SEROTONIN AND PROTEIN KINASE C-IMMUNOCYTOCHEMISTRY OF THE RETINA OF THE SKATE *RAJA ERINACEA*.

by

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Abstract:

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Adviser: Professor Richard L. Chappell

1) The skate retina contains at least two different classes of bipolar cell, one of which conforms to the rod bipolar cell known in mammalian retinas. A similar cell is also found in the teleost retina. All these cells are believed to belong to the retinal ON channel and there is no indication that the skate is an exception.
2) The second class of bipolar cell described in my study has biochemical (accumulation of serotonin) and morphological features (axonal ramifications in the outer strata of the IPL) suggesting that this cell belongs to the retinal OFF channel. Glutamate-receptor agonists, however, failed to induce decrease of serotonin-immunoreactivity in these

cells, thus indicating important differences between these cells and their counterparts in other retinas.

3) The serotonin system of the skate retina can be subdivided into two distinct classes of cell with entirely different pharmacologic profiles. Amacrine cells can be depleted of serotonin by exposing the retina to glutamate receptor agonists and appear to be sensitive to the high affinity serotonin transporter blocker fluoxetine. Bipolar cells, in which serotonin release cannot be induced by receptor stimulation, are insensitive to fluoxetine, however their serotonin uptake can be completely blocked by the serotonin uptake inhibitor zimelidine.

4) The latter two points show that, within the CNS, at least two serotonin systems with markedly different pharmacologic properties may coexist. Blockage of the serotonin transporter in one part of the system may activate the other system and thus cause complex interactions between different populations of serotonergic / serotonin-accumulating cells. These interactions may exceed by far the simplified concept of increasing extracellular serotonin levels as proposed in antidepressant therapies.

Some findings presented here have been published previously in:

SCHLEMERMEYER, E. & CHAPPELL, R. L. (1991). Glutamate and serotonin immunocytochemistry indicate centrifugal fibers and a visual streak in the skate retina. *Society for Neurosciences* **17**, abstract 549.6.

SCHLEMERMEYER, E. & CHAPPELL, R. L. (1991). Two classes of bipolar cell identified in the all-rod skate retina by PKC- and serotonin-like immunocytochemistry. *Investigative Ophthalmology and Visual Science Supplement* **32**, 1264; (abstract) 2923.

SCHLEMERMEYER, E. & CHAPPELL, R. L. (1994). Differential effects of excitatory amino acids, high K^+, and an uptake blocker on the serotonergic system of the skate retina. *Investigative Ophthalmology and Visual Science Supplement* ; **35**; 1360 (abstract) 494.

SCHLEMERMEYER, E. & CHAPPELL, R.L. (1996) Two classes of bipolar cell in the retina of the skate *Raja erinacea*. *Journal of Neurocytology* **25**, 625-635.

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1. Introduction

A. Rationale

The visual system is an extremely important source of perception that enables us to transform quanta of light into electrical and chemical signals that are processed by our brains to recognize our environment. In human, the parts of the brain dedicated solely to visual input occupy more than 60% of the entire cerebral cortex, thus reflecting the dominant role of vision in our perception of the world. The first station of the visual pathway is the eye which has a thin layer of nerve cells in its posterior portion, the neural retina. The retina itself is a protrusion of the diencephalon and, although its structure is highly conserved in all vertebrates, numerous specializations are found in different species according to the different requirements of the habitats in which the animals live.

For example, species of all vertebrate orders living in light environments have developed multiple classes of cone photoreceptor with different spectral sensitivities that account for the perception of color as an additional dimension of contrast (Barlow, 1981). For other species of the same order that occupy dark environments, an elaborate color vision holds no advantage, since the threshold for cone vision is several log units above the scotopic (rod vision) range. In these animals, rods are the predominant

photoreceptors. In primates, examples for the first case are tree shrews whose retinas contain ca 95% cones (Müller & Peichel, 1989), whereas the nocturnal *lemuroidea* fall into the latter category. Interestingly, as already described by Ramón y Cajal (1892) the photopic (cone vision) and scotopic pathways remain separated at least at the level of second order neurons, in that so called **rod** bipolar cells show a different pattern of connectivity in the inner retina than so called **cone** bipolars (for review see: Wässle *et al.*, 1991).

A unique situation is found in the retina of the little skate, *Raja erinacea*, because this retina contains only rod photoreceptors. This feature offers the opportunity to study a simplified system of organization of a retina, because the additional channels for color information are not present in the skate retina. Further, at the beginning of my study, it was not clear whether the skate retina contains even ON- and OFF-channels, although some evidence that both exist were obtained from ganglion cell recordings in earlier studies (Dowling & Ripps, 1970). My aim in the study presented here was to examine the bipolar cell system of the skate retina and to morphologically and pharmacologically characterize at least one class of bipolar cell as to whether these cells belong to the ON- or OFF-channels of the vertical pathway through the retina as discussed further below.

B. Retina

The vertebrate retina follows the common aspects of organization of sensory circuits. A primary sensory neuron, in this case the cone or rod photoreceptor reacts to a stimulus (light) and this information is converted into a code of electrical nature, which is then transmitted to central stations by way of interneurons (bipolar, amacrine and horizontal cells) and output cells, the ganglion cells. The main neurons in the vertebrate retina are arranged in a layered fashion where 3 nuclear layers are segregated by two synaptic (plexiform) layers (Figure1).

The above mentioned neurons of the retina can be further subdivided into several classes, depending on their connectivity (ON, OFF, ON-OFF, cone-related, rod-related), morphology (large field, small field, displaced etc.) and pharmacology (serotonergic, glycinergic, inhibitory, excitatory etc.).

The classification of ganglion and bipolar cells into "ON" or "OFF" cells originates in the work of Hartline (1938, 1940) who found that ganglion cells of the frog can be grouped into three functional types: those that responded to an onset of light (ON cells), those that responded to an offset of light (OFF cells), and those that responded to both the onset and offset of light (ON-OFF cells). Hartline also described a phenomenon known as the "receptive field" of a given neuron, namely that region of the retina that has to be illuminated in order to receive a response from a particular neuron (Hartline,

1938). The receptive field of a neuron can further be subdivided into a "center" and a "surround" where a stimulation of the surround induces a response of opposite polarity or reduces the response to the stimulation of the center. Kuffler (1953) investigated the organization of the receptive field of ganglion cells of the cat and further grouped response types of these cells into ON-center with OFF-surround (ON cell) and OFF-center with ON-surround (OFF cell). In other words: stimulation of the receptive field center of a bipolar/ganglion cell by light can either result in a hyperpolarization of the cell (OFF cell) or in a depolarization of the cell (ON cell). Curiously, these two responses are generated by the same transmitter substance (glutamate) where at light-on glutamate transmission is reduced by photoreceptors and at light-off more glutamate is being released by photoreceptors.

In the case of bipolar cells, poikilotherm vertebrates have so called rod-dominated ON bipolar cells and rod-dominated OFF bipolar cells, indicating that the input to these second order neurons comes mainly from the rod photoreceptors of those retinas (Kaneko & Tachibana, 1978; Kaneko *et al.*, 1979; Ishida *et al.*, 1980; Saito & Kujiraoka, 1982; Marc *et al.*, 1988). In retinas of mammals, the rod pathway is served by a single, morphologically homogenous class of cell, the "rod-bipolar cell" (Dacheux & Raviola, 1986, Wässle *et al.*, 1991). Of particular interest are the subdivisions of the inner plexiform layer (IPL) into an outer sublamina a and

an inner sublamina b. It has been established that retinal interneurons that form their synapses in sublamina a belong to the off-pathway and neurons with their synapses in sublamina b are said to belong to the on-pathway (Famiglietti *et al.*, 1977). For descriptive purposes the IPL may also be subdivided into 5 strata of equal width (Ramón y Cajal, 1892) (Figures 1, 2).

As mentioned above, the retina of the little skate *Raja erinacea* is a unique preparation for the study of visual processes because it has only rods (Dowling & Ripps, 1970; Szamier & Ripps, 1983) and therefore lacks one of the major classes of retinal cells, cone photoreceptors. The simplicity of having to incorporate only one class of photoreceptor with a single peak of spectral sensitivity, into a conceptual model of retinal signal processing has fostered numerous studies taking advantage of the absence of other first order neurons (e.g. Dowling, 1987).

Of all living vertebrates, the skate retina is probably one of the closest to the ancestral neural retina (reviewed by Linser, 1991). Nevertheless skate retinal neurons show a wide spectrum of capabilities as seen in the operating range of the rod photoreceptor of this species which goes well into the range of cone photoreceptors in other species (Dowling and Ripps, 1970; 1991, Cornwall *et al.*, 1989).

With this in mind, the organization of the skate retina at the level of second order neurons is of special interest. One might wonder whether it may

contain only one class of bipolar cell, the rod ON bipolar cell, especially since this particular type of second order neuron has been found to constitute a single, homogenous class of cell dedicated to the rod pathway in the retinas of mammals (Dacheux & Raviola, 1986, Wässle *et al.*, 1991). On the other hand, it is well established that in cold blooded vertebrates which have mixed rod/cone input to their bipolar cells, both rod dominated ON and rod dominated OFF bipolar cells are present (Kaneko & Tachibana, 1978; Kaneko *et al.* 1979; Ishida *et al.*, 1980; Saito & Kujiraoka, 1982; Marc *et al.*, 1988). Therefore, in addition to ON bipolar cells, an OFF bipolar cell pathway could well exist in an all-rod retina.

C. Serotonin

A pioneering study of the skate retina (Bruun et al., 1984) provided evidence that several subsets of neuron, that is, amacrine and bipolar cells may utilize serotonin (5-HT) as neurotransmitter. For this reason, the serotonergic system of the skate retina was chosen to study the morphologic and pharmacologic properties of these cells.

Serotonin plays a variety of modulatory functions throughout the entire vertebrate organism. Originally 5-HT was recognized as a substance released from clotting blood into the serum which exerts a tonic action on the smooth musculature of blood vessels (reviewed by Green, 1989). In the CNS,

the serotonergic system is considered the phylogenetically oldest neuronal system (Page, 1968). Therefore it is not surprising that serotonin is involved in a variety of the most archaic brain functions such as sleep-wakefulness, appetite, nociception and analgesia, nausea and vomiting, thermoregulation and aggression. In today's society, serotonin has gained wide attention in the context of depression and drug abuse, and, consequently, the serotonergic system has become a primary target for pharmacological intervention with mood disorders.

Serotonin does not cross the blood-brain barrier and has to be synthesized by neurons themselves. The amino acid tryptophan (from the diet) is the primary substrate for serotonin synthesis. The key enzyme in serotonin synthesis is tryptophan hydroxylase, a soluble cytoplasmic enzyme, that is under normal conditions unsaturated in nervous tissue. Following the hydroxylation of tryptophan to 5-hydroxytryptophan, the latter is immediately decarboxylated to yield serotonin (5-hydroxytryptamine). The catabolism of serotonin involves a deamination by monoamine oxidase in the brain to 5-hydroxy-indoleacetic acid or, in the pineal organ, n-acetylation and a transferase reaction to form melatonin (Cooper et al., 1991) (Figure 3).

Many of the pioneering studies on serotonin pharmacology have employed the retina as a suitable model for the study of uptake and release of serotonin (Osborne, 1988). In the vertebrate retina serotonin was first

detected by Welsh (1964) and Quay (1965). In subsequent studies, uptake of radiolabeled 5-HT by retinal cells in a variety of mammalian and non-mammalian species was reported (reviewed by Osborne, 1988). After the introduction of immunocytochemical methods, it became possible to study its endogenous presence in greater detail than with the earlier-employed Falck-Hillarp method (Falck & Owman, 1965). These studies, however, also showed for the first time an important discrepancy between uptake of the neurotransmitter and its endogenous localization, in that many cells which, based on the uptake studies, had been classified as putatively serotonergic failed to reveal its endogenous presence (Marc *et al.*, 1988). Indeed, after ruling out some artifacts, the current conclusion is that under physiological conditions none of the serotonin-accumulating neurons of mammalian retinas contain significant amounts of serotonin (Sandell & Masland, 1989; Massey *et al.*, 1992). A variety of neurons in the non-mammalian retina contain serotonin, although, it appears that serotonin is synthesized only by one class of neuron (Zhu *et al.*, 1992, Wilhelm *et al.*, 1993; Schütte, 1994). This cell, a large field amacrine cell, is also the only neuron which possesses tryptophane hydroxylase, the key enzyme for serotonin synthesis (Jequier, 1969). Nevertheless, evidence for the presence of serotonin in *e.g.* bipolar cells, first reported in the retina of the little skate *Raja erinacea* by Bruun *et al.* (1984), has held up well. This establishes an interesting parallel to the

mammalian brain serotonin system where a similar dichotomy between serotonergic and "masked" serotonergic cells (cells that stain for serotonin after intraventricular injection of tryptophane) exists (for review see Takeuchi, 1988). Furthermore, the distribution of serotonergic cell groups in the elasmobranch CNS shows more resemblance to serotonergic brain nuclei in mammals than to the distribution pattern of serotonergic cells in reptile and amphibian CNS with the exception of the dorsal raphe nucleus (Stuesse *et al.*, 1990, Stuesse *et al.*, 1991a,b) Thus, the non-mammalian, elasmobranch retina may constitute an important model for the study of interactions between physiologically different serotonin systems.

D. Protein Kinase C:

Protein Kinase C (PKC), originally isolated from brain (Takai *et al.*, 1977), is a family of phosphorylating enzyme expressed in virtually all tissues. Its maximal activity and concentration are found in spleen and brain (Kuo *et al.*, 1984, Huang & Huang, 1986). Originally, the name PKC was coined to indicate the "C"alcium dependence of one protein kinase. By now there have been at least 10 subspecies described: α, βI, βII, γ, δ, ε, ζ, λ, η, and θ (Nishizuka, 1988, Osada *et al.*, 1990, Sanchez-Prieto *et al.*, 1994). PKC is found in the cytosol of cells or in their membrane (Forooqui *et al.*, 1988) with

the cytosolic form representing the inactive state and the membrane bound form the active state of the enzyme (Osborne, 1991) but it may also bind to the cell membrane and stay inactive (Bell, 1986). At least 4 PKC isoforms are activated by diacylglycerol. The initial rise in cytosolic Ca^{2+} induced by inositoltrisphosphate (IP_3) is thought to alter the C-kinase so that it translocates from the cytosol to the cytoplasmic face of the plasma membrane (Alberts *et al.*, 1994). There it is activated by the combination of Ca^{2+}, diacylglycerol, and the negatively charged membrane phospholipid phosphatidylserine. In neurons, PKC is thought to phosphorylate ion channels and thereby to modulate the excitability of the nerve cell plasma membrane. (Sanchez-Prieto *et al.*, 1994). One of the major consequences of PKC activation is an increase of intracellular calcium, the rate limiting ion for vesicular transmitter release.

Negishi *et al.* (1988) and Wood *et al.* (1988) first reported that an antibody, directed against the α and β subspecies of PKC, stains a class of bipolar cell in vertebrate retinas. Negishi *et al.* classified these cells as rod bipolar cells of the ON-center type. This finding was confirmed by numerous studies and this specific PKC antibody (clone MC5, Amersham) is now being used as a reliable marker for rod bipolar cells in mammals or for rod-dominant bipolar cells in a variety of non-mammalian species (Negishi *et al.*, 1988; Wood *et al.*; Suzuki & Kaneko, 1990; Suzuki *et al.*, 1990; Müller &

Peichel, 1991; Osborne *et al.*, 1991; 1992; Huwiler *et al.*, 1992; Kolb *et al.*, 1993; Fukuda *et al.*, 1994).

Serotonin immunocytochemistry exposed a rather low density of immunoreactive bipolar cells in the skate retina (Bruun *et al.*, 1984) which seemed in contrast with a high density of bipolar cells observed by methylene blue staining in other elasmobranch species (Schaper, 1899, Retzius, 1905, Witkovsky & Stell, 1973) indicating that the skate may well possess additional types of bipolar cell that were not revealed by serotonin immunocytochemistry.

I have used the immunoreaction against PKC in the skate retina in order to examine whether the skate contains "rod bipolar cells" of similar morphology as mammalian and other non-mammalian retinas.

2. Materials and Methods:

A. Animals and tissue preparation:

Adult skates of the species *Raja erinacea* were obtained from the Marine Biological Laboratories, Woods Hole (MA) and kept in a seawater (Instant Ocean, Aquarium Systems) aquarium under a steady 12h light/dark cycle. Two hours after light-ON (ca 10 am), the animals were pithed, the eyes

enucleated, the anterior chamber and lens were removed, and the remaining eyecup drained of vitreous humor. This time was chosen to guarantee that the animals were fully light adapted and to keep a variation of the state of adaptation to a minimum. The eyecups were fixed for two hours at room temperature in freshly prepared 6% paraformaldehyde in 0.1M phosphate buffer (PB) containing 3% sucrose or, for PKC-immunocytochemistry in modified Zamboni's fixative (4% paraformaldehyde, 0.24% picric acid, 3% sucrose in 0.1M PB) and then placed in a solution of 30% sucrose in PB overnight for cryoprotection, briefly incubated in Cryomatrix (Shandon, Pittsburgh, PA), and shock-frozen in liquid N_2. Eighteen to 25µm sections were cut on a cryostat (Hacker Instruments) and mounted on gelatin-coated slides. The sections were air-dried for 2-3 hours, rinsed in phosphate-buffered saline (PBS) or frozen for storage. See table 1 for composition of solutions employed.

B. Immunocytochemical procedures (general):

Immunocytochemistry is a combination of anatomical, immunological and biochemical techniques where the sensitivity and specificity of the antibody-antigen interaction is combined with the ability to visualize immunoreactivity in tissues by microscopy. In these techniques labeled antibodies are

employed to localize tissue antigens *in situ*. Antigens are rendered insoluble by fixation and sections or whole mounts of tissue are incubated in antibody solutions by one of several available techniques. The primary antibody is always directed against the antigen of interest. One-step methods use a labeled primary antibody and multistep methods employ an unlabeled antibody followed by a labeled secondary or tertiary antibody. The most common types of antibody used are polyclonal antisera that are generated in rabbits and monoclonal antibodies derived from mouse myeloma cell lines. There are many other animals used for antibody production (goat, donkey, rat, horse etc.) but, due to the limited availability of myeloma cell lines, only mice and rats are used for generating monoclonal antibodies.

C. Immunocytochemical procedures (specific):

My methods for immunocytochemical staining are the result of critical examination of various parameters (incubation time, concentration of antibodies, temperature) found in the literature. I have used blocking agents like normal goat serum and compared the results with pieces of the same retina where normal goat serum had been omitted. After careful examination, I could not see any difference in tissue appearance or staining/background intensity and decided to leave out that step from the immunocytochemical protocol. Similar experiments were made in order to obtain optimal

incubation times and antibody concentrations. In short, with each new antiserum employed I tested a dilution series of 1:50, 1:100, 1:1000, 1:2000 and 1:5000 for each primary antiserum and used the dilution that gave the clearest results, that is, the least background staining, and kept that specific concentration through all further experiments. Incubation times of serotonin-preuptake and drug addition were determined in a similar fashion with "reproducibility" as the determining factor. In general, incubation times of 20-30 minutes gave reproducible results in all cases. Control retinas were incubated in Ringer's solution only or, in some cases, in Ringer substituted with a limited drug composition.

The primary and secondary antisera used were diluted with PBS containing 0.15% Triton X-100 (Sigma, St. Louis, MO). Triton X-100 is a highly viscous solution which tends to stick to a plastic pipette tip (Eppendorf). In order not to vary the concentration of Triton X-100 too much in different batches I always dropped the used pipette tip into the solution and left it there. A small batch of Triton X-100 solution (100 ml) keeps well in the refrigerator and can be used for a long time (6 months). The retinal sections were incubated in the following primary antibodies: rabbit anti-serotonin (1:2000, Incstar, Stillwater, MN), mouse anti-protein kinase C (PKC) (1:5000, Amersham, Arlington, IL; clone MC5) or rabbit anti-glutamate (Arnel, New York, NY; a kind gift of Dr. A. Rustioni), respectively, for 2 hours

at room temperature (23 °C) or overnight at 4°C (no difference was observed with the same antisera between those two incubation procedures). After 2 rinses in PBS (10 min. each), the sections were incubated for one hour in the secondary antisera (1:50) conjugated to fluorescein isothiocyanate (FITC) (anti-rabbit or anti-mouse, Sigma, St. Louis, MO). In some cases, antibody-cocktails of the primary and subsequently of the secondary antisera were employed for double-staining with FITC anti-mouse and Texas Red anti-rabbit (1:50). A concentration ladder with different concentrations of secondary antibody (FITC, 1:25, 1:50, 1:200, 1:1000) was made only once with the concentration of 1:50 yielding the clearest results.

After two washes in PBS, the slides were cleared with glycerol (in PBS 1:1) or Vectashield (Vector, Burlingame, CA), coverslipped and examined under a fluorescent microscope (Zeiss Universal, Oberkochen, Germany) equipped with 16x and 25x multi-immersion Neofluar objectives. Vectashield mounting medium has to be kept in the dark at 4°C to keep its properties that prevent bleaching of immunostained tissue. Photographs were made using Kodak Technical Pan Film which was developed with freshly made D-19 developer (very high contrast, Kodak) for 6 minutes. For doublestaining experiments, subsequent exposures of the same retinal location were taken on black and white (Kodak Technical Pan) film using the appropriate filters and printed at the same magnification. The prints were scanned into Adobe

Photoshop (version 3.0 and version 4.0, Mountain View, CA). Pseudocolours were assigned to respective image files which then were merged to obtain a red-green-blue (RGB) color picture.

D. Serotonin- Immunocytopharmacology:

The term *immunocytopharmacology* will be used to describe immunocytochemical experiments that include a pharmacological treatment of living retinal tissue *in vitro* prior to fixation and immunocytochemical processing of the tissue. In most experiments the eyecups were incubated for 20-30 minutes at room temperature (23 °C) in Ringer's solution (table 2) with the appropriate drugs added. Drugs employed individually or in combination were: serotonin (10 μM), pargyline (1mM) (Weiler and Schütte, 1985a), N-methyl D aspartate (NMDA, 5mM), high K^+ (100mM), aspartate (5mM), zimelidine (0.5mM), fluoxetine (25-500 μM), kainic acid (5 μM) and quisqualate (30 μM). A stock solution of serotonin in PBS (10 mg/1ml) could be kept at 4°C until it turned dark in color (after ca. 3 weeks) indicating its decomposition. In experiments which relied on comparing intensity of serotonin immunoreactivity after dissimilar pharmacological treatment of the retinas among different experiments, the eyecups were incubated in serotonin (10μM) and pargyline (1mM) (inhibitor of oxidative degradation of

serotonin) for 30 min. prior to experimental drug exposure and/or fixation to establish a uniform baseline of staining-intensity in bipolar cells. In other words, before adding excitatory amino acids (like NMDA or aspartate) to the incubation Ringer, a serotonin uptake was performed with the same tissue to yield a uniform serotonin immunoreactivity in bipolar cells. After this initial serotonin-uptake, the eyecups were transferred into fresh Ringer with the respective experimental drugs added. Presumptive centrifugal fibers appeared to be insensitive to all pharmacological treatments, that is, they never changed their degree of staining and were used as a "visual control" to evaluate overall intensity of staining in the tissue.

E. Experimental Controls:

Controls for primary antibodies were made on tissue pieces that were treated simultaneously with experimental tissues except that no primary antibody was added. All other treatment remained the same. Immunocytochemistry without the primary antisera results in tissue without any stained cells but a certain amount of background staining in the photoreceptor layer.

Control experiments for drug treatments were done in a similar way, always leaving out the drug in question in the control but otherwise treating the tissue in the same fashion (incubation time, temperature, rinses) as the experimental tissues.

At the time of microscopic observation one can get a quick overview of the nonspecific background staining by switching the excitation/emission filters of the microscope to a filtersystem that is specific for a wavelength other than the fluorescence observed. For instance if a FITC stained tissue shows fluorescence with a Texas Red filter that fluorescence may be an artifact and not a true immunoreactive staining.

3. Results:

A. Serotonin Immunocytochemistry:

Serotonin accumulating bipolar cells:

Serotonin-accumulating bipolar cells were found to be unevenly distributed throughout the retina with their highest density in a horizontal band: the visual streak (Brown, 1969;originally described in this species as "horizontal streak" by Brunken, 1983) which runs in a wide horizontal arc throughout the entire width of the dorsal retina and follows the course of the tapetum lucidum. The density of serotonin-accumulating bipolar cells peaked at *ca.* 5000 cells/mm^2 in the visual streak and declined to *ca.* 670 cells/mm^2 in the periphery (Figure 4).

In the visual streak, the somata were oval in shape, measured 5-7μm across and usually occupied the third or fourth tier of somata from the OPL, slightly distal from the midline of the inner nuclear layer (INL). In central retinal areas, the dendrites emanated from a single primary stalk which crossed the outer INL perpendicularly and branched out before reaching the OPL (Figure 5a).

Farther away from the visual streak, the morphology of stained bipolar cells changed, in that, as a function of increasing eccentricity, the somata became much thicker and both dendritic and axonal arbors covered a larger area, concomitant with a reduced density of stained cells in the retinal periphery. Also, with increasing eccentricity, the distal portion of the soma itself became the site of origin of several primary dendrites heading towards the OPL at oblique angles (Figure 5b). These primary dendrites repeatedly ramified within the distal INL before splitting up into their terminal branches which ran horizontally at the level of the OPL. Axons of serotonin-accumulating bipolar cells crossed the INL in a more or less oblique fashion, depending on their retinal location, before they dipped perpendicularly into the IPL (Figures 5a-c). A detailed examination of the axonal arborizations was difficult because numerous stained amacrine cell processes (Figure 5d) obscured the fine branchlets of serotonin-accumulating bipolar cell axon terminals. It was, however, possible to determine that, after entering the IPL, the main axon became varicose and branched into very fine terminals in the border of strata 1 and 2 and in stratum 4 (Figure 5c). Immunostaining in this class of bipolar cell appeared to be uniform throughout the soma and processes.

Dendritic fields were hard to distinguish from one another within the visual streak. In midperipheral areas of the retina or in the retinal periphery,

however, it was possible to obtain a clear view of the stained arborizations in the OPL. Dendrites of neighboring serotonin-accumulating bipolar cells overlapped considerably, that is, in most cases, they reached at least as far as to the dendritic field center of the nearest neighbor serotonin-accumulating bipolar cell (Figure 5e). The overall coverage of the retina by these dendritic fields stayed the same over the whole retina. In streak areas the somata are closer together and bear shorter dendrites than in the periphery where the somata are larger with longer dendrites reaching up to the receptive field center, marked by the position of the soma, of the neighboring cell of the same type.

Serotonergic amacrine cells and centrifugal fibers:

Serotonergic amacrine cells in the skate *Raja erinacea* have been previously described (Bruun *et al.*, 1984; Brunken *et al.*, 1986). In short, I found at least one type of serotonergic amacrine cell with short stout processes in the innermost tier of the INL which branched out several 100 μm and formed a dense network of very fine-caliber fibers in the outer half of the inner plexiform layer (IPL) (Figures 5d,6a,b). Their somata measured ca 10-15 μm across with the expected variation in size and shape depending on retinal

location. The density of immunoreactive amacrine cells showed a similar distribution pattern as serotonin-accumulating bipolar cells (Figure 7).

One class of very varicose, strongly stained fibers that could be followed radiating from the optic nerve head (Figure 8) were tentatively classified as centrifugal fibers. The serotonin immunoreactivity of these fibers was not affected by any of the drugs used which made them a welcome control in pharmacological experiments to evaluate the overall staining intensity of the tissue. This finding is consistent with the notion that these fibers originate in the brain and receive all their input there, whereas the axons, terminating in the retina are exclusively presynaptic, that is, they lack postsynaptic receptor specializations. These fibers could be followed several 100 μm in the nerve fiber layer before they ascended into more distal layers of the retina. After turning into the INL, these fibers made close contact to some serotonergic amacrine cell somata (Figure 9). Other serotonergic amacrine cell perikarya appeared not to be contacted but the lack of observation does not exclude that a higher than noticed number of amacrines were "in touch" with the putative centrifugal fibers. In some instances, the fibers could be followed meandering through the distal portions of the INL up to the level of the OPL where they seemed to end without further branching.

Serotonin in the Photoreceptor Layer:

The outer nuclear layer in the photoreceptor layer of the skate often showed non-specific background staining. Serotonin-immunostaining or serotonin uptake by photoreceptors has been reported (Redburn & Mitchell, 1989) but this was not followed in this study.

B. Protein Kinase Cilmmunocytochemistry:

PKC-immunocytochemistry revealed one class of bipolar cell as the only cellular elements stained by the specific antibody employed, no other cells or fibers exhibited immunostaining (Figure 10). PKC-immunoreactive bipolar cells have a distribution pattern similar to that of serotonin-accumulating bipolar cells. However, based on double staining experiments (Figure 11), their number was approximately twice as great, independent of the retinal location. Like the serotonin-accumulating bipolar cells, PKC-immunoreactive bipolar cells showed considerable variability in size and spatial arrangement as a function of retinotopic location. The somata of PKC-immunoreactive bipolar cells were located distal to those of serotonin-accumulating bipolar cells (Figure 11). In central retinal areas, they were spindle-shaped with one very prominent dendrite which did not ramify before it reached the OPL. In cross sections, individual dendritic branches were difficult to discern, rather,

they appeared to fuse into a thin feathery band of fluorescence (Figure 10). The axons of PKC-immunoreactive bipolar cells were much thinner than the primary dendrites. Like serotonin-accumulating bipolar cell axons, they crossed the IPL in an oblique manner, depending on their retinal location. The PKC-immunoreactive bipolar cells displayed a typical club-shaped terminal in stratum 5 of the IPL (Figure 10), similar to that of mammalian rod bipolar cells (Negishi *et al.* 1988; Wässle & Boycott, 1991). The club-like endings usually consisted of 3 to 4 bulbous terminals closely associated with each other.

In retinal wholemounts, the spatial geometry of the PKC-immunoreactive bipolar cells and the relation of their dendrites to one another could be observed in greater detail (Figure 12a). Secondary dendrites in the OPL were of extremely fine caliber. Still it was possible to observe that, like the dendrites of serotonin-accumulating bipolar cells, they reached at least to the center of the neighboring PKC immunoreactive bipolar cell and thus overlapped to at least 300% (Figure 13) (Wässle & Riemann, 1978; Schütte & Weiler, 1987; Young & Vaney, 1991; see also Jeon & Masland, 1995, Fig. 8a). Figure 12b shows the same retinal spot, focused on the somata. In retinal wholemounts, also the arrangement of the terminals could be studied in greater detail (Figure 12c). In addition to the bulbous terminals, fine varicose processes which remained at the vitread border of

the IPL were stained, similar to those described in the goldfish (Ramón y Cajal, 1892; Suzuki & Kaneko, 1990). The intensity of PKC immunoreactivity was strongest in somata and terminals, leaving the nuclei of the cells as markedly less stained structures. In no case did any bipolar cell display both serotonin and PKC-like immunoreactivity (Figure 11).

C. Serotonin Immunocytopharmacology:

Incubation time and pargyline

During the process of determining a favorable incubation time for serotonin uptake, I observed that in control retinas incubated without the addition of serotonin or pargyline to the Ringer, bipolar cells and amacrine cell processes often showed increased serotonin-like immunoreactivity after ca 35 to 40 minutes. For example, incubation in Ringer alone for one hour at room temperature (n=5) resulted in heavily labeled bipolar and amacrine cells (Figure 14). Almost identical results were obtained by incubating the tissue in Ringer containing only pargyline (n-=2) for 1-2 hours (Figure 15). I noted, however, that these results were sometimes variable. In addition, I was concerned that the viability of the tissue might have been compromised by a prolonged incubation time which could interfere with subsequent

pharmacological experiments. On the other hand, consistently strong and evenly distributed labeling in amacrine and bipolar cells was obtained after 20 minutes of incubation of the eyecup in Ringer's solutions containing 1 mM pargyline and 10 μM serotonin. I used this procedure to establish uniform baseline labeling and will refer to such pretreated eyecups as "preloaded".

Untreated retina

Control retinas which were not treated with any drugs or preloaded with serotonin in the presence of pargyline have been described above. Briefly, for a comparative purpose, they showed strongly stained large soma amacrine cells (Figures 5d, 6a,b,9,16), the processes of which generated a dense network of stained fibers in the distal inner plexiform layer (IPL).

Serotonin uptake

Preloading of the eyecup with 5μM serotonin and inhibition of its oxidative degradation by monoamine oxidase by pargyline changed the retinal distribution of 5-HT in that now the amacrine cells and the bipolar cells displayed strong labeling. The morphology and distribution of these serotonin-accumulating bipolar cells have also been described in detail above. Briefly, their somata are oval in shape, measure 5-7μm across and

usually occupy the third or fourth tier of somata from the outer plexiform layer (OPL), slightly distal to the midline of the inner nuclear layer (INL). Preloading resulted in uniform labeling throughout the entire cell including the dendritic ramifications in the OPL and part of the distal INL (Figure 5). Immunoreactivity in presumptive centrifugal fibers appeared not to be affected by the preloading, however, since the labeling was always at saturating levels, a minor increase might have gone unnoticed.

Release studies:

A number of earlier studies had shown that it is possible, in some species, to induce depletion of retinal cells of serotonin by exposure of the retina to glutamate receptor agonists or by non-specific depolarization of the retina with high K^+ (Osborne and Patel, 1984; Weiler and Schütte, 1985a,b; Schütte and Witkovsky, 1990; Yaqub and Eldred, 1993). To investigate whether similar results could be obtained in the skate retina, I investigated the effects of several excitatory amino acids on the retinal distribution of serotonin.

1) NMDA

Incubation of preloaded eyecups in Ringer containing 5mM NMDA (n=4) caused loss of immunostaining in the IPL. The primary amacrine cell processes appeared to be depleted of serotonin, that is, they were very thin and often immunostaining stopped abruptly a few μm away from the soma (Figure 17, curved arrow). Labeling in serotonin-accumulating bipolar cells seemed to be unaffected by NMDA treatment (Figure 17, open arrow).

2) Aspartate

Aspartate, when applied in the same dose as NMDA (5mM; n=3), resulted in an appearance of the retinas similar to those treated with NMDA, with pronounced loss of immunostaining in the IPL and loss of staining in primary processes of serotonergic amacrine cells (Figure 18). In addition, a qualitative difference regarding the distribution of serotonin immunoreactivity in amacrine cells was obvious. The somata often showed large areas in their distal pole which were completely devoid of staining, giving the cells a vacuolized or swollen appearance (Figure 18, large arrows). Staining in bipolar cells appeared to be largely unchanged, in some cases their somata were stained more strongly than the axons (Figures 19, 20).

3) high K^+

Non-specific depolarization of the entire retina by elevated potassium caused an almost complete loss of serotonin-like immunoreactivity in the IPL except for a few fine processes of amacrine cells (Figure 21a). Bipolar cell axons appeared very thin (Figure 21b). In contrast, their somata showed enhanced serotonin-staining, comparable now to that of amacrine cell bodies (Figure 21b). The appearance of centrifugal fibers was unchanged.

4) Kainic acid

I could not observe any changes in retinas that were incubated in Ringer's solution with addition of kainic acid in comparison to control retinas. Bipolar cells did not lose or gain any significant amount of serotonin immunoreactivity and the stained amacrine cells showed the same intensity of staining in both somata and processes in experimental and control retinas.

5) Quisqualate

In order to assure that the skate serotonin-accumulating (presumed OFF) bipolar cell does not differ from OFF bipolar cells found in other species by

expressing a variant of the post synaptic receptor in the OPL with decreased sensitivity to kainate, retinas were incubated with the selective AMPA receptor agonist quisqualate. In quisqualate treated retinas, however, serotonin immunoreactivity was affected as little as in the kainate treated retinas, that is, no noticeable difference between treated retinas and the control tissue could be established. Thus, the idea that a glutamate receptor mutation may account for the lack of kainate effects appears unlikely.

Uptake blocker

Surprisingly, the only treatment possibly inducing depletion of bipolar cells of serotonin, and this only at their axon terminals was high K^+, while none of the glutamate receptor agonists showed any visible effects. This finding strongly suggests that serotonin may not be released by bipolar cells of the skate retina, or if release occurs, it may be compensated for by a powerful re-uptake mechanism. This possibility and the fact that uptake of serotonin by these cells occurs even without its exogenous (without preincubation in Ringer with added serotonin) addition suggested an interesting phenomenon which I decided to investigate further.

1) Zimelidine

The selective serotonin uptake blocker and antidepressant (Cox et al., 1978) zimelidine was applied to previously untreated retinas simultaneously with 10μM serotonin and 1mM pargyline to evaluate its effect on serotonin uptake by bipolar cells. Five hundred μM zimelidine completely inhibited uptake of serotonin by bipolar cells as inferred from their complete lack of staining (Figure 22). Amacrine cells were not affected by zimelidine and displayed their normal staining pattern with their processes occupying the outer half of the IPL (Figure 22). Figure 23 shows the control retina of the same animal which was treated identically, only that zimelidine was omitted from the bathing solution.

2) Fluoxetine

Fluoxetine (prozac®), a blocker of the high affinity serotonin transporter with a similar selectivity ratio (serotonin:norepinephrine:dopamine) as zimelidine (Wong *et al.*, 1995) did not inhibit serotonin uptake by bipolar cells (Figures 24a,b). On the other hand, fluoxetine caused, in some experiments (3/7), a decrease of serotonin immunoreactivity in amacrine cells. In some cases (2/3), the decrease of serotonin labeling in amacrines was concomitant with

an enhancement of labeling in bipolar cells. When the retinas were incubated in high K^+ Ringer containing 25 μM fluoxetine, bipolar cells stained more strongly than control retinas in normal Ringer with fluoxetine. High K^+/fluoxetine treatment reduced 5-HT-labelling in the IPL to a narrow band in sublayer 1(not shown).

4. Discussion

A. Bipolar Cells of the skate retina:

My data demonstrate that there are at least two different subpopulations of bipolar cell in the skate retina. One, the serotonin-accumulating bipolar cell, displays morphological features similar to those of serotonin-accumulating bipolar cells in other species (Osborne, 1984). The other type, which shows PKC immunoreactivity, conforms to the morphology of the rod bipolar cell described by Ramón y Cajal (1892) and Wässle *et al.* (1991).

In the carp retina, both ON- and OFF-bipolar cells receive signals from both rods and cones (Kaneko & Tachibana, 1978; Kaneko *et al.* 1979; Saito & Kujiraoka, 1982) and may be designated further as rod-dominant or cone-dominant types, depending on the weighting of rod and cone input they receive. Similarly, in the goldfish retina two different types of rod-dominated

bipolar cells have been described on the basis of serial, semi-thin sections of Golgi-impregnated bipolar cells (Ishida *et al.* 1980) and glutamate immunoreactivity (Marc *et al.* 1990). The rod-dominant cells are distinguished by one major feature, a thick lobular axon terminal in either sublamina a or b of the IPL. The finding of these typical rod bipolar terminals in both sublamina a and b indicates that the goldfish retina contains both ON- and OFF rod dominated bipolar cells (Sherry & Yazulla, 1993). Similar findings were obtained in the larval tiger salamander retina (Hensley *et al.*, 1993; Yang & Wu, 1993). Of the two classes of rod dominated bipolar cell only the presumed ON-type which has its terminal in sublamina b has been found to be strongly immunoreactive to an antibody against the α and β-subspecies of PKC (Suzuki & Kaneko, 1990).

In mammals, a morphologically similar type of bipolar cell is stained by PKC immunoreactivity. These cells, the mammalian rod bipolar cells, have been shown to receive all their input from rods (Wässle *et al.* 1991; Wässle & Boycott, 1991). The rod photoreceptor to rod bipolar cell system in mammals is much more restricted in its circuitry in that rod bipolar cells do not contact cones and cone bipolar cells receive no input from rods (Ramón y Cajal, 1892; Daw *et al.* 1990; Wässle & Boycott, 1991). Rod bipolar cells in mammalian retinas also appear to constitute a morphologically homogeneous class of cell, as discussed by Dolan & Schiller (1994), the physiological

properties of which were a matter of debate for some time. Karschin and Wässle (1990) originally described the isolated rat rod bipolar cell as being depolarized by glutamate and therefore belonging to the OFF-channel which seemed to fit the finding of hyperpolarizing light-responses of cat rod bipolar cells (Kolb & Nelson, 1983; Nelson & Kolb, 1983). Later, however, Yamashita and Wässle (1991) found, using the whole-cell Nystatin patch recording technique (Horn & Marty, 1988), that only channel closures were observed as a response to application of the glutamate agonist 2-amino-4-phosphonobutyrate (APB), resulting in hyperpolarization of the cells. Therefore, the rat rod bipolar cells must be considered to belong to the ON-class. The same authors also suggested that the ruptured whole-cell patch clamp technique may have been responsible for the earlier characterization of these bipolar cells as OFF-cells (Karschin & Wässle, 1990) since it may cause washout of intracellular second messenger systems into the patch electrode, which affects the response. This suggestion is supported by evidence provided by Nawy and Jahr (1990, 1991) that the postsynaptic receptor of ON-bipolar cells in tiger salamander retina is linked via a G protein to a phosphodiesterase and that the addition of cGMP into the patch pipette resulted in an enhancement of the light response. Therefore, a washout of cGMP from the cytosol into the patch pipette could inhibit the cell's response. The results of Yamashita and Wässle were confirmed by de

laVilla *et al.* (1995) in isolated rod bipolar cells of the cat retina. The morphologies of cat and rat rod bipolar cells are almost identical (Chun *et al.*, 1993), as are their responses to APB (Yamashita & Wässle, 1991; de la Villa *et al.* 1995). Therefore, all mammalian rod bipolar cells so far investigated may be considered to constitute a highly conserved class of cell (as suggested by Dolan & Schiller, 1994) with identical synaptology independent of the species studied (Daw *et al.*, 1990; Vaney *et al.* 1991; Wässle & Boycott, 1991; Kolb, 1994).

The finding of a class of bipolar cell which shows both of the structural features of "rod bipolar cells" (strong PKC immunoreactivity, thick lobular terminals at the inner margin of the IPL) does not seem at all surprising in an all-rod retina such as the skate's. However, visual function has been shown to depend on the presence of both ON- and OFF-pathways and ON-bipolar cells primarily transmit information about the increment of brightness above background. Indeed, when the ON-channel was blocked by APB, the detection of stimuli lighter than background (incremental) was severely impaired in the monkey whereas the detection of stimuli darker than background (decremental) was largely unaffected (Schiller *et al.* 1986; Dolan & Schiller, 1994). One can infer that the detection of decrements in brightness below background depends on neurons which convert darkness into an excitatory signal: the OFF-channel which, even in scotopic vision,

may act independently of the rod bipolar cell pathway (de Vries & Baylor, 1995).

Skates have highly specialized eyes with an elaborate pupil mechanism (Dowling & Ripps, 1976) and even though the retinas of *Raja erinacea* contain only rods (Szamier & Ripps, 1983) there is no indication that they have abandoned the retinal OFF-channel. According to Famiglietti *et al.* (1977), physiologic properties of inner retinal neurons are reflected in their ramification pattern in the inner plexiform layer (IPL), that is, cells that respond to a stimulus of light in their receptive field center with a depolarization (ON-center cells) branch in the inner portion of the IPL, whereas cells that are hyperpolarized by a stimulus of light falling into their respective field center (OFF-center cells) ramify only in the outer half of the IPL. This is also true for bipolar cells, if one allows for the exception that the only valid discriminating feature is the absence or presence of axonal ramifications in the outer half of the IPL (Weiler, 1981; Weiler & Schütte, 1985a; Stone & Schütte, 1991; Ammermüller & Kolb, 1995; Schütte, 1995) suggesting that OFF-center bipolar cells may have additional ramifications in the inner strata of the IPL. Thus, one can identify bipolar cells stained by a given method as either ON or OFF bipolar cells. The only exception to this rule known so far are two distinct classes of color opponent displaced bipolar cells recently shown in turtle by Ammermüller *et al.* (1995) which are

characterized by the location of their somata in the outer nuclear layer (ONL). But even those cells which do not show definite ON or OFF responses fit the general scheme in that they ramify in sublamina a and have pharmacological properties of OFF-center bipolar cells (Schütte, 1995). One has to keep in mind, however, that the above data are generally concerned with cone or mixed rod-cone bipolar cells, even though the mammalian rod bipolar cell fits into this model.

The second class of rod bipolar cell in the skate retina is characterized by the ability to accumulate serotonin from the incubating medium, as are some bipolar cells in goldfish (Marc, 1982; Marc *et al.* 1988), turtle (Weiler & Schütte, 1985 a, b), *Xenopus* (Schütte & Witkovsky, 1990), frog (Zhu *et al.* 1992), chicken and pigeon (Floren, 1979) or to accumulate it from endogenous retinal sources such as serotonin released from serotonergic amacrine cells (Negishi & Teranishi, 1990; Schütte, 1994). Interestingly, it was in studies of the skate retina that serotonin was observed for the first time in bipolar cells (Bruun *et al.* 1984) although Brunken *et al.* (1986) were unable to confirm this observation because of very weak staining in their preparations. Bruun and colleagues observed that axon terminals of serotonin-accumulating bipolar cells dipped into the inner strata of the IPL, and tentatively classified the cells as ON-bipolar cells. Given the abundance of serotonin-like immunoreactive amacrine cell processes throughout the

entire depth of the IPL, the extremely delicate ramifications of serotonin-accumulating bipolar cell axons in the distal IPL may have been difficult to resolve in the original investigation. It is interesting to note here that the particular class of bipolar cell which accumulates serotonin in the goldfish retina has been identified as the a2 bipolar cell (Marc *et al.* 1988) which receives mixed rod-cone input and is thought to hyperpolarize upon light-stimulation (Ishida *et al.* 1980). Serotonin accumulating bipolar cells in the retinas of turtle (Weiler & Schütte, 1985b) and *Xenopus* (Schütte & Witkovsky, 1990) have been classified as OFF bipolar cells on the basis of serotonin release upon exposure to kainic acid and show morphological features similar to those of serotonin-accumulating bipolar cells in the skate retina.

In the skate retina, Dowling (1974) described two types of synaptic junctions to bipolar cells in the photoreceptor terminal. One was the typical invaginating ribbon synapse and the other appeared to be analogous to the basal junction made by cone terminals. Dowling (1974) tentatively classified those two contacts as belonging to ON and OFF bipolar cells. Recently, Chappell and Rosenstein (1996) have provided electrophysiological evidence for functional ON and OFF bipolar cell pathways in the skate retina by observing selective block of picrotoxin-enhanced ON and OFF components of the skate electroretinogram by APB and kynurenic acid.

respectively. In accordance with the above findings and on the grounds of the distribution of the axonal ramifications, I interpret the serotonin-accumulating bipolar cell as an OFF bipolar cell and the bipolar cell which labels with an antiserum directed against PKC as an ON bipolar cell of the skate retina.

B. Immunocytopharmacology:

My data show that in the skate retina, serotonin-accumulating amacrine and serotonin-accumulating bipolar cells differ from each other with respect to their pharmacological properties in two ways. The first difference concerns the capability of releasing serotonin. In some other vertebrate retinas, serotonin can be released by bipolar cells when they are depolarized by excitatory amino acids (EAAs) (*Xenopus*: Schütte &Witkovsky, 1990; turtle Weiler & Schütte, 1985b; frog: Zhu *et al.*, 1992; pigeon and chicken: Floren, 1979) and also by elevated K^+ in the Ringer (Osborne and Patel, 1984; Weiler & Schütte 1985b). Serotonin release by bipolar cells is Ca^{2+} dependent (Weiler & Schütte 1985b, Schütte & Witkovsky 1990). In the present study, I have found no evidence for release of serotonin by bipolar cells upon exposure to EAAs, suggesting that serotonin may play a different

role in skate bipolar cells. Skate retinal serotonergic amacrine cells, however, were highly sensitive to NMDA and aspartate which indicates that it may function there as a neurotransmitter. This is in contrast to results obtained by Yaqub and Eldred in turtle (1993) who report that NMDA did not affect serotonergic amacrine cells but caused its accumulation by a tristratified amacrine cell (tentatively identified as the dopaminergic amacrine cell). This difference may be due to different protocols used where drug exposure time and drug concentration become important issues. Our findings, however are consistent with the results of Yaqub and Eldred (1993) regarding aspartate which also reduced immunostaining in amacrine cells in their preparation.

Vanahatalo and Soinila (1994) examined serotonin-containing fibers in the pituitary intermediate lobe that colocalize with tyrosine hydroxylase. On the basis of a series of uptake experiments and pharmacological manipulation of the tissue with a variety of uptake blockers, they suggested that those fibers take up serotonin through a dopamine transporter and do not synthesize serotonin. This idea is supported by the finding that in the CNS serotonin may be taken up by dopaminergic terminals after increasing 5-HT concentration in the synaptic cleft through uptake inhibitors (Van Praag & Ansis, 1990). Since there are no skate bipolar cells that stain for tyrosine hydroxylase (unpublished results, see also Brunken, 1983) and uptake of serotonin into these cells can clearly be blocked by Zimelidine I suggest that

the function of serotonin in these cells does not comply with the general notion of that of a "false transmitter" (a neuroactive substance, released by cells through exocytosis or by transporters, that is not synthesized in these cells, **not** a drug that has sufficient similarity to a natural transmitter to be packaged in vesicles and released as mentioned by Schwartz, 1991) as described by Vanahatalo and Soinila (1994).

High K^+ did not deplete bipolar cell somata of serotonin immunoreactivity. On the contrary, it enhanced labeling in the distal portions of these cells in that both somata and dendrites stained even more strongly after high K^+ treatment than after exposure to exogenous serotonin. The axons of these bipolar cells, however, displayed a clear loss of staining. Amacrine cells seemed to be sensitive to high K^+ because their processes in the IPL were almost completely depleted of serotonin after non-specific depolarization caused by high K^+. The finding that primarily the processes were affected by high K^+, while the somata retained immunolabeling may be explained by the fact that presynaptic sites involved in release are usually located on the terminals rather than on the soma of a neuron. Also, the larger surface to volume ratio on processes may play a role. In this regard, the functional asymmetry of bipolar cells which are predominantly postsynaptic in the outer retina and which have their ribbon synapses confined to axon terminals in the IPL may explain the "selective" loss of immunoreactivity in

their axons. Immunocytochemical studies of glutamate transporters in the retina have shown that there is no asymmetry regarding the distribution of these molecules, that is, transporters are evenly distributed over the entire surface of the bipolar cell plasma membrane (Grünert *et al.*, 1994; Rauen & Kanner, 1994). Even though no immunocytochemical studies on the subcellular distribution of serotonin transporters in the retina have been made available, it is conceivable that their distribution follows a similar layout. Consequently, uptake should occur at all parts of a cell while release only occurs predominantly at the presynaptic site. This may explain why, after depolarization of all retinal neurons by high K^+ which results in release of serotonin into the extracellular retinal space (which may reach a much higher intraretinal extracellular concentration than during uptake of external serotonin), the distal portions of serotonin-accumulating bipolar cells show enhanced labeling.

The second difference between amacrines and bipolar cells concerns the differential effects of two distinct blockers of the serotonin transporter. The fact that zimelidine selectively affected immunolabeling in bipolar cells whereas fluoxetine seemed to decrease the serotonin concentration in amacrine cells indicates the presence of different transporter molecules in their plasma membranes. This observation is in accordance with results reported by Witkovsky *et al* (1987) who found that zimelidine abated the

neurotoxic effect of 5,6 dihydroxytryptamine in bipolar cells of the turtle but not degenerative changes caused by 6-hydroxydopamine in amacrine cells. The increased serotonin immunoreactivity that I sometimes observed in bipolar cells after exposure of the retina to fluoxetine or fluoxetine in high K^+ Ringer suggests that serotonin which was released by amacrines could not be taken back up by these cells and was then accumulated by bipolars. This model of action is consistent with findings by Negishi and Teranishi (1990) who suggested transfer of exogenously applied serotonin analogs between the different classes of retinal serotonin-accumulating cells. The same mechanism of action seems to exist in the *Xenopus* retina (Schütte & Witkovsky, 1990, Schütte, 1994) where a transfer of endogenous serotonin (synthesized within the cell) from amacrines to bipolar cells ("borrowed transmitter", a term used to describe a transmitter that is being released and taken by a neuron but not synthesized in this cell) has been reported. It is noteworthy that Schütte and Witkovsky (1990) suggested that *Xenopus* bipolar cells possess a lower affinity uptake transporter for serotonin than the serotonergic amacrine cells. Zhu *et al.* (1992) reported, however, that incubation of the isolated neural retina (*Xenopus*) in amphibian culture medium including 0.5µM fluoxetine (in ambient light) markedly reduced serotonin immunoreactivity in bipolar cells compared to untreated retinas incubated under the same conditions without the addition of fluoxetine. In the

skate, fluoxetine addition never reduced bipolar cell SLI to levels as low as in untreated retinas with the same incubation time.

In conclusion, my data show that the skate retinal serotonin system consists of at least two distinct components with markedly different pharmacological properties. A similar situation may exist in the CNS where serotonergic and masked serotonergic cells have been reported (Takeuchi, 1988). Therefore, blocking of one serotonin transporter may increase the activity of the other. Further examination of these phenomena could provide new and important insights in the differential effects of antidepressant, serotonin-related drugs. I also propose that the skate retina could be employed as a simple model to study the cellular actions of antidepressants.

5. Tables:

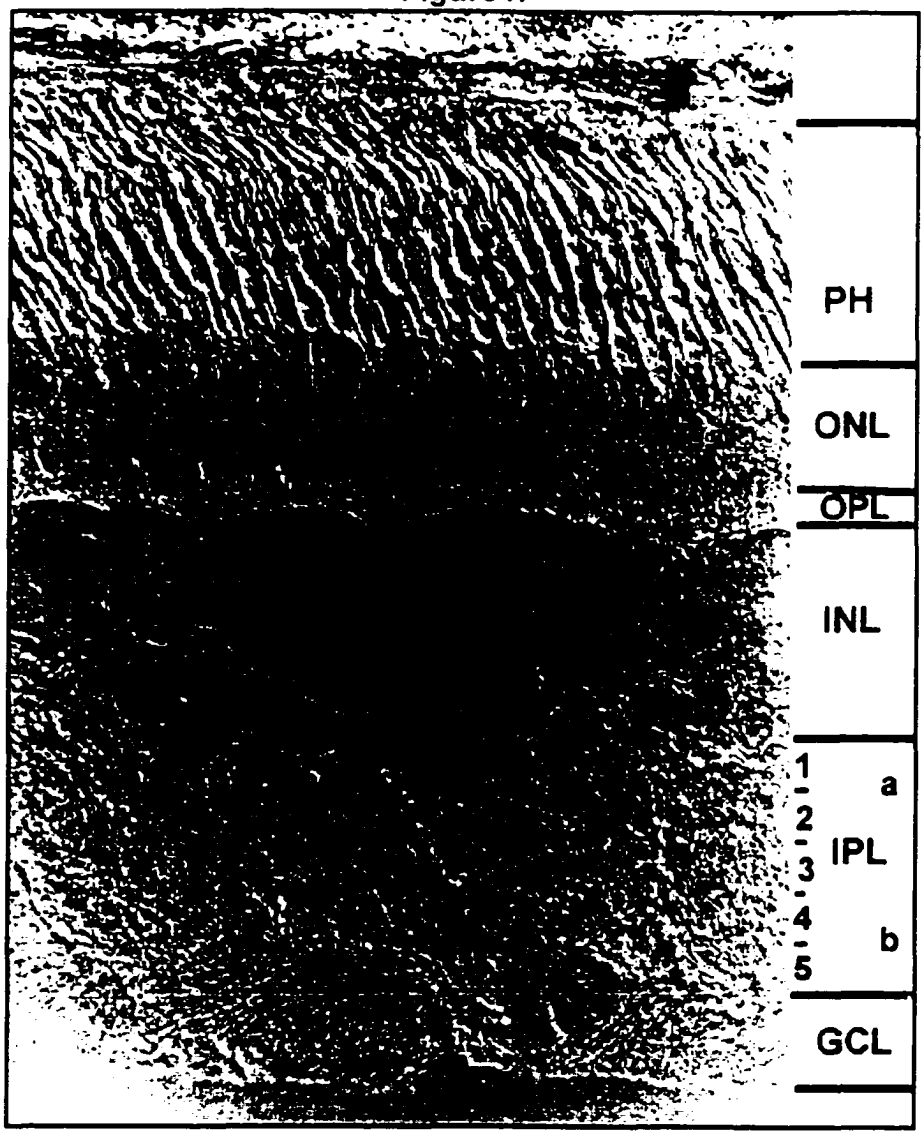
A. Composition of buffers, fixatives and slide coating solutions used

Solution	chemical composition	special
phosphate buffer (PB) yields 525ml, pH 7.87 0.2 molar	Na_2HPO_4 dibasic, 14.2g/500ml NaH_2PO_4 monobasic, 0.6g/25ml	keeps well in fridge, needs to be stirred a while before use because of crystallization in the cold
phosphate buffered saline (PBS)	7.75g NaCl/900ml H_2O + 100 ml PB 0.1 molar, pH 7.87	
paraformaldehyde fixative	6g paraformaldehyde + 50 ml H_2O	heat to 64 °C, stir heavily
	25 drops or less 1N NaOH	add slowly until clear
	50 ml 0.2 M PB 3 g sucrose	add
modified Zamboni's fixative	6g paraformaldehyde +50 ml H_2O	heat to 64 °C, stir heavily
	25 drops or less 1N NaOH	add slowly until clear
	70 ml 0.2 M PB 4.2g sucrose	add
	20ml saturated picric acid solution (1.2%)	handle with care
gelatin coating for slides	2.5g gelatine 250 mg Chromalaun (chromium potassium sulfate) 1l H_2O	heat to 55-60 °C, dip slides briefly, once, in hot solution, let dry, store slides in fridge

B. Composition of Skate Ringer's Solution

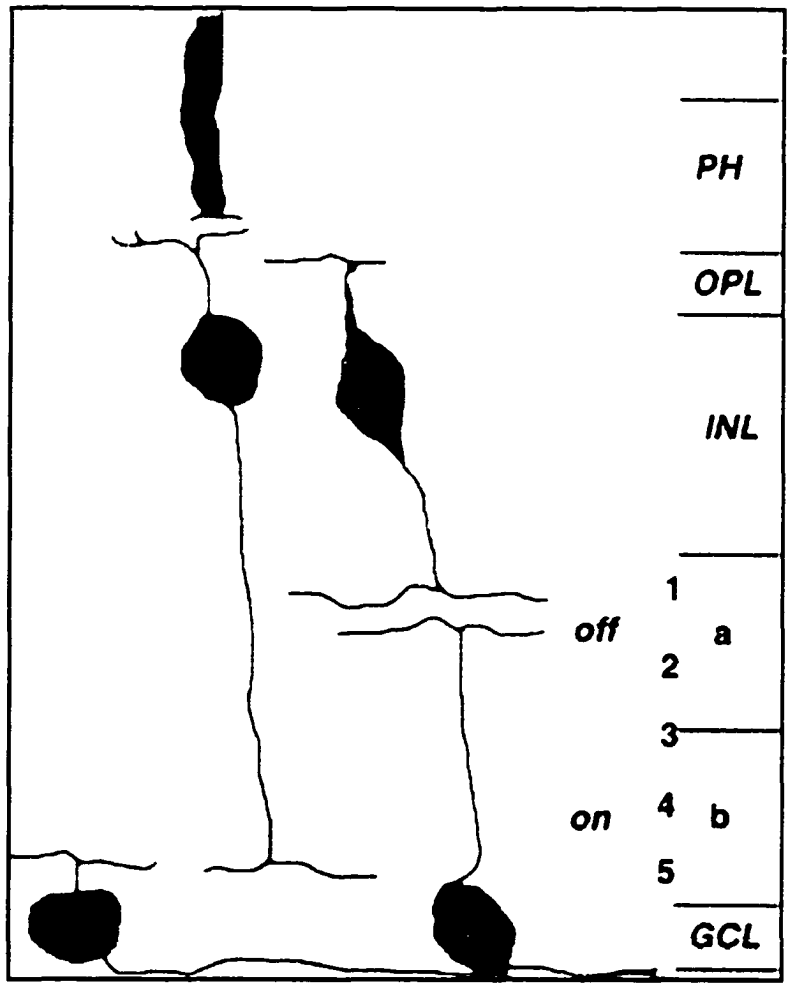
	normal/l H_2O	**high K/lH_2O**
NaCl	14.6g (250mM)	8.8g (150 mM)
KCl	0.46g (6mM)	7.45g (100mM)
$CaCl_2 2H_2O$	0.60g (4mM)	0.60g (4mM)
$MgCl_2 6H_2O$	0.20g (1mM)	0.20g (1mM)
NaH_2PO_4	0.024 (0.2mM)	0.024 (0.2mM)
$NaHCO_3$	1.7g (20mM)	1.7g (20mM)
Glucose (dextrose)	2.16g (12mM)	2.16g (12mM)
Urea	21.62g (360mM)	15.62g (260mM)
Hepes	1.2g (5mM)	1.2g (5mM)
Na-propionate $C_3H_5NaO_2$		9.61g (100mM)
NaOH 1N	ca 85 drops (towards pH 7.82)	ca 85 drops (towards pH 7.82)
note: to keep the amount of Na constant in the high K^+-Ringer 100 mM Sodium Proprionate was added (this is also a fungicide and mold-preventive), in order to keep the osmolarity I used 100mM less Urea in the high K^+-Ringer		

Figure1:



Differential interference contrast image of a ca 16 μm thick vertical section through the skate retina. Bars on the right hand side mark the approximate borders of the different layers. The three nuclear layers: ganglion cell layer (GCL), inner nuclear layer (INL) and outer nuclear layer (ONL) are divided by plexiform bands of the inner plexiform layer (IPL) and the outer plexiform layer (OPL) PH = photoreceptors, outer segments..

Figure 2:



This simplified drawing shows the different sublaminas (a and b) of the inner plexiform layer (IPL) and the arrangement of strata 1 to 5 in the IPL. One photoreceptor is shown in the photoreceptor layer (PH) and two interneurons, one terminating in sublamina a (the off-layer) and the second one terminating in sublamina b (on-layer). Two ganglion cells are shown that receive input in sublamina b or sublamina a. (GCL = ganglion cell layer, OPL = outer plexiform layer, INL = inner nuclear layer).

Figure 3:

Synthesis and metabolism of serotonin:

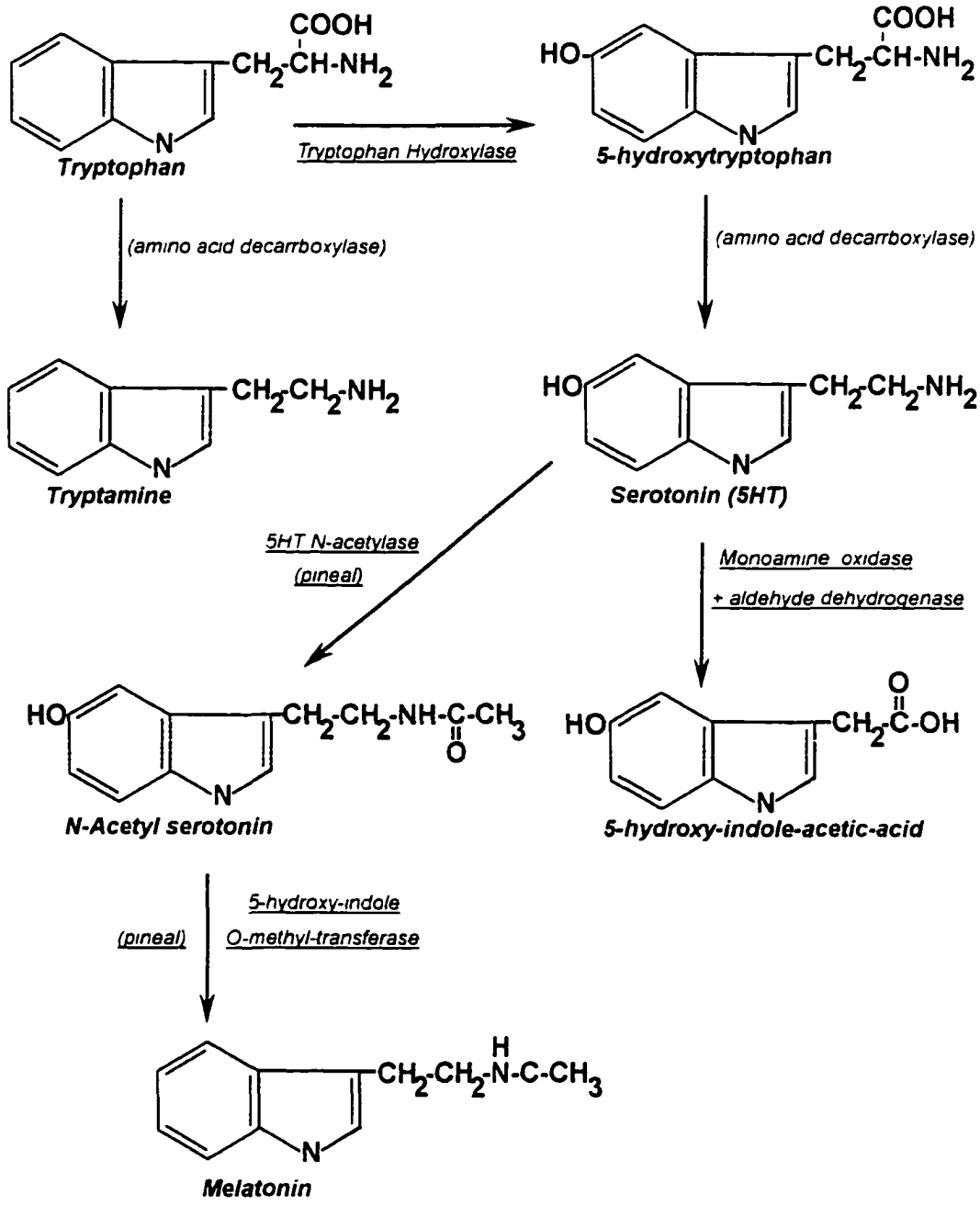
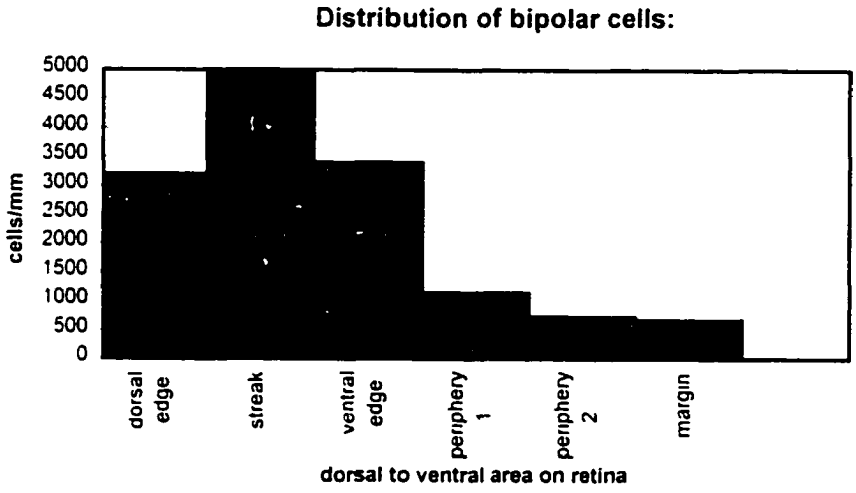


Figure 4:



The distribution of bipolar cells across the skate retina. Cells were counted in a vertical path from the dorsal edge (edge) of the wholemount to the ventral margin (margin) of the retina.

The piece of flattened retina divided into the above used areas

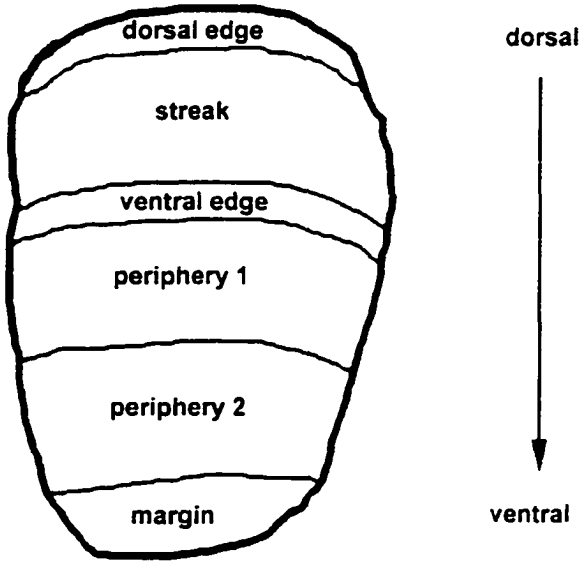


Figure 5:

Serotonin immunoreactivity in the skate retina after incubation of the eyecup in Ringer containing serotonin and pargyline.

a) Vertical section through the visual streak showing the general morphology and distribution of serotonin-accumulating bipolar cells. In the visual streak, bipolar cells usually give rise to a single dendrite (e.g. arrow) which does not ramify until it reaches the OPL. The axons (thin arrows) pass through the INL orthogonally without much deviation from the shortest course from the OPL to the IPL. Within the IPL labeling is heaviest in stratum 1 even though most of sublamina a contains some stained processes. Occasionally, thin fibers which are presumably of centrifugal origin arise from the ganglion cell layer (open arrow).

b) Serotonin-accumulating bipolar cells in the retinal periphery. These somata give rise to more than one dendrite (arow) which further ramifies shortly after emanating from the perikaryon. Axons deviate strongly from the orthogonal course and shift laterally by 50-70 μm before entering the IPL. In the IPL the bipolar cell axon terminals cannot be unambiguously identified because of the abundance of heavily labelled amacrine cell processes.

c) The fine processes and branching points (arrows) of serotonin-accumulating bipolar cell axons at the border of strata 1 and 2 and spanning strata 3 and 4 are visible in this section of an area in mid-periphery.

d) In whole mounted retinas numerous amacrine cells show strong immunofluorescence (large arrow) in somata and a network of tapering processes, branching into fine, varicose, long dendrites. The micrograph also shows the distribution of labelled bipolar cells which are slightly out of the focal plane (arrowhead).

e) Because of the dense network of labeled amacrine cell processes which add a hazy background to the whole mount staining, it is difficult to obtain a clear view of bipolar cell dendrites approaching the OPL. In many cases, however, it is possible to trace labeled bipolar cell dendrites which always end in the proximity of neighboring dendritic field centers marked by the position of the soma.

PH= photoreceptor layer, INL= inner nuclear layer, IPL= inner plexiform layer.

Figure 5:

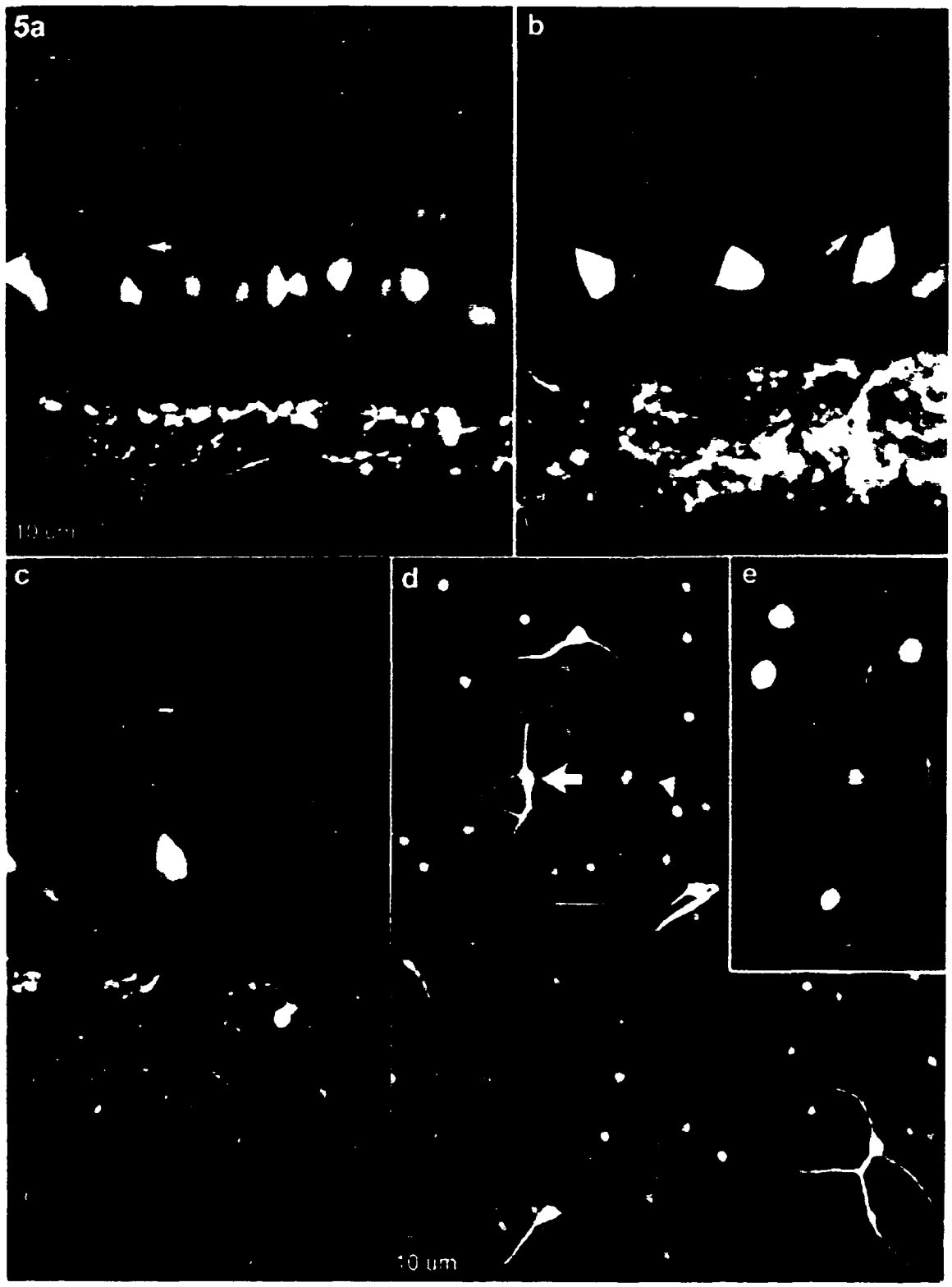
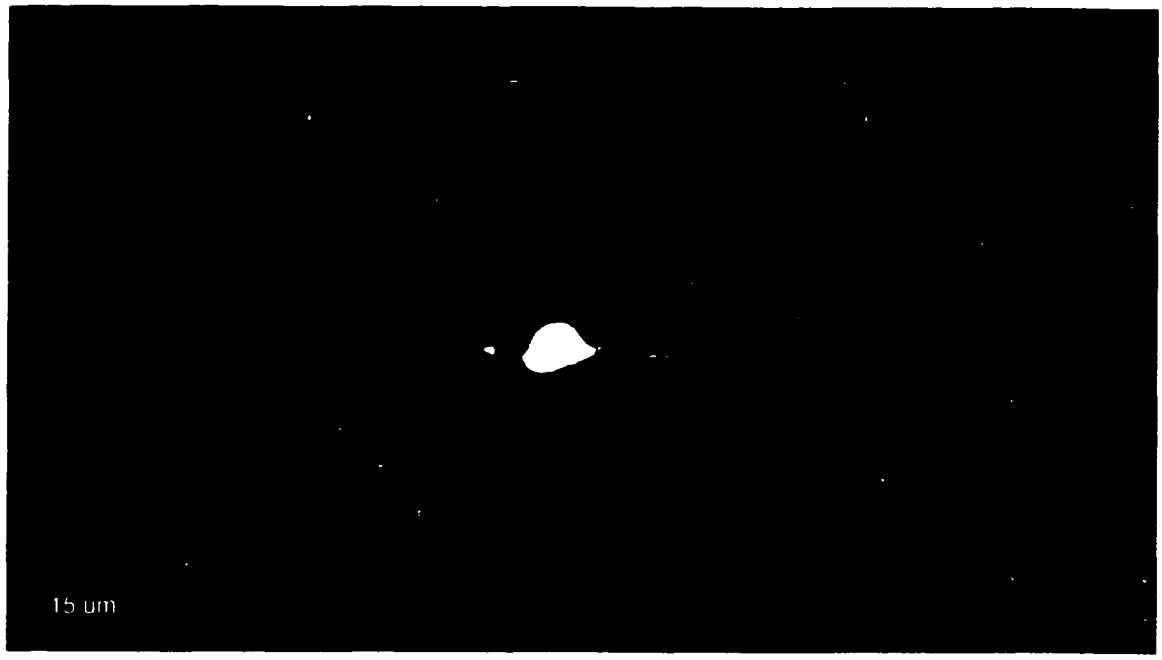
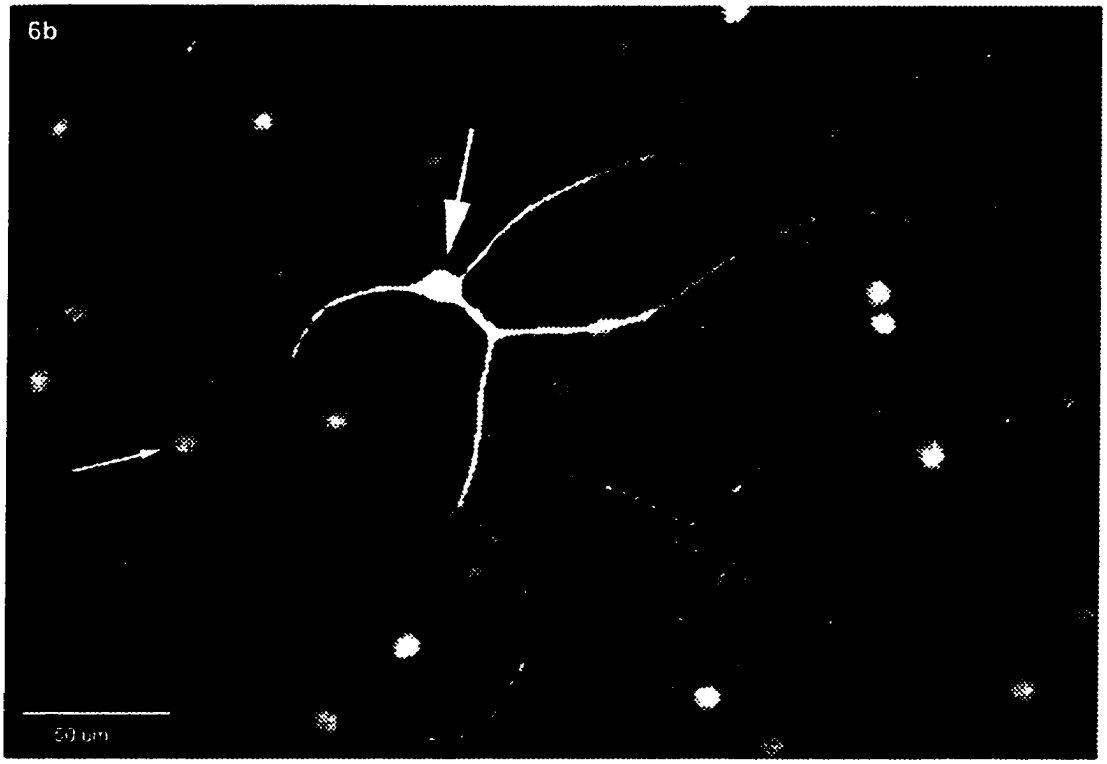


Figure 6a:



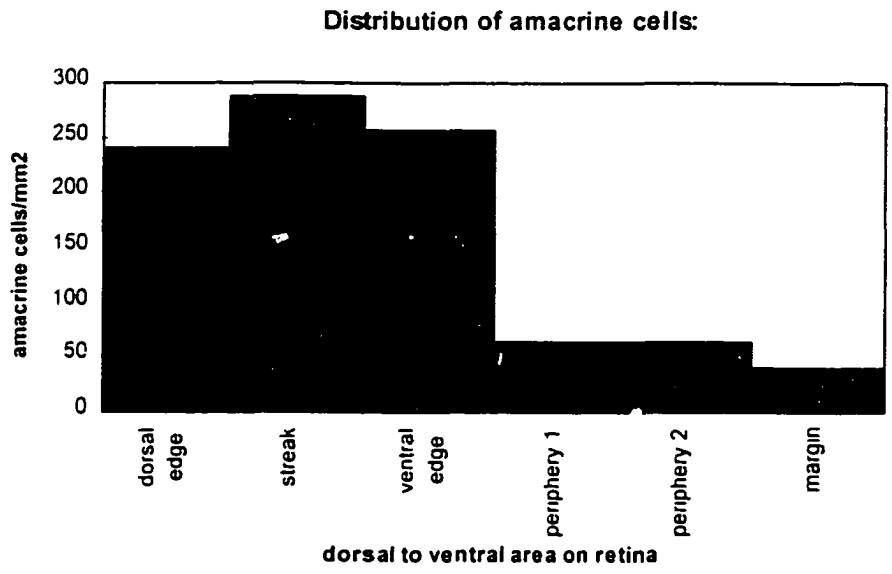
A ca. 20μm vertical section of the skate retina processed for serotonin immunocytochemistry. One amacrine cell from a somewhat peripheral area of the retina is shown. The two arrows indicate the approximate reach of the main amacrine cell processes in this plane.

Figure 6b:



A wholemounted skate retina processed for serotonin immunocytochemistry. The large arrow points to a serotonergic amacrine cell with its main thick processes that taper out long and thin to form a dense network in the inner plexiform layer. Stained bipolar cells (small arrow) are out of the focal plane.

Figure 7:

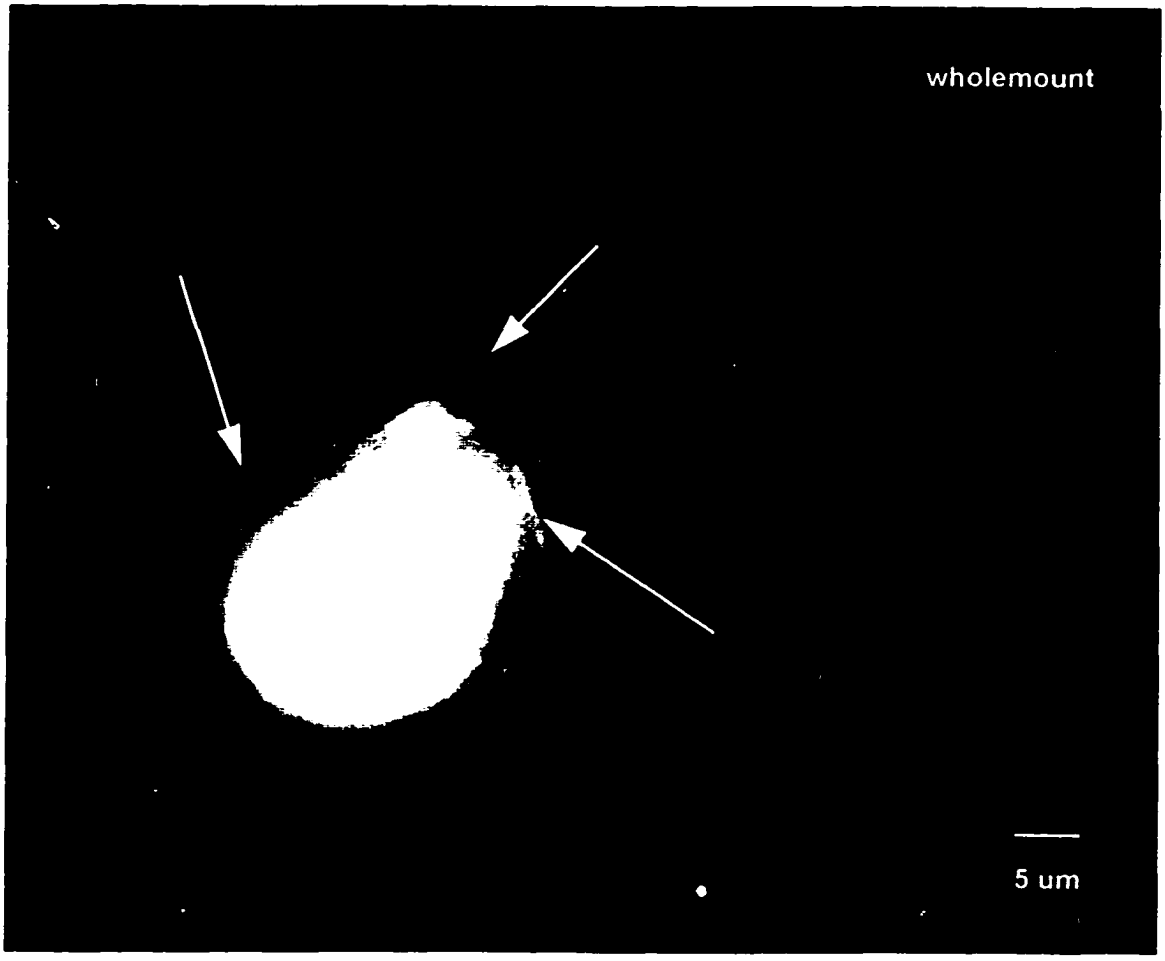


Distribution of serotonergic amacrine cells across the skate retina. The cells were counted in a whole mounted retina processed for serotonin immunocytochemistry along a vertical path starting at the dorsal edge of the retina down to the ventral margin (see figure 4).

Figure 8:

A whole piece of optic nerve (including the optic disk) was processed for serotonin immunocytochemistry. Numerous stained fibers were visible throughout the length of the nerve. These fibers, examples are indicated by the arrows, bear a very characteristic highly varicose appearance, distinguishing them morphologically from higher order branches of serotonergic amacrine cells. Staining of these fibers was always very strong and crisp and could not be influenced by pharmacologic treatment. In no case could any of these fibers be traced to a soma located in either retina or optic nerve.

Figure 9:



Retinal wholemount processed for serotonin immunocytochemistry. In the wholemount I could observe that presumptive centrifugal serotonergic fibers make close contact with serotonergic amacrine cell somata. The fibers loop around the somata often forming a branching point and then continue through the inner nuclear layer towards another serotonergic amacrine cell.

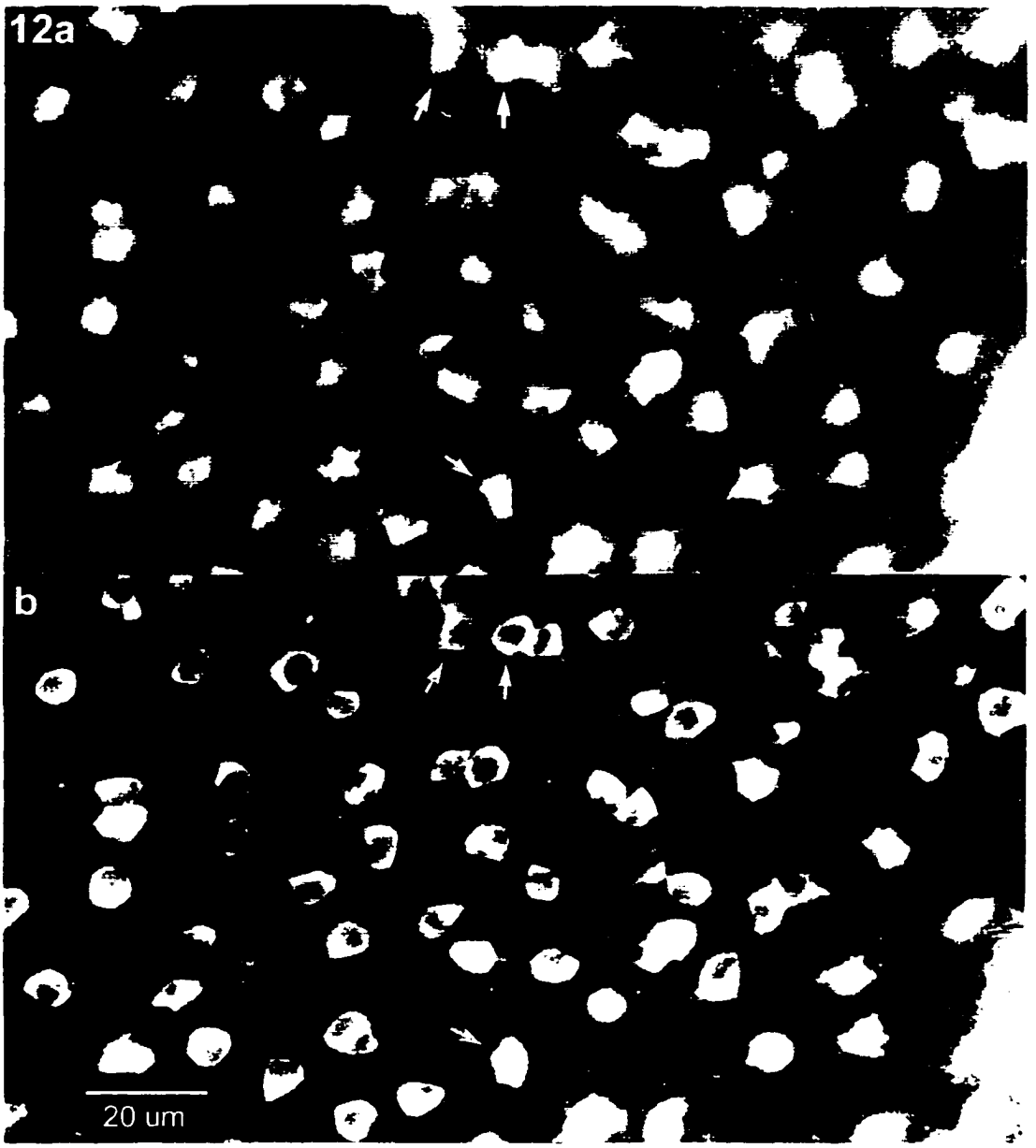
Figure 10:

Protein kinase C immunoreactivity in the skate retina reveals a homogenous class of bipolar cell with the typical appearance of rod bipolar cells. The axons end in large terminals at the proximal border of the inner proximal layer (IPL), a level of termination in the IPL that is characteristic of the ON-pathway. The dendrites of these bipolar cells form a fine feathery band at the distal edge of the inner nuclear layer. The fine, bushy appearance of these bipolar cell dendrites conforms well to the morphology of the mammalian rod bipolar cell.

Figure 11:

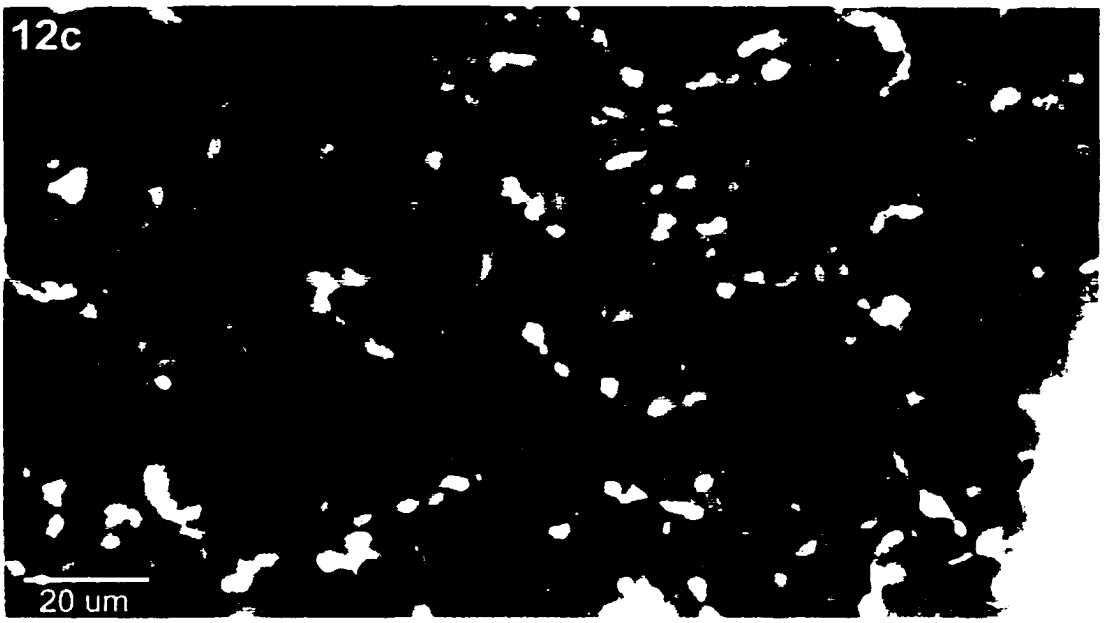
Double immunostaining for serotonin (green) and PKC (red). Colors were assigned in Adobe Photoshop before merging two exposures of the same retinal section taken with different excitation/emission filters. Note that there are roughly twice as many PKC-immunoreactive bipolar cells as serotonin-immunoreactive bipolar cells. The asterisk labels part of a serotonergic amacrine cell and the arrow points to a PKC-labeled terminal that clearly lies more proximal in the inner plexiform layer (IPL) than all serotonin-stained processes. INL=inner nuclear layer, PHL=photoreceptor layer.

Figure 12:



Protein kinase C immunoreactive bipolar cells in a wholemount of skate retina, imaged in two different focal planes. A fluorescent spot caused by a crease in the retina (due to distortion during the mounting procedure) serves as a landmark in the lower right of all photographs. Note: legend continues on next page. figure 12c!

Figure 12c:

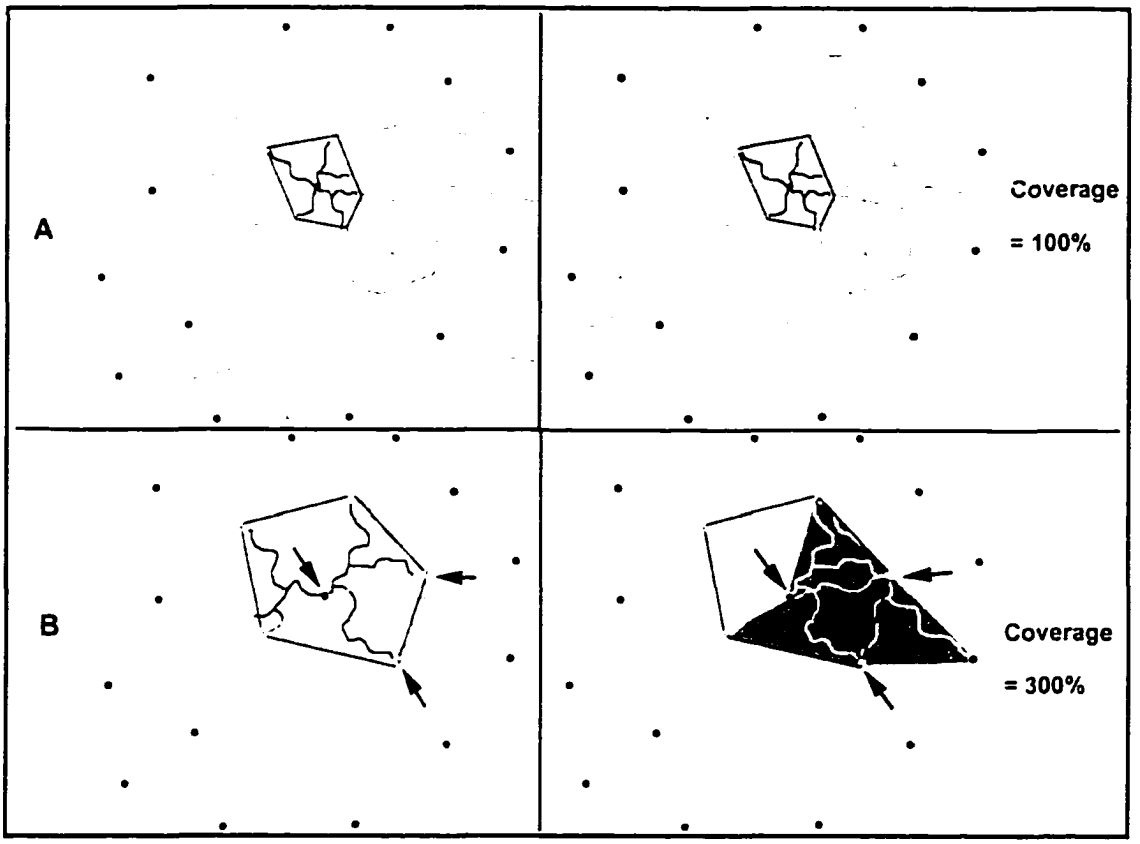


a) the focus is on the dendrites of PKC-immunoreactive bipolar cells showing their fine lateral processes. The perikarya are out of the focal plane.

b) Focus on the somata of the same bipolar cells. Arrows in a and b denote corresponding cell bodies.

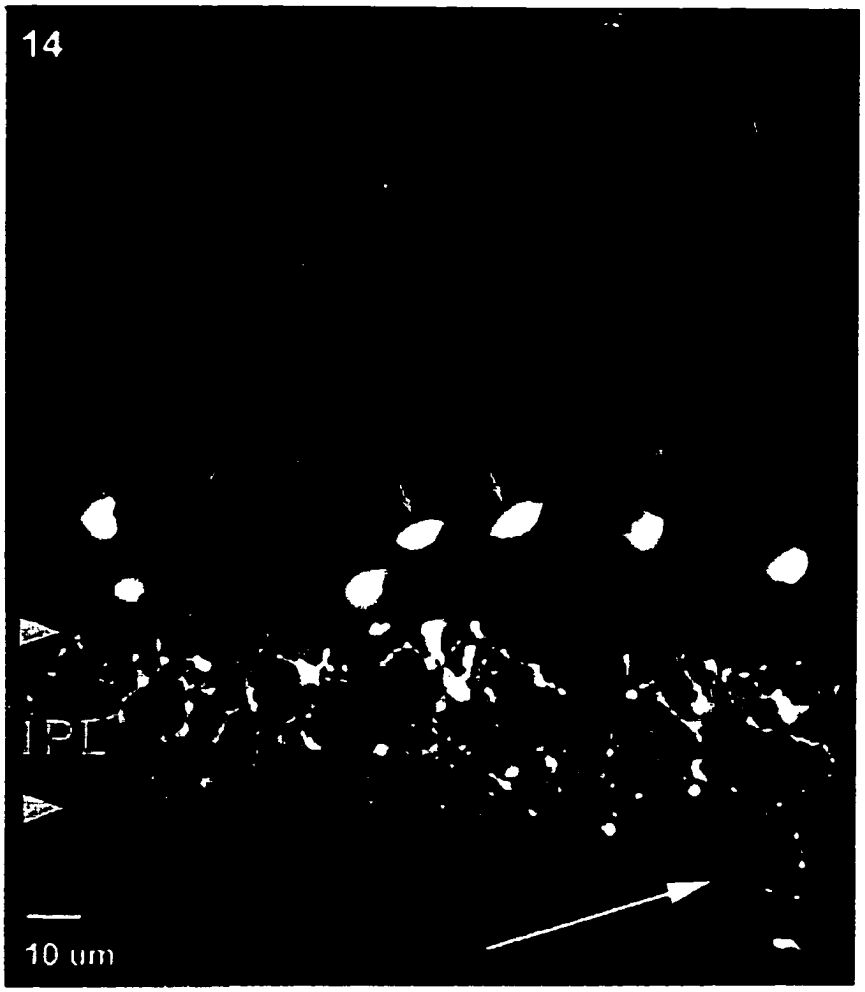
c) Protein kinase C immunoreactive bipolar cells in a wholemounted skate retina. This is the same piece of retina as in figures 12a and b, the focus is on the club-shaped terminals of PKC-stained bipolar cells. Note that because of the lateral shift oft he axons any association with their somata can not be made.

Figure 13:



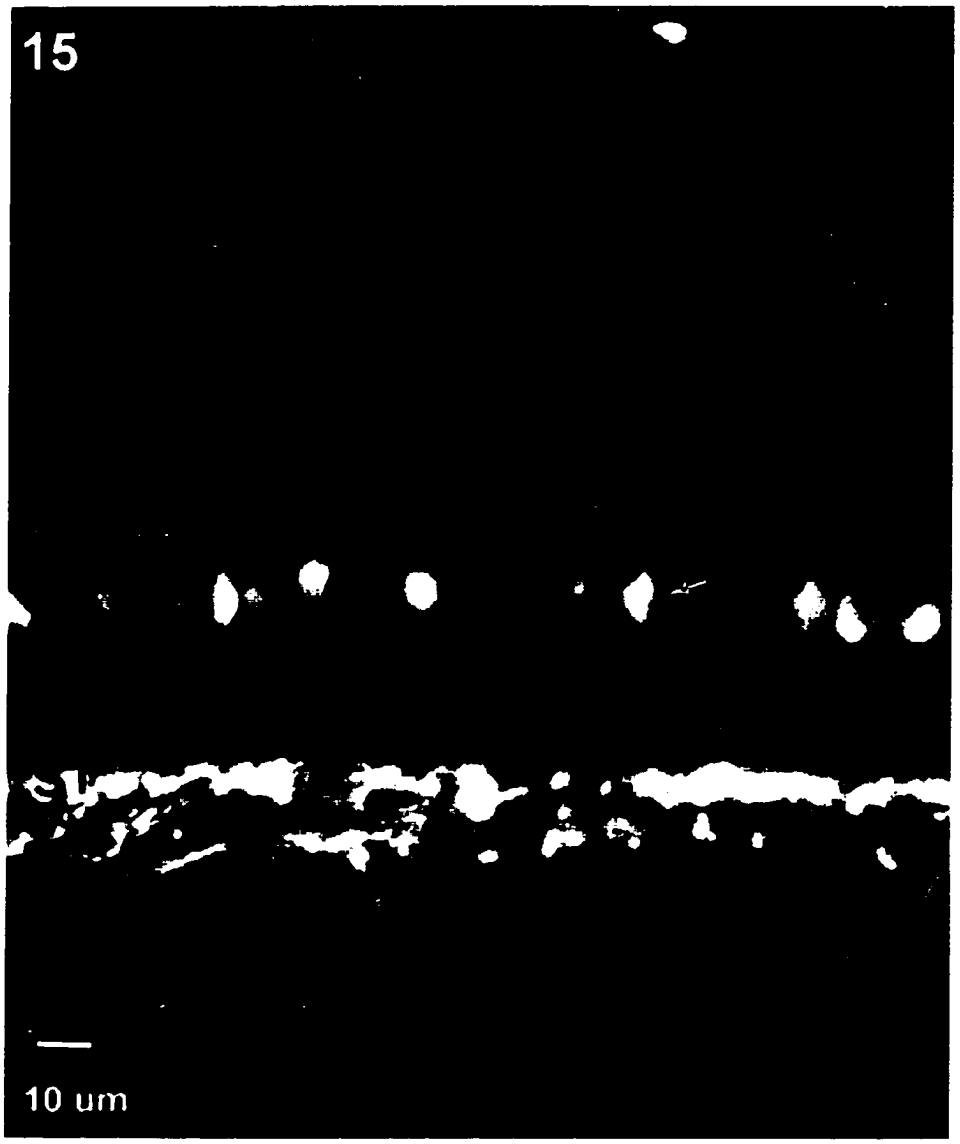
Schematic drawing of two possible coverage factors of dendritic fields. In panel A the dendrites of the cells reach as far as the tip of dendrites of neighboring cells. The retina is covered once by the entirety of all dendritic fields of this particular cell type. Panel B shows a cell type whose dendrites reach up to the center of the dendritic fields of their neighboring cells of the same type (dot, representing cell soma, arrows). As can be seen from the diagram this results in a coverage of the retina of 300% or three times, because each space on the retina is covered by dendritic fields of three cells.

Figure 14:



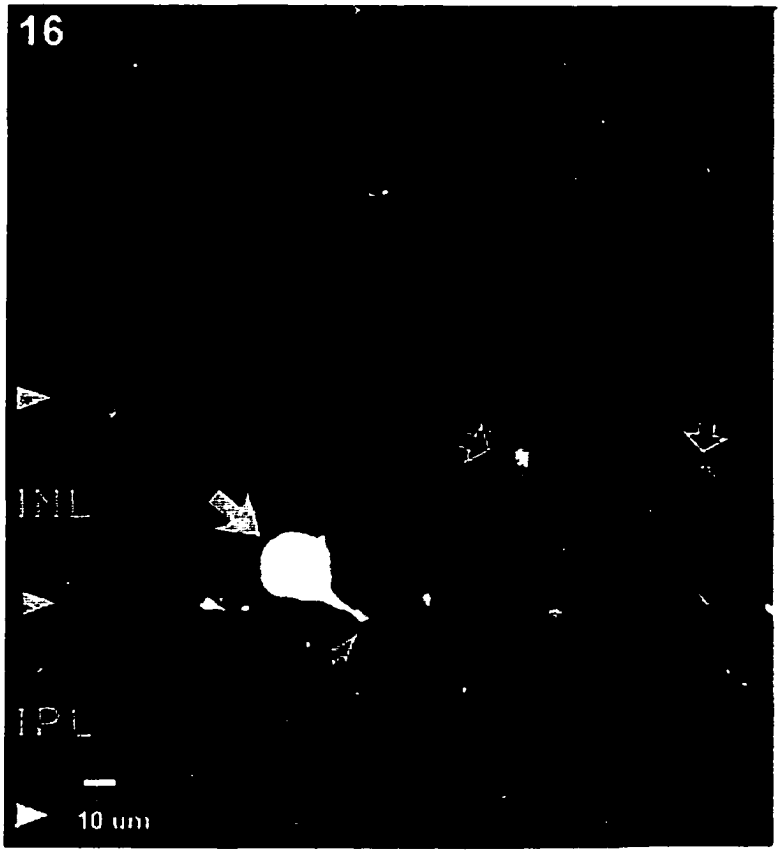
Incubation of the retina in Ringer's solution without any pharmacologic agents for one hour at room temperature results in heavily stained bipolar (small arrows) and amacrine cells and numerous processes in the IPL. A serotonergic fiber of presumptive centrifugal origin is indicated by the long arrow.

Figure 15:



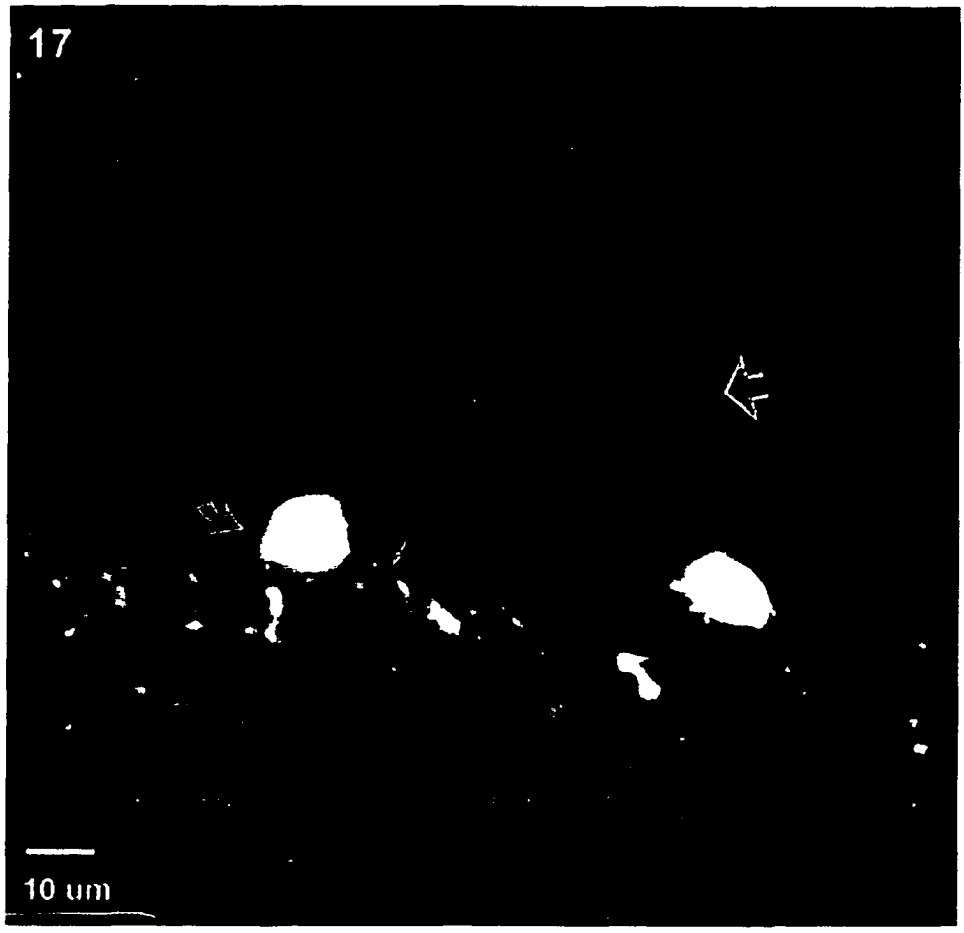
Serotonin immunoreactivity of a retina incubated for 1.5 hours in Ringer's solution containing pargyline only. Bipolar cells (arrow) stain strongly and the staining in the inner plexiform layer is strongest in stratum 1 (S1) of the inner plexiform layer.

Figure 16:



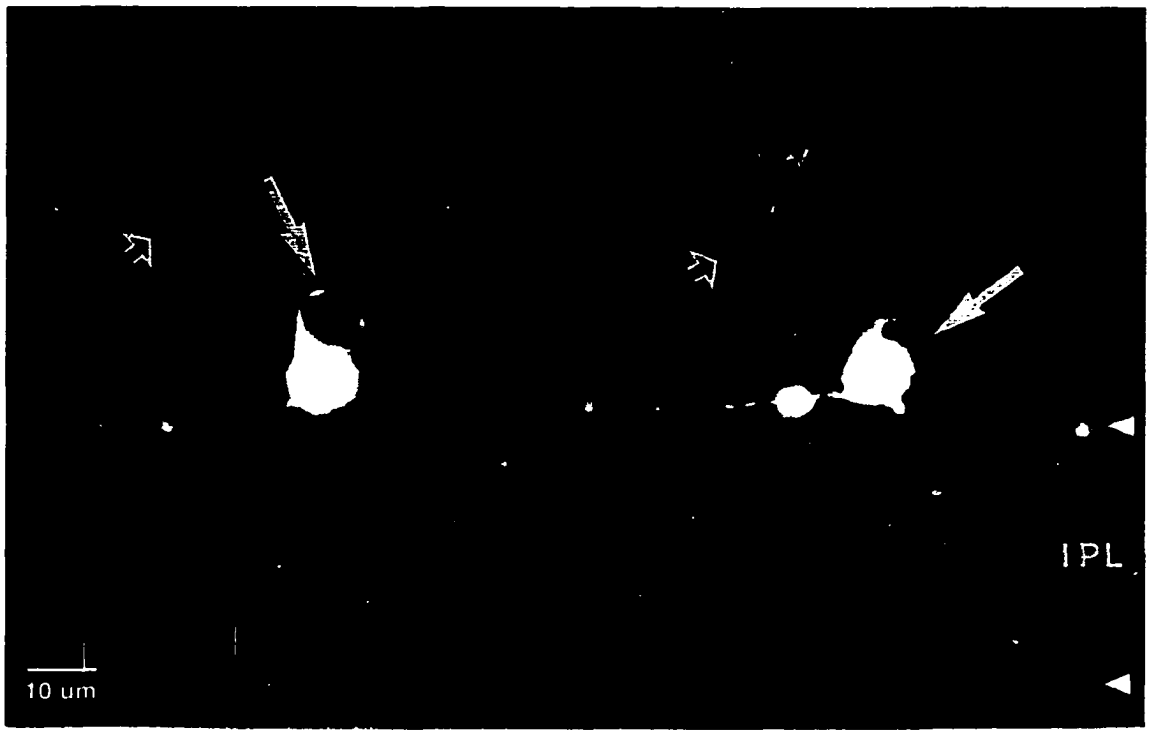
In the untreated skate retina (fixed immediately after dissection), serotonin immunoreactivity reveals large, heavily stained amacrine cells (solid arrow). Bipolar cells (open arrows) which in this picture are out of the focal plane, are labeled only faintly. (INL=inner nuclear layer, IPL=inner plexiform layer).

Figure 17:



Vertical section of a 5-HT labeled, preloaded (serotonin + pargyline) skate retina that was subsequently incubated in Ringer containing 5 mM NMDA. Serotonin immunoreactivity in bipolar cells (open arrow) remains intense throughout the entirety of the cells. Amacrine cells (curved arrow) show diminished immunoreactivity in their primary processes.

Figure 18:

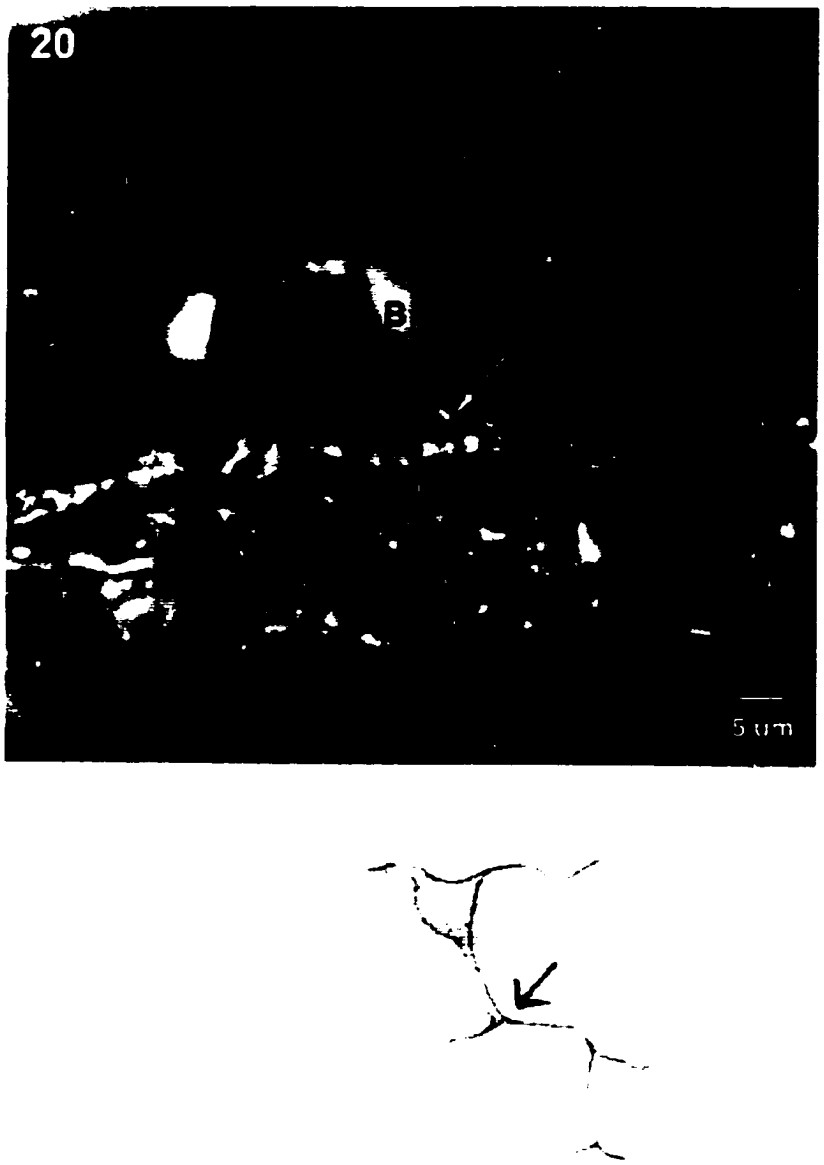


A vertical section of a skate retina treated with 5 mM aspartate after preloading the retina with serotonin. This sections shows in detail the severe loss of 5-HT immunoreactivity in large parts of serotonergic amacrine cells (arrows) and the general loss of immunoreactive fibers throughout the IPL. Serotonin immunoreactivity of bipolar cells (open arrows) remained as intense as in control retinas without the addition of aspartate but the cells are in a different focal plane in this micrograph. Arrowheads indicate the borders of the inner plexiform layer (IPL).

Figure 19:

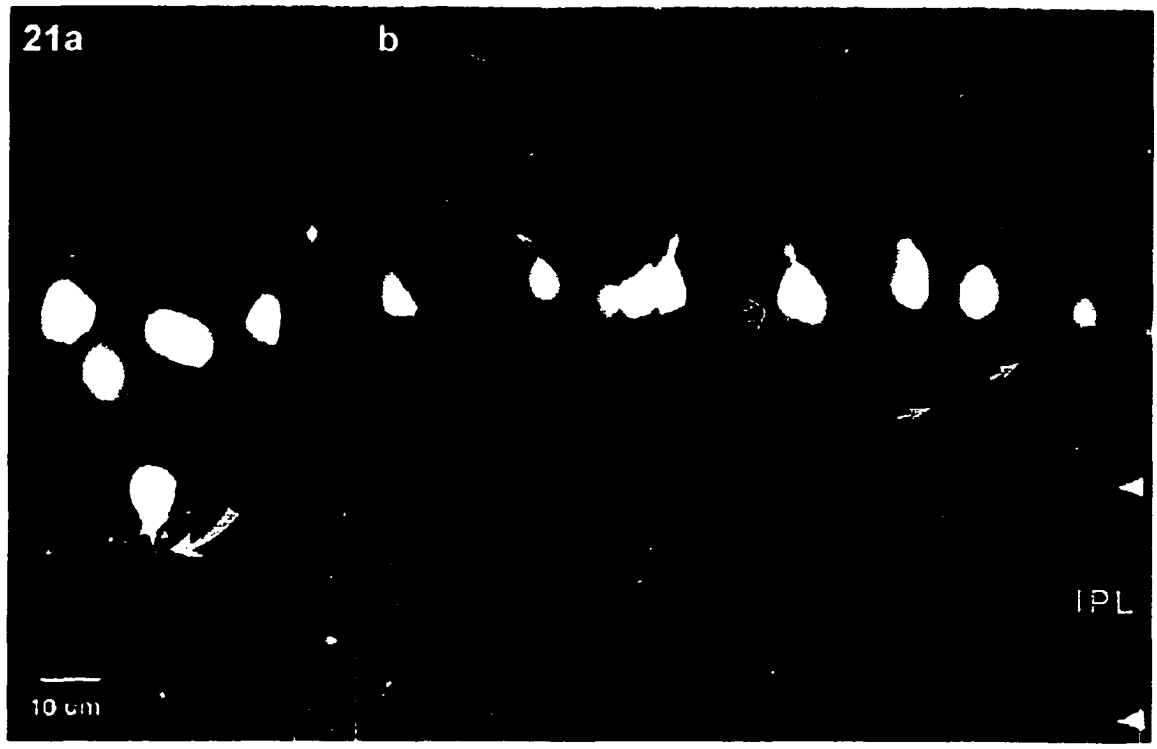
In the Aspartate treated retina (same treatment as in fig. 18) bipolar cell somata in the inner nuclear layer (INL) stained as strongly as in control retinas. The inner plexiform layer (IPL) exhibited a diminished immunoreacitivity due to loss of serotonin immnureactivity in amacrine cell processes which made it easier to follow the path of bipolar cell axons in the IPL (arrows). A clear band of immunoreactivity could be observed in stratum 1 of the IPL and a faint band in stratum 4.

Figure 20:



A vertical section of a rather peripheral area of an aspartate treated retina. Bipolar cells (B) stain strongly and due to loss of immunoreactivity in the inner plexiform layer bipolar cell axons and their bifurcations (arrow) could be followed more easily. The drawing shows how far the axons could be followed by changing the focal plane.

Figure 21:

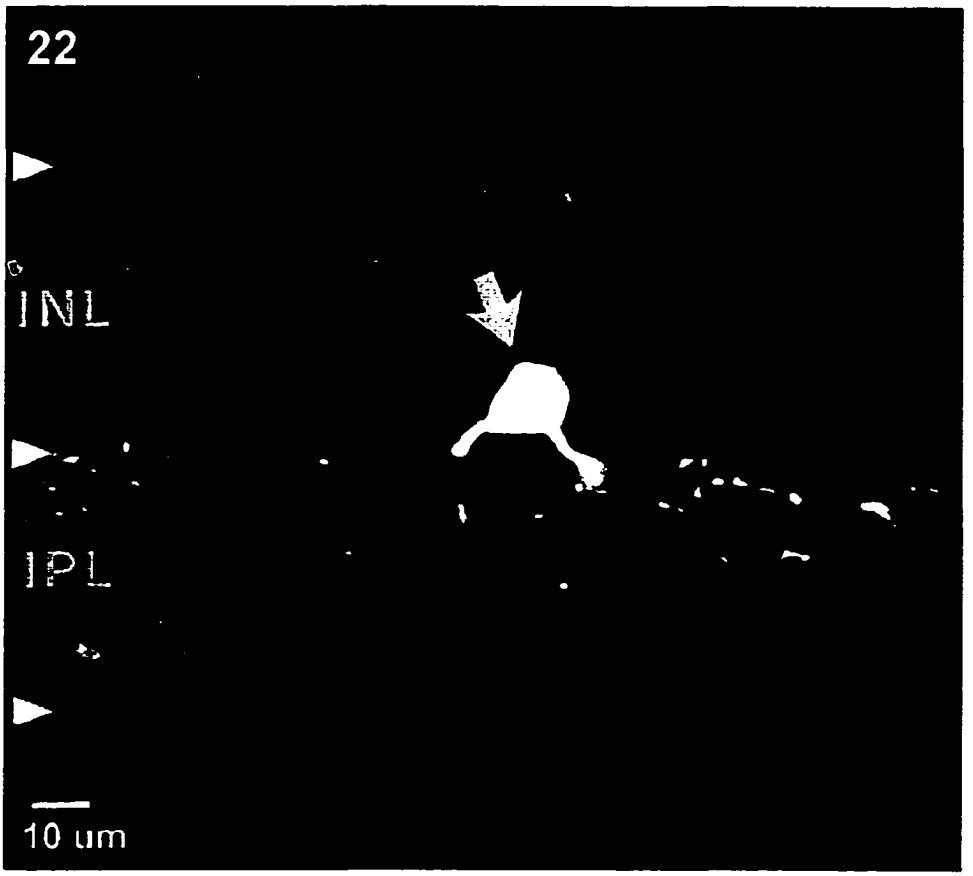


Vertical section of a skate retina incubated in high K^+Ringer after previous preloading with serotonin (and pargyline).

a) Bipolar cells as well as amacrine cells (curved arrow) have lost 5-HT immunoreactivity in all of their processes in the inner plexiform layer (IPL).

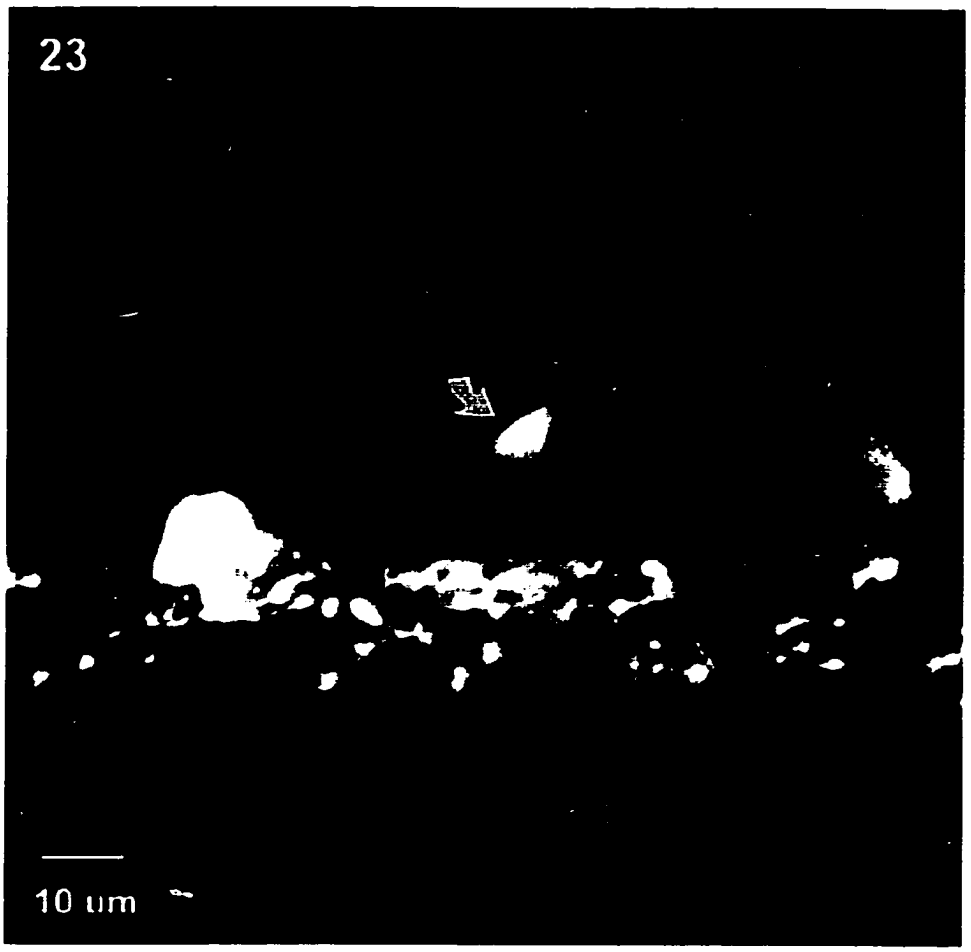
b) The IPL is almost devoid of 5-HT immunoreactive fibers and bipolar cell axons appear very thin (small arrows). In contrast, somata of bipolar cells (open arrow) exhibited an increase in serotonin staining to levels equal to the staining intensity of amacrine cell somata (see also 21a).

Figure 22:



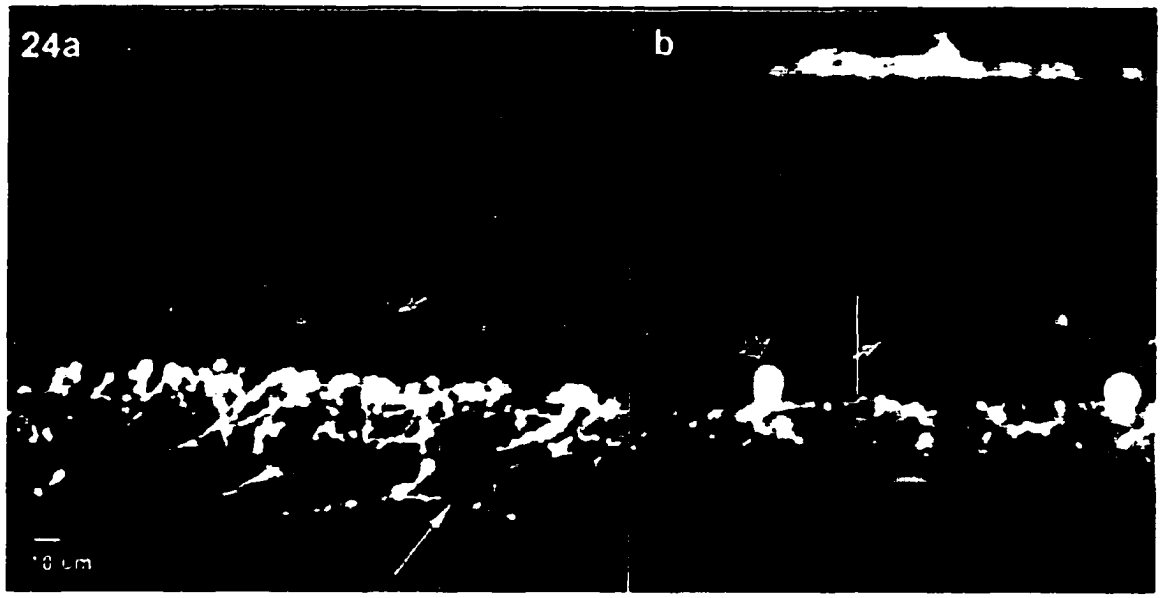
Vertical section of a skate retina that was incubated in Ringer containing serotonin and pargyline in the presence of the serotonin-uptake blocker zimelidine (1mM). 5-HT immunoreactivity is visible in the outer third of the inner plexiform layer (IPL) and in amacrine cells (arrow) only. No serotonin in bipolar cells was detected by the immunoreaction. (INL = inner nuclear layer).

Figure 23:



Vertical section of the second half of the same retina as in Figure 22 which was incubated in Ringer containing serotonin and pargyline but no zimelidine. A dense network of stained fibers occupied the outer two thirds of the inner plexiform layer (IPL). One immunoreactive amacrine cell can be seen in the left half of the micrograph and numerous bipolar cells (curved arrow) exhibited serotonin immunoreactivity.

Figure 24:



Retinas incubated in Ringer in the presence of 25 μM fluoxetine (a) and 1 mM pargyline or 500 μM fluoxetine and 1mM pargyline (b). Bipolar cells (short solid arrows) show strong to moderate labeling indicating that fluoxetine does not interfere with their accumulation of serotonin. Amacrine cells (open arrow) did lose serotonin immunoreactivity in their processes in the inner plexiform layer although this loss did not seem to be as obvious as for instance in the aspartate-treated retinas. The longer arrow in figure 20a points to an immunoreactive fiber that runs at the inner margin of the retina and has the same appearance as other fibers of presumptive centrifugal origin.

Figure 24b is from a more peripheral area of the retina, hence with a thinner inner plexiform layer and a lower density of stained bipolar cells.

7. Bibliography

ALBERTS, B, BRAY, D., LEWIS, J, RAFF, M., ROBERTS, K. & WATSON, J.D. (1994) The molecular biology of the cell. 3rd edition, Garland Publishing, New York.

AMMERMÜLLER, J., KOLB, H. (1995). The organization of the turtle inner retina. I. ON- and OFF-Center Pathways. *Journal of Comparative Neurology* **358**, 1-34.

AMMERMÜLLER, J., MULLER, J., KOLB, H. (1995). The organization of the turtle inner retina. II. Analysis of colour-coded and directionally selective cells. *Journal of Comparative Neurology* **358**, 35-62.

BARLOW, H.B. (1981). The Ferrier lecture: Critical limiting factors in the design of the eye and visual cortex. *Proceedings of the Royal Society of London B* **212**, 1-34.

BELL, R. (1986) Protein kinase C activation by diacylglycerol second messengers. *Cell* **45**, 631-632.

BROWN, K. T. (1969). A linear area centralis extending across the turtle retina and stabilized to the horizon by non-visual clues. *Vision Research* **9**, 1053-1062.

BRUNKEN, W. J. (1983). Amacrine and Ganglion Cell types of the Retina of the Skate, *Raja*. Ph.D. dissertation SUNY-Stony Brook, Stony Brook, New York.

BRUNKEN, W. J., JIN, X. T., PIS-LOPEZ, A. M. (1993). The Properties of the Serotonergic System in the Retina. *Progress in Retinal Research* **12**, 75-99.

BRUNKEN, W. J., WITKOVSKY, P. & KARTEN, H. J. (1986). Retinal Neurochemistry of Three Elasmobranch Species: An Immunohistochemical Approach. *Journal of Comparative Neurology* **243**, 1-12.

BRUUN, A., EHINGER, B. & SYSTSMA, V. M. (1984). Neurotransmitter Localization in the Skate Retina. *Brain Research* **295**, 233-248.

CHAPPELL, R. L. & ROSENSTEIN, F. J. (1996). Pharmacology of the Skate Electroretinogram Indicates Independent ON and OFF Bipolar Cell Pathways. *Journal of General Physiology* **107**, 535-544.

CHUN, M. H., HAN, S. H., CHUNG, J. W., WÄSSLE, H. (1993). Electron microscopic analysis of the rod pathway of the rat retina. *Journal of Comparative Neurology* **332**, 421-432.

COOPER, J.R., BLOOM, F.E. & ROTH, R.H. (1991) The Biochemical Basis of Neuropharmacology. 6th edition, Oxford University Press, New York, Oxford.

CORNWALL, M.C., RIPPS, H., CHAPPELL, R.L. & JONES, G. (1989) Membrane current responses of skate photoreceptors. *Journal of General Physiology* **94**,633-647.

COX, J.,MOORE, G. & EVANS, L. (1978) Zimelidine: a new antidepressant. *Prog. Neuro-Psychopharmacol.*: **2**, 379-384.

DACHEUX, R. F. , RAVIOLA, E. (1986) The rod pathway in the rabbit retina: a depolarizing bipolar and amacrine cell. Journal of Neuroscience **15**: 331-345.

DAW, N. W., JENSEN, R. J. & BRUNKEN, W. J. (1990). Rod pathways in mammalian retinae. *Trends in Neurosciences* **13**, 110-114.

DE LA VILLA, P., KURAHASHI, T. & KANEKO, A. (1995). L-glutamate-induced responses and cGMP-activated channels in three subtypes of retinal bipolar cells dissociated from the cat. *Journal of Neuroscience* **15**, 3571-3582.

DE VRIES, S. H. & BAYLOR, D. A. (1995). An alternative pathway for signal flow from rod photoreceptors to ganglion cells in mammalian retina. *Proc.Natl.Acad.Sci.USA* **92**, 10658-10662.

DOLAN, R. P. & SCHILLER, P. H. (1994). Effects of ON channel blockade with 2-amino-4-phosphobutyrate (APB) on brightness and contrast perception in monkeys. *Visual Neuroscience* **11**, 23-32.

DOWLING, J. E. & RIPPS, H. (1970). Visual adaptation in the retina of the skate. *J. Gen. Physiol.* **56**, 491-520.

DOWLING, J. E. & RIPPS, H. (1976). From sea to sight. *Oceanus* **19**, 28-33.

DOWLING, J. E. (1974). *Synaptic arrangements in the vertebrate retina: The photoreceptor synapse.* In: Synaptic Transmission and Neural Interaction. M. V. L. Bennet, ed. Raven Press, New York, 87-101.

DOWLING, J. E. (1987). *The retina: an approachable part of the brain.* Cambridge, Massachusetts and London, England: The Belknap Press of Harvard University Press.

DOWLING, J. E. & RIPPS, H. (1991) On the duplex nature of the skate retina. *The Journal of experimental Zoology Supplement* **5,** 55-65.

EHINGER, B. & FLOREN, I. (1978). Chemical removal of indoleamine accumulating terminals in rabbit and goldfish retina. *Expl. Eye Res.* **26**, 321-328.

FALCK, B. & OWMAN, C. (1965). A detailed methodological description of the fluorescence method for the cellular demonstration of biogenic monoamines. *Acta Universitatis Lundensis* **II**, No. 7.

FAMIGLIETTI, E. V., KANEKO, A., TACHIBANA, M. (1977). Neuronal architecture of ON and OFF pathways to ganglion cells in carp retina. *Science* **198**, 1267-1269.

FAROOQUI, A.A., FAROOQUI, T. YATES, A.J. & HORROCKS, L.A. (1988) Regulation of protein kinase C activity by various lipids. *Neuroschemistry Research* **13**, 499-511.

FLOREN, I. (1979). Indoleamine accumulating neurons in the retina of chicken and pigeon. *Acta Ophthalmologica* **57**, 198-210.

FUKUDA, K., SAITO, N., YAMAMOTO, M. & TANAKA, C. (1994). Immunocytochemical localization of the α-, βI-, βII- and γ-subspecies of protein kinase C in the monkey visual pathway. *Brain Research* **658**, 155-162.

GREEN, J.P. (1989) Histamine and Serotonin. In: Basic Neurochemistry: Eds: SIEGEL, G.J., AGRANOFF, B., ALBERS, R.W. & MOLINOFF, P. Raven Press, New York, Chapter **12**, 253-269.

GRÜNERT, U., MARTIN, P.R. & WÄSSLE, H. (1994) Immunocytochemical analysis of bipolar cells in the macaque monkey retina. *J. Comp. Neurol.* **348**, 607-627.

HARTLINE, H.K. (1938) The response of single optic nerve fibers of the vertebrate eye to illumination of the retina. *American Journal of Physiology* **121**, 400-415.

HARTLINE, H.K. (1940) The receptive fields of optic nerve fibers. *American Journal of Physiology* **130**, 690-699.

HENSLEY, S. H., YANG, X.-L. & WU, S. M. (1993). Relative Contribution of Rod and Cone Inputs to Bipolar Cells and Ganglion Cells in the Tiger Salamander Retina. *Journal of Neurophysiology* **69**, 2086-2098.

HORN, R. & MARTY, A. (1988). Muscarinic activation of ionic currents measured by a new whole-cell recording method. *Journal of General Physiology* **92**, 145-159.

HUANG, K.P. & HUANG F.L. (1986). Immunochemical characterization of rat brain protein kinase. *Journal of Biological Chemistry* **261**, 14781-14787.

HURD, L. B. & ELDRED, W. D. (1993). Synaptic microcircuity of bipolar and amacrine cells with serotonin-like immunoreactivity in the retina of the turtle, *Pseudemys scripta elegans*. *Visual Neuroscience* **10**, 455-472.

HUWILER, A., JUNG, H. H., PFEILSCHIFTER, J. & REMÉ, C. E. (1992). Protein kinase C in the rat retina: immunocharacterization of calcium-independent δ, ε and ζ isoenzymes. *Molecular Brain Research* **16**, 360-364.

KUFFLER, S.W. (1953) Discharge patterns and functional organization of mammalian retina. *Journal of Neurophysiology* **16**, 37-68.

ISHIDA, A. T., STELL, W. K. & LIGHTFOOT, D. O. (1980). Rod and cone inputs to bipolar cells in goldfish retina. *Journal of Comparative Neurology* **191**, 315-335.

JEON, C.-J. & MASLAND, R. H. (1995). A Population of Wide-Field Bipolar Cells in the Rabbit's Retina. *Journal of Comparative Neurology* **360**, 403-412.

JEQUIER, E., ROBINSON, D.S., LOVENBERG, W. & SJOERDSMA, A. (1969) Further studies on tryptophan hydroxylase in rat brainstem and beef pineal. *Biochemical Pharmacology* **18**, 1071-1081.

KANEKO, A. & TACHIBANA, M. (1978). Convergence of rod and cone signals to single bipolar cells in the carp retina. *Sensory Processes* **2**, 383-387.

KANEKO, A., FAMIGLIETTI, E. V.,JR. & TACHIBANA, M. (1979). Physiological and morphological identification of signal pathways in the carp retina. In *Neurobiology of Chemical Transmission.* eds. HALL, Z. W. & OTSUKA, M. pp. 235-251. New York: Wiley.

KARSCHIN, A. & WÄSSLE, H. (1990). Voltage- and transmitter-gated currents in isolated rod bipolar cells of the rat retina. *Journal of Neurophysiology* **63**, 860-876.

KOLB, H. & NELSON, R. (1983). Rod pathways in the retina of the cat. *Vision Research* **23**, 301-312.

KOLB, H. (1994). The architecture of functional neural circuits in the vertebrate retina. *Investigative Ophthalmology and Visual Sciences* **35**, 2385-2404.

KOLB, H., ZHANG, L. & DEKORVER, L. (1993). Differential staining of neurons in the human retina with antibodies to protein kinase C isozymes. *Visual Neuroscience* **10**, 341-351.

KUO, J.F., SCHATZMAN, R.C., TURNER, R.S., & ZEI, G.J.M. (1984). Phospholipid-sensitive Ca^{2+}-dependent protein kinase: a kinase major protein phosphorylation system. *Molecular and Cellular Endocrinology* **35**, 65-73.

LINSER, P.J. (1991) Comparative immunochemistry of elasmobranch retina Müller cells and horizontal cells. *Journal of Experimental Zoology, Supplement* **5**, 88-96.

MARC, R. E. (1982). The spatial organization of neurochemically classified interneurons in the goldfish retina. I. Local patterns. *Vision Research* **22**, 589-608.

MARC, R. E., LIU, W.-L. S., KALLONIATIS, M., RAIGUEL, S. F. & VAN HAESENDONCK, E. (1990). Patterns of Glutamate Immunoreactivity in the Goldfish Retina. *Journal of Neuroscience* **10**, 4006-4034.

MARC, R. E., LIU, W.-L. S., SCHOLZ, K. & MULLER, J. F. (1988). Serotonergic and Serotonin-Accumulating Neurons in the Goldfish Retina. *Journal of Neuroscience* **8**, 3427-3450.

MASSEY, S.C., MILLS, S.L. & MARC, R.E. (1992) All indoleamine-accumulating cells in the rabbit retina contain GABA. *Journal of Comparative Neurology* **322**, 275-291.

MÜLLER, B. & PEICHL, L. (1989). Topography of cones and rods in the tree shrew retina. *Journal of Comparative Neurology* **282:** 581-594.

MÜLLER, B. & PEICHL, L. (1991). Rod bipolar cells in the cone-dominated retina of the tree shrew *Tupaia belangeri*. *Visual Neuroscience* **6**, 629-639.

NAWY, S., JAHR, C. E. (1990). Time-dependent reduction of glutamate current in retinal bipolar cells. *Neuroscience Letters* **108**, 279-283.

NAWY, S., JAHR, C. E. (1991). cGMP-Gated Conductance in Retinal Bipolar Cells Is Suppressed by the Photoreceptor Transmitter. *Neuron* **7**, 677-683.

NEGISHI, K. & TERANISHI, T. (1990). Sequential course of uptake of intravitreal 5,7-dihydroxytryptamine by carp retinal cells. *Brain Research* **508**, 135-141.

NEGISHI, K., KATO, S. & TERANISHI, T. (1988). Dopamine cells and rod bipolar cells contain protein kinase C-like immunoreactivity in some vertebrate retinas. *Neuroscience Letters* **94**, 247-252.

NELSON, R. & KOLB, H. (1983). Synaptic patterns and response properties of bipolar and ganglion cells in the cat retina. *Vision Research* **23**, 1183-1195.

NISHIZUKA, Y. (1988). The molecular heterogeneity of protein kinase C and its implications for cellular regulation. *Nature* **334**, 661-665.

OSADA, S., MIZUNO, K., SAIDO, T.C., AKITA, Y., SUZUKI, K., KUROKI, T. & OHNO, S. (1990). A phorbol ester receptor/protein kinase, nPKCη, a new member of the protein kinase C family predominantly expressed in lung and skin. *Journal of Biological Chemistry* **265**, 22434-22440.

OSBORNE, N. N. (1984). Indoleamines in the eye with special reference to the serotonergic neurones of the retina. In *Progress in Retinal Research*, eds. OSBORNE, N. N. & CHADER, G. pp. 61-103. Oxford, UK: Pergamon.

OSBORNE, N. N., BARNETT, N. L., MORRIS, N. J. & HUANG, F. L. (1992). The occurrence of three isoenzymes of protein kinase C (α, β, and γ) in retinas of different species. *Brain Research* **570**, 161-166.

OSBORNE, N. N., BROYDEN, N. J., BARNETT, N. L. & MORRIS, N. J. (1991). Protein kinase C (α and β) immunoreactivity in rabbit and rat retina: Effect of phorbol esters and transmitter agonists on immunoreactivity and the translocation of the enzyme from cytosolic to membrane compartments. *Journal of Neurochemistry* **57**, 594-604.

OSBORNE, N.N. (1988) Retinal serotonin and the co-occurrence of serotonin with other neurotransmitters. *In* Neuronal Serotonin (ed. by N.N. Osborne and M. Hamon), Wiley & Sons, New York, London., pp. 129-152.

OSBORNE, N.N. & PATEL, S. (1984) Postnatal development of serotonin-accumulating neurones in the rabbit retina and an immunohistochemical analysis of the uptake and release of serotonin. *Experimental Eye Research* **38**, 611-620.

PAGE, I.H. (1968) Serotonin, Year Book Medical Publishers, Chicago.

QUAY, W.B. (1965) Indole derivates of pineal and related neural and retinal tissues. *Pharmacological Reviews* 17, 321-345.

RAMÓN y CAJAL, S. R. (1892). La rétine des vertébrés. *Cellule* **9**, 121-225.

RAUEN, T. & KANNER, B.I. (1994) Localization of the glutamate transporter GLT-1 in rat and macaque monkey. *Neuroscience Letters* **169**, :137-140.

REDBURN, D.A. & MITCHELL, C.K. (1989) Darkness stimulates rapid synthesis and release of melatonin in rat retina. *Visual Neuroscience* **3**, : 391-403.

RETZIUS, G. (1905). Zur Kenntnis vom Bau der Selachier-Retina. *Biologische Untersuchungen* **12**, 55-60.

SAITO, T. & KUJIRAOKA, T. (1982). Physiological and morphological identification of two types of ON-center bipolar cells in the carp retina. *Journal of Comparative Neurology* **205**. 161-170.

SÁNCHEZ-PRIETO, I., HERRERO, I. & MIRAS-PORTUGAL, M.T. (1994) Modulation of the exocytotic release of neurotransmitter glutamate by protein kinase C. in: Cirrhosis, Hyperammonemia and hepatic Encephalopathy, Plenum Press NY, Eds.:GRISOLIA, S. & FELIPO, V., 95-105.

SANDELL, J.H. & MASLAND, R.H. (1989) Indoleamine accumulation by retinal neurons exposed to blood. *Histochemistry* **92,** 57-60.

SCHAPER, A. (1899). Die nervösen Elemente der Selachier-Retina in Methylenblaupräparaten. *Festschrift zum 70sten Geburtstag von Karl Kupffer* 1-10.

SCHILLER, P. H., SANDELL, J. H. & MAUNSELL, J. H. R. (1986). Functions of the ON and OFF channels of the visual system. *Nature* **322**, 824-825.

SCHÜTTE, M. & WEILER, R. (1987). Morphometric analysis of serotonergic bipolar cells in the retina and its implications for retinal image processing. *Journal of Comparative Neurology* **260**, 619-626.

SCHÜTTE, M. & WITKOVSKY, P. (1990). Serotonin-like immunoreactivity in the retina of the clawed frog *Xenopus laevis*. *Journal of Neurocytology* **19**, 504-518.

SCHÜTTE, M. (1994). Serotonergic and serotonin-synthesizing cells of the *Xenopus* retina. *International Journal of Neuroscience* **78**, 67-73.

SCHÜTTE, M. (1995). Effects of kainic acid and piperidine dicarboxylic acid on displaced bipolar cells in the turtle retina. *Journal of Neurocytology* **24**, 361-369.

SCHWARTZ, J.H. (1991) Synaptic Vesicles. In: Principles of neural science. 3rd edition, Eds.: KANDEL, E.R., SCHWARTZ, J.H. & JESSELL, T.M., Elsevier, New York, Chapter 15, 225-234.

SHERRY, D. M. & YAZULLA, S. (1993). Goldfish Bipolar Cells and Axon Terminal Patterns: A Golgi Study. *Journal of Comparative Neurology* **329**, 188-200.

STONE, S. & SCHÜTTE, M. (1991). Physiological and morphological properties of OFF- and ON-center bipolar cells in the *Xenopus* retina: Effects of glycine and GABA. *Visual Neuroscience* **7**, 363-376.

STUESSE, S.L., CRUCE, W.L.R. & NORTHCUTT, R.G. (1990) Distribution of tyrosine hydroxylase- and serotonin-immunoreactive cells in the central nervous system of the thornback guitarfish, *Platyrhinoidis triseriata*. *Journal of Chemical Neuroanatomy* **3**, 45-58.

STUESSE, S.L., CRUCE, W.L.R. & NORTHCUTT, R.G. (1991a) Localization of serotonin, tyrosine hydroxylase, and leu-enkephalin immunoreactive cells in the brainstem of the horn shark, *Heterodontus francisci*. *Journal of Comparative Neurology* **308**, :277-292.

STUESSE, S.L., CRUCE, W.L.R. & NORTHCUTT, R.G (1991b) Serotonergic and enkephalinergic cell groups in the reticular formation of the bat ray and two skates. *Brain, Behavior and Evolution* **38**, 39-52.

SUZUKI, S. & KANEKO, A. (1990). Identification of bipolar cell subtypes by protein kinase C-like immunoreactivity in the goldfish retina. *Visual Neuroscience* **5**, 223-230.

SUZUKI, S., TACHIBANA, M. & KANEKO, A. (1990). Effects of glycine and GABA on isolated bipolar cells of the mouse retina. *Journal of Physiology* **421**, 645-662.

SZAMIER, R. B. & RIPPS, H. (1983). The visual cells of the skate retina: structure, histochemistry, and disc-shedding properties. *Journal of Comparative Neurology* **215**, 51-62.

TAKAI, Y., KISHIMOTO, A., INOUE, M. & NISHIZUKA, Y. (1977) Studies on a cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissues, I: Purification and characterization of an active enzyme from bovine cerebellum. *Journal of Biological Chemistry* **252**, 7603-7609.

TAKEUCHI, Y. (1988) Distribution of serotonin neurons in the mammalian brain. *In* Neuronal Serotonin (ed. by N.N. Osborne and M. Hamon), Wiley & Sons, New York, Londc-., pp. 25-56.

TAUCHI, M. (1989). Displaced and indoleamine-accumulating bipolar cells in the turtle retina. *Neuroscience Research* **7** Supplement **10**, S57-S66.

TAUCHI, M. (1990). Single cell shape and population densities of indoleamine-accumulating and displaced bipolar cells in Reev-s' turtle retina. *Proceedings of the Royal Society of London B* **238**, 351-367.

VAN PRAAG, H.M. & ANSIS, G.M. (1990) Monoamines and abnormal behaviour: a multi-aminergic perspective. *British Journal of Psychiatry* **157**, 723-734.

VANEY, D. I., YOUNG, H. M. & GYNTHER, I. C. (1991). The rod circuit in the rabbit retina. *Visual Neuroscience* **7**, 141-154.

VANHATALO, S. & SOINILA, S. (1994) Pharmacological characterization of serotonin synthesis and uptake suggest a false transmitter role for serotonin in the pituitary intermediate lobe. *Neuroscience Research* **21**, 143-149.

WÄSSLE, H. & BOYCOTT, B. B. (1991). Functional Architecture of the Mammalian Retina. *Physiological Reviews* **71**, 447-480.

WÄSSLE, H. & RIEMANN, H. J. (1978). The mosaic of nerve cells in the mammalian retina. *Proceedings of the Royal Society of London B* **200**, 441-461.

WÄSSLE, H., YAMASHITA, M., GREFERATH, U., GRÜNERT, U. & MÜLLER, F. (1991). The rod bipolar cell of the mammalian retina. *Visual Neuroscience* **7**, 99-112.

WEILER, R. & SCHÜTTE, M. (1985a). Morphological and pharmacological analysis of putative serotonergic bipolar and amacrine cells in the retina of a turtle, Pseudemys scripta elegans. *Cell and Tissue Research* **241**, 373-382.

WEILER, R. & SCHÜTTE, M. (1985b). Kainic acid-induced release of serotonin from OFF-bipolar cells in the turtle retina. *Brain Research* **360**, 379-383.

WEILER, R. (1981). The Distribution of Center-Depolarizing and Center-Hyperpolarizing Bipolar Cell Ramifications Within the Inner Plexiform Layer of Turtle Retina. *Journal of Comparative Physiology* **144**, 459-464.

WELSH, J.H. (1964) The quantitative distribution of 5-hydroxytryptamine in the nervous system, eyes and other organs of some vertebrates. *Comparative Neurochemistry*, Proc. 5th Int. Neurochem. Symp., 355-366.

WILHELM, M., ZHU, B., GABRIEL, R. & STRAZNICKY, C. (1993) Immunocytochemical identification of serotonin-synthesizing neurons in the vertebrate retina: a comparative study. *Experimental Eye Research* **56**, 231-240.

WITKOVSKY, P. & STELL, W. K. (1973). Retinal structure in the smooth dogfish *Mustelus canis*: light microscopy of bipolar cells. *Journal of Comparative Neurology* **148**, 47-60.

WITKOVSKY, P., ELDRED, W. D. & KARTEN, H. J. (1984). Catecholamine- and indoleamine-containing neurons in the turtle retina. *Journal of Comparative Neurology* **228**, 217-225.

WITKOVSKY, P. ALONES, V. & PICCOLINO, M. (1987) Morphological changes induced in turtle retinal neurons by exposure to 6-hydroxydopamine and 5,6-dihydroxytryptamine. *Journal of Neurocytology* **16**, 55-67.

WONG, D.T., BYMASTER, F.P. & ENGLEMAN, E.A. (1995) Prozac (Fluoxetine, Lilly 110140), the first selective serotonin uptake inhibitor and an antidepressant drug: Twenty years since its first publication. *Life Sciences* **57**, 411-441.

WOOD, J. G., HART, C. E., MAZZEI, G. J., GIRARD, P. R. & KUO, J. F. (1988). Distribution of protein kinase C immunoreactivity in rat retina. *Histochemical Journal* **20**, 63-68.

YAMASHITA, M. & WÄSSLE, H. (1991). Responses to the glutamate agonist 2-amino-4-phosphonobutyric acid (APB) of rod bipolar cells isolated from the rat retina. *Journal of Neuroscience* **11**, 2372-2382.

YANG, X.-L. & WU, S. M. (1993). Synaptic transmission from rods to rod-dominated bipolar cells in the tiger salamander retina. *Brain Research* **613**, 275-280.

YAQUB, A. & ELDRED, W.D. (1993) Effects of excitatory amino acids on immunocytochemically identified populations of neurons in turtle retina. *Journal of Neurocytology* **22**, 644-662.

YOUNG, H. M. & VANEY, D. I. (1991). Rod-Signal Interneurons in the Rabbit Retina: 1. Rod Bipolar Cells. *Journal of Comparative Neurology* **310**, 139-153.

ZHU, B., GABRIEL, R. & STRAZNICKY, C. (1992). Serotonin synthesis and accumulation by neurons of the anuran retina. *Visual Neuroscience* **9**, 377-