early stage: These cells were slightly larger in the proto-INL than in the proto-GCL (Fig. 3A). In the distal one-third of the developing retina, scattered cells were GABA-/GAD_{67}-IR (Fig. 3B). These cells appeared to be horizontal cells based on their location. Note that the position of these GABAergic cells was not different from that at S15.

S18–S23 (approximately embryonic days 25–40). S18 (Fig. 4) was a period of rapid development (see Nguyen et al., 2000). The majority of ChAT-IR cells in the INL also were GABA-/GAD_{67}-IR. However, in the GCL, only a small percentage of ChAT-IR cells was also labeled by GABA/GAD_{67} antibodies. Near the optic nerve head, where development was more advanced (Fig. 4D–F), the colabeled cells were distributed on both sides of the IPL (Fig. 4F). Furthermore, in these advanced regions, ChAT-IR dendritic stratifications were present in the IPL (Fig. 4D,F). By contrast, in regions located farther from the optic nerve head and, thus, less advanced regions (Fig. 4A–C), the IPL still was forming, ChAT-IR cells still were in the process of migration, and GABA-/GAD_{67}-IR dendritic stratifications were present (Fig. 4B). However, ChAT-IR dendritic stratifications were absent in the IPL (Fig. 4A,C). Cells in the position of horizontal cells were

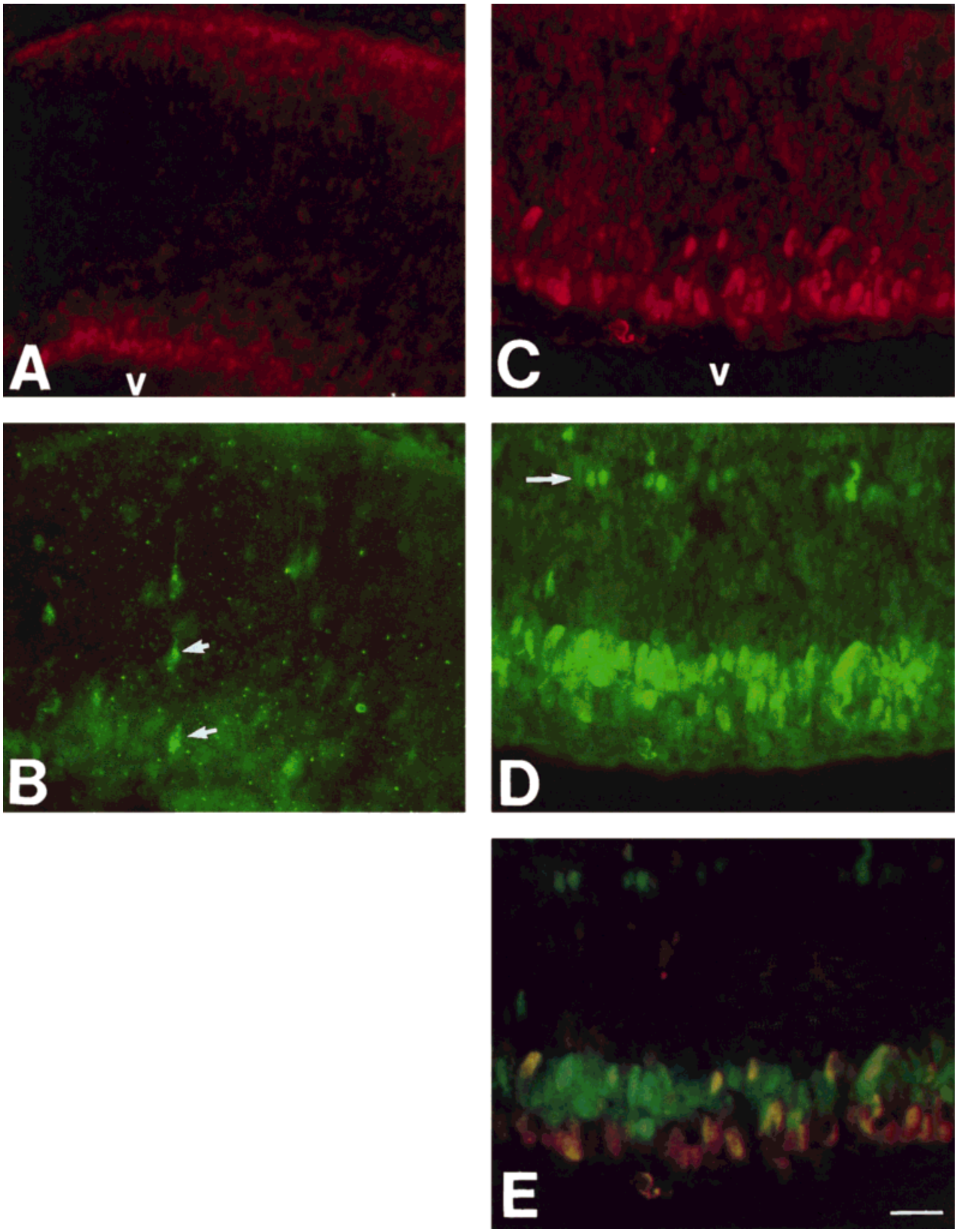

Fig. 2. **A–E:** Fluorescence photomicrographs of stage 14 (S14; A,B) and S15 (C–E) retinas demonstrating labeling for ChAT (A,C) and GABA (B,D) and colocalization of ChAT and GABA for the S15 retina (E). The retinas at these stages are not subdivided into nuclear and plexiform layers. At S14, cells in the retina are not ChAT immunoreactive (-IR; A), whereas GABA shows immunoreactivity in some migrating cells (B, arrowheads). At S15, cells near the vitreous (v) are ChAT-IR. GABA-IR cells at this stage are located in two regions of the retina: near the vitreal border and in the distal one-third (D, arrow). At S15, most of the ChAT-IR cells (C) colocalize GABA (D). E shows the colocalization. Scale bar = 20 μm.

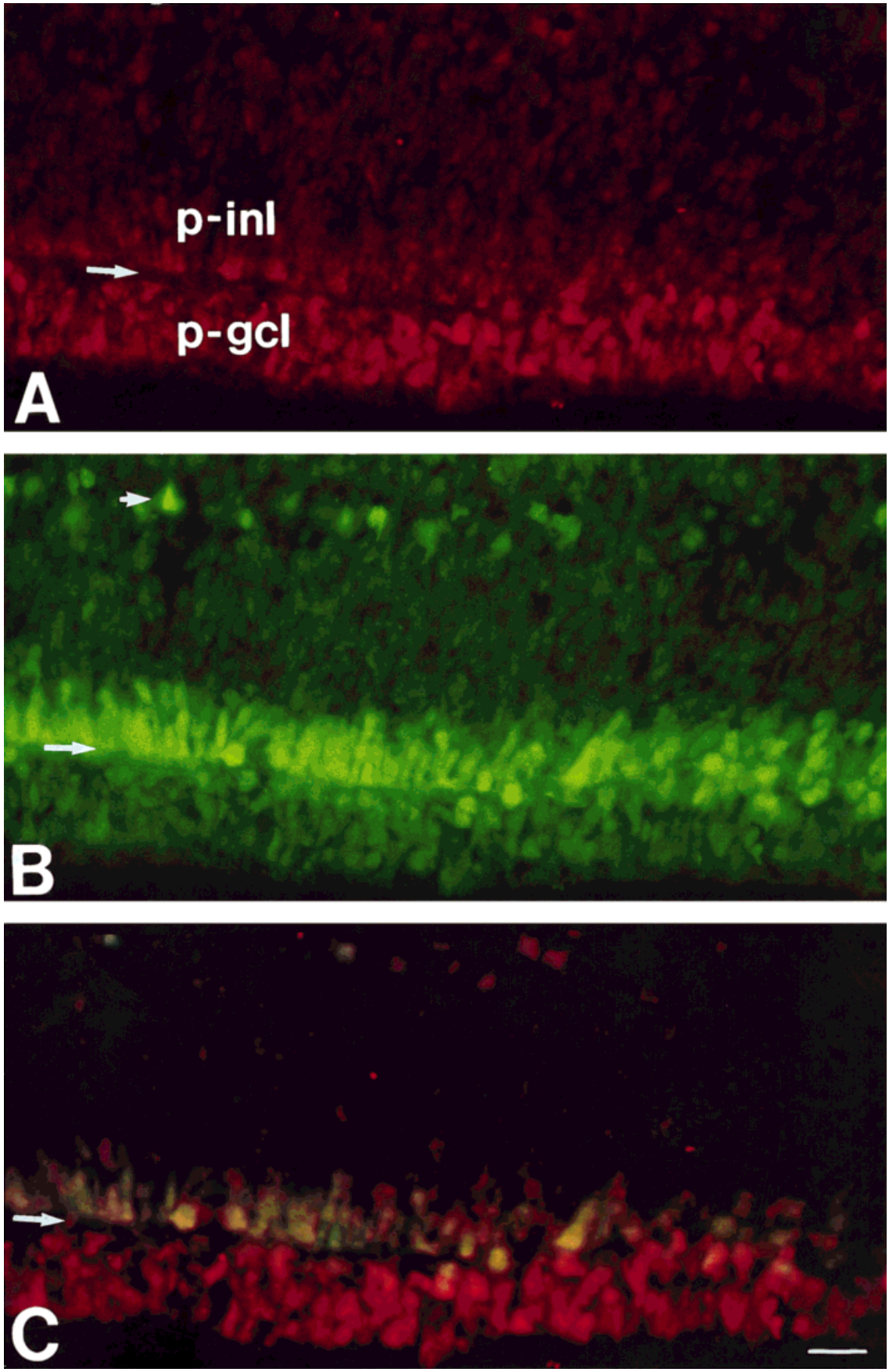

Figure 3

immunoreactive to GABA/GAD_{67}, and, approximately at this stage, their processes that extended laterally within the outer plexiform layer were first observed (not shown).

By S20 (not shown), migration of cells appeared to have ceased, and the retina contained all cellular and plexiform layers. The pattern of cells that were ChAT-IR and GABA-/GAD_{67}-IR resembled regions of a S18 retina (Fig. 4F) that were more developed. Within the IPL, ChAT-IR and GABA-/GAD_{67}-IR dendritic strata were observed. In the GCL, multiple rows of cells continued to be labeled by ChAT antibodies. However, only the most distal positioned cells among this population and, thus, those closest to the GCL/IPL border also were GABA-/GAD_{67}-IR.

At S22 (not shown) and S23 (Fig. 5), although the retina continued to be growing and maturing, the morphology and pattern of cells showing colocalization of ChAT and GABA/GAD_{67} remained similar to what was seen at S20. However, as shown in the accompanying paper (Nguyen et al., 2000), the density of ChAT-IR cells in the GCL was declining.

S25 (approximately embryonic day 52). At S25 (not shown), the distribution and labeling pattern of the adult-like cholinergic amacrine cells that were ChAT-IR and GABA-/GAD_{67}-IR resembled those in the adult retina. These cells, partly due to the significant decrease in the density of ChAT-IR cells in the GCL, were easier to identify as belonging to the presumed starburst amacrine cell population than in the previous stages. At this stage, the identification of these cells was easier and was possible with ChAT immunoreactivity alone. Also at this stage, the type III cholinergic amacrine cells were first observed toward the middle of the INL, and, generally, they were colabeled by antibodies to GABA/GAD_{67}.

Birth to postnatal day 28. At birth [postnatal day 0 (P0)], the morphology of the retina with respect to ChAT and GABA/GAD_{67} immunoreactivity resembled that of the adult retina. One day after birth, at P1 (not shown), some retinas appeared to contain slightly more ChAT-IR cells in the GCL than at birth, and this was reflected in a lower percentage of colocalization of GABA/GAD_{67} (see discussion in Nguyen et al., 2000). Although the density of ChAT-IR cells in the GCL had increased, the pattern of colocalization did not change from the previous stages. Curiously, at P1, a higher density of colabeled cells appeared on both sides of the IPL. In addition, the type III cholinergic amacrine cells were observed with a higher frequency than at S25 or subsequent stages. Beginning at P1 and observed at subsequent stages, GABA (but not GAD_{67}) antibodies labeled axons in the nerve-fiber layer. The colocalization of ChAT and GABA/GAD_{67} in retinas from P7 to P28 (not shown) resembled that seen in the adult retina.

Fig. 3. Fluorescence photomicrographs of an S16 retina demonstrating the labeling for ChAT (**A**) and GABA (**B**) and colocalization of ChAT and GABA (**C**). At this stage, a groove (left), although it is not yet apparent throughout the retina, indicates the forming IPL (A, arrow), which subdivides the immediate regions into a proto-INL (p-inl) distal to this groove and a proto-GCL (p-gcl) proximal to this groove. More ChAT-IR cells are located in the proto-GCL than in the proto-INL. GABA-IR cells are located predominantly distal to this groove (B). An arrowhead in B indicates the GABA-IR cells in the distal third of the retina, similar to those at S15 (see Fig. 2D). Colocalization of ChAT and GABA are observed in cells distal to this grove (C). Scale bar = 20 μm.

Quantitative analysis

Surprisingly, from the time that the presumed starburst amacrine cells were present in the developing retina, they were GABA-/GAD_{67}-IR. These cells were found to be stable to adulthood. Their numbers grew only slightly from S15 to S18 but were constant from S18 onward. Figure 6 shows that the majority of cells in the INL that were ChAT-IR also were GABA-/GAD_{67}-IR. However, due to the presence of the population in the GCL that transiently expressed ChAT, the relative percentage of colocalization was low there during the early developmental stages (up until S25). Around birth, as this transient population started to decline significantly and eventually disappeared from the GCL, the relative percentage of colocalization significantly increased. At P1, a "recurrence" of the transient population was observed in the GCL, causing the dip in the graph (see discussion in Nguyen et al., 2000).

The distribution of colocalization on both sides of the IPL was reflected in the mirror-like symmetry of the presumed starburst amacrine cells (Fig. 7). By labeling these cells with GABA/GAD_{67} antibodies, their populations were distinguished easily. Although there was a slight predominance of the colocalization in the GCL at S16, at subsequent stages, the colocalizing cells were relatively stable in their relative frequency and distribution in the INL and GCL.

In the GCL, the majority of cells that were labeled by GABA/GAD_{67} antibodies belonged to the adult-like displaced cholinergic amacrine cell population from S16 to adulthood (Fig. 8). For retinas younger than S16, there were still no subdivisions into nuclear and plexiform layers; therefore, categorizing the colabeled cells into either the INL or the GCL was not possible. By contrast, in the INL, whereas there were many different types of amacrine cells that were labeled by GABA/GAD_{67} antibodies, only a small percentage of these were the presumed starburst amacrine cells.

DISCUSSION

This study has demonstrated that, in the turtle retina, a population of amacrine cells, presumably the starburst amacrine cells, is both ChAT-IR and GABA-/GAD_{67}-IR early in development. This occurs as early as S15, when the retina is not yet divided into nuclear and plexiform layers. With the formation of the IPL, the relative density of the colabeled cells in the GCL is similar to that in the INL from S18 onward. Moreover, by S18, these cells are distributed on both sides of the IPL in a mirror-like symmetry (Brecha et al., 1988; Kosaka et al., 1988; Guiloff and Kolb, 1992b), bearing a striking resemblance to those in the mammalian species described as the starburst amacrine cells. This observation was not obvious during the earlier developmental stages, as described in the accompanying paper (Nguyen et al., 2000), because immunohistochemistry was performed only for ChAT. Therefore, in that study, only with the decline and eventual disappearance of the transient cholinergic population were the presumed starburst cells identified clearly. Moreover, at S18, the cholinergic dendritic strata make their appearance in the IPL, and they remain stable through adulthood, that is, they essentially maintain their position in the IPL to adulthood (Nguyen et al., 2000). Taken together, the data

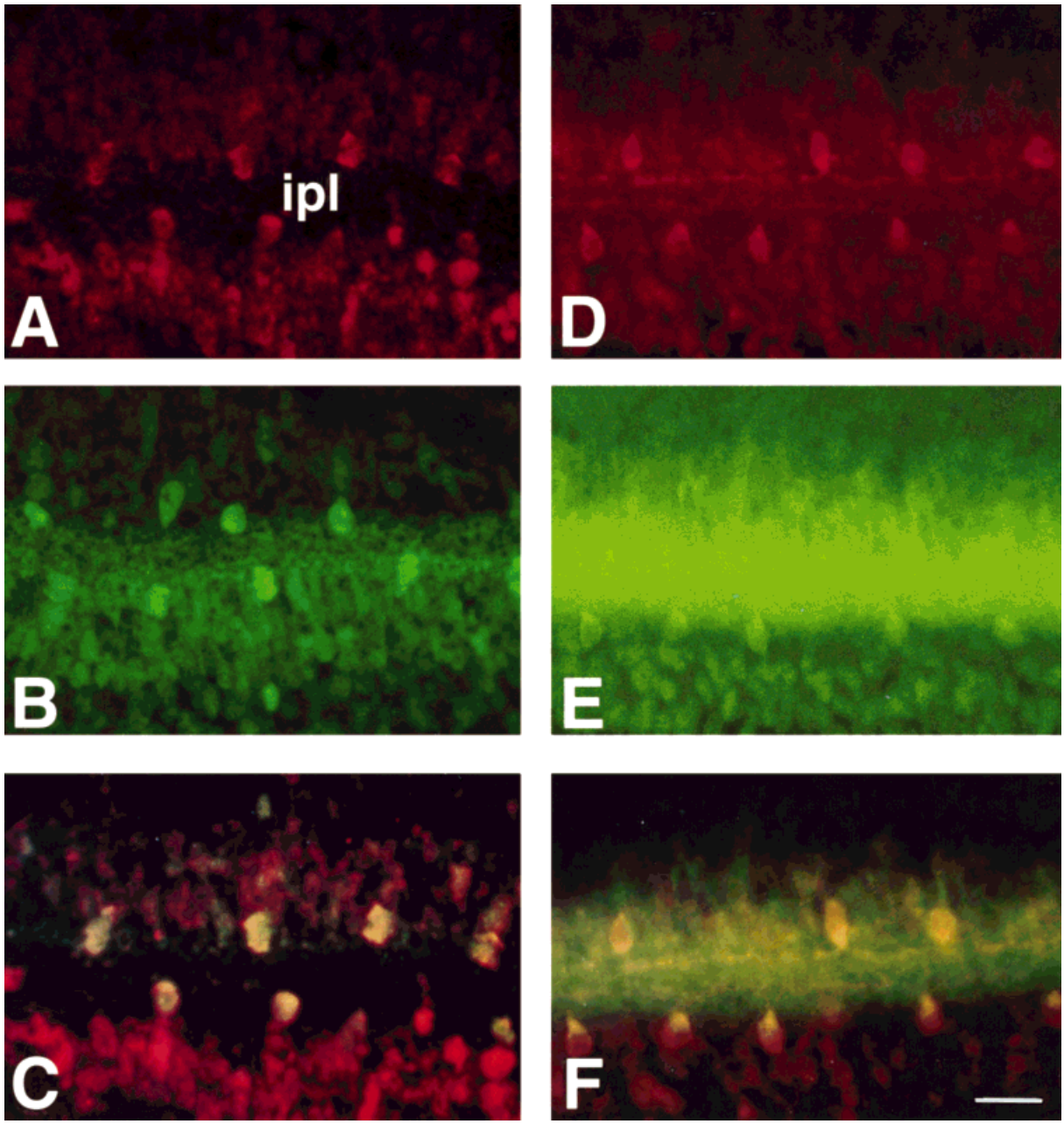

Fig. 4. **A–F:** Fluorescence photomicrographs of an S18 retina demonstrating labeling for ChAT (A,D), GABA (B), and glutamic acid decarboxylase (GAD; E) and colocalization of ChAT and GABA/GAD_{67} (C/F). A–C show an area in the retina that is less developed (farther away from the optic nerve head). Here, cholinergic cells still are in the process of migration across the inner plexiform layer (ipl; A). D–F show an area (near the optic nerve head) that is a little more advanced in its development, as indicated by the formation of the cholinergic strata in the ipl (D). Even in a region that is less developed, cells already are immunoreactive for ChAT and GABA antibodies (C). Scale bar = 20 μm.

from this and the previous study (Nguyen et al., 2000) demonstrate that the putative starburst amacrine cells are born early in retinal development and are stable into adulthood. Furthermore, the colocalization of ChAT and GABA/GAD_{67} in the turtle retina, in agreement with observations in other vertebrates (Brecha et al., 1988; Kosaka et al., 1988; Vaney and Young, 1988; Criswell and Brandon, 1992; Brandon and Criswell, 1995), suggests that the starburst amacrine cells are conserved phylogenetically (Famiglietti, 1983; Voigt, 1986; Millar et al., 1987; Guiloff and Kolb, 1992b; for review, see Vaney, 1990).

Transient cholinergic population

The stable population of presumed starburst amacrine cells is in contrast to a population of cells in the GCL that

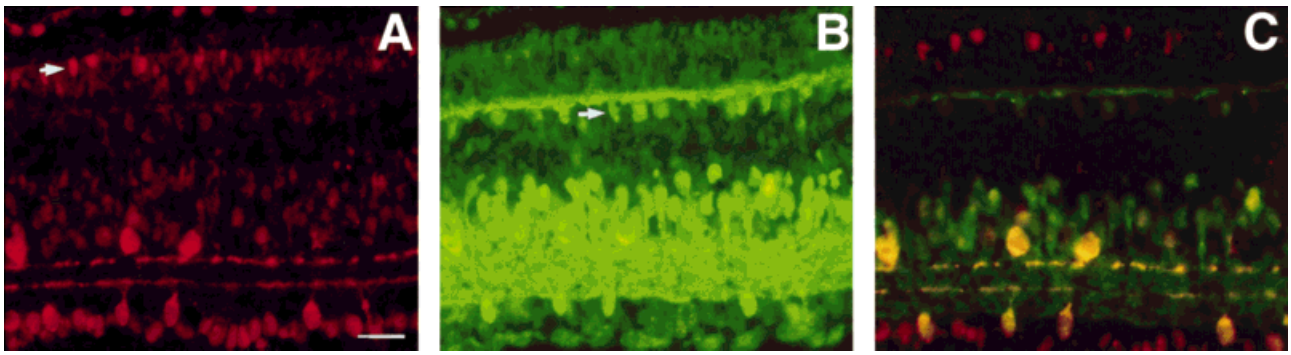

Fig. 5. Fluorescence photomicrographs of a S23 retina demonstrating labeling for ChAT (**A**) and GABA (**B**) and colocalization of ChAT and GABA (**C**). The layers in the retina at this stage are formed and resemble those in the adult retina (see Fig. 1). ChAT-IR cells are located on both sides of the inner plexiform layer (IPL), but there are more present on the ganglion cell layer side. GABA also label cells on both sides of the IPL but predominantly in the inner nuclear layer. Some photoreceptor nuclei are seen to be ChAT-IR (A, arrowhead). An arrowhead in B indicates GABA-IR horizontal cells. Scale bar = 20 μm.

appears to express ChAT only transiently. This transient cholinergic population is not GABA-/GAD_{67}-IR. This transient ChAT-IR population in the GCL does not decline and disappear smoothly but experiences some fluctuations, first around S22 and then around P1 (Nguyen et al., 2000). The reasons for these fluctuations are not known, but there are physiologic developmental patterns that occur at these stages that are worth discussing. At S22, spontaneous activity arises in the turtle retina (Sernagor and Grzywacz, 1995), and this activity is dependent on acetylcholine (Masland, 1977; Sernagor and Grzywacz, 1995, 1996, 1999; Feller et al., 1996). Thus, the increase in the number of cells that are ChAT-IR at S22 may be related to a supply-demand relation that is necessary to propagate these waves of correlated activity. At P1, there is another rise in the number of cells that are ChAT-IR. The same increase and subsequent decline postnatally has been observed elsewhere (Feller et al., 1996). A plausible reason may be that exposure to light triggers this rise in cell density, because cholinergic pathways also contribute to light responses in the retina (Masland and Ames, 1976; Ariel and Daw, 1982; Ariel and Adolph, 1985).

To what type of cells does the population of cells that transiently express ChAT belong? Birth dating studies have shown that ganglion and amacrine cells are the first cells in the retina to be born (Zimmerman et al., 1988; Dann, 1989). Once cells become differentiated, they migrate from the outer limiting membrane, the equivalent of the ventricular zone elsewhere in the developing central nervous system (Polley et al., 1989). In the current study, at S15, ChAT-IR cells are observed in the middle of the retina, possibly at a late stage of migration, if not already completed. During S18, ChAT-IR cells migrate across the IPL, but it appears that only the presumed starburst amacrine cells migrate and not the population that transiently localizes ChAT. Consequently, given the location of the transient cells and the early stages (S15–S18) of their appearance, they are likely to be ganglion and/or amacrine cells. Also, the high density of the transient population early in development makes it unlikely that it is comprised solely of amacrine cells. The idea that ganglion cells may transiently express ChAT early in development is plausible given the ubiquitous and trophic nature of acetylcholine.

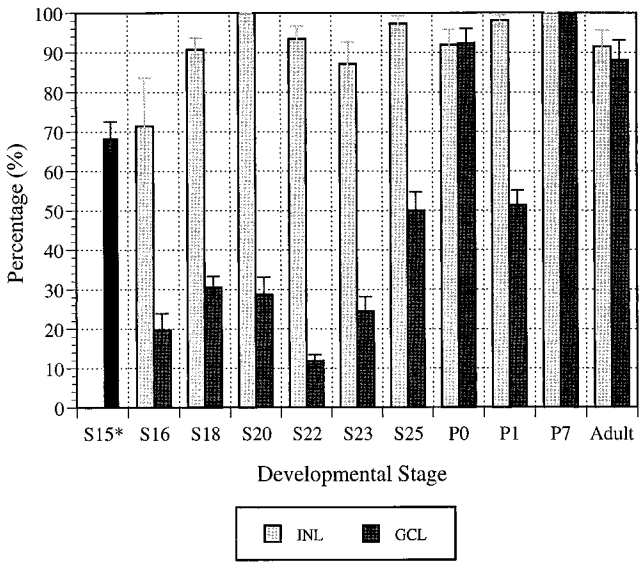

Fig. 6. Histogram of the relative percentage of ChAT-IR cells in the inner nuclear layer (INL) and the ganglion cell layer (GCL) that also express GABA/GAD_{67}. The high percentage in the INL demonstrates that the majority of ChAT-IR cells in this layer also are GABA-/GAD_{67}-IR. However, due to the presence of the transient cholinergic population in the GCL during the early developmental periods, the relative percentage of colocalization in the GCL is low until approximately S25. The dip in percentage at postnatal day 1 (P1) is discussed in the text and in the accompanying paper (Nguyen et al., 2000). Asterisk for S15 indicates that a differentiation between INL and GCL is not made.

The fate of the population that transiently localizes ChAT is being examined currently in this laboratory, and preliminary data indicate that this transient population does not experience programmed cell death (Grzywacz and Nguyen, 1998). Moreover, from this study, the decline and disappearance of cells that transiently expresses ChAT are not likely due to the growth and expansion of the retina with age, resulting in a "spreading out" of these cells, as suggested elsewhere (Greiner and Weidman, 1982; Mitrofanis et al., 1988). Consequently, the most

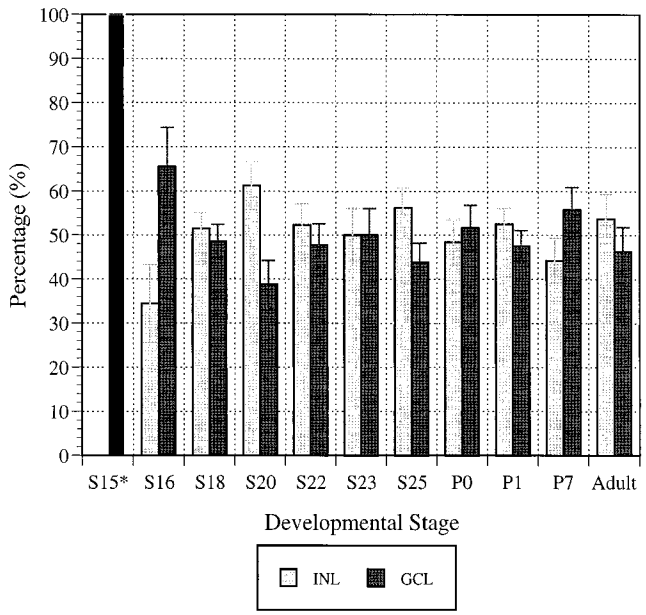

Fig. 7. Histogram of the relative percentage of colocalization of ChAT and GABA/GAD_{67} on both sides of the IPL (the sum of the colocalization in the INL and GCL percentages at each stage is 100% at every stage). This histogram reflects the mirror-like symmetry that is characteristic of the cholinergic starburst amacrine cells. Frequency and distribution of colocalized cells in the INL and the GCL are relatively stable from S18 to adulthood.

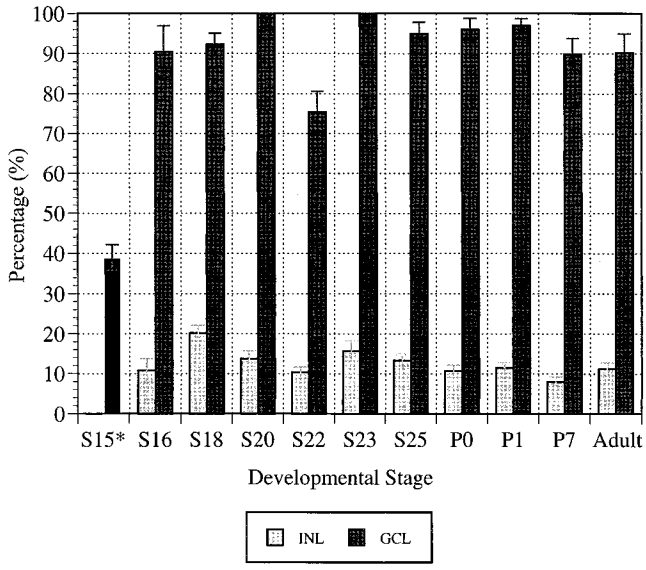

Fig. 8. Histogram of the relative percentage of GABAergic cells in the INL and the GCL that also are ChAT-IR. In the GCL, the majority of GABA-IR/GAD_{67}-IR cells are ChAT-IR. However, in the INL, because there are many types of amacrine cells that are GABAergic, the percentage of those that are ChAT-IR is low but is consistent with age.

likely explanation is that the expression of ChAT is downregulated during late embryogenesis. During development of the vertebrate retina, there have been observations of transmitters being expressed transiently, and they are thought to have neurotrophic roles. A few studies have even suggested that cells switch neurotransmitters. For example, early in development in the rabbit, retinal ganglion cells contain GABA but then switch to glutamate postnatally (Mitrofanis et al., 1988). Additional support for the transient expression of GABA has been observed in the zebrafish GCL and optic nerve (Pow et al., 1994). The question, then, becomes: What functions do the ganglion cells serve by transiently expressing GABA? GABA is suggested to have a neurotrophic role during development, promoting synaptogenesis, growth, and differentiation of neurons (Sandell et al., 1994; Buznikov et al., 1996). Therefore, perhaps the population that transiently expresses ChAT observed in the current study also subserves a neurotrophic function in the developing retina. One such function may be to help mediate the waves of spontaneous activity after S22 (Sernagor and Grzywacz, 1995). These waves appear to be crucial for the control of receptive field growth (Sernagor and Grzywacz, 1995). However, the waves become weaker around birth (Sernagor and Grzywacz, 1996), coinciding with the disappearance of the transient cholinergic population. In addition, the transient cholinergic population is not necessary for the waves, because the waves continue to exist until approximately P21, well past the time that the transient cholinergic population disappears. Conversely, the transient cholinergic population may have trophic roles other than those mediated by the waves, because these cells appear long before the waves in development.

Another component of the cholinergic system that was not explored in this study but that is worth mentioning is acetylcholine esterase (AChE). Because the transient expression of ChAT has been observed in the developing retina (Nguyen et al., 2000), AChE is expressed transiently in embryonic nervous tissues more than in the adult tissues (for review, see Small et al., 1996). In the developing chick nervous system, a transient increased expression of AChE is observed during the period of migration and differentiation (Spira et al., 1987; Layer, 1991). This transient expression may play a role in cellular adhesion relating to neurite growth (Layer, 1991; Layer et al., 1993; for reviews, see Layer and Willbold, 1994; Small et al., 1996). Therefore, perhaps acetylcholine and its esterase, AChE, work in unison during the development of the retina to promote cellular differentiation, migration, and synaptogenesis.

Type III cholinergic amacrine cells

The turtle retina contains a third population of cholinergic amacrine cells that is located toward the middle of the INL (Guiloff and Kolb, 1992b). This type III cholinergic amacrine population is present from S25 to adulthood, and it is similar to that found in the dogfish (Brandon, 1991), chicken (Millar et al., 1987), and tree shrew (Conley et al., 1986) retinas. In the current study, it was found that type III cholinergic amacrine cells were colabeled by GABA/GAD_{67} antibodies. The significance of the type III cholinergic amacrine cells has not been established. Their processes are known to ramify in multiple strata in the IPL (Guiloff and Kolb, 1992b) and, thus, may act in a relatively nonspecific and/or global manner.

GABA-/GAD$_{67}$ immunoreactivity in the retina

The localization of GABA/GAD$_{67}$ in cells appears early in the developing turtle retina (as early as S14), prior to the appearance of ChAT-IR cells. In addition, prior to the establishment of ChAT-IR dendritic strata in the IPL, GABA-/GAD$_{67}$-IR dendritic strata already are present. The early appearance of GABA in the retina has been attributed to its neurotrophic functions (Cherubini et al., 1991; Rowe-Rendleman et al., 1996). With the formation of the IPL, GABA/GAD$_{67}$-IR cells take their positions in the retina as a single row in the GCL and multiple rows in the INL. In the GCL, almost all GABA/GAD$_{67}$-IR cells belong to the presumed starburst amacrine cell population. However, there may be some GABA/GAD$_{67}$-IR cells in the GCL that are ganglion cells (there are axons in the fiber layer that are GABA-/GAD$_{67}$-IR; Yu et al., 1988; Guiloff and Kolb, 1992a,b; Versaux-Botteri et al., 1994). In contrast to the GCL, in the INL, the presumed starburst amacrine cells represent only a small subpopulation of the GABA-IR amacrine cells. This is because there are up to 37 types of amacrine cells identified in the turtle (Ammermuller and Kolb, 1995).

Finally, in the distal one-third of the retina, GABA/GAD$_{67}$ immunoreactivity is present at S15 in cells that are in the position of horizontal cells. These cells are not ChAT-IR, and their position is relatively stable in the outer retina. The early localization of these GABA-/GAD$_{67}$-IR cells in the distal one-third of the retina corresponds to findings by others (Versaux-Botteri et al., 1994; Ammermuller and Kolb, 1995). In the postnatal rabbit retina, GABAergic horizontal cells may influence maturation and synaptogenesis of cone photoreceptors (Redburn and Madtes, 1986; Messersmith and Redburn, 1993). In the turtle retina, it is likely that the early localization of GABA-/GAD$_{67}$ in horizontal cells may serve a similar purpose.

Significance of the putative starburst amacrine cells

It is intriguing that a cell contains and perhaps releases both excitatory and inhibitory neurotransmitters. In the adult retina, acetylcholine and GABA are important transmitters. The former is important for sensitivity of motion (Masland et al., 1984; Schmidt et al., 1987; Grzywacz et al., 1997), whereas the latter contributes to a host of functions, such as surround inhibition (Amthor and Grzywacz, 1993; Massey et al., 1997; Grzywacz et al., 1998) and response transience (Werblin et al., 1988). Moreover, the simultaneous action of both transmitters is necessary for the normal operation of at least one retinal function: directional selectivity (Ariel and Adolph, 1985; Grzywacz et al., 1997). However, directionally selective responses in the turtle retina do not exist at S18 (Sernagor and Grzywacz, 1995), at the time when the presumed starburst amacrine cells and their processes first are positioned in the retina. In addition, mature directional selectivity does not emerge until postnatal ages in the turtle retina (Sernagor and Grzywacz, 1995). Furthermore, in the turtle, synaptogenesis does not begin until S22 (Sernagor and Grzywacz, 1995). Therefore, the significance of presumed starburst amacrine cells in the early stages of the developing turtle retina is intriguing. It has been suggested that acetylcholine and GABA have neurotrophic functions; therefore, perhaps by design, the presumed starburst amacrine cells use these two transmitters synergistically to achieve particular neurotrophic roles in the developing retina. Hence, the role of colocalization of acetylcholine and GABA may change during life, switching from epigenetic control of development to visual processing.

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