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SHORT COMMUNICATION

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Reorganization of horizontal cell processes in the developing FVB/N mouse retina

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Abstract We investigated the morphological changes of horizontal cells after postnatal photoreceptor degeneration in the developing FVB/N mouse retina, using immunocytochemistry with anti-calbindin D-28K. From postnatal day 14 (P14) onwards, processes emerging from horizontal cells descend into the inner plexiform layer (IPL) and ramify mainly in stratum 1 of the IPL. Electron microscopy revealed that the descending processes make synaptic contacts with bipolar cells in the outer plexiform layer. Our results clearly demonstrate that loss of photoreceptor cells induces the reorganization of horizontal cell processes in the retinas of FVB/N mice as they mature.

Keywords Horizontal cells · Immunocytochemistry · Retinal degeneration · Plasticity · Mouse (FVB/N)

Introduction

The retinal degeneration (*rd*) mouse has an inherited retinal dystrophy, in which the rod photoreceptors start to degenerate after birth, followed by the degeneration of cone photoreceptors as a secondary effect of the disease (Farber et al. 1994). Over the years, many studies have used the *rd* mouse as a model for the human rod dystrophy retinitis pigmentosa, which ultimately leads to blindness (Berson 1996). In addition, the model has been used to study the etiology of this disease and for developing strategies to prevent photoreceptor loss or to restore function once vision has been lost, because the pattern of photoreceptor loss is similar to that in affected humans (Farber et al. 1994). In the *rd* mouse, altered numbers and a 10–20% reduction in the size of ganglion cells were reported by Grafstein et al. (1972). These results were further corroborated by Ward (1982), who reported a reduction in the thickness of the inner plexiform layer and in the density of ganglion cells in the *rd* mouse. A loss of retinal ganglion cells and axons in the optic nerve fiber layer, and neurite sprouting of rods, amacrine or horizontal cells have been also described in the retinas for humans with retinitis pigmentosa (Stone et al. 1992; Santos et al. 1997; Fariss et al. 2000). Stone et al. (1992) suggested that this ganglion cell loss might be an effect of transneuronal degeneration.

The FVB/N mouse is one of the strains developed by inbreeding Swiss mice N:GP (NIH general-purpose mouse) and selecting for mice sensitive or insensitive to pertussis toxin. One group of sensitive mice was shown to be trophic for the B strain of Friend leukemia virus. The strain designation is derived from its description as Friend virus B-trophic. This strain exhibits rapid degeneration of photoreceptor cells identical to that seen in *rd* mice (Taketo et al. 1991).

In the mammalian retina, horizontal cell processes form two elements of the postsynaptic triad at the ribbon synapse of photoreceptor cells, and are involved in the formation of the antagonistic surroundings of the ganglion cell receptive field (Mangel 1991). As horizontal cells are the main postsynaptic cells to lose their primary input in the FVB/N mouse, it is important to determine whether the horizontal cells show morphological changes by way of transneuronal degeneration, as commonly shown in other areas of the central nervous system (Cowan 1970).

In the present study, we investigated the morphological changes of horizontal cells, using immunocytochemistry with antisera against calbindin D-28K, a specific marker

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for horizontal cells (Röhrenbeck et al. 1987), in developing FVB/N mice.

Materials and methods

Ten litters of FVB/N mouse pups for the experimental groups, and two litters of Swiss mouse pups for control groups, were used. The 1st day after birth was taken as postnatal day 0 (P0). All animals were housed and treated according to the regulations of the Catholic Ethics Committee of the Catholic University of Korea, Seoul, which conform to the NIH guidelines for the use of animals in research. The animals were deeply anesthetized by intraperitoneal injection of 4% chloral hydrate (1 ml/100 g body weight). The eyes were enucleated, and the animals were killed by an overdose of 4% chloral hydrate. Afterwards, the anterior segments of the eyes were removed, and the eyecups were fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer (PB: pH 7.4), for 2–3 h. Following fixation, the retinas were carefully dissected and transferred to 30% sucrose in PB for 24 h at 4°C. They were then frozen in liquid nitrogen, thawed, and rinsed in 0.01 M phosphate-buffered saline (PBS; pH 7.4).

For immunocytochemistry, the avidin-biotin-peroxidase complex (ABC) method was used. Whole-mount preparations and 40-µm-thick vibratome sections were prepared from FVB/N mice at postnatal days (P) 12, 13, 14, 15, 16, 18, 21 and 56. They were then incubated in 10% normal goat serum (NGS) and 1% Triton X-100 in PBS for 1 h at room temperature, to block nonspecific binding sites. The sections were then incubated with a mouse monoclonal antibody to calbindin (Sigma, St. Louis, MO; dilution 1:3000) in PBS containing 0.5% Triton X-100, for 3 days at 4°C. Retinas were washed in PBS for 45 min (3×15 min), incubated for 12 h in biotinylated goat anti-mouse IgG (Vector Laboratories, Burlingame, CA) with 0.5% Triton X-100 at 4°C, rinsed in PBS, and subsequently incubated in ABC solution (Vector) in PBS for 12 h at 4°C. Retinas were then rinsed in two changes of PBS and three changes of 0.05 M TRIS-HCl buffer (TB), pH 7.6, for 5 min each at room temperature, and incubated in 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB) in TB for 10 min. Hydrogen peroxide was added to the incubation medium, giving a final concentration of 0.01%, and the container was gently shaken as the reaction proceeded. The reaction was stopped with several washes of TB and PB after 1–2 min, as determined by the degree of staining. The retinas were mounted on gelatin-coated slides with the ganglion cell layer (GCL) facing upwards, and coverslips were applied with glycerol.

For electron microscopy, three FVB/N mice at day P56 were anesthetized and put to death as described above. The eyecups were fixed in a mixture of 4% paraformaldehyde and 0.2% picric acid in PB for 30 min at room temperature. The retinas were then carefully dissected, and small pieces were taken from the central region and fixed for an additional 2 h at 4°C. After being washed in PB, the retinal pieces were transferred to 30% sucrose in PB for 6 h at 4°C, rapidly frozen in liquid nitrogen, thawed, and embedded in 4% agar in distilled water. The pieces were cut into 40-µm sections using a vibratome, and sections were placed in PBS. They were incubated in 10% NGS in PBS for 1 h at room temperature, to block nonspecific binding, and were then incubated in calbindin antibody diluted 1:3000 for 12 h at 4°C.

The following immunocytochemical procedures were carried out at room temperature. The sections were washed in PBS for 45 min (3×15 min), incubated in biotin-labeled goat anti-mouse IgG for 2 h, and then washed 3 times in PBS for 45 min (3×15 min). The sections were incubated in ABC solution for 1 h, washed in TB, and then incubated in 0.05% DAB solution containing 0.01% H_2O_2. The reaction was monitored using a low-power microscope, and was stopped by replacing the DAB and H_2O_2 solution with TB. The stained sections were postfixed in 1% glutaraldehyde in PB for 1 h. They were washed in PB containing 4.5% sucrose for 15 min, then postfixed in 1% OsO_4 in PB for 1 h, dehydrated in a graded series of alcohol, and flat-embedded in Epon 812. After the sections had been cured at 60°C for 3 days, well-stained areas were cut out and attached to an Epon support for further ultrathin sectioning (Reichert-Jung, Germany). Ultrathin sections (70–90 nm thick) were collected on one-hole grids coated with Formvar, and examined using a transmission electron microscope (JEOL 1200EX, Tokyo, Japan).

Results and discussion

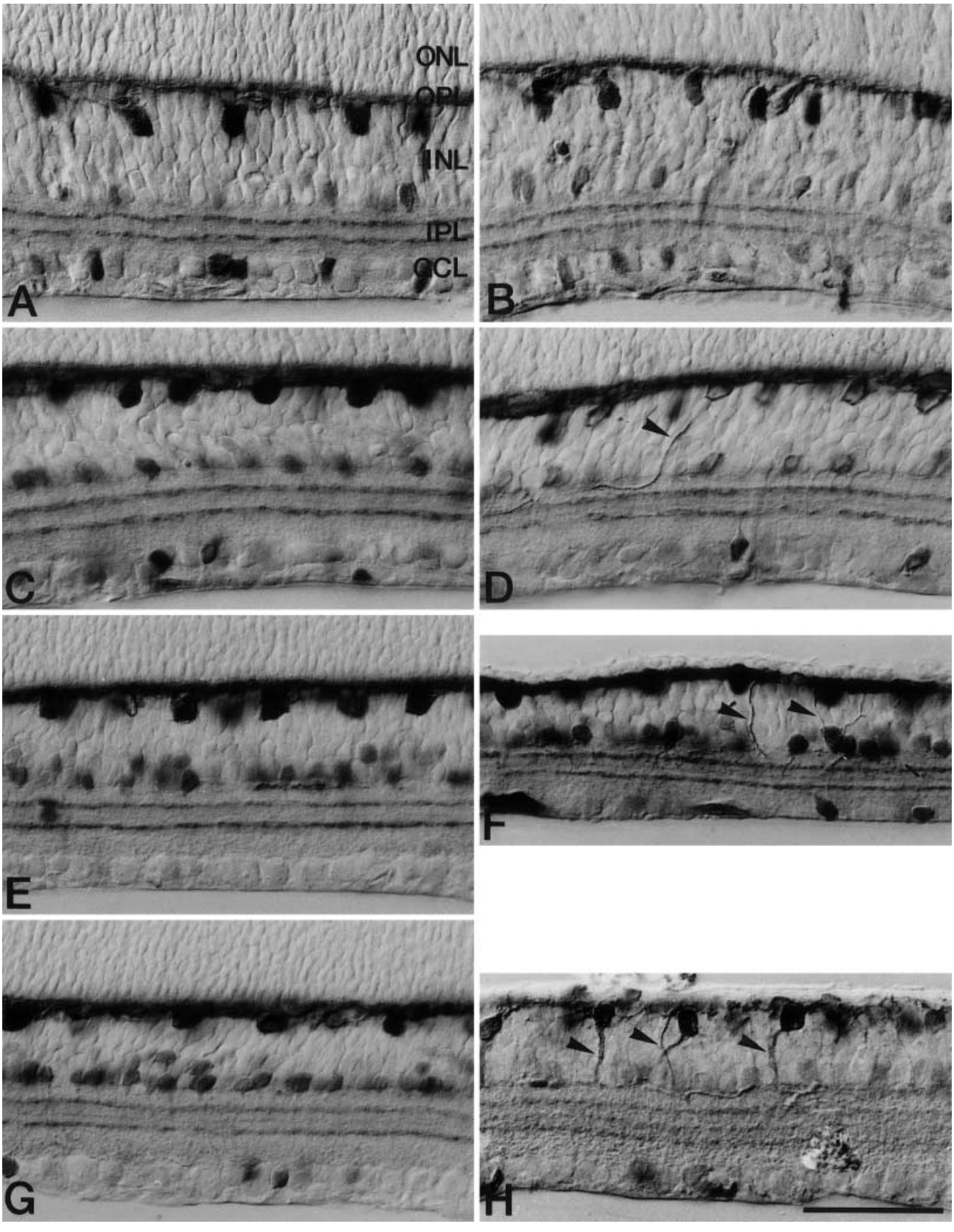
In the present study, we investigated the changes of general structure and calbindin-immunoreactive horizontal cells in FVB/N mice at different developmental stages.

The general morphological changes of retinal tissues of FVB/N mice could be identified in the vibratome sections. At P12, the retina showed five well-organized layers: ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), and outer nuclear layer (ONL) (Fig. 1A). Afterwards, the retinas of FVB mice decreased in thickness with increasing age, mainly through the loss of photoreceptor cells. By P21, the ONL consisted of only a single cell layer (Fig. 1E). These results are in agreement with a previous report that photoreceptor nuclei began to become pyknotic at P10, and that by P21 only a single row of sparse nuclei remains (Caley et al. 1972).

When retinal tissues were stained immunocytochemically with antibodies against calbindin, dense labeling was found in the OPL and in the cell bodies located at the outer margin of the INL (Figs. 1, 2) in both FVB/N and control mice. During the developmental period, no descending processes were observed in the control mice (Fig. 1A, C, E, G). However, in the FVB/N mice, horizontal cell dendrites descended into the IPL and ramified mainly in the IPL close to the INL from P14 onward (Fig. 1D, F, H). In P56 mice, sprouting of horizontal cell dendrites into the IPL is frequently seen. Figure 2 shows a whole-mount of a P56 FVB/N mouse retina immunostained with antisera against calbindin. Labeled horizontal cell bodies and their processes are seen (Fig. 2A). When the plane of focus was shifted from the OPL to the IPL, descending processes, which ramified in the IPL close to the INL, could be observed on some of the horizontal cells (Fig. 2B).

To identify whether there is synaptic plasticity in the FVB/N mice retina, we examined the anti-calbindin-stained ultrathin sections using electron microscopy. Figure 3 is an electron micrograph taken from the outer

Fig. 1 Light photomicrographs taken from vertical vibratome sections (40 µm) of the control **(A, C, E, G)** and FVB/N **(B, D, F, H)** mouse retinas at postnatal days P12 **(A, B)**, P14 **(C, D)**, P21 **(E, F)** and P56 **(G, H)**. These were processed for calbindin D-28K immunoreactivity. **A, C, E, G** Labeled horizontal cell somata are seen at the outer margin of the inner nuclear layer (*INL*). In addition, labeled amacrine and displaced amacrine cells are visible. **B** The morphology of the labeled horizontal cells is similar to those shown in **A**. **C** A descending process (*arrowhead*) is clearly seen to emerge from the horizontal cell and ramify in stratum 1 of the inner plexiform layer. **F, H** Several processes descending into the IPL are seen (*arrowheads*) (*ONL* outer nuclear layer, *OPL* outer plexiform layer, *GCL* ganglion cell layer). *Scale bar* 50 µm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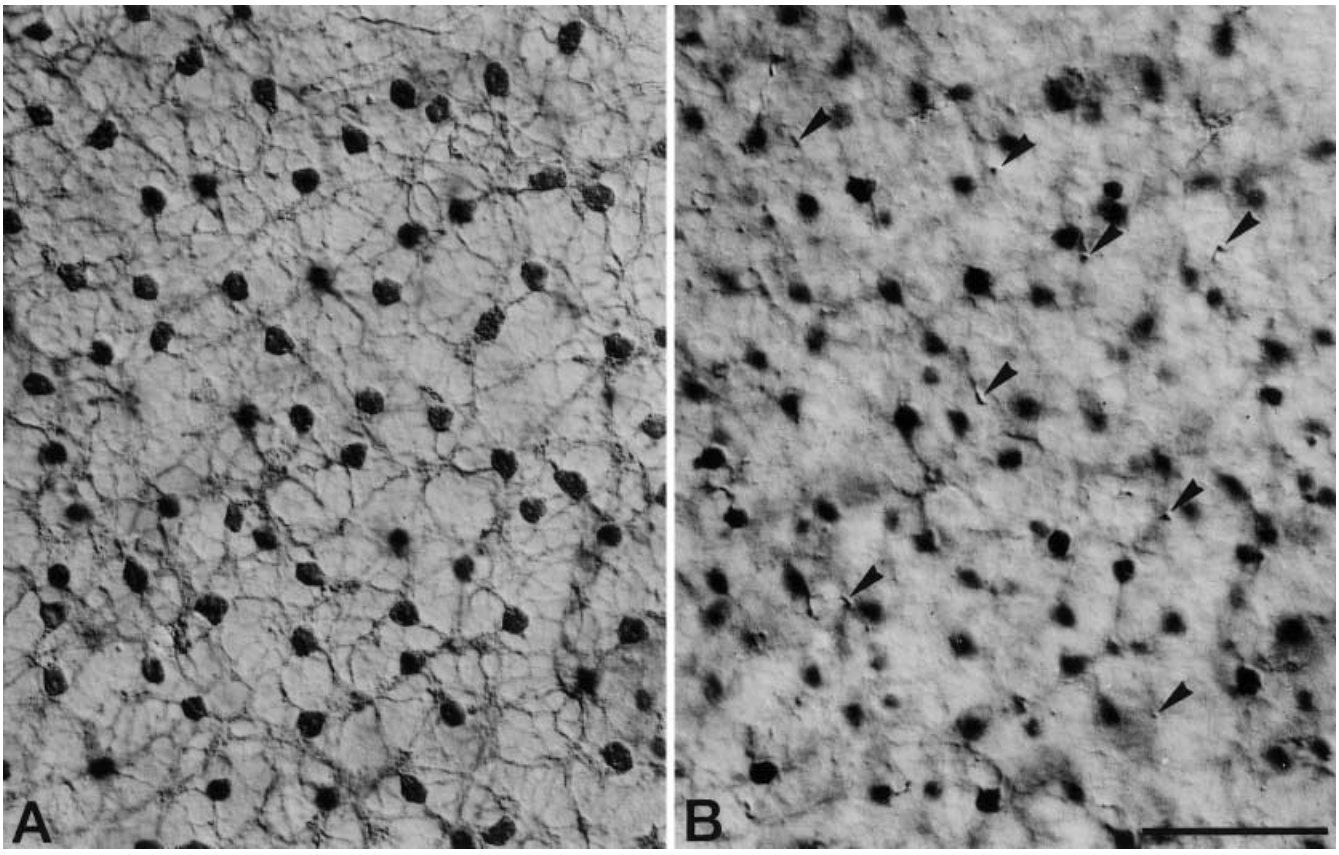

Fig. 2A, B Light micrographs taken at different focal planes in the same field of a whole-mount of P56 FVN/N mouse retina processed for calbindin immunoreactivity. **A** The focal plane is at the outer margin of the INL. Calbindin-labeled horizontal cells are seen. **B** The focal plane is at the IPL close to the INL. Descending processes (*arrowheads*) are seen in the IPL. *Scale bar* 50 µm

margin of the INL. The descending process, marked by the frame in Fig. 3, is shown at higher magnification in the inset. The labeled process with longitudinally running neurofilaments is shown to make a conventional output synapse with the bipolar cell soma in the OPL.

In the present study, we found that horizontal cells of the FVB/N mice send their dendrites into the IPL and their descending processes make unusual synaptic connectivity with bipolar cells in the OPL during developmental periods. Horizontal cells are interneurons modulating the transfer of information between photoreceptors and bipolar cells in the OPL of the vertebrate retina. In rat, mouse and gerbil retinas, only one type of horizontal cell, the B-type cell, is known to exist (Peichl and González-Soriano 1993), unlike other mammals, which have two types: A-type and B-type horizontal cells (Boycott et al. 1987; Peichl and González-Soriano 1993).

In this study, the horizontal cells of control mice lacked descending processes, strongly suggesting that loss of photoreceptors induces sprouting of the horizontal cell dendrites in the FVB/N mice. Similar sprouting of horizontal cell processes has also been noted in Royal College of Surgeons rats (Chu et al. 1993), in adult cat retinas after experimental detachment (Lewis et al. 1998; Marc et al. 1998), in human retinas with retinitis pigmentosa (Fariss et al. 2000), and in retinas of *rd* mice (Strettoi and Pignatelli 2000). This suggests that horizontal cells retain a remarkable potential for morphological plasticity to respond to injury and disease.

In the mammalian retina, glutamate mediates synaptic transmission from the photoreceptor cells to bipolar and horizontal cells (Massey 1990). Kainate subunits GluR6/7 are localized to horizontal cell processes postsynaptic to both rod spherules and cone pedicles (Brandstätter et al. 1997). Although the mechanisms responsible for the sprouting of horizontal cells into the IPL remain unclear, excitotoxicity by glutamate released from damaged photoreceptors might play an important role in the sprouting of horizontal cell dendrites. This explanation is further supported by the results of Peichl and Bolz (1984), which showed that an application of kainic acid, an excitotoxic drug, into the adult cat and rabbit retina induces sprouting of horizontal cell dendrites into the IPL. Such massive release of glutamate affects horizontal cells by altering their intracellular balance through the passage of excess Na^+ and Ca^{2+} across the plasma membrane. Recently, Wässle et al. (1998) suggested that calbindin may control the level of intracellular calcium within the first 100 ms following calcium influx in retinal horizontal cells, as shown in cerebellar Purkinje cells (Airaksinen et al. 1997). Therefore, it could be inferred that the sprouting of horizontal cell dendrites may be due to their greater capacity to buffer

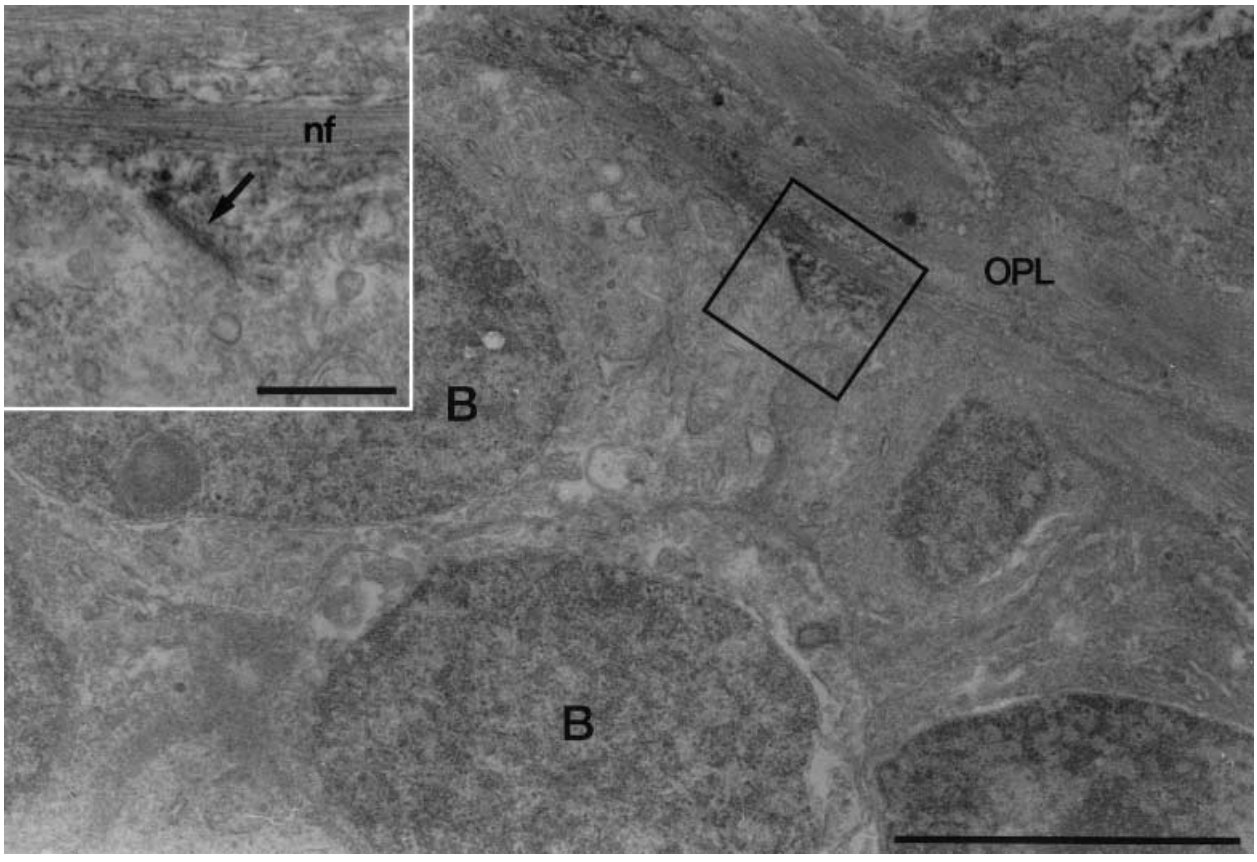

Fig. 3 Vertical ultrathin sections through the OPL of the retina of a P56 FVB/N mouse, processed for calbindin immunoreactivity. One unlabeled bipolar cell soma (*B*) is postsynaptic to a labeled cell process (see *frame*). *Inset*: The magnified process, presumably a horizontal cell process, because of its location and amount of neurofilaments (*nf*), makes a synapse with a bipolar cell soma. *Scale bars* 3 µm (*main figure*), 0.5 µm (*inset*)

the intracellular calcium induced by excitotoxicity. However, other mechanisms cannot be excluded. For example, neurofilaments, which are present in rat and rabbit horizontal cells (Peichl and González-Soriano 1993, 1994; Lörke et al. 1995), might be involved in the process of buffering the bulk of Ca^{2+} entry (Shaw 1991). While our results clearly show that FVB/N mice horizontal cells have the potential for morphological plasticity, further studies are needed to elucidate the actual mechanisms responsible for the reorganization of horizontal cell processes in the developing FVB/N mice.

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