Effect of retinal ablation on the expression of calbindin D_{28k} and GABA in the chick optic tectum

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The effect of retinal ablation on qualitative and quantitative changes of calbindin D_{28k} and GABA expression in the contralateral optic tectum was studied in young chicks. Fifteen days old chicks had unilateral retinal ablation and after 7 or 15 days, calbindin expression was analyzed by Western blot and immunocytochemistry. Neuronal degeneration was followed by the amino-cupric silver technique. After 15 days, retinal lesions produced a significant decrease in calbindin immunostaining in the neuropil of layers 5-6 and in the somata of neurons from the layers 8 and 10 of the contralateral tectum, being this effect less marked at 7 days post-lesion. Double staining revealed that 50-60% of cells in the layers 8 and 10 were calbindin and GABA positive, 30-45% were only calbindin positive and 5-10% were only GABAergic neurons. Retinal ablation also produced a decrease in the GABA expression at either 7 or 15 days after surgery. At 7 days, dense silver staining was observed in the layers 5-6 from the optic tectum contralateral to the retinal ablation, which mainly represented neuropil that would come from processes of retinal ganglion cells. Tectal neuronal bodies were not stained with silver, although some neurons were surrounded by coarse granular silver deposits. In conclusion, most of calbindin molecules are present in neurons of the tectal GABAergic inhibitory circuitry, whose functioning apparently depends on the integrity of the visual input. A possible role of calbindin in the control of intracellular Ca^{2+} in neurons of this circuit when the visual transmission arrives to the optic tectum remains to be studied.

Key words: calbindin D_{28k}, tectum opticum, chicks, retinal ablation, GABA

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Calbindin D_{28k} (CB) is a protein discovered in 1966 by Wasserman and Taylor in the chick intestine. This protein is absent or negligible in the intestine from rachitic chicks and its synthesis is induced by vitamin D or calcitriol (Taylor, 1974). In contrast, this protein is expressed in brain from different birds and mammals, independently of vitamin D status (Li, 1998). Although CB is widely distributed in the central nervous system, its physiological role in the brain remains unclear. Certain evidences suggest that CB acts as a Ca^{2+} buffer preventing deleterious changes of excessive Ca^{2+} accumulation (Batini, 1993). The overexpression of CB gene increases the survival of hippocampal neurons in vitro following energetic or excitotoxic insults (Phillips, 1999). On the contrary, it fails to protect hippocampal neurons against ischemia in spite of its calcium buffering properties in CB knockout mice (Klapstein, 1998). Jouvenceau et al. (2002) have demonstrated that CB plays a critical role in long term synaptic plasticity in the hippocampal CA1 area of mice by limiting the elevation of calcium in the cytosol. On the other hand, the cytoprotective role of CB has also been found in astrocytes where CB suppresses apoptosis induced by a calcium ionophore and the amiloid β-peptide (Wernyj, 1999). It has also been recently reported that CB overexpression protects striatal neurons from transient focal stroke (Yenari, 2001). Airaksinen et al. (1997) have demonstrated that CB is necessary for an integrated nervous system function; they have shown that null mutants are severely impaired in tests of motor coordination, which suggests these mutants have functional deficits in cerebellar pathways.

The immunolocalization of CB has been described in retina and other structures of the visual pathways in different species (Hamano, 1990; Johnson, 1995; Mize, 1999). In our laboratory, it has been demonstrated that CB appears very early in the embryonic

period of chick retina and shows changes in distribution along development and after hatching, which indicates that Ca^{2+} homeostasis in retina is well regulated, probably to avoid excessive accumulation of the cation (Tolosa de Talamoni, 2002). In calbindin-null mutant mouse, CB immunoreactivity was absent in retina; however, horizontal cells were present and normal, an indication that CB is not required for the maintenance of retinal structure (Wäsle, 1998). The exact role of CB and other calcium binding proteins in retina remains to be elucidated. Different responses of CB expression have been demonstrated after enucleation or deafferentation of visual pathways from different species (Miguel-Hidalgo, 1991; Mize, 1992a ; Britto, 1994). We have observed that all neural structures of the chick tectofugal and thalamofugal visual pathways were positive for CB staining showing depletion of CB immunoreactivity in the contralateral tectum and nucleus rotundus after monocular enucleation (Díaz de Barboza, 2000). Monocular enucleation in hamsters provokes a marked reduction in the number of CB immunoreactive neurons on the experimental side of the superior colliculus (Kang, 2002).

The association between CB and GABA has been found in a variety of neurons from diverse areas, which is a subject of speculation and, in certain cases, controversial. In the lateral geniculate nucleus and visual cortex of adult primates, CB and GABA immunoreactivity was detected, as well as in other subcortical visual structures of rhesus monkey (Mize, 1992a ; Mize, 1992b). However, Behan et al. (1992) found a lack of co-localization of CB and GABA in the hamster superior colliculus by using a double fluorescent labeling technique and confocal laser microscopy. In the cat pretectum, CB is a precise marker of neuron clusters that overlap the retinal projection zones, but none of those CB cells contain GABA (Nabors, 1991). On the other hand, Zhou and Galligan (2000) found GABA receptors on CB-immunoreactive myenteric neurons of guinea pig intestine. Taking into account the previous papers, it is reasonable to think that CB and GABA expressions could be both altered in the optic tectum by damaging the integrity of the visual pathway. Therefore, we analyzed the effect of retinal ablation on qualitative and quantitative changes in the CB and GABA expression in the chick tectum.

Materials and Methods

Two weeks after hatching, Cobb Harding chicks (INDACOR, S.R.L.) were anesthetized with 30 mg of chloral hydrate/100 g of body weight, and had unilateral retinal ablation. First, an incision in the cornea was performed and the lens and vitreous humor were removed. The neural retina was completely destroyed and removed with common swabs. Hemostatic sponge of collagen (Hemostop, Herix S.A. Laboratory, Montevideo, Uruguay) was placed inside the eye, and the eyelids were sutured closed. Survival times were either 7 or 15 days. Following the experimental period, chicks were deeply anesthetized and perfused through the ascending aorta with normal saline, and fixative of 4% paraformaldehyde in 0.01 M sodium phosphate buffer pH 7.3. Brains were soaked in the same fixative for 5 hours, and then treated with 30% sucrose for cryoprotection. Sections of 30 µm of each brain were cut in a coronal plane by using a freezing microtome.

Antisera. The polyclonal antiserum anti-calbindin D_{28k} produced in rabbits against chicken intestinal calbindin D_{28k} was generously donated by Dr. Curtis Fullmer (Department of Physiology, Cornell University, Ithaca, NY, USA). An affinity-purified rabbit anti-GABA antiserum was purchased from Chemicon Int. Inc, Temecula, CA, USA.

Immunoblotting. Fresh superficial layers (1-10) from the ipsilateral and contralateral optic tectum to the retinal lesion were removed 7 and 15 days after surgery and homogenized with water. The proteins (40 µg) were separated by SDS-PAGE with 15% acrylamide and 0.1% SDS (Laemmli, 1970), running in the same gel protein standards of known molecular weight (Amersham Int., Little Choulfout, England). The proteins were transferred to nitrocellulose sheets (Towbin, 1979). The blots were incubated at 37°C for a couple of hours with Tris-buffered saline solution (TBS) containing 0.02 mol/L Tris, 0.5 mol/L NaCl pH 7.4 and anti-CB antibody (1:500 dilution). The secondary antibody, peroxidase conjugated goat anti-rabbit immunoglobulins (1:500 dilution in TBS) (Amersham Pharmacia Biotech UK limited, Little Chalfont, Buckinghamshire, England), was incubated at room temperature for two hours. Non-specific binding on the nitrocellulose was blocked with nonfat dry milk in TBS and washes between incubation steps were accomplished with TBS. The detection was performed by using 3,3´-diaminobenzidine (DAB) as a chromogen.

Immunocytochemistry. Calbindin localization was carried out in free floating brain sections by the

streptavidin-biotin-peroxidase technique. Anti-CB antibody (1:1000 dilution) was incubated with the sections overnight at 4°C. After rinsing, the solution containing the biotinylated secondary antibody (Histostain-SP, Zymed Inc., San Francisco, USA) was added to the tissue, and after a 2-hour incubation period, the sections were rinsed. The streptavidin-peroxidase conjugate solution was immediately added, incubated for 90 min, rinsed, and the color was developed by incubation with 0.05% DAB solution and 0.01% H_2O_2 for 1-2 min. In the negative controls, the primary antibody was substituted for nonimmune serum. This completely eliminated the staining. Double labeling of CB and GABA was performed by sequential incubations with each primary antibody. Calbindin staining was done as described above. Afterwards, peroxidase remaining was suppressed by incubation in 0.5% H_2O_2 in methanol for 45 minutes at room temperature and washed in 0.01M phosphate buffered saline (PBS) pH 7.4 several times. This step was followed by incubation with PBS plus 5% normal serum for 1 h to suppress non specific binding. For GABA labeling, the primary antibody was incubated 48 hours at 4 °C. The anti-GABA was visualized by using an avidin-biotin system (Vectastain ABC-Alkaline Phosphatase System, Vector Laboratory Inc., Burlingame, CA, USA) containing a biotinylated goat anti-rabbit secondary antibody. Alkaline phosphatase activity was revealed by using a Vector Blue Alkaline Phosphatase Substrate kit III (Vector Inc.). The inhibition of endogenous alkaline phosphatase was accomplished by reaction with levamisol. The sections were mounted onto slides covered with gelatin, air dried and coverslipped with VectaMount (Vector Inc.).

Amino-cupric-silver technique. The amino-cupric silver protocol described by De Olmos et al. (1994) was used to detect neuronal degeneration in the optic tectum after retinal deafferentation. Nissl staining was employed as counterstaining.

Data analysis. Quantitative measurements of number of stained cell bodies and layers´s width were accomplished on digital images from tectal sections of 4 or more animals employing the KS Lite 2.0 computer program (Kontron Elektronik, Eching, Germany). Three images of representative sections from the rostral and caudal regions of optic tectum from each animal were obtained with a Leica DC 200 Twain camera (Heerbrugg, Switzerland) attached to a Zeiss Axiolab microscope (Jena, Germany) Comparisons were made only within matching layers at both sides of optic tectum sections of each animal. The results were expressed as number of immunostained cells/mm^2 of area from each layer. Statistical comparisons were made using a paired t-test. Differences were considered significant at $p < 0.05$.

Results

Calbindin D_{28k} expression in chick optic tectum at 7 and 15 days after retinal ablation is shown in Figure 1. As previously reported (Díaz de Barboza, 2000), the positive staining was present in several layers but the intensity was higher in Cajal's layers 5 and 6, mainly in dendrites and axons. A few cells from the same laminas were also positively stained in their somata. An important number of cell bodies were CB-positive in layers 8 and 10. A reduction in the immunoreactivity of CB can be seen in the optic tectum (Figure 1b and 1d) contralateral to the retinal ablation as compared to the ipsilateral optic tectum (Figure 1a and 1c). The width of the layers 1-10 showed a tendency to decrease either in the rostral optic tectum or in the caudal optic tectum, and this was significantly different in the rostral tectum 7 and 15 days post-lesion and in the caudal tectum 15 days after surgery (Table 1). In the contralateral rostral optic tectum, the width of layers 5-6 was decreased at 7 and 15 days after retinal ablation, while in the contralateral caudal optic tectum, a significant reduction was only observed after 15 days of deafferentation (Table 2). Although the width of layers 5-6 was smaller in the contralateral optic tectum than that of the same layers from the ipsilateral tectum, the intensity of the staining was apparently not different (Figure 1). The number of CB-positive cells in deeper layers (8 and 10), was smaller in the contralateral tectum than that from the ipsilateral tectum at 7 and 15 days after retinal deafferentation (Table 3). Western blot assays revealed only one fraction of 28 kDa that corresponds to calbindin D_{28k}, whose expression decreased in the tectum contralateral to the retinal lesion as compared to the ipsilateral tectum 15 days post-surgery. This effect was not evident at earlier times (Figure 2).

Silver staining of contralateral and ipsilateral optic tectum 7 days after retinal ablation is shown in Figure 3a and 3b. Dense silver staining is observed in the layers 5-6 from the optic tectum contralateral to the retinal deafferentation, which represents mainly neuropil that originate from processes of retinal ganglion cells. The layer 9, which is not a retinorecipient

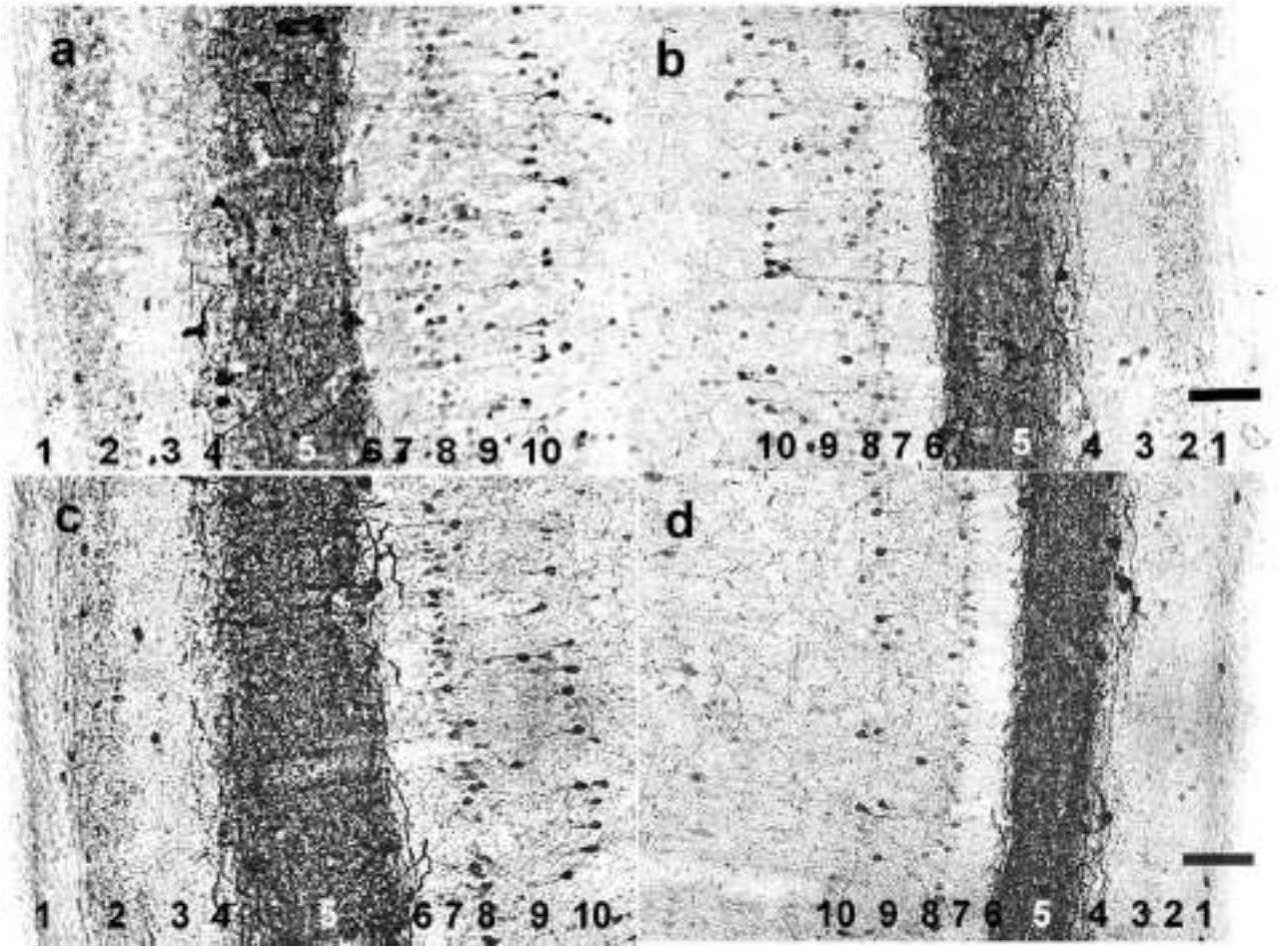

Figure 1. Calbindin D_{28k} immunolocalization in chick optic tectum. Ipsilateral (a) and contralateral (b) optic tectum 7 days after unilateral retinal ablation, ipsilateral (c) and contralateral (d) optic tectum 15 days after unilateral retinal ablation. Scale bar: 50 µm.

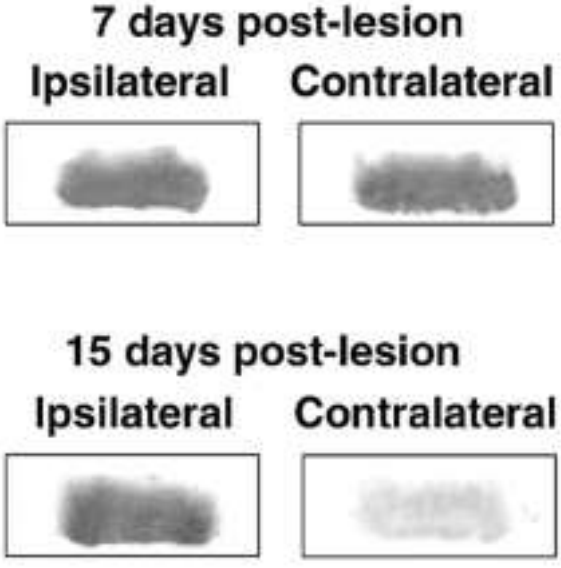

Figure 2. Western blots analysis of optic tectum homogenates 7 and 15 days after unilateral retinal ablation. Each band shows CB immunoreactivity of homogenates from the ipsilateral or contralateral optic tectum. Proteins of homogenates (40 µg) were separated by SDS/PAGE, transferred to nitrocellulose sheet, and treated with a polyclonal anti-calbindin antibody.

Table 1. Width (µm) of layers 1-10 of optic tectum after retinal lesions

Time after lesion	*Rostral Tectum*		*Caudal Tectum*	
	Ipsilateral	*Contralateral*	*Ipsilateral*	*Contralateral*
7 days	409.4±22.7 (n = 4)	334.7±37.3* (n = 4)	450.4±71.1 (n = 4)	405.5±25.6 (n = 4)
15 days	382.5±9.5 (n = 4)	338.5±16.5* (n = 4)	454.3±53.1 (n = 5)	334.4±35.5** (n = 5)

Table 2. Width (µm) of layers 5-6 of optic tectum after retinal lesions

Time after lesion	*Rostral Tectum*		*Caudal Tectum*	
	Ipsilateral	*Contralateral*	*Ipsilateral*	*Contralateral*
7 days	111.2±2.5 (n = 4)	72.4±1.9* (n = 4)	133.5±31.0 (n = 4)	109.1±19.1 (n = 4)
15 days	106.6±7.6 (n = 4)	73.2±2.5* (n = 4)	116.3±9.4 (n = 5)	83.4±4.4** (n = 5)

*Values are means±S.E., (n) = number of animals. Statistical comparisons were made using a paired "t" Test. Differences were considered significant at *p < 0.05 (contralateral vs ipsilateral) and **p < 0.005 (contralateral vs ipsilateral).*

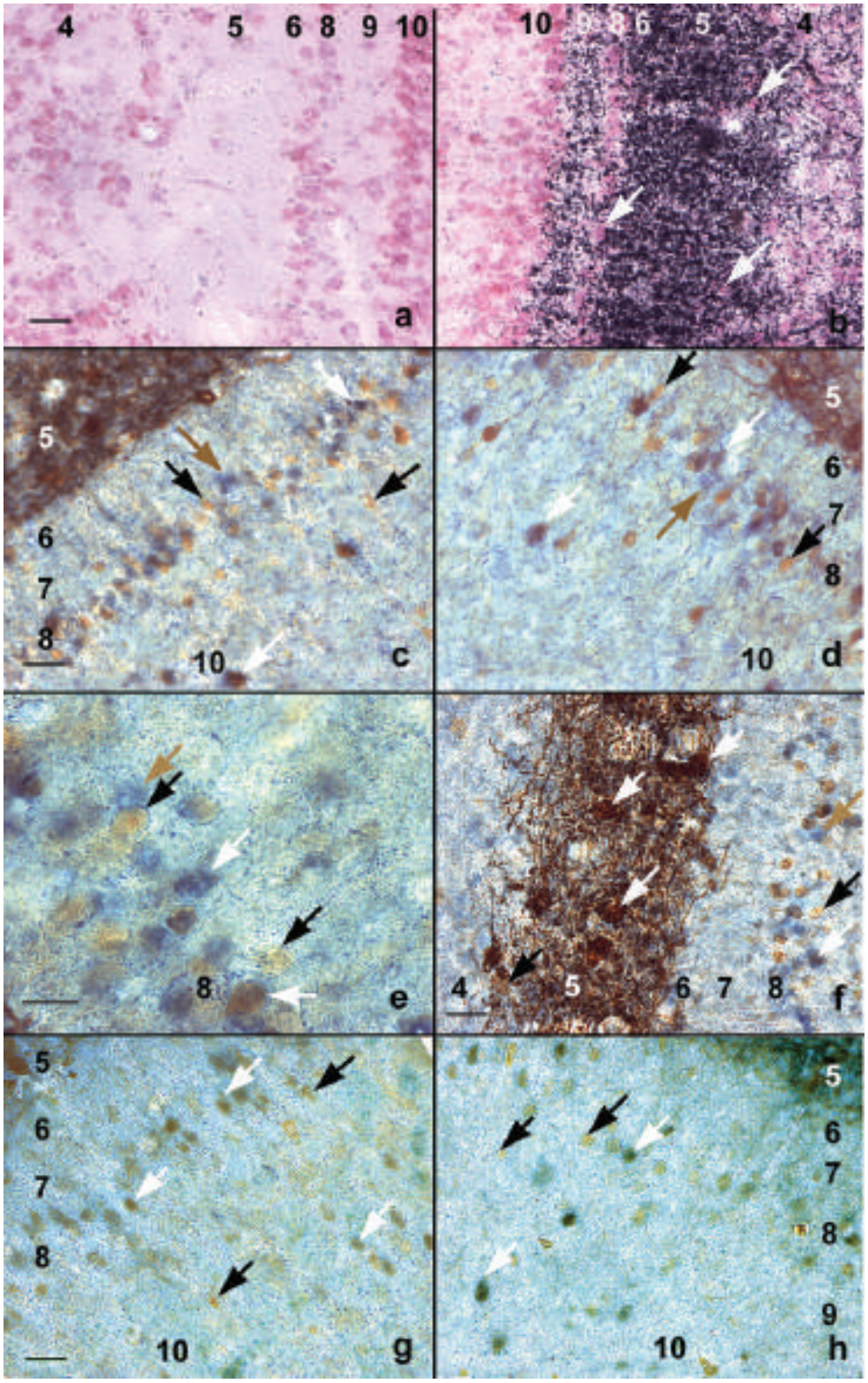

Figure 3. Amino-cupric-silver stain of ipsilateral optic tectum (a) and contralateral optic tectum (b) 7 days after unilateral retinal ablation. White arrows show the Nissl stain neurons. Scale bar in a and b is 20 µm. Colocalization of CB (brown) and GABA (blue) in the ipsilateral (c, e, f , g) and in the contralateral (d, h) to the unilateral retinal ablation, 7 days (c, d, e, f) and 15 days (g, h) post-lesion. White arrows show CB and GABA immunoreactive neurons, black arrows show CB immunoreactive neurons and brown arrows show GABA immunoreactive neurons. Scale bar in c, d, f, g and h is 20 µm, in e is 10 µm.

layer, was also stained with silver. Nissl counterstaining indicates that tectal neuronal bodies were not stained with silver, although some neurons were surrounded by coarse granular silver deposits. Degenerating axons can also be observed. Neuropil degeneration in layers 5-6 began 48 hours post surgery, then increased for several days and attained a maximum at 7 days. Afterwards, at day 15, the neurodegeneration had declined (data not shown).

The double labeling reveals GABA neurons with a

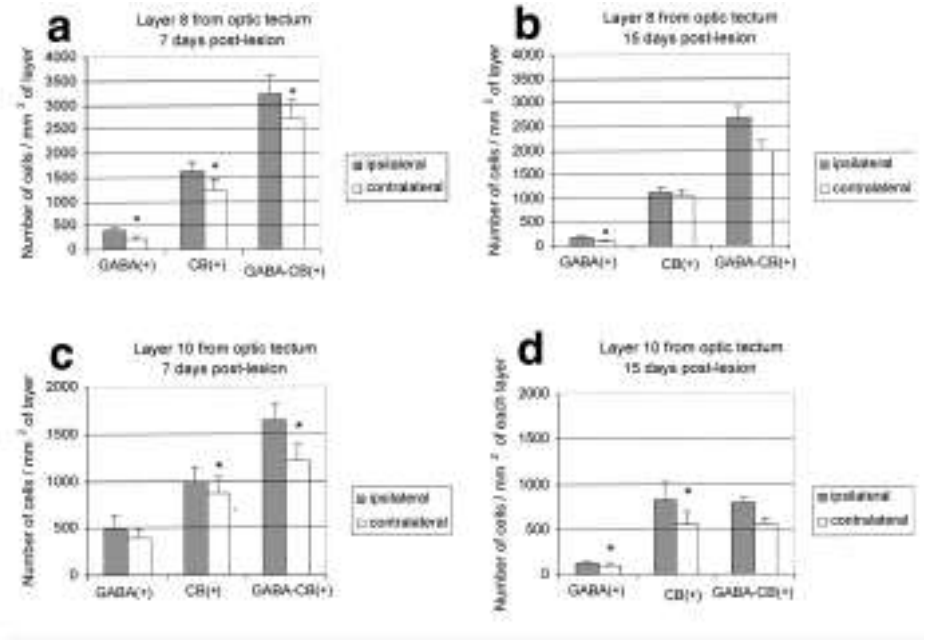

Figure 4. Effect of unilateral retinal ablation on the number of CB, GABA or CB-GABA immunoreactive cells /mm² of area from layers 8 and 10 of chick ipsilateral and contralateral optic tectum. The bars represent media ± SE of 3 or 4 animals. Statistical comparisons were made using a paired "t" test. Differences were considered significant at *$p < 0.05$.

blue staining, CB cells in brown and GABA + CB cells in brown bluish, which can be more appreciated in the Figure 3e. The colocalization of CB and GABA was most prominent in layers 5-6 (Figure 3f). In the layers 8 and 10, some neurons were CB positive (30-45%), some were GABA positive (5-10%) and some were both CB and GABA positive (50-60%) (Figure 3c). GABA immunoreactivity was significantly reduced in layer 8 on the contralateral side to the retinal lesions at 7 (Figure 3d, Figure 4a) and 15 (Figure 3h, Figure 4b) days after deafferentation as compared to the corresponding ipsilateral sides (Figure 3c and Figure 3g), and in the layer 10 after 15 days post-lesion (Figure 4d). CB staining was reduced in the layer 8 only at 7 days post-lesion (Figure 4a), while in layer 10 the reduction was observed at both 7 and 15 days after surgery (Figure 4c and 4d). Double labeling of GABA and CB was reduced in layers 8 and 10 at 7 days after retinal ablation (Figure 4a and 4c).

Discussion

The present report shows, either by Western blot analysis or immunocytochemistry, a significant reduction of CB expression in the optic tectum contralateral to the retinal ablation 15 days after surgery. The reduction was mainly produced in the neuropil of layers 5-6 and in the somata of neurons from the layers 8 and 10. One week earlier similar changes occurred affecting mainly CB expression in layers 8 and 10.

The width of layers 1-10 and 5-6 was also reduced by the retinal ablation showing some differences in the time of appearance between the rostral and the caudal tectum, probably as a consequence of differences in the topographic distribution of retinal fibers coming from the retinal ganglion cells.

Different responses in the CB expression after injury of diverse neural pathways have been found in several systems of different animal species. Blümke et al. (1994) observed a discrete reduction of CB and parvalbumin immunoreactivity in the dorsal lateral geniculate nucleus and in the striate cortex of adult macaque monkeys after monocular enucleation. Britto et al. (1994) demonstrated that retinal lesions in pigeons produce a marked depletion of somata and neuropil staining for both CB and parvalbumin immunoreactivities in the contralateral optic tectum. Dassesse et al. (1998) found a differential expression of CB in motoneurons after hypoglossal axotomy. They observed low neuronal CB mRNA and protein content in normal state, transiently increased at 8 days post operation and declining to normal again at 28 days after surgery. The authors think that upregulation of CB expression might facilitate the survival of injured motoneurons. The axotomy of sciatic motoneurons in the adult rat produced downregulation of CB immunoreactivity in putative Renshaw cells, while the median and ulnar nerve lesions at different ages induced CB immunoreactivity in cervical ventral horn (Fallah, 1999).

The avian tectofugal pathway includes optic fibres

of retinal origin that terminate on the contralateral tectum in its external layers (2-7), called the optic layers. These terminals are large and of varied appearance in the different areas. After internal elaboration in the tectum, large neurons of the layer 13 project prominently to the nucleus rotundus (Ngo, 1992), which constitute the main efferent pathway of the avian optic tectum. Terminal ramifications of the dendrites of tectal ganglion cells of layer 13 terminate into bunches in the middle of the layers 4-5 of the optic tectum. The optic terminals and the dendritic terminals of tectal ganglion cells form synaptic glomeruli, which present GABA-positive terminals that may originate from the axons of local neurons or from axons of neurons of magnocellular isthmic nucleus (Tömböl, 1999). This GABAergic circuit could inhibit or modulate the optic transmission in the tecto-rotundus-ectostriatal pathway. It has also been suggested that through the action of inhibitory neurons from layers 4 and 5, visual attention might be attenuated on one side remaining elevated on the other side (Tömböl, 1998). In this paper, we show by double labeling for CB and GABA that the retinorecipient layers from optic tectum present CB positive cells, GABA positive cells and double labelled CB and GABA positive cells. Retinal ablation also decreased GABA immunoreactivity in the contralateral optic tectum. It appears that the integrity of the retinal afferents is necessary to keep the steady-state levels of GABA in the optic layers from the optic tectum. The reduction in the GABA expression in the layers 5-6 might occur as a consequence of neuropil degeneration from retinal ganglion cells as shown by the silver staining. The reduction in the GABA immunoreactivity in neurons from layer 8 was not caused by neuronal degeneration, at least at the studied times, but apparently by the lack of direct input from the retina or from other layers of superficial tectum. Due to the fact that layer 10 does not receive a direct input from the retina, the GABA immunoexpression reduction might be a result of indirect rearrangements in the gabaergic local circuit from tectum because of the retinal ablation. It has been shown that GABA, glutamate decarboxylase and other immunochemical markers related to the GABA circuit are regulated by the visual input in the adult monkey visual cortex (Cellerino, 1992).

Co-localization of CB and GABA in different neuronal systems has been also shown in a variety of species such as salamander, mudpuppies (Deng, 2001) and cat (Mize, 1992b). The physiological role of this co-localization remains unknown. By using the oligonucleotide antisense strategy for CB mRNA, Vig et al. (1999) have found that Purkinje cells treated with that antisense oligonucleotide showed a significant decrease in GABA uptake as compared with the control cells. The authors conclude that the expression of CB may have a significant participation in Purkinje cell viability. As we did not search biochemical signals of neuronal degeneration, we can not rule out the possibility that the neurons exhibiting less CB and GABA are committed to cell death. On the other hand, some trophic factors emerging from the glial cells could protect CB cells from injury as was previously observed in other neural systems (Pérez-Navarro, 1996).

As earlier mentioned, CB has been reported to protect neurons from degeneration by buffering intracellular calcium. A lesion-induced increase in the proportion of CB immunoreactive neurons should be expected if CB is neuroprotective. However, our data mainly show a significant reduction in the CB expression in the optic tectum after monocular retinal ablation, which discards a protective role of this protein.

In conclusion, monocular retinal ablation produces a significant reduction in the CB expression in several layers from chick optic tectum. This effect, that alters CB immunostaining of neuropil from layers 5-6 and somata of neurons from layers 8 and 10, is mainly observed 15 days after surgery. Wallerian degeneration of axons from retinal ganglion cells but not somata degeneration from tectal neurons occurs at the same time. Double labeling of CB and GABA reveals that a high proportion of CB neurons are GABAergic, and retinal ablation also produces GABA depletion, including in some CB negative cells. It appears that in chick optic tectum, most of CB molecules form part of neurons from the tectal GABAergic inhibitory circuit, whose functioning depends on the integrity of the visual input. Further studies are necessary to elucidate if calbindin plays a role in the intracellular Ca^{2+} homeostasis in the GABAergic tectal cells when the visual input arrives to the optic tectum.

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