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Calcium-binding proteins in the retina of a calbindin-null mutant mouse

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Abstract Calcium-binding proteins are abundantly expressed in many neurons of mammalian retinae. Their physiological roles are, however, largely unknown. This is particularly true for calcium-modulating proteins ("calcium buffers") such as calbindin D28k. Here, we have studied retinae of wildtype (+/+) and calbindin-null mutant (–/–) mice by using immunocytochemical methods. Although calbindin immunoreactivity was completely absent in the calbindin (–/–) retinae, those cells that express the protein in wildtype retinae, such as horizontal cells, were still present and appeared normal. This was verified by immunostaining horizontal cells for various neurofilament proteins. In order to assess whether other calcium-binding proteins are upregulated in the mutant mouse and may thus compensate for the loss of calbindin, mouse retinae were also immunolabeled for parvalbumin, calretinin, and a calmodulin-like protein (CALP). In no instance could a change in the expression pattern of these proteins be detected by immunocytochemical methods. Thus, our results show that calbindin is not required for the maintenance of the light-microscopic structure of the differentiated retina and suggest roles for this protein in retinal function.

Key words Calbindin · Parvalbumin · Calretinin · Neurofilament protein · Calmodulin-like protein · Mouse [calbindin null mutant (–/–)]

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Introduction

The calcium-binding protein calbindin D28k is a member of the large family of "EF-hand" proteins, which bind Ca^{2+} with high affinity (Persechini et al. 1989; Heizmann and Hunzicker 1991; Baimbridge et al. 1992; Polans et al. 1996; Schäfer and Heizmann 1996). The EF-hand consists of two α-helices, viz., "E" and "F", joined by a Ca^{2+}-binding loop. The expression of calbindin in the retina has been studied in a variety of mammalian species; however, the pattern of expression varies remarkably between different species and even at different retinal locations. In the monkey retina, for example, calbindin is present in cones outside the fovea but is absent from foveal cones (Haley et al. 1995). In the inner nuclear layer (INL), subpopulations of horizontal, bipolar, and amacrine cells express calbindin. In the ganglion cell layer, some displaced amacrine cells show strong immunoreactivity for calbindin, whereas ganglion cells are only weakly labeled (Verstappen et al. 1986; Röhrenbeck et al. 1987, 1989; Pasteels et al. 1990; Grünert al. 1994). In the rat retina, on the other hand, calbindin labeling is most prominent in horizontal cells (Rabié et al. 1985; Pasteels et al. 1990; Pochet et al. 1991; Feigenspan et al. 1993; Peichl and González-Soriano 1994). Distinct label is also present in amacrine cells, in three horizontal bands in the inner plexiform layer (IPL), and in ganglion cells and displaced amacrine cells.

Further EF-hand proteins, which have characteristic distributions in various mammalian retinae are parvalbumin (PV), calretinin, recoverin, neurocalcin, and visinin-like proteins. Again, the cell types labeled with specific antibodies against these different calcium-binding proteins vary between different species (Endo et al. 1985, 1986; Rogers 1987; Pasteels et al. 1990; Sanna et al. 1990; Pochet et al. 1991; Nakano et al. 1992; de Raad et al. 1995). PV for instance, is strongly expressed in the horizontal cells of the primate retina, whereas very weak expression is found in the horizontal cells of the rat retina (Röhrenbeck et al. 1989; Peichl and González-Soriano 1994). AII amacrine cells of the rat retina are in-

tensely labeled for PV, whereas the same type of neuron is immunoreactive for calretinin in the monkey retina (Wässle et al. 1993, 1995).

Recently, calbindin-null mutant mice have been generated by gene-targeting (Airaksinen et al. 1997a,b). In the present immunocytochemical study, we demonstrate the pattern of calbindin expression in wildtype and mutant mouse retinae and show that there are no gross structural changes in the retinae of null mutant mice. The apparent incoherence in the expression pattern of various calcium-binding proteins, when homologous cell types are compared across species, and the variations in the expression patterns with retinal location suggest that there might be a mutual regulation of these proteins. We have therefore compared PV, calretinin and calmodulin-like protein (CALP) immunoreactivities in normal and mutant retinae.

Materials and methods

Animals and tissue preparation

Principles of laboratory animal care and specific national laws were followed. The generation of calbindin-deficient mice has been described elsewhere (Airaksinen et al. 1997a,b). Sex-matched wildtype (+/+) and homozygous calbindin-null mutant (–/–) littermates (aged 6–7 months and weighing 30–40 g), which were derived from heterozygous females and males, were used. Mice were killed by an overdose of CO_2. The eyes were quickly enucleated and transferred to a Petri dish containing 0.1 M phosphate buffer (PB, pH 7.4, 4°C). The eyes were opened and the eye cups were immersion-fixed in 4% paraformaldehyde (PA) in 0.1 M PB (pH 7.4, 4°C) for 2 h. After fixation, the retinae were washed in PB for 2 h and immersed in 30% sucrose in PB at 4°C overnight. The retina of a (+/+) mouse and the retina of a (–/–) mouse were mounted and frozen on top of each other in freezing medium (Reichert-Jung) in the form of a sandwich and sectioned vertically at 14 µm on a cryostat. Sections were collected on gelatin-coated slides and stored at –20°C before use. With this sandwich method, each slide had sections of (+/+) and (–/–) mice retinae next to each other. This ensured that the sections experienced exactly the same treatment throughout the immunostaining protocol and allowed a direct detailed comparison of the labeling patterns and intensities.

Immunocytochemistry

Antibodies

Calbindin was detected with three mouse monoclonal antibodies: a commercially available antibody anti-calbindin-D (dilution 1:1000; clone CL-300, Sigma) and two antibodies (15C3 and 13D3, dilution 1:2000) kindly provided by W. Hunziker at Hoffmann-La Roche, Basel (Pinol et al. 1990). PV was detected with a commercially available mouse monoclonal antibody (dilution 1:1000; clone PA-235, Sigma) and a rabbit antiserum PV 4064 (dilution 1:500; kindly provided by M. R. Celio, Fribourg). Calretinin was detected with a rabbit antiserum Ab6C (dilution 1:2000) kindly provided by J. H. Rogers, Cambridge (Rogers 1987; Pasteels et al. 1990). Calmodulin-like proteins were detected by a rabbit antiserum against CALP (dilution 1:1000) kindly provided by M. Kreutz, Magdeburg (Seidenbecher et al. 1997). The neurofilament triplet protein consists of a light (NF-L), a medium (NF-M), and a heavy (NF-H) subunit. We used the mouse monoclonal antibody 2F11 (dilution 1:10; no. MON 3004, Sanbio) recognizing NF-L and NF-H, the mouse monoclonal antibodies N52 (dilution 1:200) and NE14 (dilution 1:500; both from Sigma) recognizing NF-H, and the mouse monoclonal antibodies DA2 (1:500, against NF-L) and 3H11 (1:500, against NF-M) kindly provided by G. Shaw, Gainsville (Harris et al. 1991).

Immunostaining

The immunoperoxidase staining procedure of the sections was as follows: (1) wash in PB; (2) preincubation in PB with 10% normal goat serum (NGS) and 0.5% Triton X-100 for 30 min; (3) overnight incubation in the primary antibody/antiserum, diluted in PB containing 3% NGS, 0.5% Triton X-100 and 0.01% sodium azide; (4) wash in PB; (5) 1 h incubation in goat anti-mouse IgG or goat anti-rabbit IgG (for primary antibodies/antisera from mouse or rabbit, respectively), diluted in PB with 0.5% Triton X-100; (6) wash in PB; (7) 1 h incubation in mouse peroxidase-antiperoxidase complex (PAP) or rabbit PAP, respectively, diluted in the same medium as step (5); (8) wash in PB; (9) visualization of the peroxidase with diaminobenzidine (DAB) and H_2O_2. All steps were carried out at room temperature. Control sections were processed as above, except that the primary antibodies were omitted. They showed no specific immunoreactivity with either the protocol for mouse antibodies or that for rabbit antisera.

Results

Calbindin immunoreactivity

In the calbindin (+/+) mouse retina, calbindin immunoreactivity was most prominent in horizontal cells (Fig. 1A,C). Their large cell bodies at the outer margin of the INL and their processes in the outer plexiform layer (OPL) were strongly labeled (Pochet et al. 1991). At high magnification (Fig. 1C), the terminal boutons of horizontal cell axons, which form the contacts with rod spherules, could be detected. In the inner retina, several types of amacrine cells, displaced amacrine cells, and probably also ganglion cells were labeled with variable intensities (Fig. 1A). Two narrow bands were labeled within the IPL. This pattern of immunoreactivity was observed with three different antibodies against calbindin.

No specific labeling for calbindin could be detected in the calbindin (–/–) mouse retina (Fig. 1B). Again, this was confirmed with all three antibodies used. Occasional immunoreactivity of blood vessels was attributable to endogeneous peroxidases. By high-power Nomarski microscopy, cell bodies of unlabeled horizontal cells could be detected (Fig. 1D), showing that the horizontal cells were still present in the (–/–) mouse retina.

The presence of horizontal cells was further corroborated by immunostaining the retinae of (+/+) and (–/–) mice for neurofilament proteins. It has been previously shown that, in certain rodents including mice, horizontal cell axon terminals can be stained with antibodies against neurofilament (NF) proteins (Dräger 1983, 1985; Dräger et al. 1984; Hammang et al. 1992, 1993; Peichl and González-Soriano 1993, 1994). Altogether five antibodies that recognize the three subunits of the NF triplet were applied in the present study. Figure 2A shows a section of (+/+) mouse retina immunolabeled for the light (NF-L) and heavy (NF-H) subunits. Label in the OPL was confined

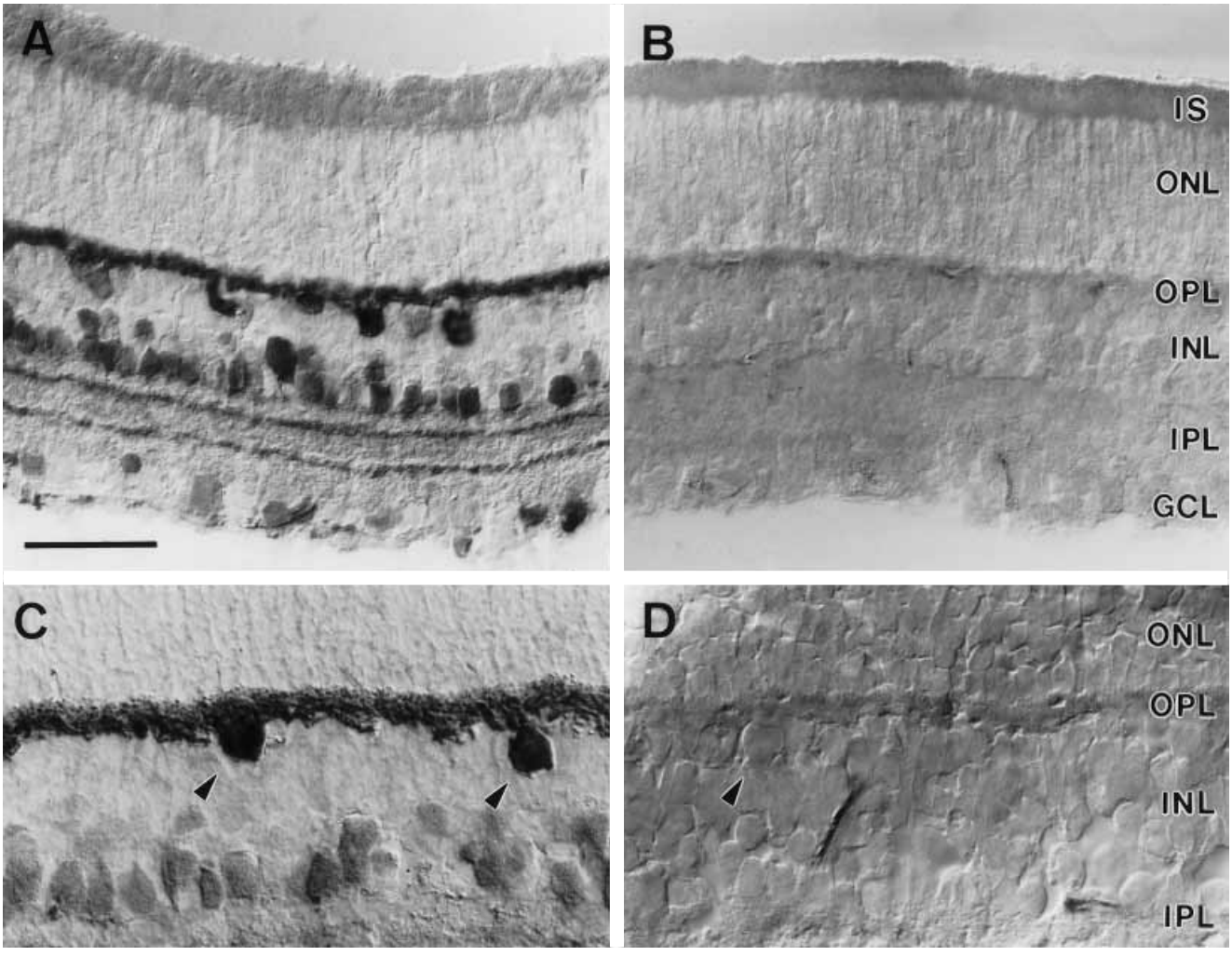

Fig. 1A–D Micrographs of vertical cryostat sections through mouse retinae immunolabeled for calbindin. Immunoreactivity was visualized with the peroxidase-anti-peroxidase (PAP) technique, and the micrographs were taken with Nomarski optics. *IS* Inner segments, *ONL* outer nuclear layer, *OPL* outer plexiform layer, *INL* inner nuclear layer, *IPL* inner plexiform layer, *GCL* ganglion cell layer. **A** Calbindin immunoreactivity in a normal (+/+) mouse retina is found in horizontal, amacrine, displaced amacrine, and ganglion cells. **B** Calbindin immunoreactivity is absent from the calbindin-null mutant (–/–) mouse retina. **C** Horizontal cells (*arrowheads*) and their terminals are labeled most intensely in the calbindin (+/+) mouse retina. **D** An unlabeled horizontal cell body (*arrowhead*) shows that horizontal cells are still present in the calbindin (–/–) retina. *Bar* in **A** 50 μm for **A**, **B** and 35 μm for **C, D**

to the axons of horizontal cells (Peichl and González-Soriano 1993, 1994). The same labeling pattern was also found in the (–/–) mouse retina (Fig. 2B). In sections taken from the vicinity of the optic nerve head, prominent labeling was also found in the optic nerve fibre layer (not shown). Immunolabeling for the medium (NF-M) subunit in the (–/–) mouse (Fig. 2C) also revealed horizontal cell axons in the OPL and optic nerve fibres. The heavy (NF-H) subunit (Fig. 2D) was similarly expressed in horizontal cell axon terminals, in large ganglion cells, and in optic nerve fibre bundles.

With all the antibodies used here, the labeling of neurofilament proteins in (+/+) and (–/–) mouse retinae was indistinguishable. This is strong evidence that the retinal horizontal cells are still present in the (–/–) mouse, and that their neurofilament proteins are normal.

Other calcium-binding proteins

Parvalbumin

Horizontal cells of primate and cat retinae are stongly immunoreactive for PV (Röhrenbeck et al. 1987). In the rat retina, horizontal cells showed strong calbindin immunoreactivity; however, weak PV immunoreactivity was also observed in horizontal cells when postembedding immunocytochemistry was applied to semithin sections (Fig. 6 of Peichl and González-Soriano 1994). Hence, there is the possibility that mouse horizontal cells also express PV, and that the PV level is upregulated in calbindin (–/–) mice.

Figure 3A,B shows vertical sections of retinae that were immunostained for PV. In the (+/+) mouse (Fig. 3A), PV immunoreactivity is found in the ganglion

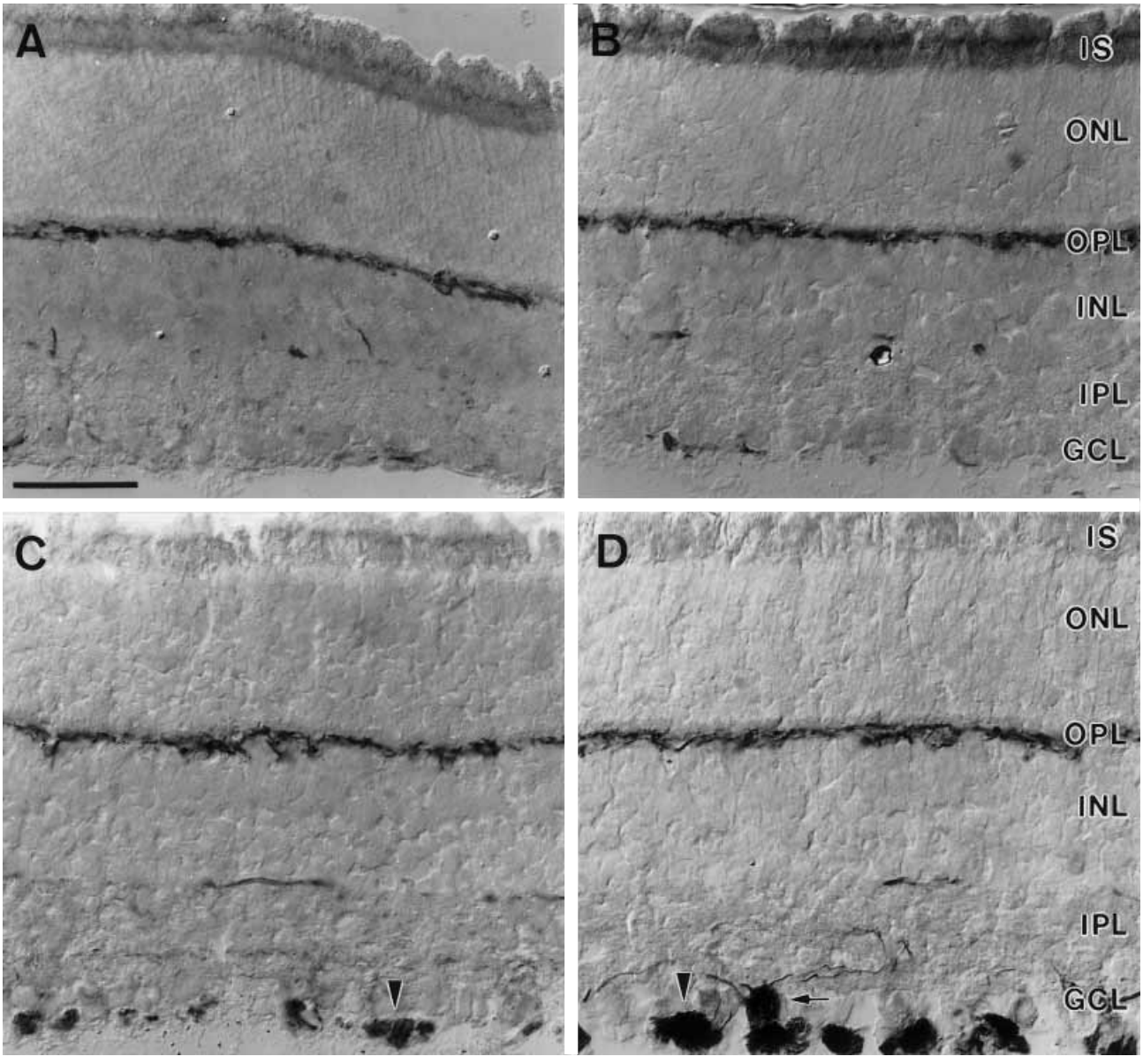

Fig. 2A–D Micrographs of vertical cryostat sections through mouse retinae immunolabeled for neurofilament (NF) proteins. **A** Horizontal cell axons are labeled in the OPL of the normal (+/+) mouse retina by antibody 2F11, which recognizes the light (NF-L) and the heavy (NF-H) subunits of the neurofilament triplet protein. **B** In the calbindin (–/–) mouse retina similarly prominent labeling of horizontal cell axons is found with antibody 2F11. **C** Antibody 3H11 against the medium (NF-M) subunit labels horizontal cell axons and optic nerve fibre bundles (*arrowhead*) in the calbindin (–/–) retina. **D** Antibody N52 against NF-H labels horizontal cell axons, large ganglion cells (*arrow*), and optic nerve fibres (*arrowhead*) in the calbindin (–/–) retina. *Bar* 50 µm for **A–D**, abbreviations as in Fig. 1

cell layer, i.e., in ganglion cells and possibly displaced amacrine cells. Their dendrites ascending into the IPL are also labeled, as are bundles of optic nerve fibres. In the OPL, only very weak PV immunoreactivity can be detected. The labeling pattern of the (–/–) mouse retina (Fig. 3B) is indistinguishable and, in particular, there is no evidence for an increase in PV immunoreactivity in the OPL.

Calretinin

Calretinin labeling in the (+/+) mouse retina was very strong in the ganglion cell layer (Fig. 3C). Ganglion cell bodies and putative displaced amacrine cell bodies showed variable labeling intensities. In the IPL, three narrow bands were strongly imunoreactive, and some amacrine cells were found to be labeled in the INL. The labeling pattern for calretinin in the (–/–) mouse (Fig. 3D) is similar to that of the (+/+) mouse (Fig. 3C). Thus, there is no apparent upregulation of calretinin in the calbindin-deficient mouse.

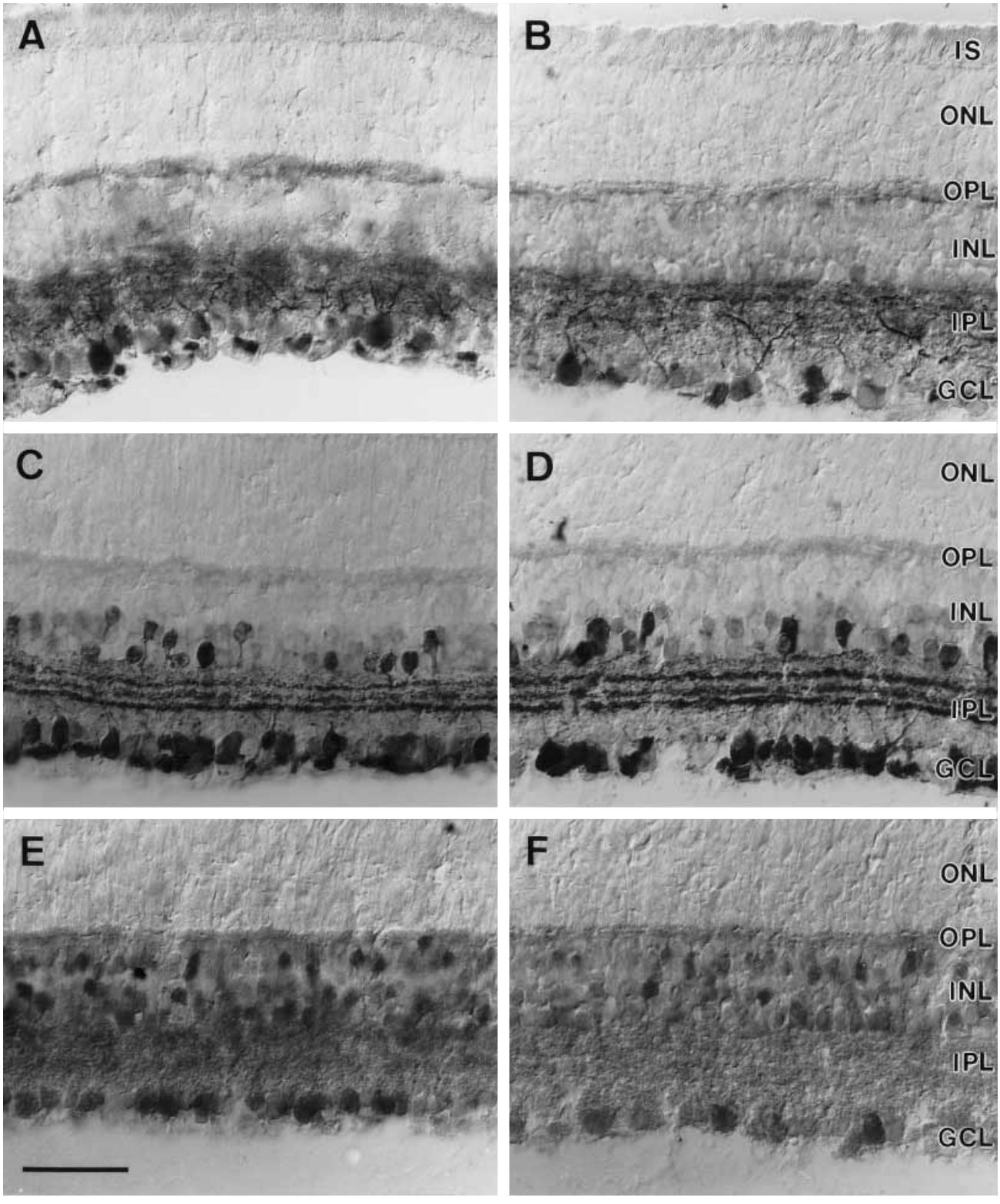

Fig. 3 Micrographs of vertical cryostat sections through mouse retinae immunolabeled for PV (**A,B**), calretinin (**C,D**), and CALP (**E,F**). **A** In the normal (+/+) mouse retina, PV immunoreactivity is found in cell bodies of the ganglion cell layer and in their dendrites in the IPL. Weak immunoreactivity is also present in the OPL. **B** The identical pattern is observed in the calbindin (–/–) mouse retina, suggesting that there is no upregulation of PV. **C** Cell bodies of amacrine, displaced amacrine, and ganglion cells are intensely labeled for calretinin in the normal (+/+) mouse retina. Their dendrites and processes form three distinct bands in the IPL. **D** The identical pattern is found in the calbindin (–/–) mouse retina, suggesting that there is no upregulation of calretinin. **E** CALP immunoreactivity in the normal (+/+) mouse retina is found in some bipolar, amacrine, displaced amacrine, and ganglion cells. **F** The identical labeling pattern is seen in the calbindin (–/–) retina, suggesting that there is no upregulation of CALP. *Bar* 50 µm for **A–E,** abbreviations as in Fig. 1

Calmodulin-like protein

CALP labeling of the (+/+) and (–/–) mouse retina is shown in Fig. 3E,F. Immunoreactivity was never as clear as for the other calcium-binding proteins, suggesting that low levels of CALP are expressed by many neurons of the INL and ganglion cell layer. Many bipolar cells, amacrine cells, and cells in the ganglion cell layer are more intensely labeled. Comparison of the immunoreactivities in Fig. 3E and F shows no apparent difference between (+/+) and (–/–) mice.

Discussion

Morphological appearance of calbindin (–/–) mice retinae

The general appearance of the retina is the same in calbindin (–/–) and (+/+) mice. There are no differences in the thickness or structure of the retinal layers. In normal mouse retina, the horizontal cells show the highest level of calbindin immunoreactivity. Amacrine cells, displaced amacrine cells, and ganglion cells are only moderately labeled. Since altered levels in the expression of calcium-binding proteins are thought to be responsible for many pathological changes in cells (Schäfer and Heizmann 1996), we have evaluated whether horizontal cells and amacrine cells survive the deletion of calbindin. Inspection of vertical sections of (–/–) mouse retinae by Nomarski optics (Fig. 1D) has shown that horizontal cell bodies, which are substantially larger than the surrounding bipolar cell bodies, are present at the outer margin of the INL. Immunostaining for neurofilament proteins has demonstrated that horizontal cells in (–/–) mouse retinae express all three neurofilament protein subunits, and the staining pattern is identical to that of (+/+) mouse retinae. The retinae of murid rodents have only one type of horizontal cell, and the neurofilaments are confined to the horizontal cell axon (Peichl and González-Soriano 1993, 1994).

Many different types of amacrine cells, possibly including normal and displaced cholinergic amacrine cells, express calbindin. The location of the two calbindin-immunoreactive IPL bands and the shapes of some of the labeled amacrine cell bodies (Fig. 1A) are reminiscent of the cholinergic amacrine cells in rat retina (Voigt 1986). The immunostaining for calretinin (Fig. 3C) shows that amacrine cells in (–/–) mice retina remain otherwise normal. Most ganglion cells are immunoreactive for PV (Fig. 3A,B), and the ganglion cell layers in (+/+) and (–/–) mice do not seem to be different. In conclusion, no apparent morphological differences are found between calbindin (–/–) and (+/+) mice retinae.

Other calcium-binding proteins are not upregulated in calbindin (–/–) mice retinae

The immunocytochemical staining applied in the present study is not a quantitative method defining the precise content of the various calcium-binding proteins in retinal neurons. However, with the precautions of staining sandwiched sections of (+/+) and (–/–) retinae together and of using the eyes of littermates of the same age and sex, we are confident that substantial differences in the expression patterns of the various calcium-binding proteins should have become apparent.

In the rat retina, horizontal cells showed strong immunoreactivity for calbindin and weak immunoreactivity for PV that could only be detected with the more sensitive postembedding method (Peichl and González-Soriano 1994). This might be the reason that we did not see PV immunoreactivity in mouse horizontal cells. However, as can be seen in Fig. 3, horizontal cells are devoid of PV both in (+/+) and in (–/–) retinae, suggesting that there is no detectable upregulation of PV in horizontal cells of calbindin (–/–) retinae. The same holds for amacrine cells; there is a lack of PV immunoreactivity both in (+/+) and (–/–) retinae.

Calretinin was found to be strongly expressed in various types of amacrine cells (Fig. 3C,D). The staining pattern is similar to that found in the rat retina (Pasteels et al. 1990). We have performed double-labeling for calretinin and choline acetyltransferase (ChAT) in the rat retina and have seen that the outer and inner bands labeled by calretinin in the IPL correspond to the two bands formed by the cholinergic amacrine cells, and that their cell bodies are also double-labeled (unpublished observations). This suggests that the cholinergic amacrine cells of the mouse retina also express calretinin and that the outer and inner labeled bands in the IPL (Fig. 3C,D) correspond to the cholinergic strata. Judging from their location, these two calretinin strata also appear to express calbindin (Fig. 1A). Goebel and Pourcho (1997) have found that distinct subpopulations of amacrine cells co-localize calretinin, calbindin, and PV in the cat retina. As the labeling pattern of calretinin is the same in (+/+) and (–/–) retinae (Fig. 3C,D), it may be concluded that the cholinergic amacrine cells are present in the (–/–) mouse and that their calretinin expression is unaffected.

Finally, we have also stained normal and mutant mice for the calmodulin-like protein CALP and have observed no differences (Fig. 3E,F). In conclusion, no immunocytochemically detectable upregulation of PV, calretinin or CALP is found in calbindin (–/–) mice.

Possible functional role of calcium-binding proteins in the retina

Recoverin seems to be involved in the regulation of phosphodiesterase (PDE) in photoreceptors and in the phosphorylation of activated rhodopsin (Polans et al. 1996). In rods of recoverin-null mutant mice, PDE activity fails to light-adapt normally and flash responses are faster than those of control rods (Baylor 1996). PV, calbindin, and calretinin are also abundant in retinal neurons but are of unknown function (Heizmann and Hunzicker 1991). The calbindin-null mutant mice used in the present study are severely impaired in tests of motor coordination. Confo-

cal imaging of Purkinje cells in cerebellar slices of calbindin (–/–) mice has revealed marked changes in postsynaptic calcium currents: their fast, but not slow, decaying component has a larger amplitude in null mutant than in wildtype mice (Airaksinen et al. 1997b). Thus, calbindin seems to control the level of intracellular calcium within the first 100 ms following calcium influx. If calbindin has a similar function in retinal horizontal cells, their light responses in (–/–) mice may show subtle changes, possibly being more transient. There are two physiological approaches for studying such changes. One method is to record the electroretinogram (ERG) and the flicker responses in normal and mutant mice. Unfortunately, the contributions of horizontal cells to the ERG are thought to be relatively small (Falk and Shiells 1986; Sieving et al. 1994). However, complete degeneration of horizontal cells in transgenic mice expressing the early region of simian virus 40 under the control of the promoter for phenylethanolamine-N-methyltransferase causes severe changes to the ERG (Hammang et al. 1993; Peachey et al. 1997). The other approach involves intracellular recordings from horizontal cells of the mouse retina in an in vitro preparation (Suzuki and Pinto 1986). Such experiments should be undertaken in the future in order to understand the functional role of calcium-binding proteins in the retina in more detail.

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