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Long-Term Treatment of the Developing Retina with the Metabotropic Glutamate Agonist APB Induces Long-Term Changes in the Stratification of Retinal Ganglion Cell Dendrites

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Key Words

Cat retina · a and b sublaminae · Inner plexiform layer · Glutamate receptors

Abstract

The gradual restriction of initially multistratified retinal ganglion cell (RGC) dendrites into ON and OFF sublaminae of the inner plexiform layer (IPL) can be effectively blocked by treating the developing retina with 2-amino-4-phosphonobutyrate (APB), the metabotropic glutamate agonist, or by light deprivation. Previous studies have focused on the short-term consequences of such manipulations, so the long-term effects of arresting dendritic stratification on the structural development of RGCs are as yet unknown. In the present study, we have addressed this issue by performing a morphological analysis of α RGCs labeled by retrograde transport of horseradish peroxidase injected into the dorsal lateral geniculate nucleus of adult cats that received monocular injections of APB from postnatal (P) day 2 until P30. A large proportion of the α cells in the APB-treated eye (44%) were found to have multistratified dendrites that terminated in both the ON and OFF sublaminae of the IPL. The dendritic arborization pattern in the sublaminae of the IPL of these cells was asymmetric, showing a variety of forms. Immunolabeling of retinal cross-sections showed that mGLUR6 receptors appeared normal in density and location, while qualitative observation suggested an increase in the axonal arborization of rod bipolar cells. These findings indicate that long-term treatment of the neonatal retina with APB induces a long-lasting structural reorganization in retinal circuitry that most likely accounts for some of the previously described changes in the functional properties of RGCs.

Introduction

The way in which neurons acquire their complex and precise morphological and functional specificity is of considerable interest. The visual system has long been a favorite model for developmental studies and the mechanisms that regulate dendritic and axonal development of retinal ganglion cells (RGCs) are beginning to emerge

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[Wingate, 1996; Wong and Ghosh, 2002; Chalupa and Huberman, 2004]. Early in development the dendrites of RGCs ramify throughout the inner plexiform layer (IPL), and the restriction of these processes into ON and OFF sublaminae occurs mainly during the period when bipolar cells form synapses with RGCs [Maslim and Stone, 1986, 1988], implying a causal link between these two developmental events [for review see Chalupa and Gunhan, 2004].

There is evidence that the stratification of RGC dendrites depends on glutamate-mediated activity since intraocular treatment of the developing retina with 2-amino-4-phosphonobutyrate (APB), a metabotropic glutamate agonist that hyperpolarizes ON bipolar and rod bipolar cells, thereby preventing their release of glutamate [Slaughter and Miller, 1981], perturbs the stratification of RGC dendrites [Bodnarenko and Chalupa, 1993; Bodnarenko et al., 1995]. Moreover, such treatment during the critical period (first month of postnatal life) effectively modifies responses to light. An abnormally high percentage (37%) of RGCs were found to respond to both light onset and offset and a detailed analysis of receptive field organization of such neurons suggested changes in synaptic connectivity [Bisti et al., 1998]. The cells driven by the treated eye did not show a suppressive surround even though they responded to a single polarity (ON or OFF) in response to luminance increments or decrements. Taken altogether, these observations suggested a substantial reorganization in retinal circuitry following long-term blockade of glutamate release by retinal interneurons.

In an effort to relate these functional modifications to morphological changes, the dendritic trees of RGCs were filled with horseradish peroxidase (HRP). The enzyme was injected at the end of the recording session into the dorsal lateral geniculate nucleus (dlgn) of adult animals in which the release of glutamate from ON and rod bipolar cells was blocked in one eye during the first postnatal month. In addition frozen sections of retinal tissue taken from treated and untreated eyes were immunostained to assess whether APB treatment induced changes at the level of second-order neurons. Some of our findings have been summarized in abstract form [Deplano et al., 1999; Deplano et al., 2002].

Materials and Methods

Retinae analyzed in the present experiments were obtained at the end of the recording session from 4 animals which received intraocular injections of APB during the first postnatal month in one eye [Bisti et al., 1998]. All procedures were in compliance with National Institutes of Health guidelines and approved by the Animal Care and Use Committee of the Consiglio Nazionale delle Ricerche.

Intraocular Injections

APB was injected into the right eyes of newborn cats at P2 and continued daily until P32, with a 2 days respite for the weekends. The animal was anesthetized with 4% halothane in oxygen, and intraocular injections were made with a 10-μl syringe, containing 0.092 mg of APB diluted in sterile saline, and a 28-gauge needle. This dosage was the same as that used in previous electrophysiological and anatomical studies [Slaughter and Miller, 1981; Bodnarenko et al., 1995]. The injections were made into the temporal portion of the sclera at the level of the ora serrata, and great care was taken to insert the needle through the initial opening for all the subsequent injections. Although we could not verify the diffusion of APB across the retina, it has to be pointed out that neither single unit recordings [Bisti et al., 1998] nor the analysis of RGCs' dendritic stratification patterns showed major differences between cells sampled from nasal and temporal retinal regions, suggesting an even distribution of the drug. After the last injection at P32, the animals were allowed to reach maturity in the colony.

Animal Preparation and HRP Injections

When an animal was at least 4 months of age, it was used for the electrophysiological and morphological experiments. Anesthesia was initially induced by an injection of ketamine (Ketalar; Parke-Davis, Courbevoic, France; 30 mg/kg, i.m.), and an endotracheal tube and venous cannula were inserted. Anesthesia was maintained by Farmotal (i.v. sodium thiopental; Famitalia, Italy; 1.5 mg/kg/h) during surgery and throughout the recording session. Bilateral openings were made in the skull to allow insertion of microelectrodes into the dlgn and the optic tract, contralateral and ipsilateral to the treated eye. In 2 animals, after recordings were completed on one side of the brain, a pressure injection of HRP was made into the dlgn before recordings were made from this structure on the other side. The electrode was removed and a Hamilton syringe [10 μl, filled with 30% HRP (Sigma, St. Louis, Mo.) in 2% dimethylsulfoxide (DMSO; Sigma) and 0.5% poly-*L*-ornithine] was positioned at the same depth. A total of 27 injections (3 μl each) were performed at nine positions of both dlgns (3 injections at each position 1 mm apart in depth). After 2 days, a lethal dose of Ketalar was injected; the animals were perfused, and the retinae were removed, processed for HRP and whole-mounted.

We analyzed a total of 2,207 α cells along the horizontal and vertical meridian in both control (1,058) and treated (1,149) retinae and the spatial coordinates of each neuron were determined and fed to the computer. These neurons were chosen for analysis because they were found to be exceptionally well-filled by HRP injections [Kirby and Chalupa, 1986]. Dendritic arborizations of α cells were imaged at high magnification and the analysis of their distribution at different depths of the IPL was conducted by means of a computer-aided system [Deplano et al., 1994].

Immunohistochemistry

In 2 animals, at the end of the recording session, the eyes were enucleated immediately after euthanasia. The posterior eye cups were immersion fixed in 4% buffered paraformaldehyde for 30 min, washed in 0.1 *M* phosphate-buffered saline (pH 7.4), the retinae

were dissected from the eye cup and cryoprotected by immersion in 15% sucrose overnight. Retinae were then embedded in Tissue Tek OCT compound (Miles Inc., USA), snap frozen in liquid nitrogen/isopentane and cryosectioned at 20 μm. Sections were collected on gelatinized slides coated with poly-*L*-lysine. They were then immunolabeled for one or two of the following proteins: mGLUR6 to label glutamate receptors on rod and ON bipolar cells and protein kinase C (PKC) to label rod bipolar cells. The antibody for the mGLUR6 was a rabbit polyclonal (gift of Prof. Noga Vardi); it was used at a dilution of 1:5,000. The antibody for PKC was a mouse monoclonal (Sigma) 1:200, the secondary antibody for mGLUR6 was Alexa 594-conjugated goat anti-rabbit IgG (Molecular Probes, Inc., USA). The secondary antibody for PKC was Alexa 488-conjugated goat anti-mouse (Molecular Probes, Inc., USA). Confocal microscopy (Leica TCS-NT equipped with a krypton-argon laser) was used to take images of immunolabeled sections. High-resolution scanning was performed with a Plan-Apochromat 63×/1.4 and 40×/1.4 objective at a resolution of 1,024 × 1,024 pixels. The brightness and the contrast of the final images were adjusted using Adobe Photoshop 6.

Results

HRP-Filled Dendritic Trees

RGCs were analyzed from 4 retinae (2 control and 2 APB-treated). As may be seen in figure 1 long-term treatment did not alter the overall appearance of the ganglion cells in terms of the overall density of α and β cells. A different pattern became clear at a higher magnification as shown for 2 α cells in figure 2. As may be seen, the dendrites of the 2 ganglion cells (A, B and C, D) ramify in more than one layer of the IPL, but the stratification pattern is different for each neuron. For one of the cells (A), the dendritic arborization appears to be evenly divided into two strata, while the other cell (C) stratifies mainly at one level of the IPL, with a single dendrite reaching a deeper layer (D). For all the analyzed cells with a multistratified appearance, there was a main level of stratification with a variable number of dendrites ramifying at a different level of the IPL.

The percentage of multistratified RGCs (reported in table 1) was 44.5% and 44.4% in cat 1 and cat 2, respectively, comparable to the percentage of ON-OFF responding neurons found in these animals [Bisti et al., 1998]. The RGCs were analyzed in corresponding retinal regions along the horizontal and vertical axis in control and treated retinae; an example is shown in figure 3 with each symbol in the retina corresponding to the actual position of each analyzed neuron. In control retinae we did not find any multistratified RGCs. Since the periphery develops later than the central retina with respect to dendritic stratification [fig. 2 of Bodnarenko et al., 1995], we evaluated the percentage of multistratified neurons separately in central and peripheral retinal regions. Figure 4 shows the sampled RGCs in comparable dorsal areas of treated (RE) and untreated (LE) retinae, while table 2 shows the results in terms of the stratification patterns exhibited by these neurons. The sample from the central region is smaller than that obtained from the periphery, and most likely this reflects the fact that fewer RGCs were completely labeled in the central retina. Nevertheless, the percentage of multistratified RGCs is lower in the center compared to the periphery, suggesting that the more immature peripheral retina was affected more by APB treatment during the first postnatal month than the earlier maturing central portion of the retina.

To further analyze the RGCs in treated and untreated retinae, we defined the levels of dendritic stratifications within the IPL of the analyzed neurons. Examples are

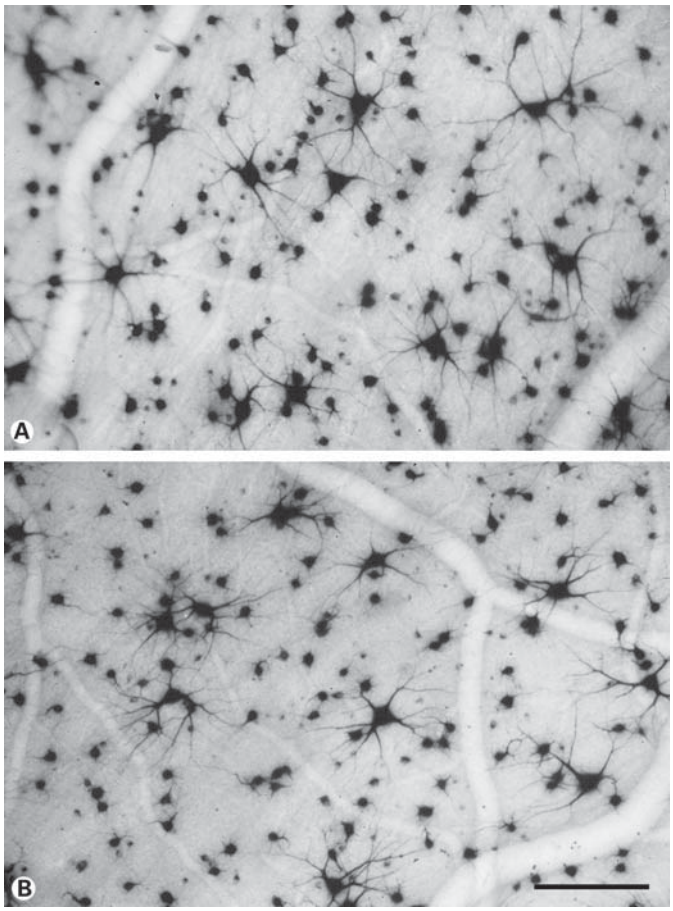


Fig. 1. Low-power photomicrographs taken in corresponding regions (9 mm dorsal to the optic disk) in the untreated (**A**) and treated retina (**B**) (cat 1). Scale bar = 250 μm.

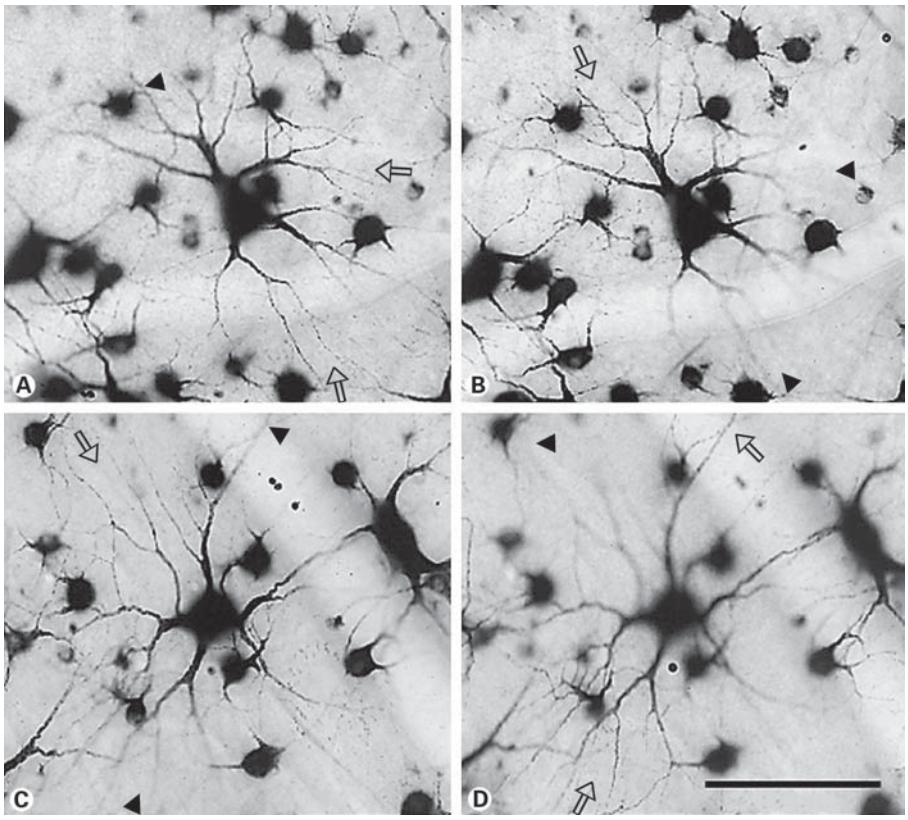


Fig. 2. Photomicrographs of 2 α cells (**A**, **B** and **C**, **D**) from treated retinae at two levels of focus. Two types of arrows indicate two levels of focus (the arrow tip points to out-of-focus dendrites). Scale bar = 100 μm.

Table 1. Incidence of multi-stratified RGCs in APB treated retinae

APB-treated retina	Total (α cells)	Mono-stratified	Multi-stratified	Multi-stratified
Cat 1	609	338	271	44.5%
Cat 2	540	300	240	44.44%

shown in figure 5. The thickness of the IPL was normalized, with the outer and inner limits of this synaptic layer being defined by the level where the cell bodies of amacrine and ganglion cells were brought into sharp focus. The thickness of the IPL, at matched eccentricities, was identical in treated and untreated retinae. For each neuron we measured the depth at which the dendritic arborization occurred. In control retinae (upper panel), the level of stratification for ON and OFF cells (represented by a rectangle) is clearly separated. It occurs for ON cells between 30 and 50% and for OFF cells between 70 and 90% of the total thickness of the IPL. In the treated retinae (lower panel) the stratification of ON cells corre-

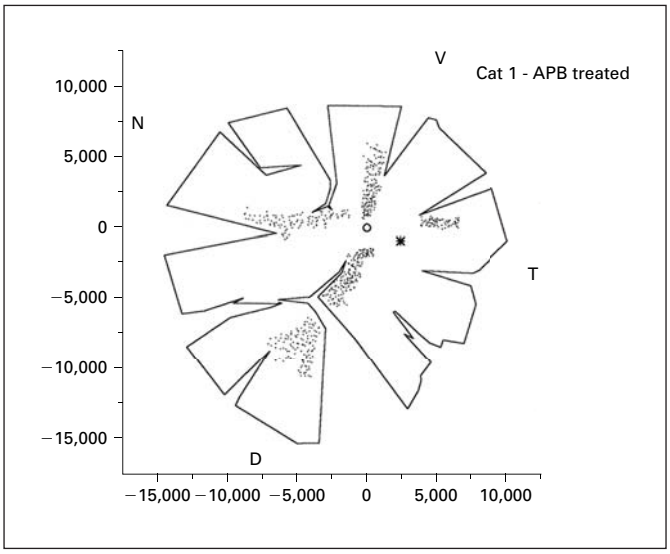


Fig. 3. Computer drawing of the right (treated) retina of cat 1. Symbols in the retina correspond to the position of the analyzed α cells. * = Area centralis; ○ = optic disk.

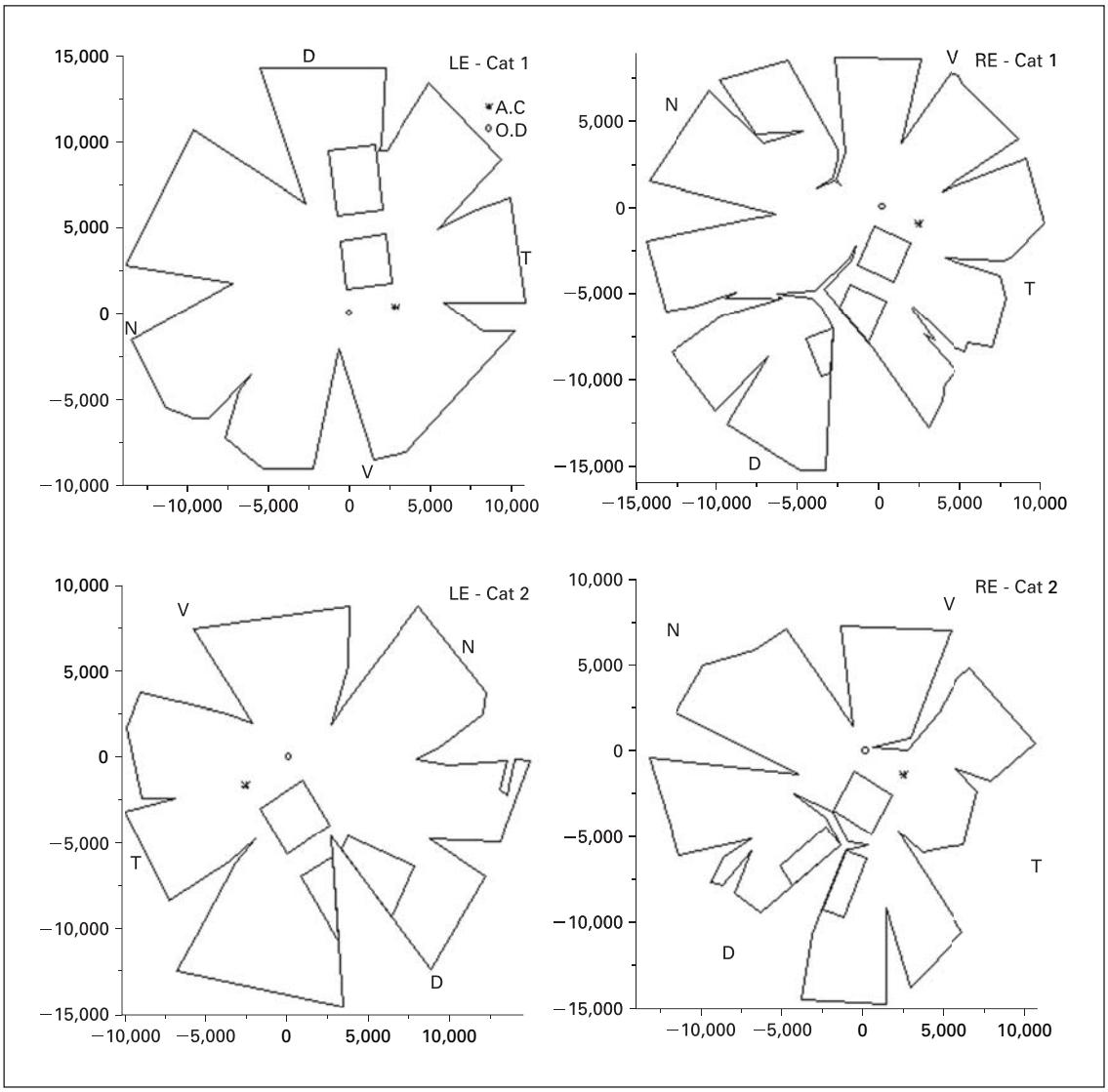


Fig. 4. Computer drawings of the 4 analyzed retinae, the two marked regions in each retina correspond to the dorsal sampling areas (central and peripheral) reported in table 2. LE = Untreated retina; RE = APB-treated retina. * = Area centralis; ○ = optic disk.

Table 2. Central vs. peripheral differences in the incidence of multi-stratified RGCs in the APB treated retinae

	Dorsal peripheral			Dorsal central		
	distance area considered – A.C. center, μm	total (α cells)	multistratified %	distance area considered – A.C. center	total (α cells)	multistratified %
Cat1, RE, treated	9,700	212	46.2	3,350	173	21.4
Cat1, LE, control	9,700	331	–	3,350	181	–
Cat2, RE, treated	6,500	220	47.3	3,350	181	23.2
Cat2, LE, control	6,500	298	–	3,350	240	–

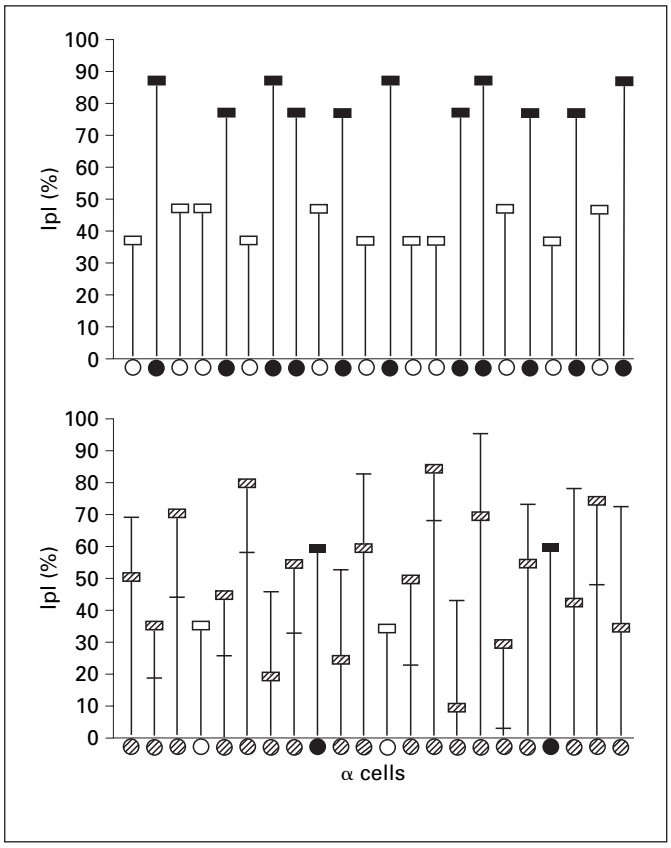


Fig. 5. Examples of stratification of α cell dendrites in the IPL of cat 2 (each circle represents one cell). The thickness of IPL was normalized, the outer and inner limits were defined by the level where the cell bodies of amacrine and ganglion cells went into focus. Upper panel (control), lower panel (treated). Filled circles = OFF cell; open circles = ON cell; shaded circles = multistratified cell.

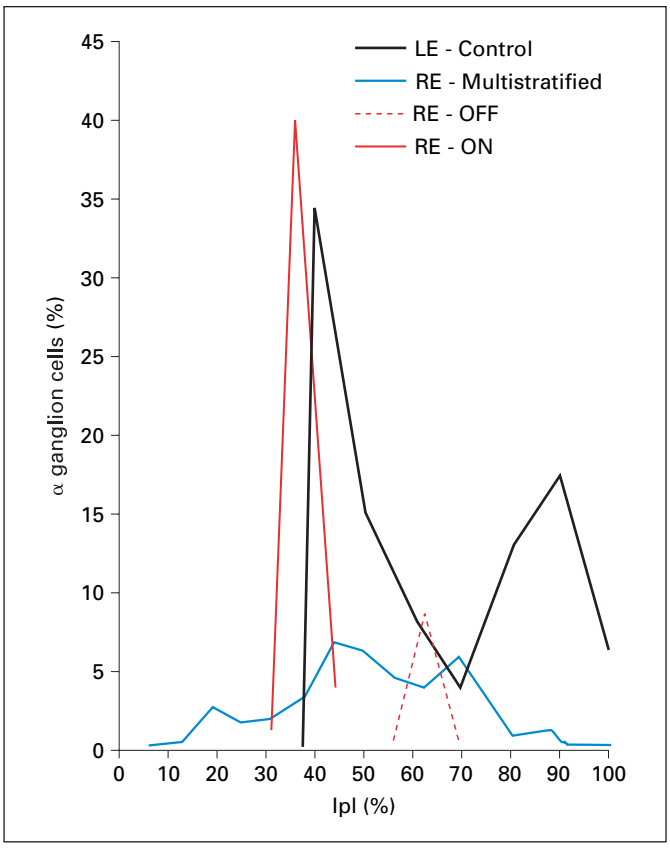


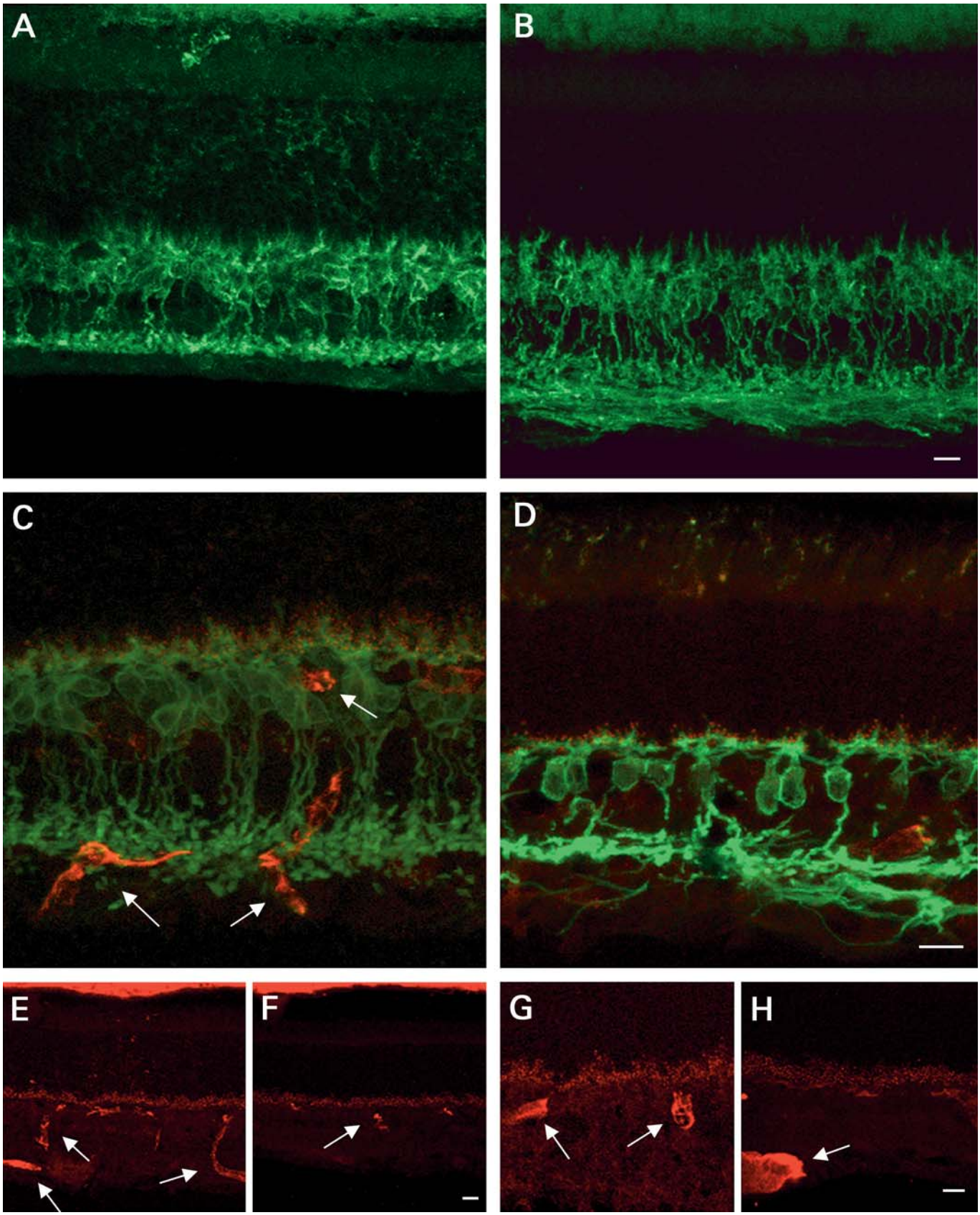
Fig. 6. Percentage of distribution of α cell dendrites as a function of IPL depth for the whole sample analyzed in treated (1,149 cells) and untreated (1,058 cells) retinae. 0 = inner limit RGC layer; 100 = outer limit INL.

sponds to that observed in control retinae, with OFF cells ramifying nearer to their cell bodies. Multistratified cells had a variable number of dendrites at various levels, the most conspicuous is represented by a rectangle, while the second level is represented as a line. Sometimes only a single dendrite ramified at a different depth than the main body of the dendritic tree (fig. 2C, D). All these types of neurons were classified as multistratified.

Figure 6 shows the level of stratification in the IPL for ON, OFF and multistratified cells in control and treated retinae. Depicted in this schematic drawing is only the main level of stratification for the multistratified cells (reported as a rectangle in fig. 5). In the control retinae, dendrites were clearly confined to two sublaminae of the IPL, outer (OFF) and inner (ON). In the APB-treated retinae, the proportion of α cells with dendrites in the b-sublamina (ON) was approximately normal, while the proportion of cells with dendrites in the a-sublamina (OFF) was less than normal. Note that the dendrites of multistratified cells were randomly distributed across the thickness of the IPL.

Immunohistochemistry

APB, an analog of glutamate, activates postsynaptic receptors that maintain the ON class of second-order neurons continuously hyperpolarized. These neurons normally respond to an increment of light and give rise to the ON pathway [for recent review, see Nelson and Kolb, 2004]. At birth, when the APB treatment was started, the outer segment of photoreceptors was immature, and it takes at least 10–15 days for the phototransductive cascade to be capable of responding to light. At the same


A
B
C
D
E
F
G
H

time, both the expression and the localization of postsynaptic receptors for glutamate (mGLUR6) on ON cone and rod bipolar cells undergo major changes. Receptors are initially expressed on both the soma and the dendritic arborization of bipolar cells and become restricted to the postsynaptic site at the end of the first postnatal month [Nomura et al., 1994; Ueda et al., 1997; Vardi et al., 2000].

We wondered whether perturbation of RGCs' dendritic stratification would be associated with changes in the development of mGLUR6 receptors and second-order neurons in the ON pathway activated by the mGLUR6. We immunolabeled rod bipolar cells of the ON pathway to visualize the localization of mGLUR6 receptors on their dendrites and soma. Confocal images of immunolabeled retinae are shown in figure 7. Rod bipolar cells, stained with an antibody against PKC, widely used as a selective marker of these classes of neurons, are presented in panels A and C for control and B and D for treated retinae. The sections shown in A and B are taken from corresponding retinal regions (central). As may be seen there is an extension of the axonal arborization of rod bipolar cells and this abnormal extension is maintained at all retinal eccentricities (the extreme periphery is shown in panel D). This observation needs to be studied in greater detail. On the other hand mGLUR6 receptors appear normally localized on rod bipolar dendritic arborizations in the OPL (panels C control and D treated), and they look similar in control and treated retinae, both in the center (panel E and F) and in periphery (G and H).

Discussion

In a previous study we showed that blocking release of glutamate from second-order retinal neurons during the critical period induced long-lasting modifications in the response properties of RGCs [Bisti et al., 1998]. The main effect was a substantially higher than normal incidence of cells that responded to a flashing spot of light with ON-OFF discharge patterns. We provide new evidence that these functional changes are associated with a substantial reorganization in the dendritic tree arborizations of RGCs. In addition, the present results offer interesting suggestions on a critical step in the retinal developmental plan, showing that APB treatment permanently modifies the axonal arborizations of rod bipolar cells. This qualitative observation needs additional studies to be accurately quantified and to be extended to ON bipolar cells.

In APB-treated eyes about 44% of RGCs were characterized as having multistratified dendritic arbors, and these were distributed across the retina in a clear central to peripheral gradient. This effect most likely reflects the retinal developmental plan, where the central retina matures earlier than the periphery, a gradient that was present at the time the APB treatments were initiated in our study. Indeed, the stratification of RGC dendrites begins in the fetal cat in the central retina about 2 weeks before birth [Bodnarenko et al., 1995]. This observation helps to clarify the small discrepancy between the electrophysiological and anatomical results: single unit recordings showed a sampling bias in the mid-periphery as observed from mapping the positions of receptive fields.

Electrophysiological data showed peculiar characteristics in response properties and receptive field organization of RGCs, some of which could be explained on the basis of the dendritic tree reorganization. The classification of receptive fields into 'uniform' and 'patchy' according to their responses to spots of flashing light can be better understood by taking into account the pattern of dendritic stratification. It was impossible to classify RGCs into discrete classes according to this parameter, but it is reasonable to assume that a neuron which ramifies mainly in one sublayer with only one branch crossing the entire IPL may have a 'patchy' receptive field showing ON-OFF responses in one subregion of the receptive field and ON or OFF in another subregion. If this line of reasoning is correct, the extensive branching throughout the IPL may subserve a 'uniform' receptive field.

The analysis of dendritic arborization does not offer any explanation for the reported absence of a suppressive periphery, even in RGCs showing a clear ON or OFF response with dendrites supposedly restricted to one sublamina. As has been suggested previously [Bisti et al., 1998], the consequence of APB treatment may extend beyond the effect on RGC dendrites inducing a reorganization in the synaptic contacts of horizontal and amacrine cells which are responsible for generating the receptive field surrounds.

Fig. 7. Confocal micrographs of sections through the cat retina labeled for PKC (**A**, **B**) and for mGLUR6 (**E**–**H**) and double-labeled for PKC and mGLUR6 (**C**, **D**). **A**, **C**, **E** and **G** = control retina. **B**, **D**, **F** and **H** = APB-treated retina. In **C** and **D** all mGLUR6 immunoreactive puncta in the OPL coincide with the dendritic arborization of PKC-labeled rod bipolar cells. The arrows point to nonspecific red fluorescence on blood vessels due to the secondary antibody as it is seen in control section stained without primary antibody. Scale bar = 10 μm.

It should be noted that while APB selectively blocks the ON pathway in the mature retina, this glutamate analog has a strikingly different effect on the visual responses of RGCs in the developing retina. Immature RGCs with multistratified dendrites have been shown to respond to both light onset as well as light offset [Wang et al., 2001]. Moreover, application of APB has been found to abolish both ON and OFF discharges in ganglion cells with multistratified dendrites in the developing retina. This means that our application of APB during the critical period affected the maturation of both the ON and the OFF pathway, and this is entirely in line with our observation that pairs of adjacent α cells were similarly affected by APB treatment (fig. 2). In the normal retina one member of such a pair is typically an ON cell while the other is an OFF cell.

We also observed an abnormal increase in the axonal arborization of rod bipolar cells in the APB-treated retinae, but it is unclear how this relates to a change in functional properties of the treated retina. Since depletion of RGCs does not alter the development of normal projections of cone bipolar cells [Gunhan-Agar et al., 2000], we have to ascribe the observed modification in rod bipolar cells to either a presynaptic effect or, as we already discussed, to a postsynaptic unbalance (reduced availability of glutamate). An additional observation of interest comes from our immunostained retinal sections. The mGLUR6 receptors on rod bipolar cells did not show any major modification after long-term APB treatment. This reinforces the idea that ganglion cell dendritic reorganization is controlled by the release of glutamate by second-order retinal neurons. This hypothesis is also supported by recent data showing that afferent activity can selectively and differentially regulate dendritic structure by modulating Ca^{2+} release [Lohmann et al., 2002]. Moreover, in knockout mice lacking mGLUR6 receptors stratification of RGC dendrites occurs normally [Tagawa et al., 1999], so in these animals the glutamate released by photoreceptors is unable to hyperpolarize rod bipolar and ON cone bipolar cells, and presumably, these retinal interneurons would be in a constantly depolarized state. The results of the present study demonstrate clearly that blocking such glutamate release during the critical period causes a long-lasting, and most likely, a permanent change in this process. It would now be of interest to determine the consequences of such a seemingly permanent reorganization in retinal circuitry on visual information processing.

The results of the present study dealing with long-term APB treatment (i.e., 30 days) offer an interesting comparison with the previously reported effects of short-term (4–11 days) APB application [Bodnarenko and Chalupa, 1993; Bodnarenko et al., 1995]. In both cases the APB treatments were initiated at P2, but two striking differences in the results are worth underscoring. First, after short-term treatment the effects of APB are virtually completely reversible by prolonged normal experience. Thus, the incidence of multistratified cells was found to return to normal levels 80 or more days after APB treatment was terminated at P10 [fig. 7 of Bodnarenko et al., 1995]. By contrast, in the present study we found that treating the retina with APB from P2 to P32 resulted in a much higher than normal incidence of multistratified cells in the treated eye of mature animals (i.e., older than 4 months of age). Indeed, the incidence of multistratified cells was nearly equivalent immediately after short-term and long-term APB treatment, being nearly 40% in both instances. This brings up the second notable difference between the effects of short-term and long-term APB treatment. After short-term treatment the dendrites of the multistratified cells ramified throughout the extent of the IPL, comparable to the state evident during normal development. The impression one gets is that the stratification process was effectively arrested by APB treatment [for example, fig. 1 of Bodnarenko and Chalupa, 1993 and also fig. 1 of Bodnarenko et al., 1995]. By contrast, in mature retinae that sustained long-term APB treatment, the extension of multistratified dendrites was much less pronounced; typically only one or a few dendritic arborizations extended into presumably inappropriate portion of the IPL.

Collectively, these observations indicate that even after long-term APB treatment of the developing retina considerable restructuring of dendritic arbors occurs in older animals (more than a month of age) once normal visual experience is permitted. Thus, long-term APB treatment did not simply 'freeze' the immature multistratified state, but rather a substantial retraction of widespread dendritic processes occurred, even after the time period when dendritic restructuring is normally largely completed.

Recently, Tian and Copenhagen [2001, 2003] showed that rearing mice in constant darkness resulted in a significant increase in the number of multistratified RGCs and a corresponding increase in the number of ON-OFF responsive ganglion cells in the dark-reared animals. These results demonstrate that the functional and morphological maturation of ON-OFF pathways in the retina requires light stimulation. In these studies, the dark-reared mice were examined near the end of the first postnatal month, shortly after the end of the dark-rearing period. In view of the long-term morphological changes in the retina of APB-treated eyes described in the present

study, it would be of interest to determine whether comparable long-term consequences are apparent in mature animals that underwent dark rearing early in development.

Acknowledgments

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