

# Changing distribution of GABA-like immunoreactivity in pigeon visual areas during the early posthatching period and effects of retinal removal on tectal GABAergic systems

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## Abstract

The distribution of GABA-like immunoreactivity in the pigeon visual system was studied during the first 9 days after hatching using a mouse monoclonal antibody, mAb 3A12, to glutaraldehyde linked GABA (Matute & Streit, 1986). GABA-like immunoreactivity was seen in cell bodies as well as in neuropil at the level of both the retina and central visual regions at any posthatching age. However, the distribution of putative GABAergic cells and processes varied with age reaching the adult pattern at around 9 days. As a general observation, almost no cell bodies in the retina (except for some perikarya in the ganglion cell layer) were labeled at hatching but densely packed immunostained processes were present in the inner plexiform layer. During the next few days, GABA-immunoreactive amacrine and horizontal cells appeared and the adult distribution of GABA-like immunoreactivity was reached at around 9 days. In the other visual regions examined, the general trend in the variation of GABA-like immunoreactivity included: (1) a progressive decrease in the density of immunostained cell bodies and (2) an almost parallel increase in the concentration of stained neuropil. Since in pigeons the adult organization of visual pathways and the final distribution of putative GABAergic systems are reached at around the same age, we suggest the possibility that incoming ganglion cell axons play a role in regulating the distribution of GABA-like immunoreactivity in visual areas. This hypothesis is supported by the fact that the distribution of GABA-like immunoreactivity in the superficial layers of the optic tectum was altered following ablation of the contralateral retina immediately after hatching.

**Keywords:** GABA immunohistochemistry, Maturation, Retinal ablation, Plasticity, Avian visual system

## Introduction

Gamma-aminobutyric acid (GABA) is the best described inhibitory transmitter in the vertebrate brain. Methods for microscopical localization of GABA and related substances have been developed and include local uptake of [<sup>3</sup>H]-GABA (Kelly & Dick, 1976; Kisvarday et al., 1986; Neale et al., 1983; Ottersen et al., 1987; Storm-Mathisen, 1975), retrograde labeling of cell bodies using [<sup>3</sup>H]-GABA injections into their terminal fields (Cuenod et al., 1982; Cuenod & Streit, 1983; Somogyi et al., 1984), as well as immunohistochemical localization of glutamic acid decarboxylase (GAD) (Mugnaini & Oertel, 1985; Oertel et al., 1981; Oertel et al., 1983) or GABA (Seguela et al., 1984;

Somogyi et al., 1985; Storm-Mathisen et al., 1983). Recently, a mouse monoclonal antibody (mAb) to glutaraldehyde-linked GABA has been developed (mAb 3A12) (Matute & Streit, 1986) and utilized to study the distribution of GABA-like immunoreactivity (GABA-LI) in insect optic lobes (Meyer et al., 1986), pigeon brain (Domenici et al., 1988), rat cerebral cortex (Celio, 1986), cerebellar cortex, olfactory bulb, and hippocampal formation (Liu et al., 1989).

Although a number of studies have been aimed at elucidating the distribution of GABAergic systems in the adult brain, relatively little is known about the maturation of GABAergic cells and processes. Nonetheless, anatomical and biochemical studies in mammals demonstrated that GABAergic systems appear early in development (Coyle & Enna, 1976; Hatten et al., 1984; Lauder et al., 1986; Wolff et al., 1984). In addition, GAD immunoreactivity has been found in growth cones of GABAer-

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gic neurons in the developing cerebellum (McLaughlin et al., 1975). This early appearance suggests the possibility that, apart from being an inhibitory neurotransmitter, GABA may play a role in synaptogenesis (Wolff, 1981).

Less is known about the maturation of GABAergic systems in the avian brain. Recently, the ontogeny of GABA receptor complex (Tehrani & Barnes, 1986) and GABAergic neurons (Jong et al., 1986) has been studied in the brain of chick embryos. In addition, development of GABA-LI in chick hyperstriatum ventrale and cerebellum has been investigated (Curtis & Stewart, 1986).

With respect to studies concerning maturation and plasticity of the brain, the pigeon visual system serves as a useful model among nonmammalian species. Indeed, its maturation is not completed at hatching and continues to take place during the early period after hatching (Bagnoli et al., 1985; Bagnoli et al., 1987; Porciatti et al., 1985). In addition, removal of one retina at hatching has been shown to induce an abnormal maturation of primary visual pathways which persists in the adult stage (Bagnoli et al., 1989a).

In the present study, we have used mAb 3A12 to describe the location of putative GABAergic cells and processes in the retina and some central visual areas of pigeons during the first 9 days after hatching. In addition, in order to investigate the possible role of incoming retinal axons on the distribution of GABA-immunoreactive (GABA<sub>ir</sub>, i.e. containing GABA-LI) cells and processes, we have also studied the pattern of GABA-LI in the optic tectum (TeO) of adult pigeons which had undergone unilateral retinal removal immediately after hatching (i.e. when axonal invasion of tectal layers was not yet completed; see Bagnoli et al., 1987).

## Methods

### Materials

Materials used were as follows: Chloral hydrate (Codex, C. Erba, Milano, Italy), goat anti-mouse IgG (H+L; Kirkegaard and Perry, Gaithersburg, MD), mouse peroxidase-antiperoxidase (PAP) complex (Sternberg-Meyer, Jarrettsville, MD), and mouse monoclonal anti-GABA antibody, mAb 3A12, as described by Matute and Streit (1986).

### Animals, retinal removal, fixation procedures

Twenty eight pigeons (*Columba livia*) ranging in age from 6 h to 9 days were used in this study. Since differences in GABA-LI were found to depend not only on the posthatching age but also on the duration of the incubation *in ovo*, we used animals which had an incubation period of 18–20 days, unless otherwise stated. Eight adult pigeons were used, four of which had been subjected to the removal of the left retina immediately after hatching. In addition, two pigeons aged 3 weeks were also used. Of these two pigeons, one was normal and the other had the left retina removed immediately after hatching. Retinal removal was performed following a procedure previously described (Bagnoli et al., 1989a). Briefly, after local infiltration of Novocain in the orbital tissue, a thin glass cannula was inserted through the corneal surface and the optical media as well as the underlying retina were aspirated under microscopic control. The eye was then stuffed with a small cotton-wool ball, and the pigeons were

returned to their nest. The completeness of retinal ablation was examined at the time when the brains were removed from the skull. In two pigeons, the operated eyes were dissected from the skull, incised in the equatorial plane, placed in 10% formalin, processed for paraffin embedding, and serially sectioned in the transverse plane at 8  $\mu$ m. Sections were then mounted on plastic-coated glass slides and alternately stained with Bodian and toluidine blue. Examination under microscopic view revealed no residual retinal tissue.

All pigeons were deeply anesthetized with chloral hydrate (20% in physiological solution, 0.25 ml/100 g body weight) and transcardially perfused with a 6% Rheomacrodex solution followed by the fixative.

Various fixatives were applied, namely: glutaraldehyde alone; glutaraldehyde in combination with formaldehyde; formaldehyde alone. Table 1 shows which fixatives were applied at which stages of development.

The removed brains were postfixed in the same solution used for the perfusion (3–5 h) and then stored in phosphate-buffered saline (PBS) at 4°C. Brains were enclosed in 20% gelatin and coronally cut on a vibratome (Oxford, Model E) at 30  $\mu$ m. Floating vibratome sections at every 90  $\mu$ m were processed for immunohistochemistry. Sections adjacent to those processed for immunocytochemistry were stained with cresyl violet.

Retinal tissue was rapidly removed from perfused pigeons and postfixed overnight at 4°C. To remove the retina, the eyes were enucleated and hemisected at the level of the ora serrata to remove cornea, iris, and lens. The vitreous humor was carefully aspirated in a Petri dish containing fixative solution, and the retina was peeled from the choroid. After cutting the pecten at its base, the retina was separated from the pigment layer by a fine brush. The apex of the pecten was taken as a landmark to identify the laterally located fovea centralis which was investigated immunohistochemically at all developmental stages indicated above. Retinal specimens were dehydrated in 2,2-dimethoxypropane (Muller & Jacks, 1975) and embedded flat in Epon 812. Semithin (1  $\mu$ m) sections were mounted on gelatinized slides. Some of these sections were stained with toluidine blue. For immunohistochemistry, sections were etched for 7 min in a mixture of methanol, propylene oxide, and potassium hydroxide (Maxwell, 1978) and washed in methanol, methanol/PBS, and PBS.

**Table 1.** Experimental parameters: number of animals, stages, and fixation procedures<sup>a</sup>

Fixation	Number of animals at each stage						
	6 hours	3 days	4 days	6 days	9 days	21 days	180 days
5% GA <sup>b</sup>	1	1	--	1	1	--	--
2.5% GA	1	1	--	1	1	--	--
2.5% GA + 2.5% FA <sup>c</sup>	3	3	2	3	3	2	8
4% FA	1	1	--	1	1	--	--

<sup>a</sup>All fixatives were diluted in phosphate buffer 0.1 M, pH 7.4. The time of hatching was defined with a precision of  $\pm 2$  h.

<sup>b</sup>GA = glutaraldehyde.

<sup>c</sup>FA = formaldehyde freshly generated from paraformaldehyde.

### Tissue processing

The immunohistochemical staining procedure was similar to that applied in an investigation of adult pigeon brains (Domenici et al., 1988). Thus, in all experiments, three washes of 5 min each followed the incubation with antibodies. Antibodies were diluted in a solution containing 10% normal bovine serum in PBS. This solution was also used prior to the application of antibodies. Briefly, the following incubations were performed on vibratome and semithin sections (at room temperature unless otherwise stated): overnight at 4°C in mAb 3A12 diluted 1:8000 for vibratome sections and 1:1000 for semithin sections; 1 h in goat anti-mouse IgG antibody diluted 1:50 for vibratome sections and 1:20 for semithin sections; and 1 h in mouse peroxidase-antiperoxidase (PAP) complex diluted 1:100. The production and characterization of the "anti-GABA" antibody, mAb 3A12, used in the present study had been described elsewhere (Matute & Streit, 1986). Briefly, the immunological properties of mAb 3A12 were determined in an enzyme-linked immunosorbent assay (ELISA). The antibody showed a high degree of preference of GABA amongst a series of amino acids conjugated to bovine serum albumin (BSA) by means of glutaraldehyde. Furthermore, mAb 3A12 revealed patterns of immunoreactivity in rat brain tissue which were consistent with available information on the localization of neurons using GABA as neurotransmitter (Matute & Streit, 1986).

To reveal the sites of immunoreactivity, sections were treated with 0.05% 3,3'-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO) and 0.01% H<sub>2</sub>O<sub>2</sub> in 0.05 M Tris buffer (pH 7.6) for 4–6 min. Sections were then washed in PBS and rinsed in water. Floating vibratome sections were mounted on gelatinized slides. All sections were dehydrated and coverslipped with Eukitt. To increase the penetration of the primary antibody, alternate vibratome sections were incubated in a solution in which 0.1% Triton X-100 had been added to the primary antibody.

In several experiments, the specificity of the immunostaining was tested using different procedures, namely: (1) mAb 3A12 containing ascitic fluid diluted 1:1000 was preincubated with GABA-BSA-glutaraldehyde complex (6–6000 µg/ml) for 2 h at room temperature and then applied to the sections to test for inhibition of immunostaining; (2) 4% formaldehyde alone was used in order to test whether mAb 3A12 reacted with antigenic sites on molecules fixed by formaldehyde; and (3) in all experiments some sections were incubated with 10% normal bovine serum in PBS instead of the primary antibody and then processed as described above.

### Quantitative analysis

Quantitative evaluations were performed from coronal sections of the different visual regions at corresponding anterior–posterior levels of brains from different age groups. Retina coronal sections were also used. Sections were examined with a Zeiss microscope equipped with 5× (n.a. = 0.08) and 80× (n.a. = 0.65) (plan) objectives, respectively, and with 10× (plan) oculars. A video camera (MW-V3E Panasonic, Osaka, Japan) was attached to the microscope and interfaced with the computer-assisted image analyzer. The images were automatically digitized, and a digitizing tablet supplied with a pen to manipulate a screen sensor was used to manually determine the boundaries of the analysis field as well as to mark, trace, and count

the cells. According to the microscope objectives, spatial measurements were calibrated to any scale (e.g. µm, mm) with one simple command and the system read directly in the calibration units. The size of visual nuclei was determined by measuring their rostrocaudal axis and volume. The rostral and caudal borders of the visual regions were assessed by determining the sections in which the first and the last labeled cells were present. The volume of visual regions was determined by integrating their area measurements from sections that contained labeled cells. The number of sections ranged from 7–11 per animal, according to the different areas. Ten area measurements in the same section on consecutive days revealed a measuring error of 1.57% (S.E.M. expressed as percentage of the mean value). Both the soma size (expressed as cross-sectional area in square micrometers) and the shape factor for labeled cells was determined. The shape factor is a two-dimensional index which is indicative of round shaped cell bodies when approaching 1. Fifty cells that had at least one nucleolus were measured in each region to obtain the soma size and shape factor for each animal. In addition, the number of cells (either labeled or unlabeled) per cubic millimeter of tissue (numerical density, *N<sub>v</sub>*) was determined by using stereological methods (O'Kusky & Colonnier, 1982; Weibel & Bolender, 1973; Weibel & Gomez, 1962). Six to nine sections for each region were used to obtain *N<sub>v</sub>* for each animal. The percentage of labeled cells was calculated by measuring the ratio of labeled cell *N<sub>v</sub>* to total cell *N<sub>v</sub>*. Total cell *N<sub>v</sub>* was obtained from cresyl-violet stained sections.

In the retinal ganglion cell layer, the soma size of labeled and unlabeled cells as well as shape factor and *N<sub>v</sub>* of labeled cells were also determined.

### Statistical analysis

Statistical analysis was performed by using Crunch Statistical Package (Crunch Software Corp., Oakland, CA). The effect of the independent variable (age) on dependent variables was assessed using the analysis of variance (ANOVA). The Student–Newman–Keuls (SNK) multiple range test (0.05 level of significance) was used to identify difference between age groups for each variable in each visual region.

### Abbreviations

For the abbreviations used in the photomicrographs of transverse sections of the pigeon brain the reader should refer to the stereotaxic atlas of Karten and Hodós (1967). Denomination of tectal layers are taken from Cowan et al., 1961.

- FRL = formatio reticularis lateralis mesencephali
- GLv = n.geniculatus lateralis, pars ventralis
- Imc = n.isthmi, pars magnocellularis
- Ipc = n.isthmi, pars parvocellularis
- LA = n.lateralis anterior thalami
- nBOR = n.basal optic root
- PT = n.pretectalis
- QF = tractus quinto frontalis
- Rt = n.rotundus
- SAC = stratum album centrale
- SGC = stratum griseum centrale
- SOP = stratum opticum centrale
- TeO = optic tectum
- TrO = tractus opticus
- VLT = n.ventrolateralis thalami



## Results

### Specificity of antibodies

According to Domenici et al., 1988, no patterns of immunoreactivity could be detected in formaldehyde-fixed tissue. Preincubation of mAb 3A12 with increasing amount of GABA-BSA (6–30  $\mu\text{g/ml}$ ) before its application to sections gradually diminished the immunostaining and made it to disappear completely at 30  $\mu\text{g/ml}$ , as previously reported (Domenici et al., 1988). There were no indications of immunostained cell bodies and processes in control sections in which the primary antibody had been omitted. Moreover, addition of 0.1% Triton X-100 to the solution containing the primary antibody consistently caused a decrease in immunostaining in cell bodies and an increase in the number of stained processes as compared to those obtained in sections incubated without this detergent.

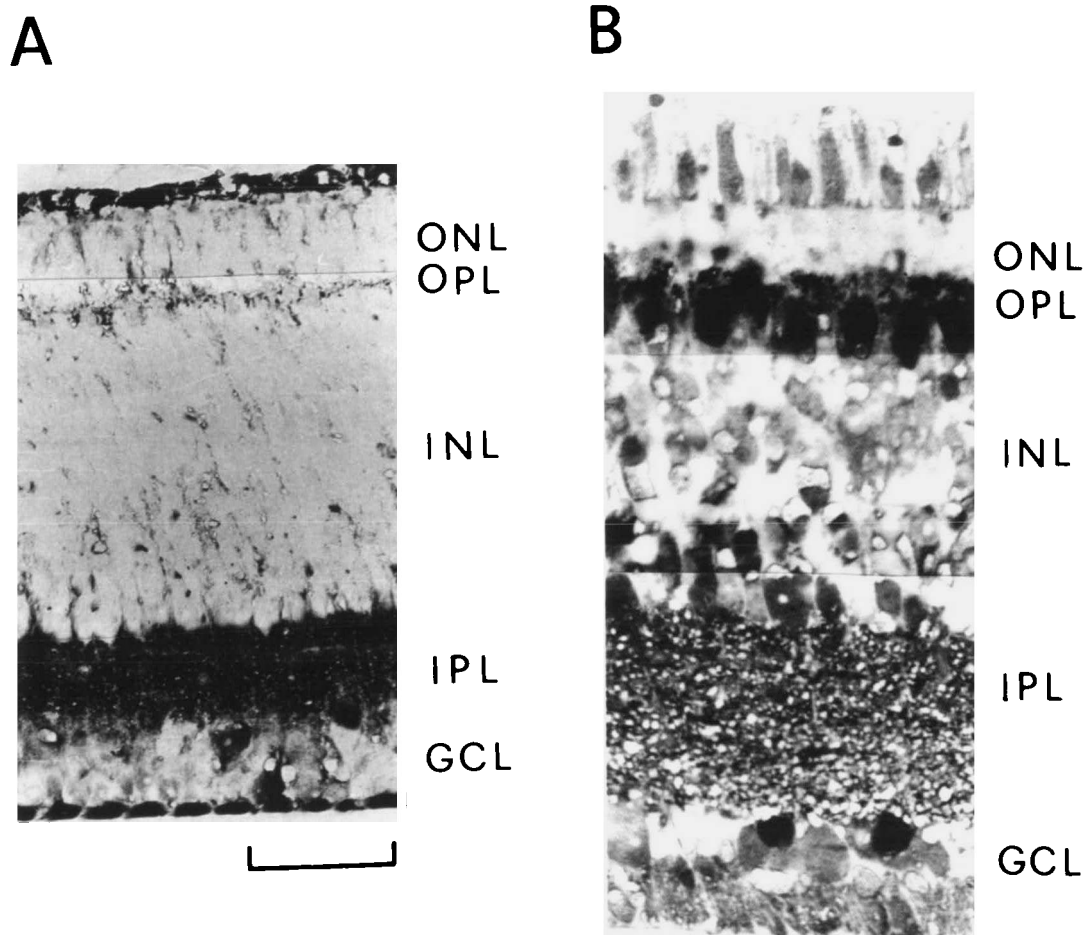
### Distribution of GABA-LI during the early posthatching period

Comparable patterns of immunoreactivity were found in brain material treated with different percentages of glutaraldehyde as

well as glutaraldehyde added to formaldehyde. In this material, a similar distribution of GABA-LI was obtained in the visual areas of animals belonging to the same age group independently of the fixative used. According to previous studies (Domenici et al., 1988; Matute & Streit, 1986), brain materials fixed with either 2.5% or 5% glutaraldehyde did not show nonspecific staining at the antibody dilution used in this study. Tissue sections for brains fixed with glutaraldehyde added to formaldehyde consistently displayed lower levels of background staining. Thus, results reported below refer to glutaraldehyde-formaldehyde fixed materials.

### Retina

The inner plexiform layer (IPL) of newly hatched pigeons contained densely packed GABA<sub>A</sub> processes which occupied most of this area (Fig. 1A). Labeling in IPL was rather uniform and did not display any lamination. Weakly immunopositive terminal-like elements were also present in both the innermost portion of the inner nuclear layer (INL) and the outer plexiform layer (OPL). Except for some GABA<sub>A</sub> perikarya which were consistently present in the ganglion cell layer (GCL), no labeled cell bodies could be observed. Prominent staining of ovoid pro-



**Fig. 1.** GABA immunohistochemistry of pigeon retina at two stages after hatching. A, at hatching and B, at 6 days. Stained processes appear throughout the inner plexiform layer (IPL) in both cases. No stained cells are present at hatching except for some GABA<sub>A</sub> cell bodies in the ganglion cell layer (GCL). Numerous GABA<sub>A</sub> amacrine cells in the inner nuclear layer (INL) and presumed horizontal cells which lie along the outer margin of INL are additionally present at 6 days. ONL: outer nuclear layer; and OPL: outer plexiform layer. Scale bar = 25  $\mu\text{m}$ .

files at the inner limiting membrane might be tentatively related to internal feet of Müller cells.

The retinal pattern of GABA-LI did not change significantly in the period to the third day after hatching, when GABA<sub>A</sub>IR cells began to appear in INL. Thus, at around 6–9 days (Fig. 1B), the pattern of GABA-LI was almost comparable to that observed in adult pigeons. Amacrine cell bodies situated in the innermost portion of INL as well as horizontal cells along the outer margin of INL were found to contain immunoreactivity. In addition, GABA-LI was associated with cells in GCL but not with photoreceptors nor with Müller cells. Some labeled terminal-like elements were observed in OPL and the innermost portion of INL. Dense GABA-LI was also present in IPL where clearly definable bands of immunoreactivity became evident at the adult stage. The pattern of GABA-LI in the adult retina was comparable to that previously shown (Agardh et al., 1987; Karten & Brecha, 1983; Osborne, 1987).

At the retinal level, the analysis of variance showed a significant effect of age ( $P < 0.05$ ) on  $N_V$  of labeled cells in GCL. No significant effect on soma size and shape factor was found. As shown in Fig. 2,  $N_V$  gradually increased from hatching to the adult stage (+85%;  $P < 0.001$ ), whereas no significant changes were observed in the soma size which was similar to that of unlabeled cells.

#### Central visual areas

The main primary visual regions at diencephalic and mesencephalic levels as well as the nucleus pretectalis (PT) and the nucleus isthmi, pars parvocellularis (Ipc), were examined for possible changes in the distribution of GABA-LI during the first 9 days after hatching. In all these regions, GABA<sub>A</sub>IR perikarya and processes could be observed immediately after hatching, and their distribution changed during the following 9 days when the adult pattern was reached. In general, numerous GABA<sub>A</sub>IR

round-shaped cells were present at hatching. Their density decreased progressively with a parallel increase in neuropil immunostaining. Cell size generally increased whereas cell morphology did not appear to change significantly except in PT where round-shaped cells found at hatching became ovoid during the following days.

For most regions examined critical alterations occurred between 3–4 and 6 days. Indeed, the distribution of GABA-LI did not change from hatching to 3–4 days and from 6–9 days when the adult pattern was reached. Since no discernible differences in the distribution of GABA-LI were found between 3- and 4-day-old pigeons, only the pattern of GABA-LI obtained at 3 days of age will be described.

In the rostro-dorsal thalamus, at hatching, the nucleus lateralis anterior thalami (LA) displayed numerous small stained cells (Fig. 3A). During the following week, neuropil labeling increased whereas the density of labeled cell bodies was found to be reduced (Fig. 3B). Similar temporal changes were also observed in other visual thalamic nuclei, such as the nucleus dorsolateralis anterior thalami, pars magnocellularis (DLAmc), and the nucleus dorsolateralis anterior thalami (DLL). In contrast,

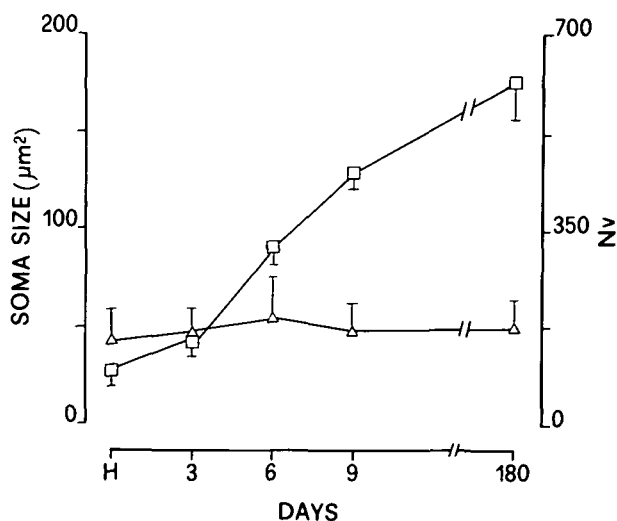


Fig. 2. Size (cross-sectional area in square micrometers, open triangles) and numerical density  $N_V$  (number of cells  $\times 10^3/\text{mm}^3$  of tissue, open squares) of labeled cell bodies in the retinal ganglion cell layers at different stages. Each point represents the mean  $\pm$  s.d. Five pigeons from hatching to 9 days and four 180-day-old pigeons were used. H: hatching.

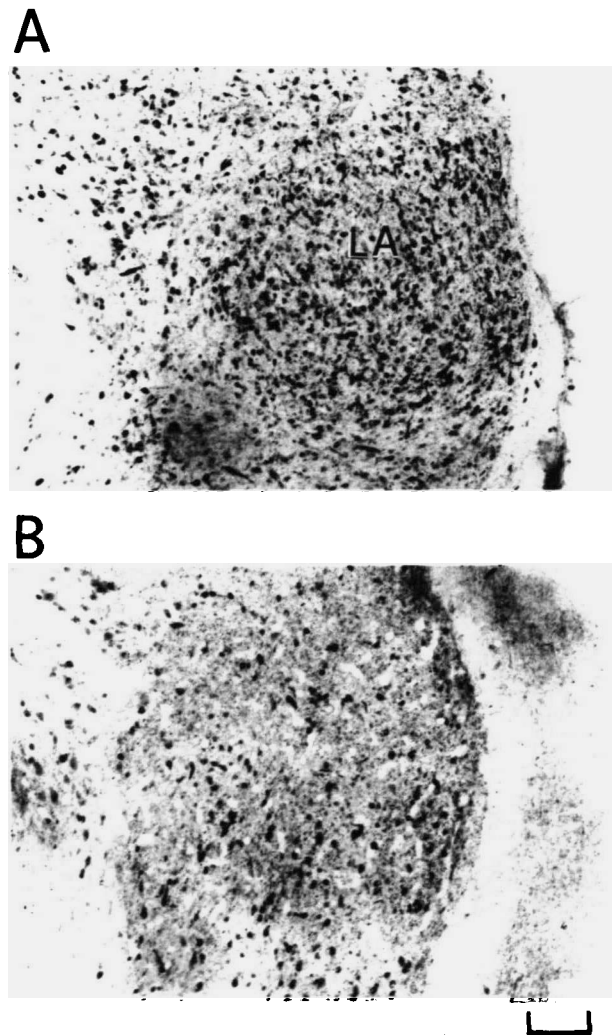
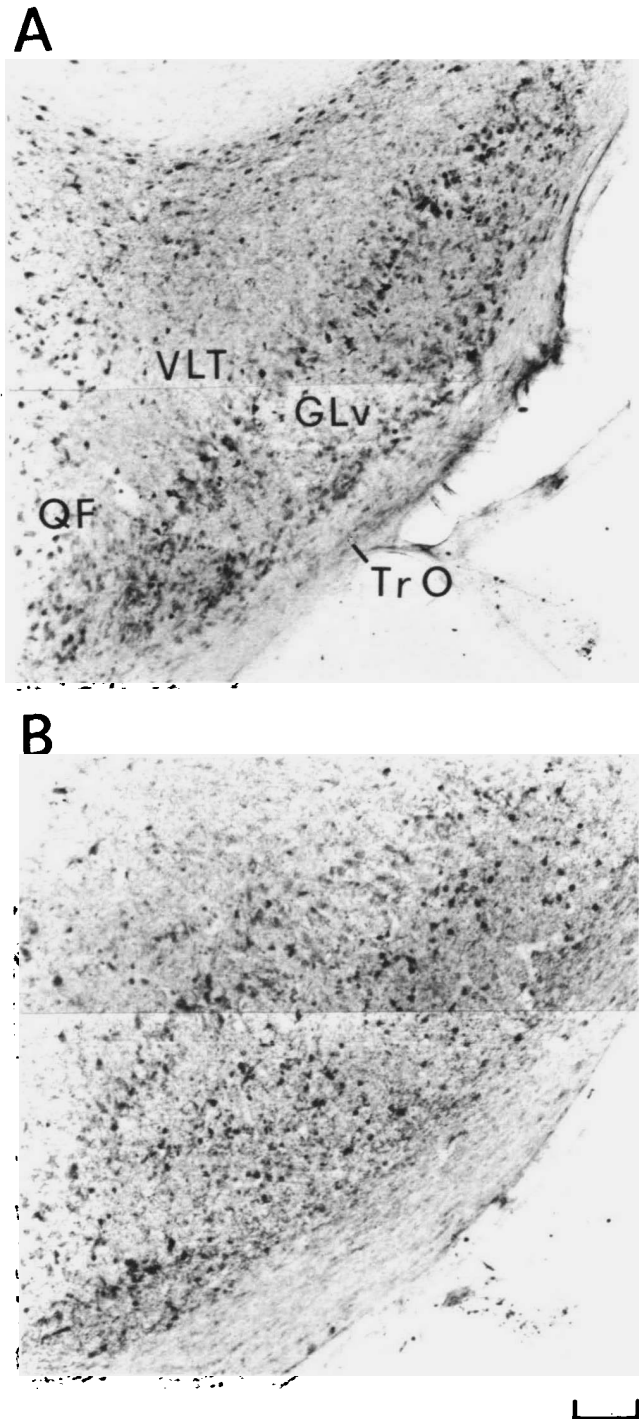


Fig. 3. GABA-LI in the rostro-dorsal thalamus showing the nucleus lateralis anterior thalami (LA). Note the increased neuropil labeling from hatching (A) to 6 days (B). Labeled cells reduce their density from hatching to 6 days. Scale bar = 100  $\mu\text{m}$ .



the nucleus rotundus (Rt) was free of labeled perikarya and neuropil.

In the ventro-medial thalamus, at hatching, small, weakly labeled cell bodies together with faintly stained neuropil were

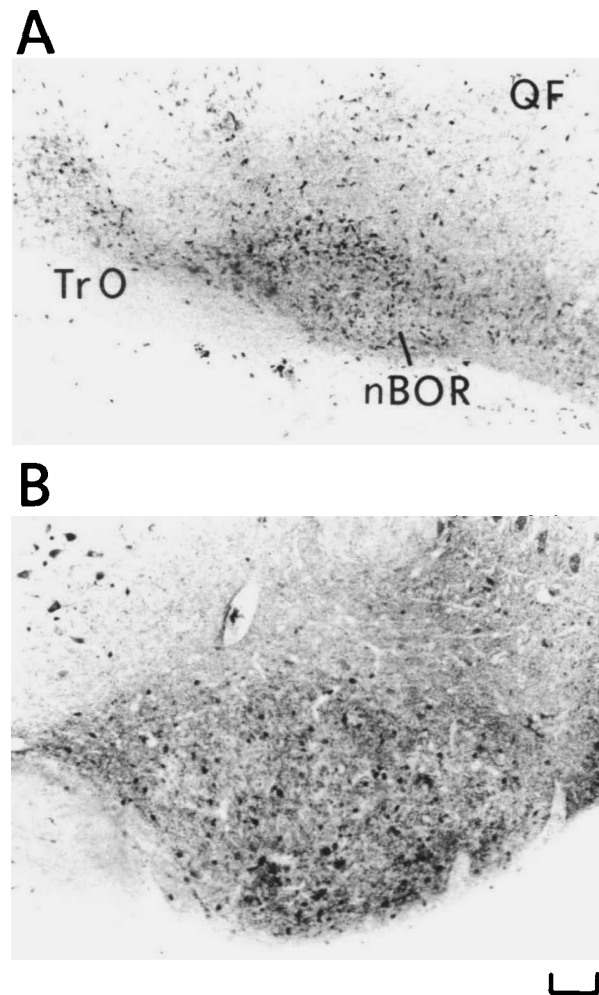


**Fig. 4.** GABA-LI in the ventro-medial thalamus showing the nucleus geniculatus lateralis, pars ventralis (GLv). Note changing distribution of GABA-LI from hatching (A) to 6 days (B) when heavily labeled cells appear in the central portion of the nucleus and a general increase in neuropil labeling can be seen. VLT = n. ventrolateralis thalami; QF: tractus quinto frontalis; and TrO: tractus opticus. Scale bar = 100  $\mu$ m.

present within the internal region of the nucleus geniculatus lateralis, pars ventralis (GLv). In contrast, the external region of GLv displayed many densely labeled cells together with numerous GABAir processes (Fig. 4A). At 6 days, heavily labeled small cells were seen scattered throughout GLv and a general increase in neuropil labeling could be observed (Fig. 4B). A further increase in neuropil labeling and a decrease in GABAir cell density was found at 9 days when the adult cytoarchitecture was reached. At the level of the thalamus, neither the optic tract nor the optic nerve showed GABA immunoreactivity at any age.

At the level of the mesencephalon, the main nuclei receiving direct retinal input showed GABAir cells and processes immediately after hatching. In general, GABA-LI varied during the first 9 days with a decreased density of stained cells and an increased neuropil labeling. This tendency is typically shown by the nucleus of the basal optic root (nBOR) presented in Fig. 5.

A similar tendency with respect to changes in perikaryal and neuropil labeling was observed in secondary visual regions of



**Fig. 5.** GABA-LI of the mesencephalon showing the nucleus of the basal optic root (nBOR). At hatching, numerous labeled cells are present (A), whereas at 9 days they appear sparsely distributed (B). Additionally, an increase in the density of labeled neuropil can be observed. QF: tractus quinto frontalis; and TrO: tractus opticus. Scale bar = 100  $\mu$ m.

the mesencephalon, namely, in PT (Fig. 6) and Ipc (Figs. 7 and 8) which have been shown to be reciprocally connected with the optic tectum (TeO) (Hunt et al., 1977; Reiner & Karten, 1982). In particular, many stained cells could be observed at hatching in Ipc, which appeared surrounded laterally by numerous stained fibers of the stratum album centralis (SAC) of TeO (Fig. 7A). The density of stained cells appeared to have decreased at 6 days mainly in the rostral division of Ipc (Fig. 7B and Fig. 8A). As shown in Fig. 8B at 9 days, no staining of neuronal perikarya was found anymore.

In TeO, GABA-LI was observed in cell bodies and neuropil at all posthatching stages, but both the distribution of GABAir cells and the density of staining in the neuropil were found to vary with age, reaching the adult pattern at around 9 days (Fig. 9). Changes in the distribution of GABA-LI were particularly evident in the tectal layers above lamina Iii as well as in layer III. The distribution of GABAir cells and processes in layers Iii and IIj was, at all ages, similar to that found in the adult pigeon. In particular, many stained cell bodies giving off

radially oriented processes could be observed in layer Iii. These processes could be more readily demonstrated in sections incubated with a solution containing the primary antibody and Triton X-100. From hatching to 9 days, three major changes in the pattern of GABA-LI occurred in the tectal layers above lamina Iii:

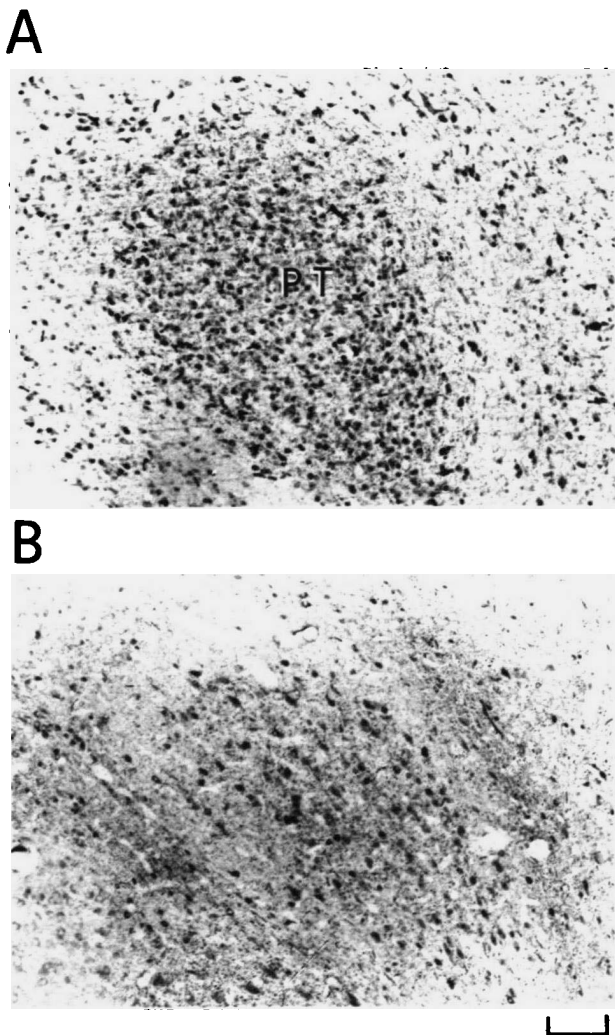
1. A progressive disappearance of stained cell bodies in that layer designated as  $L_1$  in Fig. 9A, which at 9 days was found to be occupied by unstained optic fibers (layer I in Fig. 9B). GABAir cell bodies of layer  $L_1$  could be more readily demonstrated in pigeons whose incubation period had been one week shorter than normal. As shown in Fig. 10, numerous labeled cells with associated horizontal processes embedded within a network of tangentially and radially running fibers were observed in the superficial TeO.
2. An increase in the density of neuropil labeling in the most superficial layers (designated as  $L_2$  in Fig. 9A), which by the age of 9 days have developed into sublayers Iia-c (Fig. 9B).
3. A progressive decrease in the density of labeled cell bodies in lower layers. Decrease in cell density was particularly evident in those layers (designated as  $L_3$  in Fig. 9A), which at 9 days presumably corresponded to lamina Iid (Fig. 9B) as well as in laminae Iie-h. At 9 days, laminae Iid-h showed few immunoreactive cells (Fig. 9B). In layer III, a progressive decrease in density of GABAir cells was evident from hatching to 9 days when few labeled cell bodies were detectable.

In Fig. 11, the adult distribution of GABA-LI is shown at the level of the same structures examined during the first 9 days after hatching. Their cytoarchitecture was comparable to that found at 6-9 days and is in agreement with that previously described by Domenici et al., 1988.

In the central visual regions examined, the analysis of variance showed a significant effect of age ( $P < 0.05$ ) on most dependent variables, i.e. rostrocaudal axis, volume,  $N_v$ , soma size, and percentage of stained cells. As to the shape factor of stained cell bodies, the effect of age was significant only in PT ( $P < 0.05$ ).

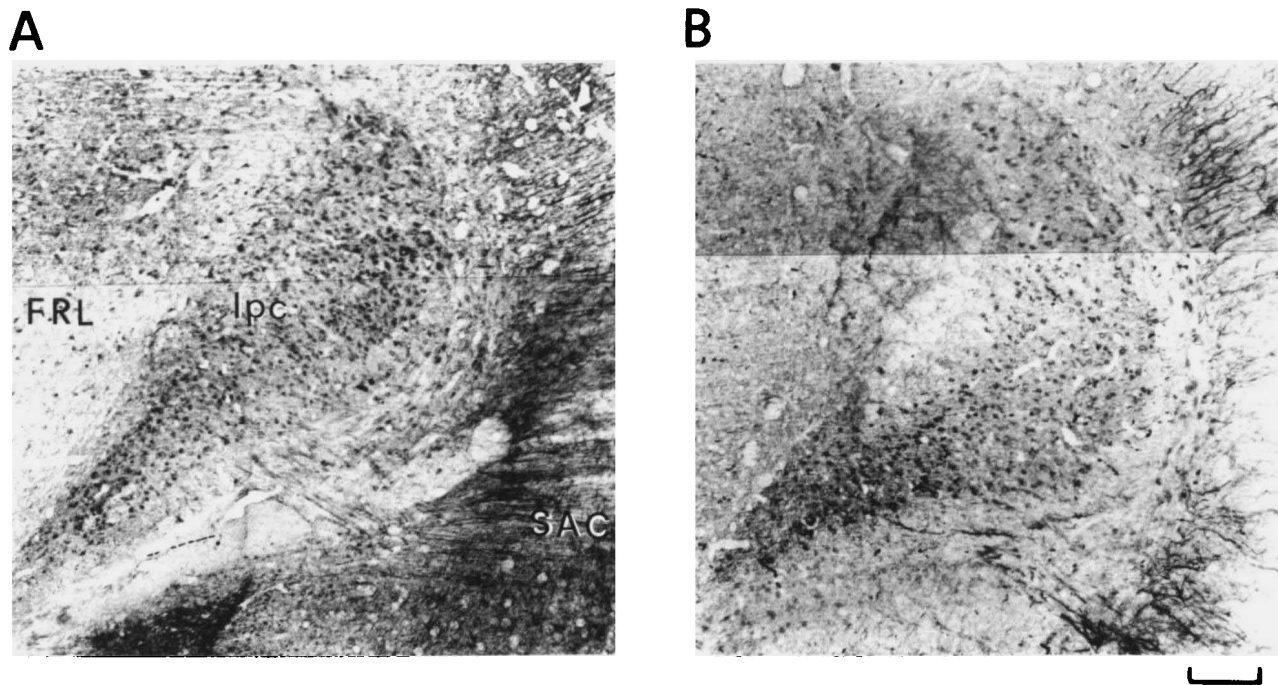
As shown in Table 2, rostrocaudal axis and volume gradually increased from hatching to the adult stage. The maximum increase in volume was shown by Ipc (+135%; difference between hatching and adult stage:  $P < 0.005$ ). Comparable differences between hatching and adult stage were observed in the other regions (PT: +68%; GLv: +64%; nBOR: +38%; LA: +36%; TeO: +68%;  $P < 0.01$ ). The maximum increase in rostrocaudal axis was observed in Ipc (+248%; difference between hatching and adult stage:  $P < 0.001$ ), whereas the minimum increase was shown by PT (+66%;  $P < 0.01$ ).

As shown in Fig. 12 in all regions examined, stained cell size gradually increased from hatching to 6-9 days. Soma-size increase ranged between a value of +20% found in TeO (difference between hatching and 9 days:  $P < 0.01$ ) and a value of +35% measured in LA (difference between hatching and 9 days:  $P < 0.001$ ). Generally, the number of labeled cells (Table 3) and  $N_v$  (Fig. 12) drastically decreased from hatching to 6 days when the adult value was reached. A further decrease both in the number of labeled cells and  $N_v$  was observed in TeO and GLv between 6 and 9 days ( $P < 0.05$ ). The highest decrease (difference between hatching and 6 days:  $P < 0.05$ ) in the number of



**Fig. 6.** GABA-LI of the mesencephalon showing the nucleus preteectalis (PT). Again, a progressive decrease in density of labeled cells with a parallel increase of stained neuropil can be observed from hatching (A) to 9 days (B). Scale bar = 100  $\mu\text{m}$ .





**Fig. 7.** Distribution of GABA-LI at the level of another mesencephalic nucleus, namely, the nucleus isthmi, pars parvocellularis (Ipc). Note that from hatching (A) to 6 days (B), stained cells appear to decrease in density mainly in the rostral part of the nucleus which is surrounded by numerous GABAir fibers of the tectal stratum album centrale (SAC). FRL: formatio reticularis lateralis mesencephali. Scale bar = 200  $\mu$ m.

**Table 2.** Volume and rostrocaudal axis of the central visual regions at different stages<sup>a</sup>

Visual regions	Volume (mm <sup>3</sup> )				Rostrocaudal axis (mm)			
	6 hours	6 days	9 days	180 days	6 hours	6 days	9 days	180 days
PT	0.178 ± 0.035	0.245 ± 0.030	0.255 ± 0.030	0.300 ± 0.057	0.450 ± 0.047	0.523 ± 0.083	0.600 ± 0.110	0.750 ± 0.135
GLv	0.206 ± 0.039	0.255 ± 0.043	0.286 ± 0.057	0.339 ± 0.055	1.024 ± 0.174	1.169 ± 0.240	1.475 ± 0.192	2.250 ± 0.360
Ipc	0.166 ± 0.030	0.223 ± 0.051	0.319 ± 0.048	0.393 ± 0.055	0.575 ± 0.113	0.983 ± 0.168	1.475 ± 0.233	2.000 ± 0.183
nBOR	0.096 ± 0.020	0.115 ± 0.014	0.127 ± 0.010	0.132 ± 0.010	0.625 ± 0.112	0.753 ± 0.093	0.825 ± 0.013	1.500 ± 0.285
LA	0.125 ± 0.030	0.154 ± 0.035	0.165 ± 0.018	0.170 ± 0.010	0.250 ± 0.045	0.380 ± 0.007	0.500 ± 0.092	0.750 ± 0.112
TeO	28.215 ± 6.053	33.623 ± 4.620	40.006 ± 6.930	42.600 ± 4.616	3.600 ± 0.530	4.103 ± 0.612	4.825 ± 0.372	5.520 ± 0.415

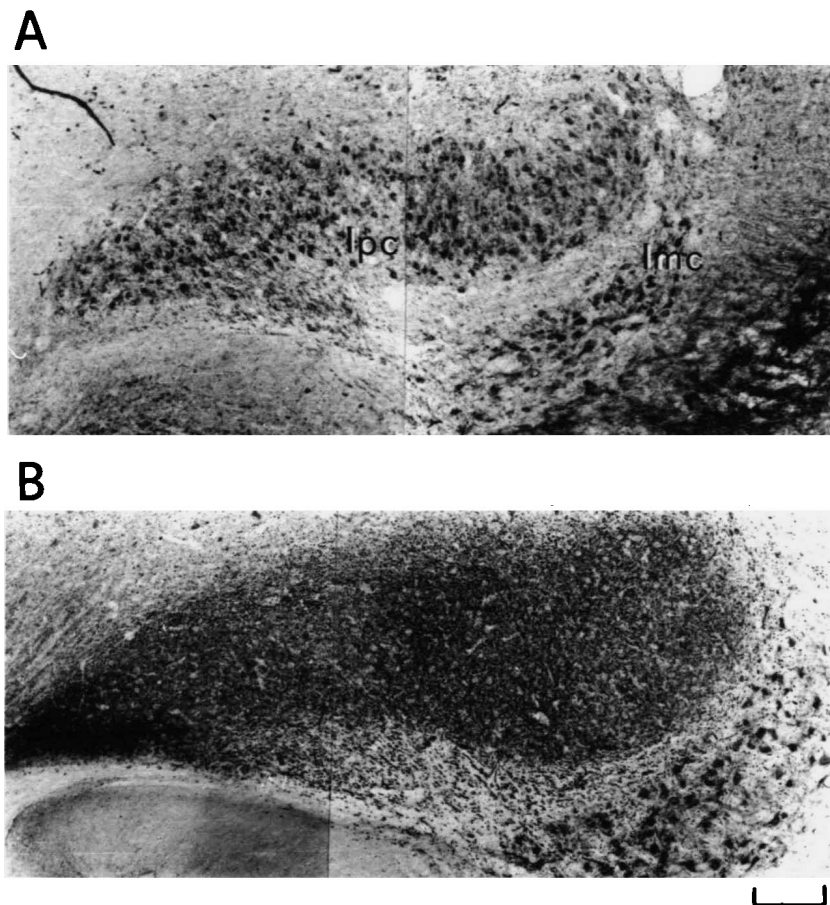
<sup>a</sup>Values are expressed as mean ± s.d. Five 6-hour-, 6-day-, 9-day-old pigeons, and four 180-day-old pigeons were used.

**Table 3.** Number of labeled cells ( $\times 10^3$ ) in central visual regions at different stages<sup>a</sup>

Visual regions	6 hours	6 days	9 days	180 days
PT	21.360 ± 0.360	2.940 ± 0.360	2.860 ± 0.494	2.400 ± 0.456
GLv	30.488 ± 5.772	15.300 ± 2.580	8.580 ± 1.710	8.475 ± 1.375
Ipc	20.750 ± 3.750	4.125 ± 0.943	0	0
nBOR	8.832 ± 1.840	4.312 ± 1.525	4.700 ± 0.112	4.880 ± 0.370
LA	19.000 ± 4.560	6.468 ± 1.470	6.600 ± 0.792	6.800 ± 0.400
TeO	16,026.120 ± 3,438.104	8,809.226 ± 1,210.440	2,400.360 ± 415.800	2,470.800 ± 267.728

<sup>a</sup>Values are expressed as mean ± s.d. Four pigeons for each age were used. Number of labeled cells was calculated by multiplying labeled cell *N<sub>v</sub>* by visual region volumes reported in Table 2.





**Fig. 8.** GABA immunohistochemistry of the nucleus isthmi, pars parvocellularis (Ipc) at 6 (A) and 9 (B) days after hatching (at a different level as compared to that shown in Fig. 7). (At this level, stained cells of the nucleus isthmi, pars magnocellularis (Imc) can also be seen.) At 9 days, stained cells of Ipc are no longer present, whereas many labeled terminal-like dots can be observed. The density of labeled cells in Imc does not appear to change significantly. Scale bar = 200  $\mu\text{m}$ .

labeled cells and  $N_V$  was found in PT (–86%) and TeO (–89%), respectively. The lowest decrease (difference between hatching and 6 days:  $P < 0.01$ ) was found in TeO (–45%) for the number of labeled cells and in nBOR (–54%) for  $N_V$ .

As shown in Fig. 13, percentage of labeled cells as compared to total cell number generally decreased from hatching to 6 days. The maximum decrease was observed in PT. Comparable decreases were observed in GLv, LA, and TeO. No changes were found in nBOR and Ipc.

The shape factor generally did not significantly change except for PT where it decreased from hatching to the adult stage ( $P < 0.001$ ).

#### *Distribution of GABA-LI in the optic tectum of adult pigeons with early unilateral retinal removal*

Pigeons which had the left retina removed on the day of hatching were examined at the adult stage in order to determine the distribution of GABA-LI in the TeO contralateral to the operated eye and to compare it to that observed in the TeO contralateral to the persisting retina. The latter was almost indistinguishable from that found in normal animals of the same age (Fig. 11).

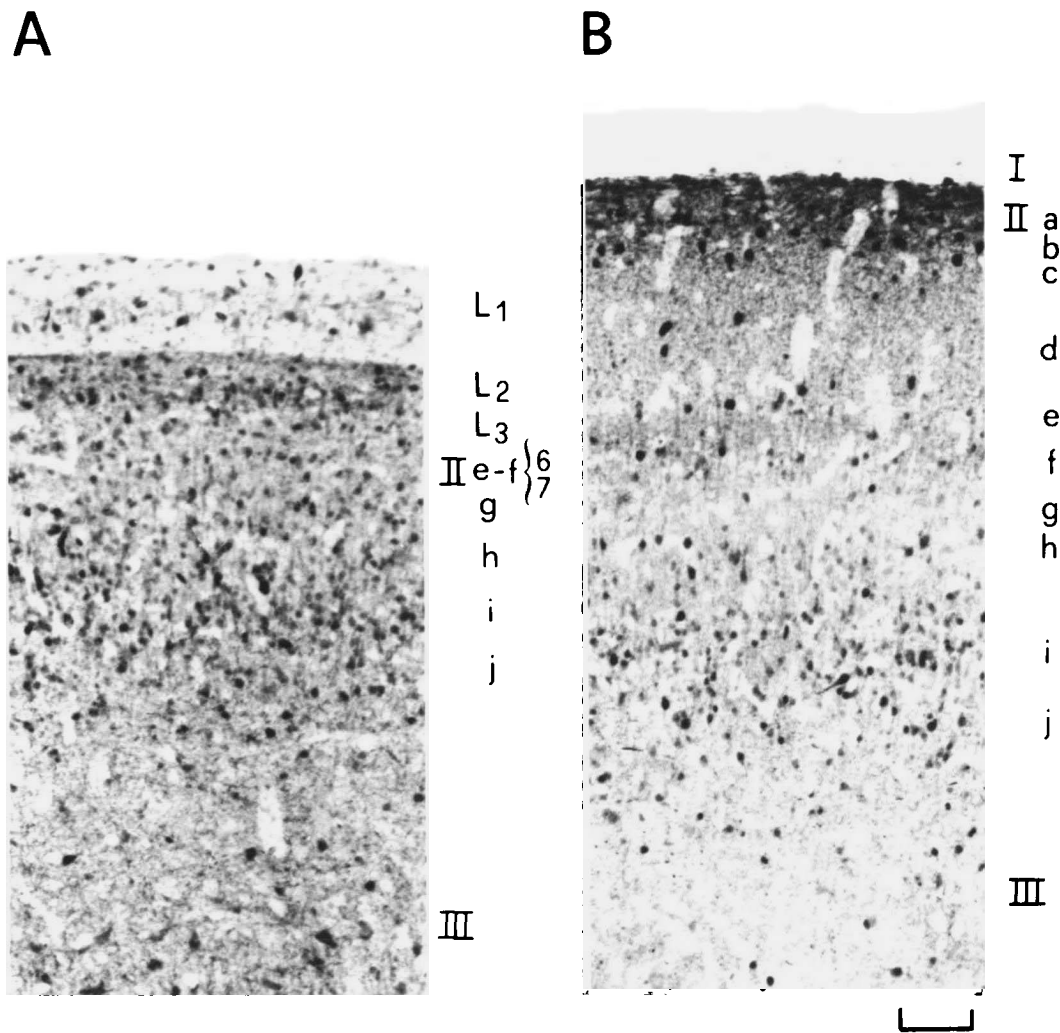
Differences in distribution of GABA-LI in the TeO contralateral to the removed retina were mainly evident in the tectal

layers above lamina III. As shown in Fig. 14, the main differences can be summarized as follows:

1. A very weak neuropil labeling and a presence of numerous small labeled cells in superficial layers (designated as  $L_2$  in Fig. 14A) which on the other side of the brain showed dense neuropil labeling and few labeled cells (laminae IIa–c in Fig. 14B).
2. A large number of GABA-positive cells in those layers ( $L_3$  and laminae IIe–h in Fig. 14A) which on the other side of the brain showed few immunoreactive cells (laminae IID–h in Fig. 14B). No major changes could be observed in layers III, IIj and III.

A similar distribution of GABA-LI was also observed after a shorter survival time, namely, in one pigeon which survived 3 weeks after retina ablation at hatching.

No significant differences in quantitative indices were found between the TeO contralateral to the persisting retina and the normal adult TeO. At the level of superficial tectal layers (from  $L_1$  to IIg in Fig. 14) contralateral to the operated eye, either the soma size or  $N_V$  (expressed by the filled square in Fig. 12) of labeled cells were similar to those found in the normal TeO at



**Fig. 9.** Distribution of GABA-LI in corresponding regions of the dorsolateral optic tectum (TeO) at hatching (A) and at 9 days after hatching (B). Main changes occur in the superficial tectal layers, namely: (1) disappearance of stained cells in that layer ( $L_1$  in A) which at 9 days is occupied by unstained optic fibers (layer I in B); (2) decrease in density of GABA<sub>A</sub> cell bodies in the tectal layers above lamina III; and (3) increase of the density of neuropil labeling in the most superficial laminae below layer I. Note that the pattern of GABA-LI at 9 days is similar to that found in adult pigeons. Scale bar = 50  $\mu$ m.

hatching. In Table 4, quantitative results from analysis of layers  $L_1$ ,  $L_2$ ,  $L_3$ , IIe-f, and IIg of the TeO contralateral to the removed retina as compared to the corresponding layers of the normal TeO at hatching are reported. For each layer, no significant differences in soma size and  $N_v$  were found between the TeO contralateral to the operated eye and TeO at hatching.

### Discussion

GABA-LI was observed in cell bodies as well as in neuropil and certain fiber tracts with a selective pattern of distribution which has been found to vary with posthatching ages. Main changes occur between 3 and 6–9 days when the adult pattern is reached. It generally corresponds to that previously described in corresponding areas of the adult pigeon brain (Domenici et al., 1988).

Before discussing in detail changes in GABA-LI distribution observed in visual areas during posthatching development, cer-

tain methodological aspects should be considered. With the antibody used in the present study, i.e. mAb 3A12, selective staining patterns had also been obtained in rats (Matute & Streit, 1986), insects (Meyer et al., 1986), and adult pigeons (Domenici et al., 1988). As shown by the present study, mAb 3A12 was able to reveal GABA<sub>A</sub> neurons in glutaraldehyde-fixed pigeon brains during posthatching development. Although information about the sensitivity to fixative during development are not presently available, the similar patterns of GABA-LI distribution obtained using either different percentages of glutaraldehyde or different fixatives at each stage seems to rule out the possibility that observed changes could be attributed to differential fixation sensitivity of GABA-like material.

According to previous results (Domenici et al., 1988), different patterns of immunoreactivity obtained following addition of Triton X-100 to the primary antibody might depend on either an increased permeability of the tissue or the loss of immunoreactive material, i.e. a decreased immunostaining in cell bodies.

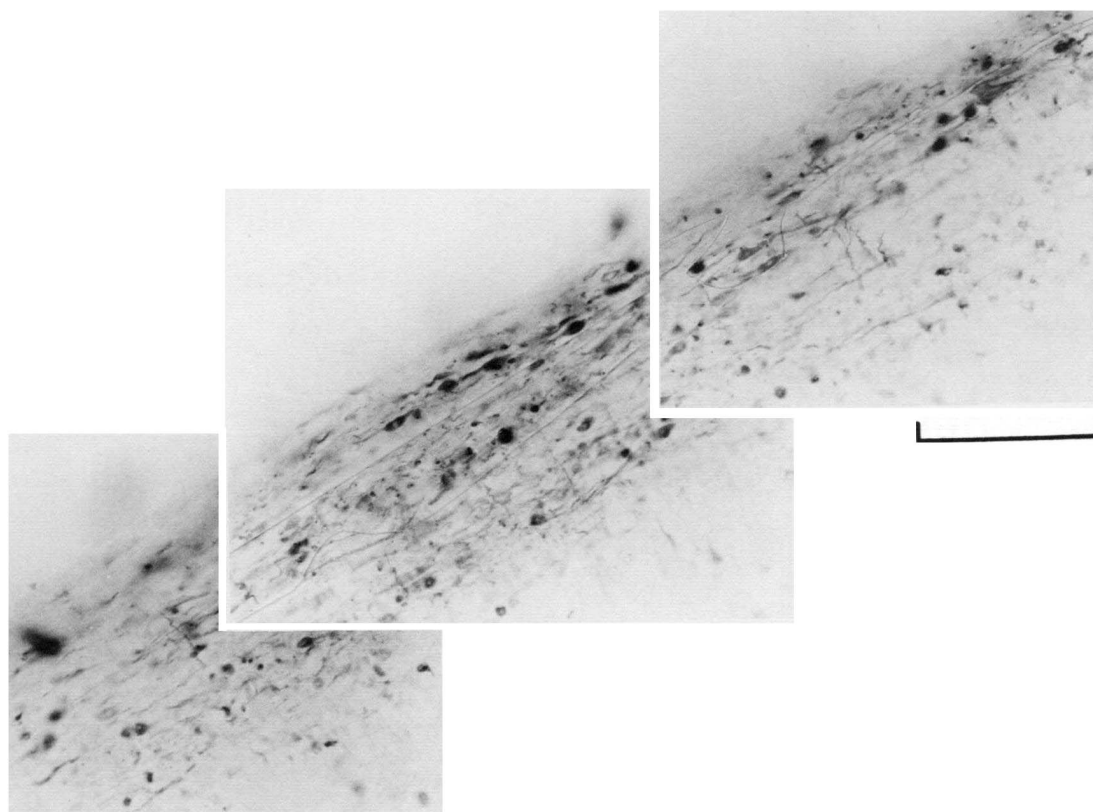


Fig. 10. GABAergic cells with horizontally oriented processes in the superficial optic tectum (TeO) of a pigeon hatched after an incubation period of 12 days *in ovo*. Scale bar = 100  $\mu\text{m}$ .

### Retina

Relative densities of GABAergic cell bodies and terminal-like elements differ strikingly according to the posthatching stage. The adult distribution of GABA-LI in the pigeon retina is reached in about 1 week after hatching. As shown by previous results (Bagnoli et al., 1985; Porciatti et al., 1985) over the same period, the pigeon retina completes its maturation, i.e. receptor outer segments as well as synapses in OPL reach their adult form and first retinal responses to light can be recorded.

The adult distribution of GABA-LI obtained in this study generally agrees with that previously described in either pigeons or other nonmammalian species with immunohistochemical methods (Agardh et al., 1987; Brandon, 1985; Brecha, 1983; Karten & Brecha, 1983; Mosinger et al., 1986; Osborne, 1987) as well as local uptake following [ $^3\text{H}$ ]-GABA application (Marshall & Voaden, 1974; Pourcho et al., 1984; Yazulla et al., 1986; Watt et al., 1984).

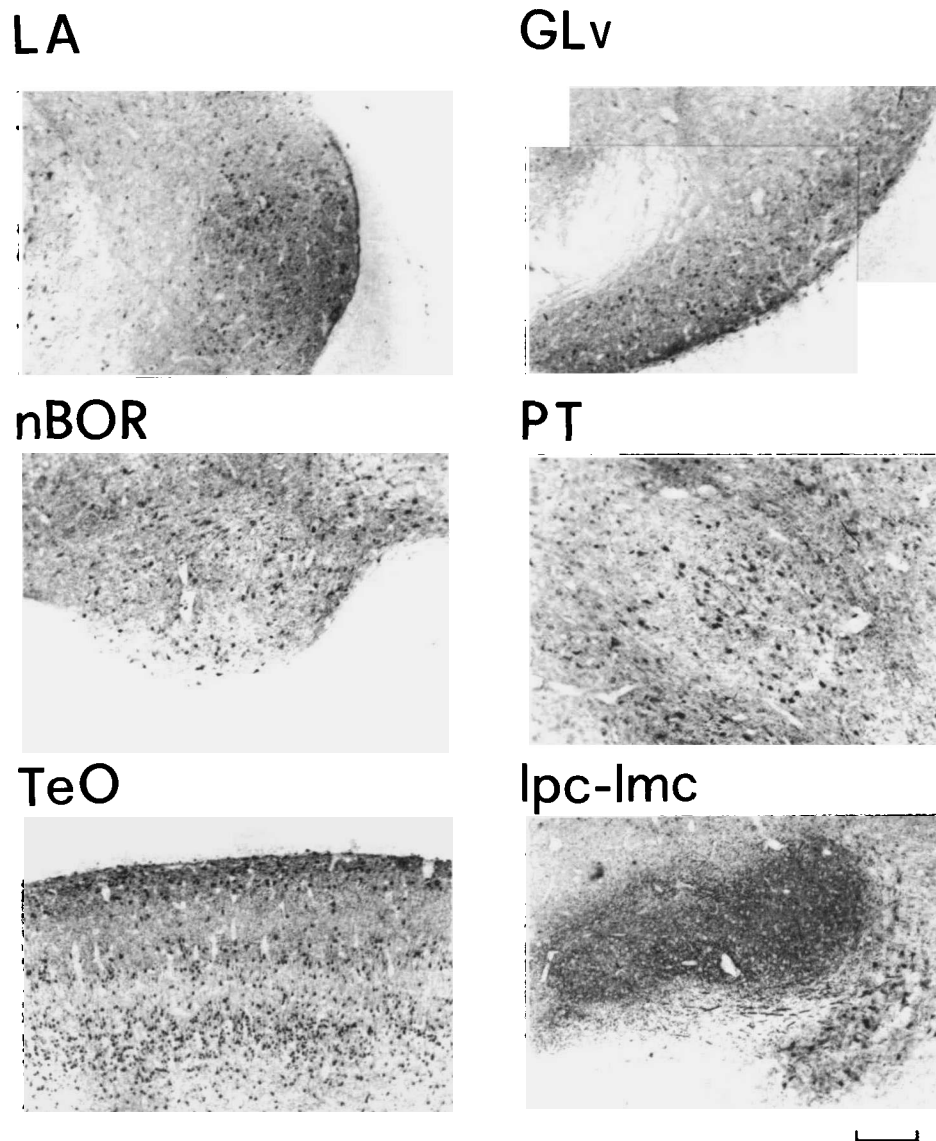
As shown by our results, numerous GABAergic perikarya are present in GCL and their density increase from hatching to

Table 4. Soma size and numerical density  $N_v$  of GABAergic cells in tectal layers at hatching and in the TeO contralateral to early unilateral retina removal<sup>a</sup>

Tectal layers	TeO at hatching		TeO contralateral to retinal removal	
	Soma size	$N_v$	Soma size	$N_v$
L <sub>1</sub>	37.10 $\pm$ 7.10	590.5 $\pm$ 14.5	40.91 $\pm$ 8.20	517.4 $\pm$ 77.6
L <sub>2</sub>	25.28 $\pm$ 8.36	925.3 $\pm$ 141.1	25.04 $\pm$ 8.02	1115.3 $\pm$ 287.9
L <sub>3</sub>	33.90 $\pm$ 4.95	1069.9 $\pm$ 76.7	29.63 $\pm$ 3.08	1248.3 $\pm$ 206.7
IIe-f	33.86 $\pm$ 5.09	1008.2 $\pm$ 144.4	38.95 $\pm$ 6.50	1368.6 $\pm$ 253.6
IIg	22.79 $\pm$ 6.57	440.8 $\pm$ 145.2	30.32 $\pm$ 7.83	273.7 $\pm$ 36.9

<sup>a</sup>Values are expressed as mean  $\pm$  s.d. Soma size was determined by measuring the cross-sectional area ( $\mu\text{m}^2$ ) of 50 cell bodies in each layer.  $N_v$ , expressed as number of cells  $\times 10^3/\text{mm}^3$  of tissue, was determined in five 6-hour-old pigeons.  $N_v$  was also determined in four 180-day-old pigeons which had been subjected to early unilateral retina removal.





**Fig. 11.** Patterns of GABA-LI in different visual regions at the adult stage. GLv: n. geniculatus lateralis, pars ventralis; Ipc: n. isthmi, pars parvocellularis; lmc: n. isthmi, pars magnocellularis; LA: n. lateralis anterior thalami; nBOR: n. basal optic root; PT: n. pretectalis; and TeO: optic tectum. Scale bar for Ipc = 300  $\mu\text{m}$ , and for the other regions, scale bar = 170  $\mu\text{m}$ .

adult stage. Based on an absence of immunostaining observed in the optic fiber layer onto the tectal surface and the optic tract, GABA<sub>A</sub> somas found in GCL can be tentatively designated as those of displaced amacrine cells. In this regard, no GAD<sub>A</sub> cells have been demonstrated in GCL (Brecha, 1983) of pigeon retina and unilateral enucleation has no effects on GAD activity of the contralateral TeO (Henke & Fonnum, 1976). In addition, GABA-LI is not observed in the rat, rabbit, pigeon, and goldfish optic nerve (Osborne et al., 1986). Moreover, rat retinæ from optic nerve sectioned eyes still reveal the presence of GABA-positive cells in GCL (Osborne, 1987). However, the presence of GAD and GABA<sub>A</sub> ganglion cells has been suggested in the retina of rabbits, monkeys (Mosinger & Yazulla, 1985; Mosinger & Yazulla, 1987; Nishimura et al., 1985; Yu et al., 1988), and larval salamanders (Yang & Yazulla, 1988). In addition, previous electrophysiological results suggest the exis-

tence of a direct inhibitory retino-tectal pathway in pigeons (Leresche et al., 1986). Therefore, it is evident that more definitive studies possibly combining GABA or GAD immunocytochemistry with the back-fill labelling of ganglion cells in the same retina are required to determine whether some GABA<sub>A</sub> ganglion cells are present in the pigeon retina.

#### Central visual regions

From hatching to 9 days, the size of GABA<sub>A</sub> cells gradually increases whereas their morphology does not change significantly except for PT where the round-shaped cells found at hatching become ovoid during the following days.

Interestingly, a reorganization of GABA<sub>A</sub> cell bodies and processes has been found after hatching and, in particular, during the first 9 days, simultaneously with changing size and shape

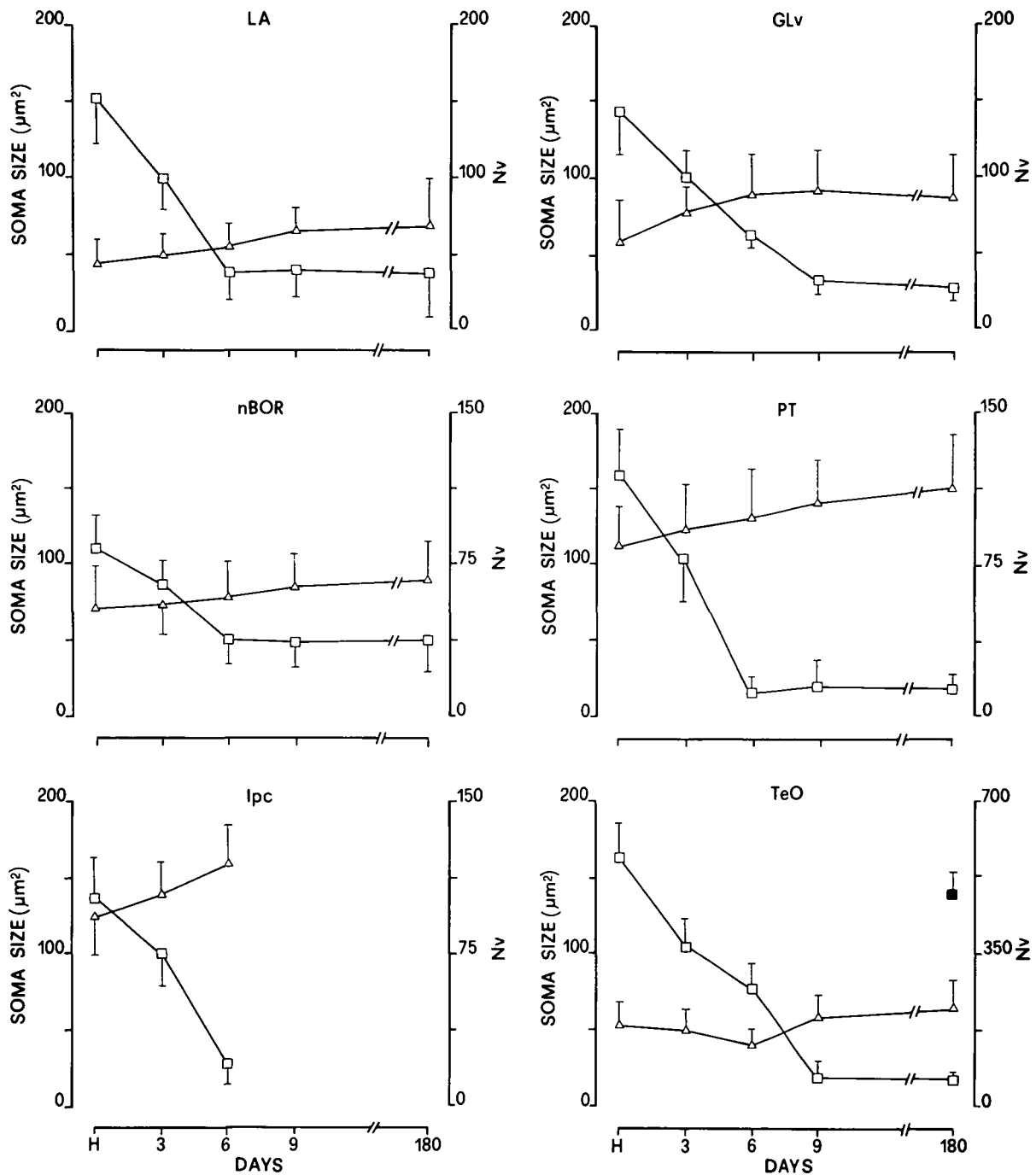


Fig. 12. Size (cross-sectional area in square micrometers, open triangles) and numerical density  $Nv$  (number of cells  $\times 10^3/\text{mm}^3$  of tissue, open squares) of labeled cell bodies in visual regions at different stages. In F, the filled square refers to  $Nv$  of labeled cells in TeO of adult pigeons with early unilateral retina removal. Each point represents the mean  $\pm$  s.d. Five pigeons from hatching to 9 days, four 180-day-old pigeons, and four pigeons with early retina removal were used. H: hatching.

of the examined nuclei, changes in the density of GABA-positive cells and processes occur. As shown by previous results over the same period, the pigeon visual system completes its maturation and primary visual areas are invaded by retinofugal axons (Bagnoli et al., 1987).

The prominent variation of GABA-LI distribution consists of a progressive decrease in the density of immunostained perikarya with an increase in the density of stained neuropil which

parallels the maturation of presumptive GABAergic cell bodies. Loss of GABAir cells is independent of the increased volume of visual regions since we found a decreased  $Nv$  which is generally considered as a good index of the relative neuronal packing density. On the other hand, the decrease in the percentage of GABAir cells we have observed after hatching seems to indicate a loss of antigenicity at increasing ages.

Loss of GABAir cell bodies which has also been described

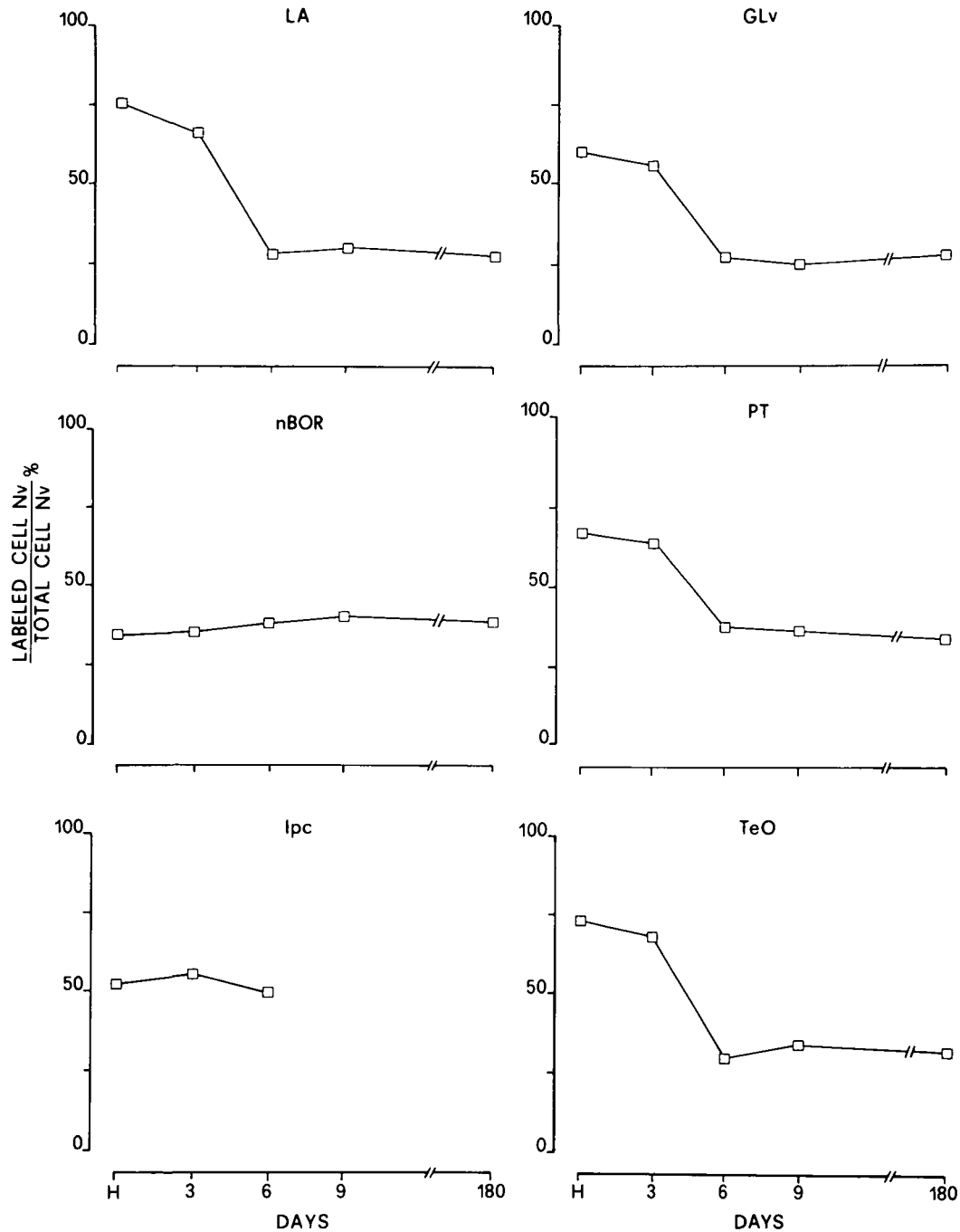


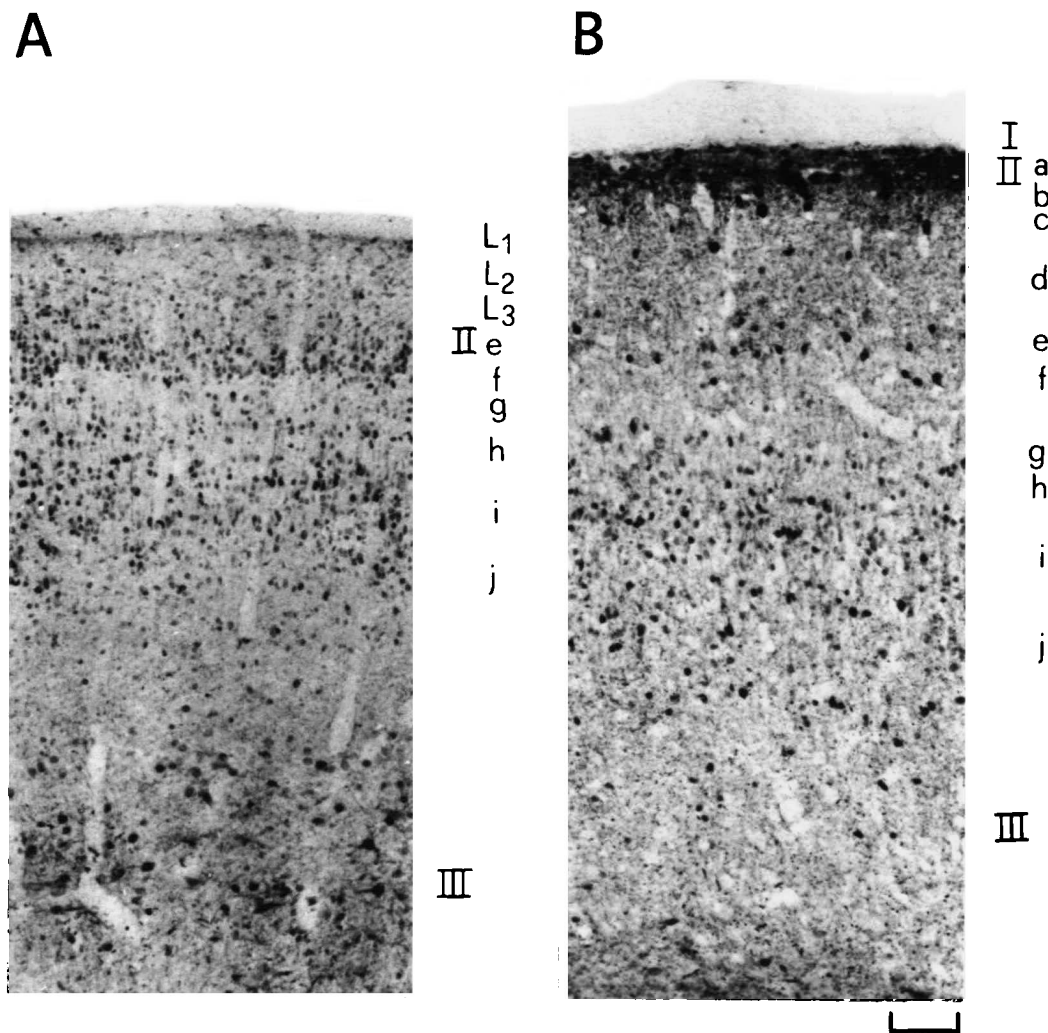
Fig. 13. Percentage of labeled cells in visual regions at different stages. Values were calculated as ratio of mean values of labeled cell *Nv* to corresponding mean value of total cell *Nv*. H: hatching.

in the ventral hyperstriatum and the cerebellum of chicks (Curtis & Stewart, 1986) might be attributed to either cell death and/or phenotypic plasticity. Indeed, a complete disappearance of perikaryal labeling and a massive increase in immunostained terminal-like elements is found in *lpc*. Similar results have been obtained with anti-GAD antibodies which show patterns of immunoreactivity similar to those obtained in this study (Bagnoli et al., 1989b). These results suggest the possibility that *lpc* neurons which produce GABA during the early maturational stages may subsequently contain another neurotransmitter. Indeed, most *lpc* cells of adult pigeons have been proposed to use gly-

cine as a neurotransmitter (Hunt et al., 1977; Streit et al., 1980; Wolfensberger et al., 1981). In addition, the presence of cholinergic neurons evidenced with antibodies to choline acetyltransferase has been widely recognized in *lpc* of fishes, amphibians, reptiles, and chickens (Brantley & Bass, 1988; Brauth et al., 1985; Desan et al., 1987; Ekstrom, 1987; Sorenson et al., 1989; Zottoli et al., 1988) as well as in *lpc* of developing pigeons (Bagnoli et al., 1989b). The persisting GABA-LI staining of terminal-like dots can be tentatively derived from GABA<sub>A</sub> cells of *lmc* and/or deep tectal layers.

At the level of the optic tectum, putative GABAergic cells





**Fig. 14.** Distribution of GABA-LI in corresponding regions of the dorsolateral optic tectum (TeO) of an adult pigeon with unilateral retinal removal at hatching. GABA-LI in TeO contralateral to the removed retina (A) strikingly differs from that in TeO contralateral to the persisting retina (B). Main differences include a very weak neuropil staining as well as a persistence of numerous stained cells in the superficial tectal layers ( $L_2$ ,  $L_3$ , and laminae IIe–h in A as compared to laminae IIa–h in B). The pattern of GABA-LI in deafferented TeO is similar to that found in normal animals immediately after hatching. Scale bar = 50  $\mu\text{m}$ .

and processes reach the adult pattern around the time when the anatomical and functional maturation of the retino-tectal system is completed (Bagnoli et al., 1987). The fact that the distribution of GABA<sub>A</sub>IR perikarya and processes in the superficial tectal layers is modified during the early period after hatching suggests a role of incoming ganglion cell axons on the maturation of putative GABAergic systems. From hatching to 9 days not only do GABA<sub>A</sub>IR cells decrease in density but also a population of GABA<sub>A</sub>IR cell bodies that is present at hatching in layer I disappears and is replaced at about 6–9 days by superficial retinal fibers. This transient population is composed of cells with processes that are most clearly seen in animals with shorter incubation period *in ovo*. In the developing cerebral cortex of cats, transient cells containing peptide immunoreactivity have been described and they have been shown to disappear while the white matter emerges (see Parnavelas & Cavanagh, 1988). On the other hand, glial cells, which are known to possess a high-affinity transport system for GABA, have been demonstrated

at hatching in the stratum opticum of the chicken TeO (Vanselow et al., 1989).

A correlation between invasion by retinal afferents and arrangements of putative GABAergic systems is supported by the effects induced by early retinal removal on the adult distribution of GABA-LI in the contralateral TeO. As shown in a previous study, retina removal has marked effects on tectal organization in pigeons (Bagnoli et al., 1989a). If performed immediately after hatching, this procedure results in a dramatic tectal shrinkage consequent to a reduction of the superficial neuropil with an associated decrease in cell number. These effects on tectal organization are secondary to the complete loss of retinal ganglion cell projections upon the superficial tectal layer.

Results from this study indicate that retinal deafferentation has marked effects on the normal pattern of GABA-LI within the superficial tectal layers. In fact, retinal ablation results in an increased density of GABA<sub>A</sub>IR cells in the adult TeO which

shows a pattern of GABA-LI comparable to that found in the normal TeO at the early stages. A similar distribution of GABA-LI was also observed shortly following retina removal, thus excluding the possibility of a long-term progressive atrophy. Modifications of GABA-LI pattern found at the level of the superficial tectal layers can be explained on hypothetical grounds by advocating the possibility that incoming retinal fibers may play a role in regulating the laminar distribution of GABAergic cells which retain their immature organization and density when the contralateral retina is early removed. Observed changes are likely to reflect an abnormal maturation of putative GABAergic systems induced by removing the retina before ganglion cell axons completely invade the superficial tectal layers (Bagnoli et al., 1987). In mammals, early retinal ablation has been shown to interfere with the normal development of GABAergic neurons in the visual cortex causing a reduction in number of GABAergic cells (Hendry & Jones, 1986) and axon terminals (Ribak & Robertson, 1986). On the other hand, transsynaptic phenomena may also explain changes in distribution of GABA-LI following retinal deafferentation, especially given the lack of convincing evidence of a retinal GABAergic contribution. Examples of transsynaptic modifications in peptide-like immunoreactivity have been previously reported in SNC (Kessler et al., 1981; Lewis et al., 1981; Schutzberg et al., 1978). Moreover, transneuronal effects of early eye removal on rat geniculate-cortical projection cells have been shown (Jeffery et al., 1984).

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