NeuroReport **8**, 1797–1802 (1997)

GABA immunoreactivity was examined in the retina of the New World monkey *Cebus apella*. Labeled cell bodies were identified as horizontal, bipolar, interplexiform, amacrine and a population of putative ganglion cells. To determine whether ganglion cells were immunoreactive to GABA, double-labeling experiments were performed using Fast Blue as retrograde neuronal tracer injected into the superior colliculus. Retinas containing FB-labeled ganglion cells were subsequently incubated with antiserum against GABA. Although retinocollicular ganglion cells were found in three different layers (ganglion cell layer, inner nuclear layer and inner plexiform layer), our experiments revealed GABA-positive ganglion cells only in the outer half of the ganglion cell layer.

Key words: Double-labeling; Fast blue, Immunocytochemistry; New World monkey; Primates

GABAergic retinocollicular projection in the new world monkey *Cebus apella*

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Introduction

GABA is an important inhibitory neurotransmitter in several types of retinal neurons in vertebrates. Electrophysiological and pharmacological studies of the retina have shown that GABA plays an important role on lateral inhibition of both plexiform layers, modifying receptive field characteristics of retinal cells under different conditions of illumination.[1,2] GABA immunoreactivity was also observed in optic nerve fibers in non-mammals, including the toad[3] and turtle[4] and more recently, in mammalian species (rabbit,[5] rat,[5] cat[5] and *Macaca*[6]). These results suggest a possible physiological role for this neurotransmitter in retinal terminals on central visual areas. Moreover, after unilateral deafferentation of the striate cortex in the Old World primates *Macaca mulatta* and *M. fascicularis*, about 20% of the remaining retinal ganglion cell axon terminals found in the degenerated dorsal lateral geniculate nucleus (dLGN) were GABAergic.[7]

Recently, it was determined that around 2.6% of the axons in the optic nerve in *Macaca* were GABA-immunoreactive (GABA-IR) and, GABA-IR axons were confined to the ventromedial part of the optic tract, suggesting that midbrain is one of the targets for the GABAergic retinal pathway.[6]

The present study aimed, first, to examine possible GABA-positive cells in the retina of the New World primate *Cebus apella*, focusing on the presence of GABAergic ganglion cells, and second, to determine whether GABA-immunopositive cells were actually ganglion cells projecting to the superior colliculus (SC).

Material and Methods

The retrograde fluorescent tracer Fast Blue (FB) was injected into the SC of two male adult *C. apella* monkeys. Ophthalmoscopic inspection of the eyes ensured that the animals were free from gross retinal abnormalities. Procedures for the use of animals were in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the commission of animal care of the IBCCF/UFRJ. Monkeys were sedated with diazepam (1 mg/kg) and restrained with ketamine hydrochloride (30 mg/kg). The animals were then intubated, secured in the stereotaxic head holder, immobilized with pancuronium bromide, and anesthetized with a mixture of halothane (1–2.5%), nitrous oxide (70%) and oxygen (30%). Temperature, EKG, pulse rate and end-tidal CO_2 were monitored throughout. Injections of 0.3 μl of a 2% suspension of FB were applied by pressure with a 1 μl Hamilton microsyringe under direct visualization. When the injections were completed and normal breathing resumed, the animals were returned to the cage. After a period of 15 days, the monkeys were deeply anesthetized, the eyes were removed, sectioned at the equator and immediately fixed in buffered 4% paraformaldehyde (0.1 M, pH 7.4) for 3 h. After enucleation, the animals

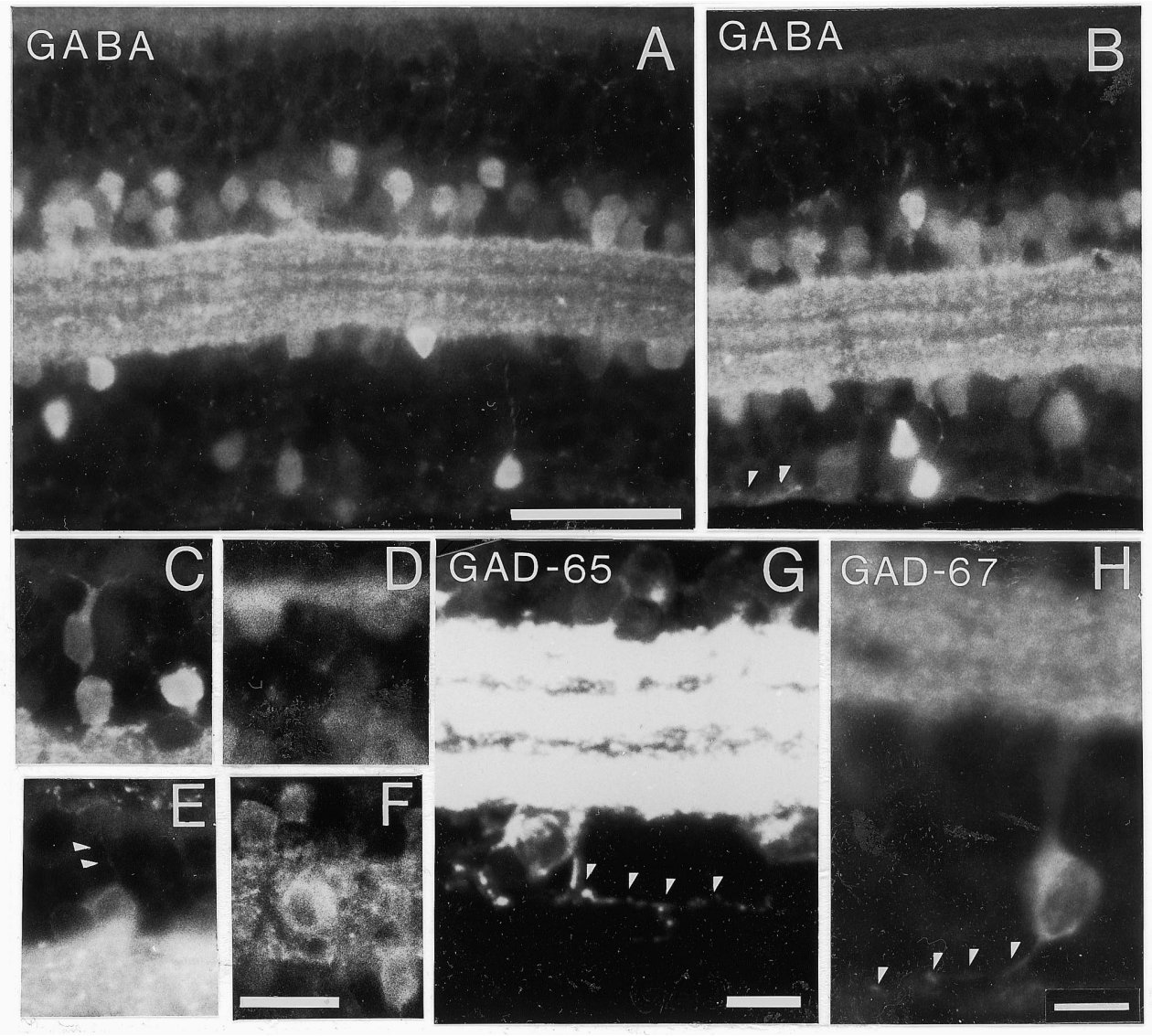

FIG. 1. Cy-3-labeled vertical sections of the retina showing GABA immunoreactivity at 1 mm (**A**) and 2 mm (**B**) of eccentricity. Ganglion cells are identified by their immunoreactive fibers in the optic nerve fiber layer (arrowheads). High magnification of GABA-positive bipolar (**C**), horizontal (**D**), interplexiform (**E**) and interstitial (**F**) cells. GAD-65-positive presumed ganglion cell reconstructed from a sequence of confocal optical sections (**G**). GAD-67-positive presumed ganglion cell (**H**). Arrowheads in (G) and (H) point to process emerging from these cells into the optic nerve layer. Bars = 50 μm (A,B), 20 μm (C–F), 10 μm (G,H).

were perfused transcardially with 4% paraformaldehyde/5% glycerol buffered solution (0.1 M, pH 7.4). Blocks were quickly frozen and cut at 40 μm on a freezing microtome. Sections of the SC containing the sites of injection were mounted on to gelatinized slides, dried on a hot plate and stored at 4°C.

After fixation, the eyes were rinsed in 0.1 M phosphate buffer (PB), pH 7.4, cryoprotected and sectioned at 10–20 μm. Sections were pre-incubated in 5% normal goat serum for 30 min and incubated with a GABA antisera;[8] GAD-65 (Hybridoma Bank) or with GAD-67 (Chemicon) overnight at 4°C. The binding sites of the primary antibody were visualized using the avidin–biotin (ABC) technique. Controls were processed at the same time using normal serum at the same final dilution as the primary antibody. In fluorescent double-labeled experiments, the primary incubation step was followed by a biotinylated secondary antiserum (IgG goat anti-rabbit, 1:200) and a tertiary step using fluorescent Cy-3 conjugated to streptavidin (1:400). Sections were observed under an Axioskop (Zeiss) microscope equipped with epifluorescent illumination with filter for viewing Cy-3 (excitation 550 nm, emission at 650 nm) and for FB fluorophore (excitation 428 nm, emission at 540 nm).

Results

GABA immunoreactivity in Cebus retina: GABA immunoreactivity in the *Cebus* retina was found mainly in cell bodies in the inner nuclear layer (INL), ganglion cell layer (GCL) and in several strata of the IPL, with bands 1 and 5 more intensely labeled in central (Fig. 1A) and peripheral retina (Fig. 1B). In the outer part of the INL, somata were found bordering the outer plexiform layer (OPL), which correspond to bipolar (Fig. 1C) and horizontal cells (Fig. 1D). In double-labeled experiments most GABA-IR bipolar cells were also labeled for PKC-α isoenzyme, suggesting that they were rod bipolar cells.[9] In the innermost part of the INL, amacrine cells containing variable levels of of GABA were found in several rows (Fig. 1A). Occasional cells extended a process back into the INL, in the direction of the OPL: these were identified as interplexiform cells (Fig. 1E). In the central and midperipheral retina, GABA-IR cells were also found in several levels of the IPL. These cells give rise to two large processes emerging and stratifying in the central stratum of the IPL and were identified as interstitial cells (Fig. 1F).

In the GCL, GABA immunoreactivity was displayed in a heterogeneous group of cells with variable soma size and immunostaining intensity. Most of the cell bodies found were characterized as displaced amacrine cells, although some seemed to be ganglion cells, since stained processes directed to the optic fiber layer were identified (Fig. 1). In the central retina, where 2–6 rows of cells are present in the GCL, GABA-labeled somata were seen in many levels, in the inner and outer rows (Fig. 1A). Some of these putative ganglion cells were also immunoreactive for the glutamate decarboxylase isoforms GAD-65 (Fig. 1G) and GAD-67 (Fig. 1H) and could be seen at the central and peripheral retina.

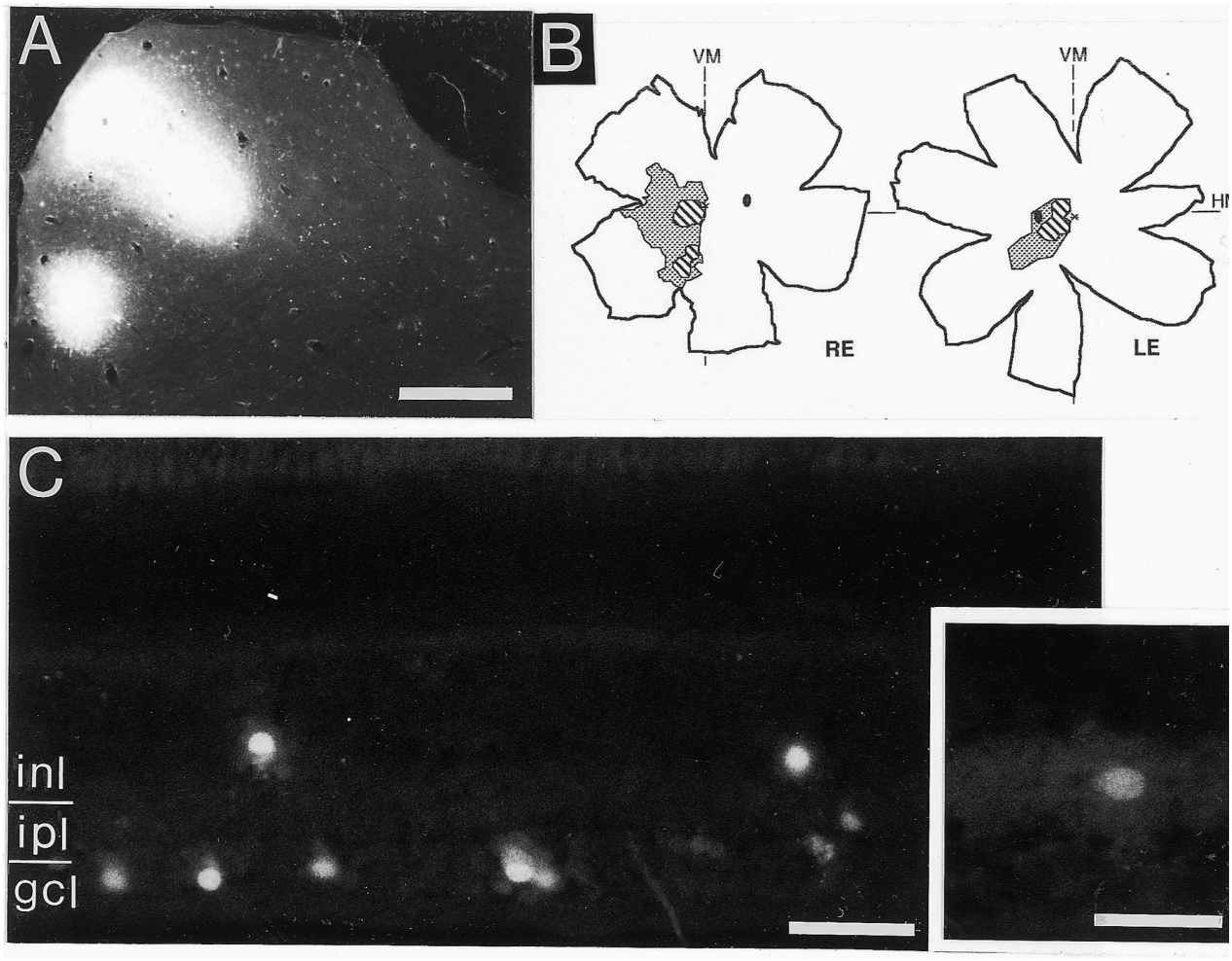

FIG. 2. Coronal section through the SC showing injection sites of Fast Blue (FB) (**A**). Topographic maps of retinal wholemounts ipsilateral and contralateral to the injected SC with regions of FB-labeled cells (dotted areas). The highest densities of labeled ganglion cells are marked by hatched areas. References for horizontal (HM) and vertical (VM) are shown for the left (LE) and right (RE) eyes. Radial section of the retina at 3 mm eccentricity showing FB-labeled ganglion cells in three different levels: inner nuclear layer (inl), inner plexiform layer (ipl) and ganglion cell layer (gcl) (**C**). Inset: High magnification of an interstitial ganglion cell at 2 mm of eccentricity. Bar 2 mm (A), 50 μm (C), 30 μm (inset).

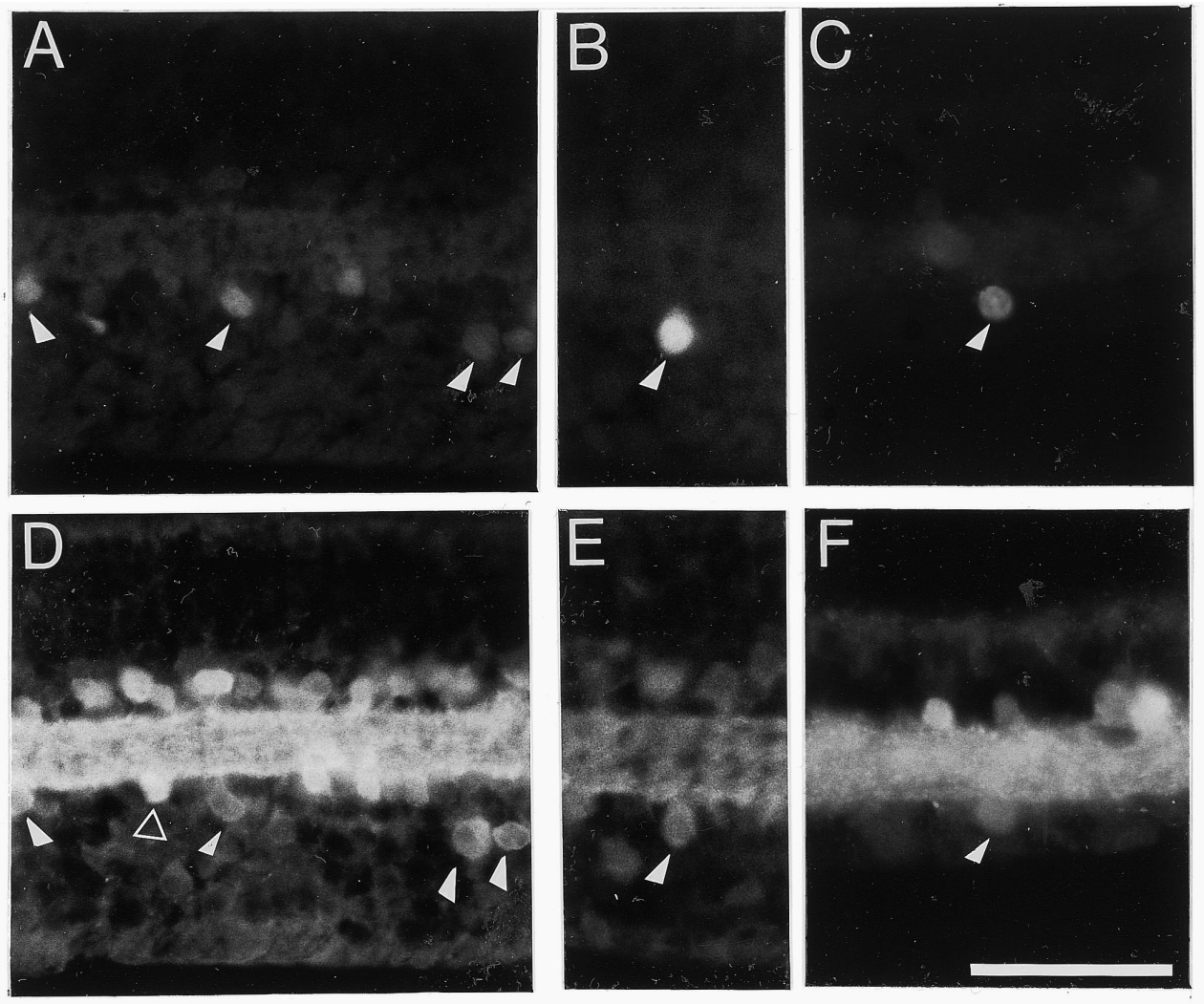

FIG. 3. Retinal radial sections double-labeled for FB (**A**–**C**) and GABA immunoreactivity (**D**–**F**) at 1 mm (A,D), 2 mm (B,E) and 6 mm (C,F) eccentricity along the vertical meridian. Arrowheads indicate double-labeled ganglion cells. Empty triangle indicates GABAergic cells that do not project to the SC. Bar = 50 μm.

Ganglion cells projecting to the superior colliculus: The identity of the GABA-immunoreactive neurons in the GCL was determined by unilateral injections of the retrograde tracer FB in the SC (Fig. 2A). We injected the SC along the representation of the horizontal meridian, which corresponded to an area in the ipsilateral and contralateral retina within 0.4–3.0 mm of eccentricity (about 2–15°) from the foveola (Fig. 2B). In the ipsilateral retina the labeled region also extended outside the horizontal meridian to a region about 6 mm of extension in the ventral vertical meridian.

FB-labeled ganglion cell bodies were found in three levels of the retina: the outer row of the GCL, the INL and in the center of the IPL (Fig. 2C). Displaced ganglion cells in the INL were scarce in both ipsilateral and contralateral retinas. Interstitial ganglion cells, detected in the IPL, were also rare and, in general, seemed to have a smaller soma than ganglion cells in the INL or GCL (Fig. 2C and inset).

GABAergic ganglion cells project to the superior colliculus: Double-label experiments in which radial sections of the retina with ganglion cells containing FB (Fig. 3A–C) were immunoreacted for GABA (Fig. 3D–F) showed co-labeled cells confined to the GCL. Within the 15° central along the horizontal meridian (in a region 0.5 mm wide), about 20% of retrograde-labeled ganglion cells in radial sections of the retina were GABA-immunoreactive. None of the displaced ganglion cells that project to the SC were double-labeled for GABA.

Many of the ganglion cells in the inner half of the GCL were not retrogradely labeled with FB, suggesting that they project to other central visual targets outside of the SC or to other parts of this

nucleus which were not injected. In fact, experiments where FB were injected in the dorsal geniculate nucleus showed some GABA-IR ganglion cells in the inner part of the GCL (not shown).

Discussion

In this study we showed that several cell types in the *Cebus* monkey retina, including ganglion cells, are GABAergic. Using the retrograde tracer FB injected into the SC, we verified that GABAergic cells projecting to this nucleus are a subset of ganglion cells situated in the GCL. GABA immunoreactivity in *C. apella* generally follows the same pattern described previously in other primates. GABA-IR amacrine cells accounted for the majority of cells in the INL and GCL and were also seen to be displaced to the IPL.[10,11] GABA-IR interplexiform, bipolar and horizontal cells, identified by criteria similar to those used in macaque monkey retina, were also evident in the *Cebus* retina. Some of the GABA-IR cells in the GCL of the *Cebus* retina gave rise to an axon directed towards the nerve fiber layer. They were few in number and identified as ganglion cells.

In Old World primates, Wilson and collaborators[6] showed a topographic distribution of GABAergic axons in the optic tract, inferring that these results could be used as evidence for the presence of GABAergic ganglion cells in these species. FB injected into the SC of *Cebus* allowed the identification of retrogradely labeled ganglion cells in several layers of the perifoveal region of the retina. Cell bodies were found mainly in the GCL and rare somata were displaced to the INL and IPL. This feature seems to be unique in this New World monkey since few reports in *Macaca* species[12–14] have shown orthotopic and displaced ganglion cells in the INL after injection of horseradish peroxidase and fluorescent dye into the SC. However, none of the studies showed retinocollicular interstitial ganglion cells. A recent study in the Cynomolgous monkey showed glutamate-immunopositive interstitial ganglion cells in the IPL of the retina; however these authors did not back-label these cells.[15]

In perifoveal regions of the *Cebus* retina, retinocollicular ganglion cells were located in the outer half of the ganglion cell layer, a pattern very similar to that seen in *Macaca mulatta*, suggesting that there is a functional lamination in the central retina of the New World primates, as for Old World primates.[16]

GABAergic retinocollicular ganglion cells: GABAergic retinal ganglion cells projecting to the midbrain were observed in retinas of turtle,[4] toad[3] (2.6% project to tectum), tiger salamander[17] (1% project to tectum), rat[18] (6% project to the SC) and ground squirrel[19] (1–3% project to the SC). A similar retinal projection was suggested for Old World primates, based on the topographic distribution of GABAergic axons in the optic tract.[6] Data presented here provide the first unequivocal evidence of GABAergic retinocollicular ganglion cells in primates. This inhibitory projection to the SC is a conserved anatomical feature in the visual system of vertebrates, independent of their diurnal or nocturnal habits, or of their position in the phylogenetic scale.

GABAergic ganglion cells in the *Cebus* retina were also immunoreactive for GAD, the biosynthetic enzyme for GABA, indicating that GABA is synthesized in these cells rather than carried from other GABAergic cells via gap junctions[20] or, alternatively, used as a metabolic substrate.[21] Thus, GABA from ganglion cells must have a functional role as a neurotransmitter in the SC. Reinforcing this idea, eletrophysiological studies in pigeon have shown a direct monosynaptic inhibitory retinal projection to the optic tectum.[22] In toads, 67% of the GABAergic retinal terminals in the optic tectum synapse on to GABA-positive dendrites of interneurons, suggesting that GABAergic optic axons could participate in a disinhibitory circuit.[23] In several mammalian species, GABAergic neurons in the SC form serial synapses, providing a possible substrate for disinhibition of other GABA terminals in this nucleus, a characteristic that seems to be conserved in all mammalian species studied.[25] It is possible that GABAergic ganglion cells projecting to the SC in the *Cebus* retina could play a role on local circuits modulating disinhibition in this nucleus. Double-label experiments identifying both GABAergic retinal terminals and local interneurons would provide a morphological basis for this hypothesis.

Conclusions

GABA-IR neurons in the *C. apella* retina are found in the INL, IPL and GCL. Retrogradely labeled retinocollicular ganglion cells are located in the INL and IPL and in the outer half of the GCL, indicating a functional lamination in the central retina of the New World monkey, similar to that in Old World primates. However, GABAergic retinocollicular ganglion cells are confined to the outer half of the GCL.

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ACKNOWLEDGEMENTS: The authors would like to thank M.M.M. Oliveira, E.S.S. Filho and G. Coutinho for their technical assistance. Dr M.C.F. de Mello has critically read the manuscript. This work was supported by grants awarded to J.N.H. and R.G. by FINEP, CNPq and FAPERJ. B.L.S.A.C. is a CAPES Ph.D. fellow.

Received 18 March 1997; accepted 5 April 1997