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Morphological and Functional Organization of ON and OFF Pathways in the Adult Newt Retina

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ABSTRACT—Morphological and functional organization of ON and OFF pathways in the adult newt retina were examined by intracellular recording and staining techniques and immunohistochemistry. Synaptotagmin immunoreactivity discriminated three broad bands within the IPL: the distal band (sublamina I), the middle band (sublamina II) consisting of two dense punctate bands (sublaminae II_a and II_b), and proximal band (sublamina III). The Lucifer-yellow labeled OFF amacrine and ganglion cells send their processes mainly in sublamina I and/or II_a where OFF bipolar cells extend their axon terminals, while ON amacrine and ganglion cells send their processes in sublamina III and/or II_b where ON bipolar cells extend their axon terminals. Processes of ON-OFF amacrine and ganglion cells ramify broadly in the whole thickness of the IPL. Many bipolar cells responded to light spot with a transient hyperpolarization at both light onset and offset. They are probably subtypes of ON bipolar cells, because their axon terminals branch mainly in sublaminae III and/or II_b, although a few cells ramified the axon at both sublaminae II_a and III. Two immunohistochemical markers for bipolar cells, PKC and RB-1, identified axon terminals in sublaminae III and/or II_b. From the ramification pattern of axon terminal, they are probably subtypes of ON bipolar cells. ChAT-ir amacrine cells ramified their dendrites in either sublamina I or II_b. Altogether, present studies support the general idea of segregation of ON and OFF pathways in sublaminae *a* and *b* of the IPL.

Key words: newt retina, intracellular recording, Lucifer-yellow staining, immunohistochemistry, ON and OFF pathways

INTRODUCTION

All known vertebrate retinas posses at least five basic types of neurons (photoreceptor, bipolar, horizontal, amacrine, and ganglion cells) and non-neuronal glial cells (Dowling, 1987). The constituent cells are arranged in a penta-lamina array: three nuclear layers [outer nuclear later (ONL), inner nuclear layer (INL) and ganglion cell layer (GCL)] and two synaptic layers [outer plexiform layer (OPL) and inner plexiform layer (IPL)]. One of the outstanding physiological features of the vertebrate retina is the segregation of information processing into separate ON and OFF pathways (Famiglietti and Kolb, 1976; Famiglietti *et al*., 1977; Nelson *et al*., 1978; for reviews see Sterling, 1986; Saito, 1987). These pathways are separated at the first synaptic zone where photoreceptor activity gives rise to two different types of bipolar cell responses. Depolarizing bipolar cells provide the ON pathways, whereas hyperpolarizing bipolar cells underlie the OFF pathways. The primary morphological distinction between ON and OFF bipolar cells is the level of their axon terminals in the IPL: ON bipolar cell axons terminate at the proximal half of the IPL (sublamina *b*) and OFF bipolar cell axons terminate at the distal half of the IPL (sublamina *a*). Correspondingly, ON amacrine and ganglion cells extend their dendrites into sublamina *b,* and OFF amacrine and ganglion cells extend their dendrites into sublamina *a*. The mechanism of the morphological segregation of ON and OFF pathways within the IPL during retinal development is not known (Bodnarenko and Chalupa, 1993; Layer *et al*., 1997; Günhan *et al*., 2002).

In most vertebrates, the central nervous system including the retina do not regenerate. However, newts and salamanders are remarkable because they possess the ability to regenerate a new functional retina after complete removal of the original retina even in adult life (Stone, 1950; Hasegawa, 1958; Keefe, 1973). Such a retinal regeneration, as well as retinal development, may be a useful tool for understanding the mechanisms of cytodifferentiation and the genesis of neural networks in the central nervous system including the retina.

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As a first step for understanding the segregation mechanisms of ON and OFF pathways during retinal regeneration of the adult newt, we are investigating the organization of ON and OFF pathways in mature newt retina and determining whether it is similar to that described in many other vertebrate retinas. Here, we report the identification of various retinal neurons by intracellular recording and Lucifer-yellow dye-injection. We also used a specific antibody for presynaptic levels and antibodies specific for some bipolar and amacrine cells to identify their stratification levels in the IPL.

MATERIALS AND METHODS

Intracellular recording and dye-injection

Adult newts (*Cynops pyrrhogaster*), 6-10 cm in body length, were obtained from a commercial supply and reared in a moist chamber at a temperature of about 22°C under a lighting cycle of approximately 12 hr light/12 hr dark. Animals were fed pieces of liver. Before electrophysiological experiments, they were kept in the dark for more than 30 min and then anesthetized with 0.1% FA100 (4-allyl-2-methoxyphenol; Tanabe, Osaka, Japan). The eye was excised and the retina was detached from pigment epithelium under dim light. The isolated retina was placed receptor-side up in a moist chamber. Microelectrodes filled with 5% Lucifer yellow CH (Sigma, St. Louis, MO) in 100 mM lithium chloride were used for intracellular recording and for iontophoretic dye injection. Electrode resistance was 150~300 MΩ. A microelectrode placed at the center of the white light spot was advanced vertically into the retina from the receptor side, while 500 msec flashes were presented at 3 sec intervals. The diameter of light spot could be changed from 0.1~0.5 mm. A spot size of 0.5 mm in diameter was usually used. Occasionally, the light spot of 0.1 mm in diameter was applied to minimize an antagonistic surround effect of horizontal cells and bipolar cells. Penetration of the cell was brought by oscillating the electrode tip with increased capacity-compensating feedback. The light intensity was attenuated by 1~5 log-neutral density filters in the light path. During electrode penetration, the light intensity of –3 log units (about 0.3 lm/m^2) was used, because it could activate both rod and cone systems without significantly light-adapting the retina. Experiments were performed at a room temperature at about 21°C.

After recording the light response of an impaled cell, the dye was passed by a steady negative current of about 1 nA for 1 to 3 min, using a bridge circuit in the recording amplifier (M701, W-P instrument, Inc, New Haven, CT). The retinal tissue including dye-injected cells was kept in the Ringer's solution for 30 min after injection before fixation and then fixed overnight at 4°C with 4% paraformaldehyde in 0.02 M phosphate-buffered saline (PBS, pH 7.4). Fixed retinas were washed in PBS at pH 7.4, equilibrated in 30% sucrose in PBS, frozen in an embedding medium (O.C.T. compound, Miles Elkhart, IN), and cryosectioned vertically through the retina at 20 μm. Sections were thaw-mounted onto gelatin-coated cover glasses, air-dried and stored at –20°C.

Immunohistochemistry

Under anesthesia, the eye was excised, fixed overnight with 4% paraformaldehyde in 0.02 M PBS (pH 7.4) at 4°C. Fixed eye was cryosectioned at 15–20 μm thickness as described above. Prior to immunohistochemistry, cryosections were rinsed in 0.02 M PBS for 30 min at room temperature. All sections were pretreated with a blocking solution which is 0.02 M PBS containing 5% bovine serum albumin (Gibco Labs, Gran Island, NY) and 5% normal rabbit serum or 5% normal goat serum (Vector Laboratories, Burlingame, CA) and 0.3% Triton X-100, and then incubated with the primary antibodies for 24–48 hr at 4°C.

Synaptotagmin: A monoclonal antibody against rat synaptotagmin was obtained from a commercial source (Calbiochem, San Diego, CA). Anti-synaptotagmin antibody was used at a dilution of 1:300 in a mixture of 3% normal rabbit serum and 0.3% Triton X-100 in 0.02 M PBS. Some sections were incubated without the primary antibody as a control. After washing to remove any unbound primary antibody, fluorescein isothiocyanate (FITC)-conjugated secondary antibody was used at a dilution of 1:150 and incubated for 3–4 hr at room temperature. Sections were examined and photographed under epifluorescence illumination by Olympus BX50 (Tokyo, Japan)

PKC: An anti-protein kinase C (PKC) antibody (α and β subspecies) was obtained from a commercial source (Calbiochem) and used as a specific marker for a subtype of retinal bipolar cells. The antibody was used at a dilution of 1:300 in a mixture of 2% normal goat serum and 0.3% Triton X-100 in PBS. The procedure of PKC immunohistochemistry is described above and also in the previous papers (Negishi, *et al*., 1992; Saito *et al*., 1994). For double labeling with synaptotagmin and PKC, sections were first incubated with the anti-PKC and then with anti-synaptotagmin as described above.

RB-1: RB-1 was obtained from mouse monoclonal antibodies against adult newt retina in our laboratory and used as a specific marker for a subtype of bipolar cells (Saito *et al*., 1994). The procedure of RB-1 immunohistochemistry is described in the previous papers (Negishi, *et al*., 1992; Saito *et al*., 1994). Because RB-1 and anti-synaptotagmin were made from the same host species, we used Fab fragments of affinity-purified secondary antibodies for double labeling of the primary antibodies. Sections were first incubated with RB-1 antibody. After washing to remove any unbound primary antibody, rhodamin (TRITC)-conjugated secondary antibody (1:150, Jackson ImmunoResearch laboratories INC. West Grove, PA.) was applied for 3–4 hr at room temperature. To saturate any open antigen binding sites on the first secondary antibody, the sections were incubated with 10% normal mouse serum (Jackson ImmunoResearch laboratories) from the same host species as the primary antibody. After several washes, the sections were incubated with excess of unconjugated Fab antibody (1:25, Jackson ImmunoResearch laboratories) for 12–16 hr at 4°C. Subsequently, the sections were incubated with anti-synaptotagmin as described above.

Choline Acetyltransferase: Polyclonal anti-goat choline acetyltransferase (ChAT) antibody was obtained from a commercial source (Chemicon International, Temecula, CA) and used as a specific marker for cholinergic amacrine cells. ChAT immunohistochemical procedure is identical to that described in Cheon and Saito (1999). For double labeling with synaptotagmin and ChAT, sections were first incubated with anti-ChAT and then incubated with anti-synaptotagmin as described above.

RESULTS

Synaptotagmin immunoreactivity and specification of the IPL

Synaptotagmin is a vesicle-associated membrane protein which has been implicated in the exocytotic and endocytotic steps of the synaptic vesicle cycle (for review, see Südhof, 1995). A monoclonal antibody against synaptotagmin cross-reacted in the adult newt retina and labeled the pre-synaptic localization of both plexiform layers, the OPL and IPL (Fig. 1). Moreover, three broad bands are clearly detected in the total thickness of the IPL, defining the INL/IPL border as 0% and the IPL/GCL border as 100%. A distal band (sublamina I) extends approximately from 0 to 20% of the IPL with a dense punctate band at the proximal

border. A middle band (sublamina II) extends from 30 to 70% of the IPL width. It includes two dense punctate bands; sublaminae II_a and II_b. A proximal band (sublamina III) extends from 80 to 100% of the IPL width without a significant dense punctate band. These synaptotagmin-immunoreactive (-ir) bands are correlated with the stratification level(s) of bipolar, amacrine and ganglion cell processes in the IPL, which were identified by physiological, morphological and

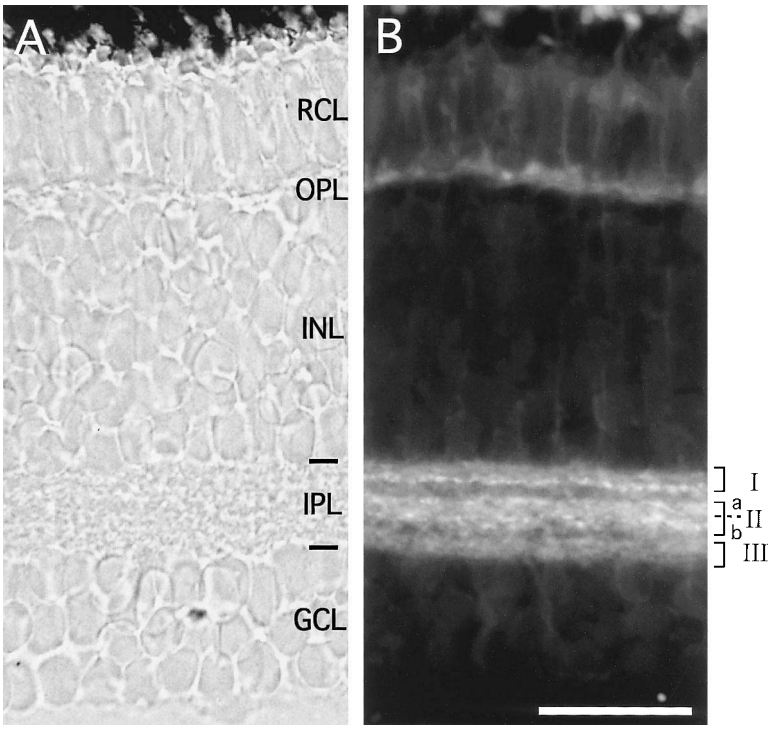

Fig. 1. A fluorescence photomicrograph of vertical section through the adult newt retina stained with a monoclonal antibody against synaptotagmin (B) and a light photomicrograph of nearby section (A). Four Synaptotagmin-ir bands (I, II_a, II_b and III) are indicated by square brackets to the right of the panel B (see text). RCL, receptor cell layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bar=50 μm in A, B.

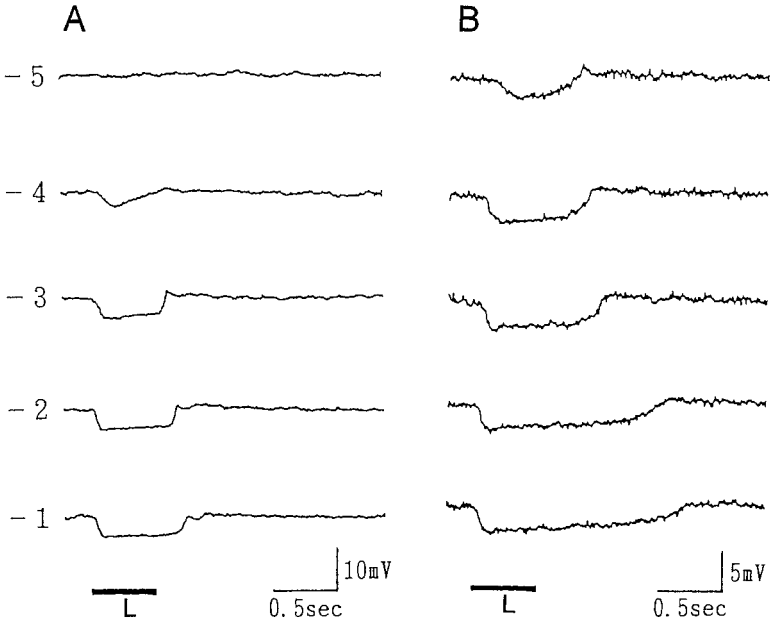

Fig. 2. Two types of OFF bipolar cell responses that are characterized by different response waveforms to spot illumination (0.5mm in diameter) of various intensities. **A:** Responses are dominated by cone input. **B:** Responses are dominated by rod input. Timing of a white light spot (L) is indicated below. The numbers by each trace indicate the negative log attenuation of the light beam.

immunohistochemical studies.

Physiological and morphological identification of bipolar cells

Seventy-five bipolar cells were stained by iontophoretic injections of Lucifer-yellow dye after recording light responses. The resting potential ranged from –10 to –40 mV with a mean value of –23.5±10.1 mV (S.D. n=26). Only the cells that continued to respond after injection of the dye were used for histological examination. Forty-four bipolar cells were successfully stained. All stained bipolar cells show a prominent Landolt club projecting into the photoreceptor layer. Twenty-two out of 44 stained cells were hyperpolarizing (OFF) bipolar cells. Depolarizing (ON) bipolar cells were less frequently encountered in the retina and only 4 cells were identified as ON bipolar cells, morphologically and physiologically. Remaining 18 cells were responded with transient hyperpolarization to onset and offset of spot illumination. We tentatively refer to these as Transient bipolar cells.

Fig. 2 shows voltage responses of two OFF bipolar cells to spot illumination of various intensities. They differ in their

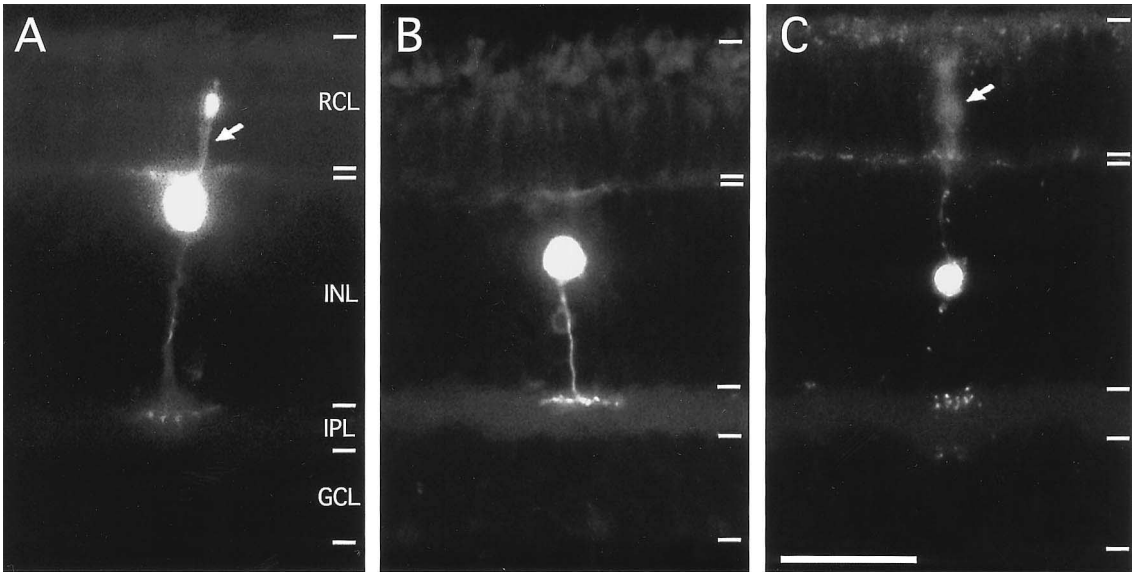

Fig. 3. Fluorescence photomicrographs of Lucifer-yellow injected OFF bipolar cells. Landolt club projecting into photoreceptor layer is seen in the panels A and C (arrow). Light response obtained from a cell A and a cell B is shown in Fig. 2B and A, respectively. Scale bar=50 μm in A–C.

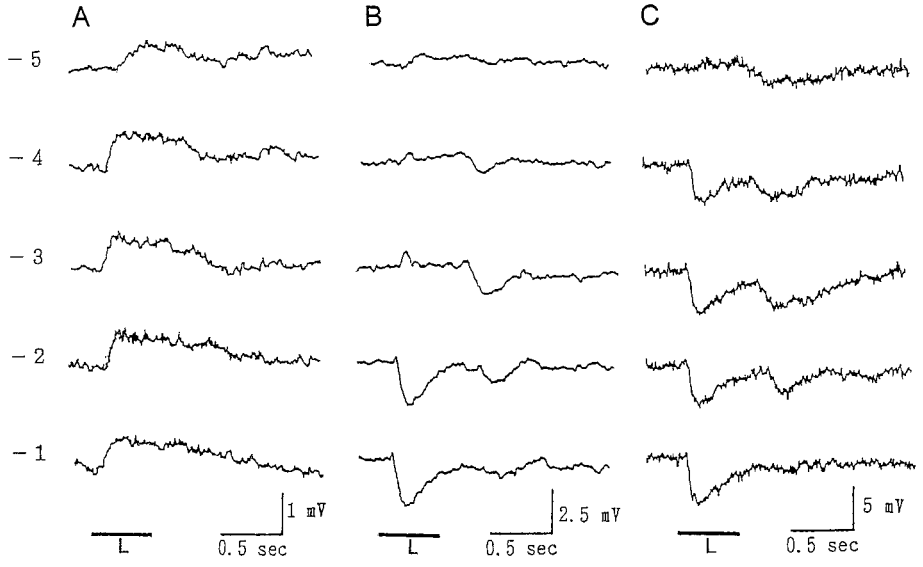

Fig. 4. Light-evoked voltage responses of an ON bipolar cell (A), and Transient bipolar cells (see text) that respond to transient hyperpolarization at light onset and offset (B and C). Timing of a white light spot (L) is indicated below. The diameter of the light spot is 0.5 mm for cell A and 0.1 mm for cell B and C. The numbers by each trace indicate the negative log attenuation of the light beam.

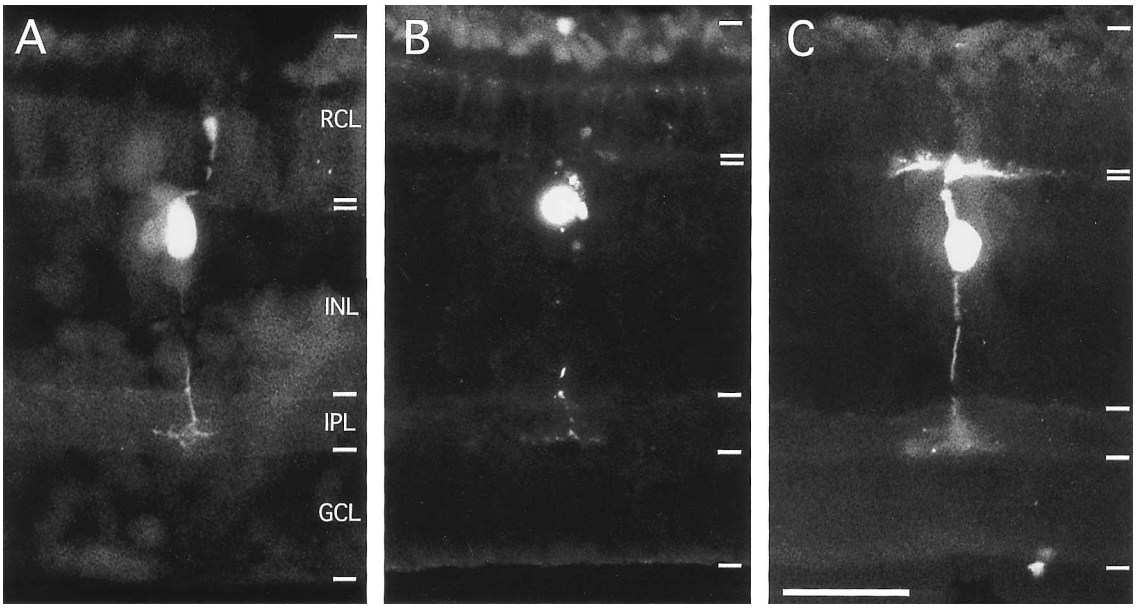

Fig. 5. Three examples of Lucifer-yellow injected ON bipolar cells. Light response obtained from a cell in panel A is shown in Fig. 4B. Scale bar=50 μm in A–C.

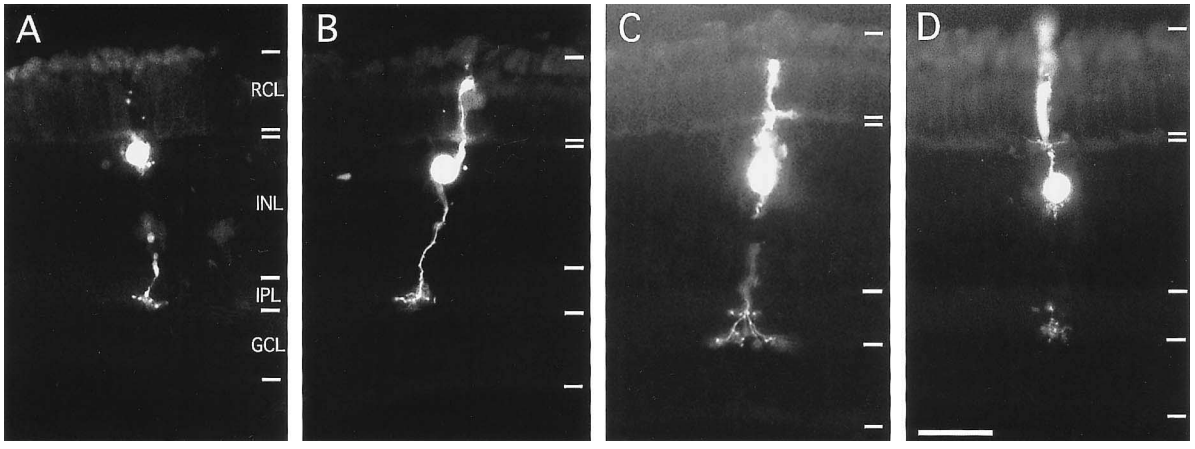

Fig. 6. Four examples of Lucifer-yellow injected Transient type bipolar cells. Light responses obtained from a cell in panel A and a cell in panel C are shown in Fig. 4B and C, respectively. Scale bar=50 μm in A–D.

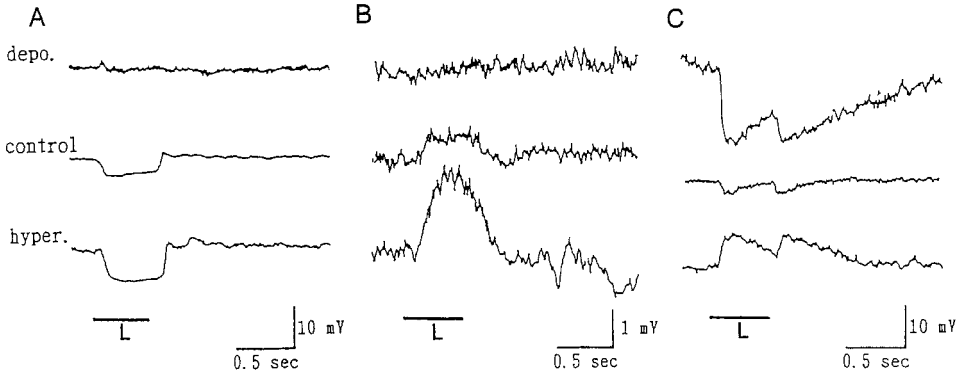

Fig. 7. Effects of the membrane polarization on the OFF bipolar cell (A), ON bipolar cell (B) and Transient type bipolar cell (C). Middle records marked as “control” are responses without application of any extrinsic current. Lower records marked as “hyper.” are responses under membrane hyperpolarization by current of about –1nA. Upper records marked as “depo.” are responses under membrane depolarization by current of about +1nA. Timing of a light spot (L) is indicated below. The diameter of light spot is 0.5mm.

sensitivity to light and response waveform. The dimmest light (–5 log unit) produced no response in Fig. 2A, but produced a small hyperpolarization in Fig. 2B. As the light intensity was increased, the response increased in the amplitude up to –3 log unit. Further increases in light intensity prolonged the response of cell B more than the response of cell A without increasing response amplitude. These results suggest that cell A is dominated by cone input, and cell B is dominated by rod input. Fig. 3 shows three representatives of Lucifer-yellow stained OFF bipolar cells. Their cell bodies were located in the most distal level (cell A), in a second cell row (cell B), and in the middle level of the INL (cell C). Axon terminals of cells A and C branched at sublaminae I and II_a of the IPL, and those of cell B branched at sublamina II_a. Rod- and cone-dominated OFF bipolar cells could not be morphologically distinguished from each other.

Fig. 4A shows voltage responses of an ON bipolar cell to spot illumination (0.5mm in diameter) of various intensities. A relatively high sensitivity to dim light intensity and

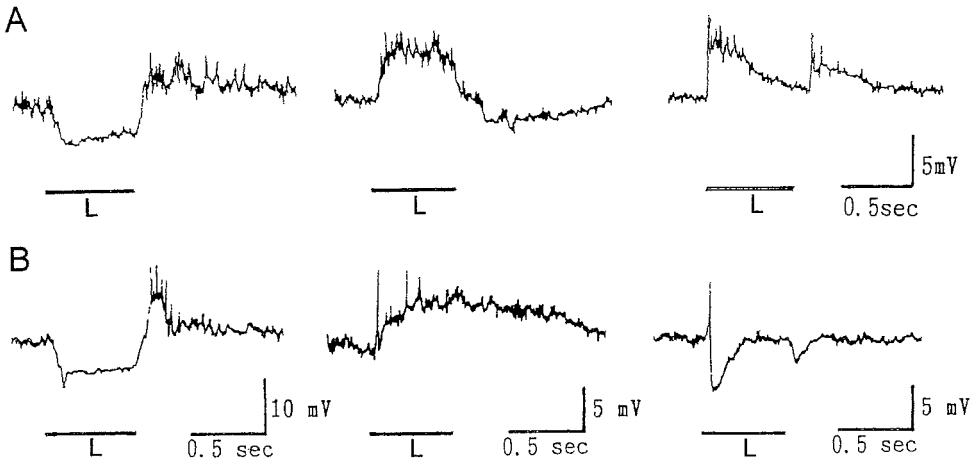

Fig. 8. Light-evoked voltage responses of amacrine cells (A) and ganglion cells (B). A: an OFF amacrine cell response (left), an ON amacrine cell response (middle) and an ON-OFF amacrine cell response (right). Calibrations in the right panel are common in the left and middle panels. B: an OFF ganglion cell response (left), an ON ganglion cell response (middle) and an ON-OFF ganglion cell response (right). Timing of a white light spot (L) is indicated below. The diameter of a light spot is 0.5mm.

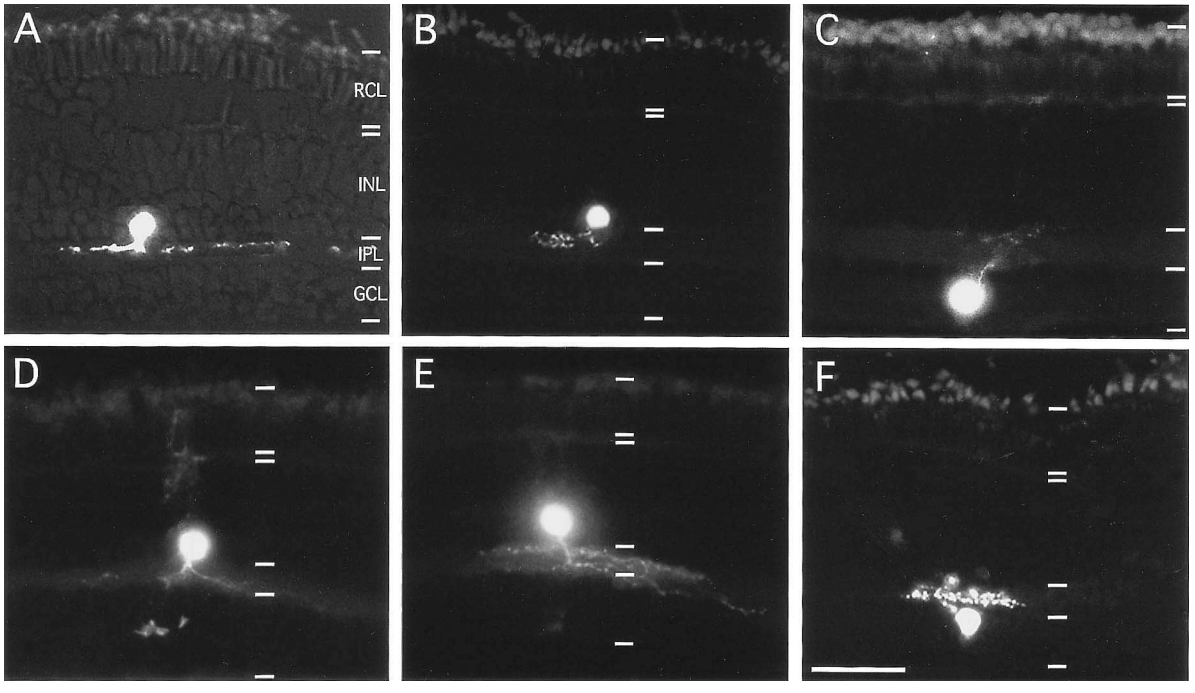

Fig. 9. Six examples of Lucifer-yellow injected amacrine cells. A–C: OFF amacrine cells. D: an ON amacrine cell. Light response is shown in Fig. 8A (middle). E-F: ON-OFF amacrine cells. Light response obtained from a cell in panel D is shown in Fig. 8A (right). Scale bar=50 μm in A–F.

prolongation of the response duration to increasing light intensity suggest that the cell receives mainly rod input. Fig. 5 shows three examples of the Lucifer-yellow stained ON bipolar cells with cell bodies located either in the most distal level (cell A), or in the second cell row of the INL (cells B and C). Axon terminals of cell A branched at sublamina II_b of the IPL, those of cell B branched at the most proximal level of sublamina III, and those of cell C branched at both sublaminae II_b and III.

Many bipolar cells (38 out of 75) examined did not respond to light with either sustained depolarization or hyperpolarization, but with a transient hyperpolarization at light onset and offset. Fig. 4B and C show two representatives of Transient bipolar cell response to spot illumination (0.1 mm in diameter) of various intensities. In Fig. 4B, the dimmest light (–5 log unit) produced a small sustained depolarization. The light intensity of –4~–3 log units produced a depolarization at light onset and then a hyperpolarization at light offset. When the light intensity was further increased, the response showed a complex waveform that is composed of a transient depolarization followed by a hyperpolarization at light onset and a hyperpolarization at light offset. Such a response pattern was observed in 6 out of 38 cells. The remaining 32 cells responded to light spot as shown in Fig. 4C. The dimmest light intensity did not produce a response at light onset but evoked an after-hyperpolarization at light offset. When the light intensity was further increased, the response at light onset became hyperpolarizing, and at light offset the hyperpolarizing response increased in amplitude. At the brightest light (–1 log unit), the response at light offset was suppressed.

We recovered 18 out of 38 stained Transient bipolar cells. Fig. 6 shows four examples of the Lucifer-yellow filled Transient bipolar cells. Their cell bodies were located either in the most distal level (cell A) or in the second cell row of the INL (cells B to D). All labeled cells ramified their main axon terminals at the proximal half of the IPL. The axon terminal of cell A ramified at sublamina III and that of cell B ramified at sublamina II_b. Cell C was characterized by a 'pyramidally branching axon' which began branching at the middle of the IPL. Axons of cells C and D ramified at more than one level within the IPL; sublaminae II_a and III.

Electrical membrane properties of bipolar cells

Fig. 7 shows the effect of polarizing current of about ±1nA on three different response types of bipolar cells. Middle traces show the control responses recorded at zero current. The hyperpolarizing current increased the amplitude of the OFF bipolar cell in A, while depolarizing current decreased it to zero, suggesting cell A has a reversal potential more positive than the dark membrane potential. Hyperpolarizing current also increased the amplitude of depolarizing response of the ON bipolar cell in B, while depolarizing current reduced it to zero, suggesting that cell B has a reversal potential more positive than the dark membrane potential. Hyperpolarization reversed the polarity the Transient bipolar cell responses in C, while depolarizing current increased the amplitude, suggesting that cell C has a reversal potential more negative than the dark membrane

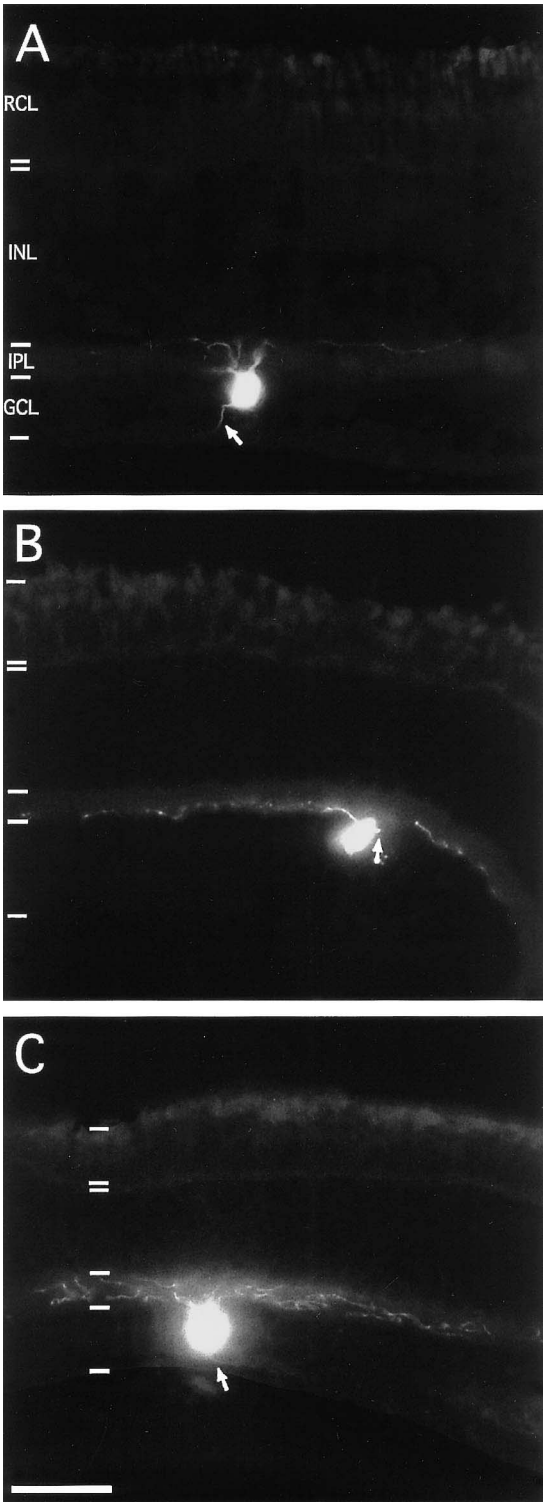

Fig. 10. Typical examples of Lucifer-yellow injected OFF ganglion cells (A), ON ganglion cell (B) and ON-OFF ganglion cell (C). Light responses obtained from a cell in panel B and a cell in panel C are shown in the middle and right of Fig. 8B. The axon is indicated by an arrow. Scale bar=50 μm in A–C.

potential.

Physiological and morphological identification of amacrine and ganglion cells

Twenty-one amacrine cells were successfully stained by iontophoretic injections of Lucifer-yellow dye after recording their response. Fifteen out of 21 cells were conventional amacrine cells whose cell bodies are located in the INL. Remaining 6 cells were displaced amacrine cells whose cell bodies were located in the GCL. All amacrine cells examined are classified into at least three types on the basis of their responsiveness to light and their level of dendritic arborization in the IPL. Five out of 21 cells are OFF amacrine cells, 3 cells are ON amacrine cells, and 13 cells are ON-OFF amacrine cells. Fig. 8A shows typical voltage responses recorded from each type of cells. An OFF amacrine cell responded to light with a sustained hyperpolarization and a disappearance of membrane potential fluctuations (Fig. 8A, left), whereas an ON amacrine cell responded to light with a sustained depolarization and an increase in membrane potential fluctuations (Fig. 8A, middle). An ON-OFF amacrine cell responded to light with a transient depolarization and spike-like potentials at light onset and offset (Fig. 8A, right). Fig. 9 shows six examples of the Lucifer-yellow filled amacrine cells. Cells A and B are conventional OFF amacrine cells with cell bodies located in the INL and dendrites ramifying in sublamina II_a for the cell A and at both sublaminae I and II_a for the cell B. They differ also in the size of their dendritic field. Cell C is a displaced OFF amacrine cell with cell body located in the GCL and dendrites ramifying at sublamina I. Cell D is a conventional ON amacrine cell with dendrites ramifying at sublamina III. Cells E and F are a conventional and a displaced ON-OFF amacrine cell, respectively. Their dendrites ramify diffusely in the IPL. They differ also in the size of their dendritic fields.

Twelve ganglion cells located in the GCL were successfully stained by iontophoretic injections of Lucifer-yellow dye after recording their response. Labeled cells without axon in the GCL were not identified as ganglion cells, because it has been reported that more than half of the cells in the GCL are displaced amacrine cells (Ball and Dickson, 1983; Gläsener *et al.*, 1988), and amacrine and ganglion cells are not spatially separated within the GCL (Gläsener *et al.*, 1988). Ganglion cells were classified at least into three types on the basis of their responsiveness to light and the level of dendritic arborization in the IPL. Spike discharges of the ganglion cell were sometimes lost during recording, because cell penetration often depolarized the cell. Fig. 8B shows typical voltage responses of each type of cell. An OFF ganglion cell responded to light with a sustained hyperpolarization and a disappearance of spike discharges (Fig. 8B, left). An ON ganglion cell responded to light with a sustained depolarization with spike discharges (Fig. 8B, middle). At light offset, the cell responded with a transient depolarization with spike discharges. An ON-OFF ganglion cell responded to light with a spike followed by the hyperpolarization both at light onset and offset (Fig. 8B, right). Fig. 10 shows three examples of the Lucifer-yellow filled ganglion

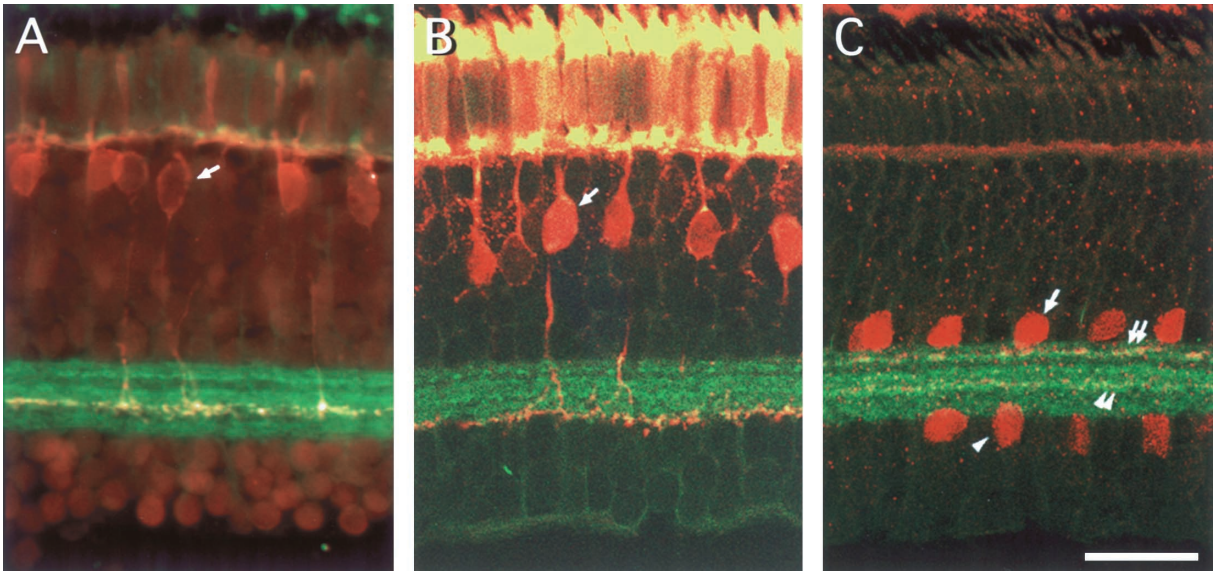

Fig. 11. Confocal images of PKC-ir bipolar cells (A), RB-1-ir bipolar cells (B) and ChAT-ir amacrine cells (C) in combination with the synaptotagmin immunoreactivity. A: A double staining of synaptotagmin-ir IPL (green) and PKC-ir bipolar cells (red). PKC-ir bipolar cells were characterized by having cell body lying at the most distal level of the INL (arrow) and axon terminating at sublamina II_b of the IPL (orange). B: A double staining of synaptotagmin-ir IPL (green) and RB-1-ir bipolar cells (red). RB-1-ir bipolar cells were characterized by having cell body lying at the second or third row of the INL (arrow) and axon terminating at the most proximal level of sublamina III of the IPL (orange dots). C: A double staining of synaptotagmin-ir IPL (green) and ChAT-ir amacrine cells (red). Two types of ChAT-ir cells, the conventional (arrow) and displaced amacrine cells (arrowhead) ramified their dendrites as single bands in sublamina I (double arrow) and sublamina II_b (double arrowhead). Scale bar=50 μm in A–C.

cells. Cell A is an OFF ganglion cell whose dendrites ramify at sublamina I, cell B is an ON ganglion cell whose dendrites ramified at sublamina III, and cell C is an ON-OFF ganglion cell whose dendrites ramify diffusely throughout the whole IPL.

Immunohistochemistry for bipolar cells and amacrine cells

At least two immunohistochemical markers, PKC and RB-1, for retinal bipolar cells have been identified in the adult newt retina (Negishi, *et al*., 1992; Saito *et al*., 1994). Fig. 11A shows a double staining of synaptotagmin-ir IPL (green) and PKC-ir bipolar cells (red). These bipolar cells have cell bodies lying at the most distal level of the INL (arrow) and axons terminating at sublamina II_b of the IPL (orange). Fig. 11B shows a double staining of synaptotagmin-ir IPL (green) and RB-1-ir bipolar cells (red). Most of these bipolar cells have cell bodies in the second or third row of the INL (arrow) and axons terminating at the most proximal level of sublamina III of the IPL (orange dots). Although their axonal processes terminate at the sublamina III, some of them, like the Lucifer-yellow labeled Transient bipolar cell shown in Fig. 6C, began branching at sublamina II_b.

Choline acetyltransferase (ChAT) is a reliable marker of cholinergic neurons in most vertebrate retinas (Voigt, 1986; Hutchins, 1987). Fig. 11C shows a double staining of synaptotagmin-ir IPL (green) and ChAT-ir cells (red). The adult newt retina has two types of ChAT-ir cells: the conventional (arrow) and displaced amacrine cells (arrowhead) with dendrites ramifying as single bands in the most distal level of the IPL (double arrow) and the lower middle of the IPL (double arrowhead). These two ChAT-ir bands correspond to sublamina I and II_b (yellow) where OFF and ON bipolar cells sent their axon terminals respectively.

DISCUSSION

Segregation of visual signals by stratification of bipolar axon terminals

In this study, we demonstrated that a monoclonal antibody against synaptotagmin cross-reacted in the adult newt retina as it does in the mammalian (Sarthy and Bacon, 1985; Koontz and Hendrickson, 1993; Greenlee *et al*., 1996) and chick retinas (Hering and Kröger, 1996). The synaptotagmin-ir staining pattern in the IPL divided into three broad bands, I, II and III. Band II was further divided into two subbands, II_a and II_b, on the basis of dense punctate labeling. The stratification level(s) of bipolar, amacrine and ganglion cell processes in the IPL corresponded with one or two bands of synaptotagmin-ir staining.

The dendrites of OFF amacrine and ganglion cells ramified in sublaminae I and/or II_a where OFF bipolar cells extend their axon terminals, whereas the dendrites of ON amacrine and ganglion cells ramified in sublamina II_b and/or III corresponding to the level of the ON bipolar cell terminals in the IPL. The dendrites of ON-OFF amacrine and ganglion cells distributed diffusely throughout the IPL. These results are in agreement with the general observation that ON and OFF pathways are segregated into the sublamina *a* and sublamina *b* (Famiglietti and Kolb, 1976; Famiglietti, *et al*., 1977; Nelson *et al*., 1978). In the case of newt retina, the sublaminae I and II_a, and the sublaminae II_b and III correspond with sublamina *a* and sublamina *b* in other vertebrate retinas.

More than half of the bipolar cells examined in this study responded with a transient hyperpolarization at both light onset and offset rather than sustained depolarization. We believe that these Transient bipolar cells are probably subpopulations of ON bipolar cells, because some of them responded with a sustained depolarization at the dimmest light, and their axon terminals ramified mainly in sublaminae II_b and/or III. Some Lucifer-yellow labeled Transient bipolar cells (Fig. 6C and D) had axons terminating not only in sublamina III, but also in sublamina II_a. Previous studies in tiger salamander and turtle retinas have shown that there are a few ON bipolar cells with axon terminals ramifying in two strata in the IPL, one in sublamina *a* and the other in sublamina *b*. (Weiler, 1981; Ammermüller and Kolb, 1995; Wu *et al*., 2000).

Although the mechanisms underlying ON-OFF hyperpolarization of bipolar cells were not well examined in this study, the results obtained by applying polarizing currents on ON-OFF responses suggest the involvement of two ion channels with different reversal potentials. Lasansky (1992) found that ON bipolar cell responses in salamander retina include two components: one mediated by direct photoreceptor input and another mediated by ON-OFF amacrine cells or interplexiform cells. He also suggested that ON-OFF responses were produced by chloride-dependent conductance increase at light onset and offset. Numerous bipolar-to-amacrine synapses have been reported (Dowling and Werblin, 1969; Wong-Riley, 1974). Also, bipolar cell terminals receive a GABAergic input (Marc *et al*., 1978; Vallerga, 1981; Wu *et al*., 1981). Thus, Transient bipolar cells may receive chloride-mediated inhibition at both light onset and offset, and much of this inhibition may be generated by ON-OFF amacrine cells, which ramify in both sublamina *a* and sublamina *b* (Lasansky, 1992; Maguire *et al*., 1989; Wu *et al*., 2000).

Two immunohistochemical markers, PKC and RB-1, identified newt bipolar cells (Saito *et al*., 1994). PKC-ir bipolar cells shown as rod-connected ON bipolar cells in other vertebrate retinas (Negishi *et al*., 1988; Yamashita and Wässle, 1991) are similar in morphological appearance to the Lucifer-yellow labeled bipolar cells in Figs. 5A and 6B. Their axon terminals ramify exclusively in sublamina II_b of the IPL. Some RB-1-ir bipolar cells possessed pyramidally branching axons that eventually terminated at the most proximal level of sublamina III. The ramification pattern of their axon terminals was similar to that of Lucifer-yellow labeled bipolar cells shown in Figs. 5C and 6C, suggesting

that they are probably a subpopulation of ON bipolar cells. These results confirm again the idea of segregation of ON and OFF pathways in the IPL.

Morphological diversity and classification of amacrine cells

Amacrine cells differ from one another in light response characteristics, neurotransmitter contents, level of cell body, size of dendritic fields and level of dendritic stratification (Yang *et al*., 1991; Pang *et al*., 2002). Therefore, classification of amacrine cells is highly dependent on the parameters chosen. We have already shown neurochemical diversity of newt amacrine cells with respect to GABA (Chiba *et al*., 1997), acetylcholine (Cheon and Saito, 1999), and several neuropeptides (Negishi *et al*., 1992). In newt retina, like other urodele species (Gläsener, *et al*., 1988), GABAergic amacrine cells are most abundant and located in the INL and GCL in two to three cell rows close to the IPL. The high density and homogeneous of labeling in the IPL precludes any hope of determining the ramification pattern of a single cell type. Yang *et al*., (1991) have addressed this issue in tiger salamander retina and analyzed the retinal slice preparation by GABA immunostaining of Lucifer-yellow filling cells after recording photocurrents with whole-cell voltage-clamp techniques. They identified GABA-ir ON amacrine cells with processes ramifying in sublamina *b*, ON-OFF amacrine cells with processes ramifying in both sublaminae *a* and *b,* and further predicted the existence of GABA-ir OFF type amacrine cells ramifying in sublamina *a* from a result obtained in other study (Maguire *et al*., 1989).

Only a small percentage of cells in both the INL and GCL of urodele amphibian's retinas is a neuropeptide-containing amacrine cells (Yang and Yazulla, 1986; Gläsener, *et al*., 1988). We have detected glucagon-, serotonin-, substance P- and neuropeptide Y-ir amacrine cells in newt retina (Negishi *et al*., 1992). Among them, the glucagon-ir amacrine cells showed the most intense dendritic labeling in the IPL. They ramified mainly both sublaminae I and III with a pattern similar to the Lucifer-yellow labeled ON-OFF amacrine cell shown in Fig. 8E and to that observed in another urodele species, *Triturus alpestris*, (Gläsener, *et al*., 1988). However, they differed in other urodele species, such as a tiger salamander, which showed diffusely ramified dendritic pattern in the full depth of the IPL (Yang and Yazulla, 1986; Gläsener, *et al*., 1988). Glucagon-ir amacrine cells obtained by combination of patch-clamp recording and dye-injection with immunohistochemistry exhibited ON-OFF responses in tiger salamander (Yang *et al*., 1991).

There are two cholinergic amacrine cells in the vertebrate retina: the conventional amacrine cells in the INL and the displaced amacrine cells in the GCL. Two ChAT-ir bands have been commonly reported in many vertebrate retinas including goldfish (Tumosa *et al*., 1984), newt (Cheon and Saito, 1999), chick (Millar *et al*., 1987; Spira, *et al*., 1987; Reiss *et al*., 1996), cat and rat (Criswell and Brandon, 1993), tree shrew (Conley *et al*., 1986; Sandmann *et al*., 1997), rabbit and monkey retinas (Famiglietti and Tumosa, 1987). In the mammalian retina, cholinergic amacrine cells in the INL ramify in the distal half of the IPL where OFF bipolar cell axons terminate and those in the GCL ramify in the proximal half where ON bipolar cells terminate (Masland and Tauchi, 1986). In newt retina, however, no such correlation between the location of cell body and the level of synaptic bands was observed (Cheon and Saito, 1999). Nevertheless, the ChAT-ir amacrine cells are one of the useful tools to investigate the segregation of ON and OFF pathways in the developing and regenerating retinas because their dendrites ramify in either sublamina I or sublamina II_b where populations of ON and OFF bipolar cells terminate.

Morphological and functional organization of ON and OFF pathways in the adult newt retina were examined by intracellular recording and staining techniques and by immunohistochemistry. Results obtained in this study provide the foundation for examining the appearance and maturation of ON and OFF pathways during regeneration of the newt retina.

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