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ASPECTS OF NEUROTRANSMISSION IN THE RETINA OF THE NEWT (*Notophthalmus viridescens*)

by

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ABSTRACT

Newt photoreceptor synaptic terminals undergo significant morphological changes over a 24 hr period (LD12:12). When not releasing neurotransmitter, during the day, photoreceptor dense-cored synaptic vesicles increase in number, supporting the hypothesis that these vesicles become "supercharged" with transmitter substance at this time. During the night, when photoreceptors release neurotransmitter, the synaptic terminal morphology indicates that synaptic vesicle release is occurring at a rapid rate, especially toward the end of the dark phase. These findings suggest that the "quanta" of transmitter in photoreceptor synaptic terminals varies over a natural day-night cycle.

Although acetylcholine has been suggested to be the photoreceptor neurotransmitter, nicotinic and muscarinic acetylcholine binding sites could not be unequivocally localized to photoreceptor postsynaptic membranes. Amino acid analysis of long-term light and dark-adapted retinas suggests that glutamate or certain sulphur-containing amino acids are likely candidates for the photoreceptor transmitter. However, autoradiographic studies show that glutamate is not accumulated by photoreceptors. Autoradiographic studies have, however, shown that a slight uptake of aspartate is exhibited by newt photoreceptors, indicating that aspartate may be the photoreceptor transmitter. But because histochemical studies have localized unusually high concentrations of -SH groups in photoreceptor synaptic vesicles, the sulphur-containing amino acids cysteate and cysteine sulphinate should be considered as likely candidates as well.

Light and electron microscope autoradiographic localization of the cells which accumulate glycine, taurine and GABA indicate that these inhibitory amino acids may be used as neurotransmitter substances by distinct populations of amacrine and ganglion cells in the inner layers of the newt retina.

Furthermore, autoradiographic studies, combined with HRP transport studies, show that the taurine-accumulating cells in the ganglion cell layer of the newt retina are, in fact, displaced amacrine cells. These studies are corroborated by optic nerve axon versus ganglion cell counts, showing that only 50-60% of the cells in the ganglion cell layer of the newt retina are truly ganglion cells.

ABBREVIATIONS USED

ACh	Acetylcholine
BTX	α-bungarotoxin
^{o}C	Degrees Celsius
Ci	Curie
cm	Centimeter
DAB	Diaminobenzidine
DAEM	N-(7-dimethylamino 4-ethyl coumarinyl) maleimide
DTE	Dithioerythrotol
g	gram
GABA	γ-aminobutyric acid
hr	Hour
HRP	Horseradish peroxidase
HRP-BTX	Horseradish peroxidase conjugated α-bungarotoxin
m	Meter
mepps	Miniature end plate potentials
mg	Milligram
ml	Milliliter
mm	Millimeter
mmol	Millimole
μCi	Microcurie
μg	Microgram
μl	Microliter

µm	Micrometer
µmol	Micromole
MO	Mercury Orange
mOsM	Milliosmole
NEM	N-ethyl maleimide
nm	Nanometer
nmol	Nanomole
QB	Quinuclidinyl benzylate
RPM	Revolutions per minute
TAS	Tris acetate saline

Explanatory Note: Both the terms "synaptic lamellae" and "synaptic ribbon" have been used in this thesis to refer to the specialized organelle found at synaptic sites in the terminals of photoreceptor and bipolar cells. Wherever possible, the term "synaptic lamellae" has been used.

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This investigation has been made possible by the kind assistance of many people to whom the author is indebted.

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This thesis is dedicated to my wife, Thelma, and to my parents.

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INTRODUCTION

Although most of the cell types in the vertebrate neural retina have been clearly identified, the method by which these cells interact in transmitting information from the photoreceptors to the brain is still poorly understood. The comparative study of the vertebrate retina by Cajal (1893) was a study partly from which his "neuron doctrine" arose. In it he demonstrates that there are five major neuronal cell types, and one type of glial cell in the retina; photoreceptors, bipolar cells, ganglion cells, horizontal cells, amacrine cells and Müller cells. It was not until recently, that another retinal neuron was discovered, the interplexiform cell (Dowling et al., 1976). The photoreceptors, bipolar cells and the ganglion cells are direct conducting neurons and are responsible for relaying visual stimuli to the brain. The horizontal cells, interplexiform cells, and the amacrine cells comprise the lateral conducting system of neurons, and are responsible for the processing of visual information within the retina. The cell bodies of these cells are organized into three nuclear layers and their synaptic contacts with one another are confined to two synaptic areas, the outer and inner plexiform layers. Much of this information has come from light microscope studies of silver impregnated retinal tissues, which were begun in the late nineteenth century and are still providing critical information today. More

recently, electrophysiological and pharmacological studies have attempted to determine the function of these identified cells (Dowling, 1970; Werblin, 1971). Because anatomical and physiological data imply that transmission in most retinal connections is chemically, rather than electrically mediated, histochemical and pharmacological studies have complemented classical anatomical studies on the functional organization of the vertebrate retina.

In addition to questions concerning the organization of the retina, there are many unsolved questions regarding the capability of the retina to adapt itself to changing light conditions in order to optimize visual acuity. Many studies have equated changes in the sensitivity of the visual system with the bleaching and regeneration of visual pigments (for a review, see Goldsmith, 1973). However, it is possible that there are neural mechanisms involved in visual adaptation as well. These mechanisms may range from a modification of synaptic contacts (ie. synaptic plasticity; Eccles, 1979) in response to diurnal variations in lighting conditions, to permanent modifications of the basic organization of the retina to suit a species' particular visual needs.

The purpose of this thesis is to explore the morphological aspects of some of the adaptive changes in the newt (Notophthalmus viridescens*) retina, especially with respect to neurotransmission between receptor cells and their postsynaptic elements.

The retina of the newt is particularly useful for the study of neuronal interactions because of the large size of the retinal elements and the ease with which the animals may be light or dark adapted. Furthermore, these animals are interesting because they possess a complex retina (Dubin, 1970) which is capable of a high degree of visual processing within the retina itself. As the basic anatomical organization of the newt retina has been thoroughly described at both the light and electron microscope levels (Dickson and Hollenberg, 1971; Keefe, 1971), this thesis will further characterize the newt retina by localizing specific neurotransmitter candidates to neuronal populations. Studies of this kind are helping to elucidate the neural pathways involved in retinal processing of visual information.

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* Notopthalmus was known as Triturus and Diemictylus prior to the 1962 decision of the International Commission on Zoological Nomemclature.

CHAPTER 1

CYCLIC VARIATIONS AND MEMBRANE RECYCLING IN THE PHOTORECEPTOR SYNAPTIC TERMINALS OF THE NEWT RETINA

INTRODUCTION

In 1952 Fatt and Katz revealed that a multimolecular amount of acetylcholine produced miniature end plate potentials (mepps) of constant amplitude at the neuromuscular junction. The packet of transmitter producing the mepps was defined as a 'quantum'. Three years later, electron microscope studies revealed that small vesicles were aggregated near the synaptic site, and it was suggested that each vesicle contained a multimolecular packet of the synaptic transmitter, acetylcholine (Del Castillo and Katz, 1954; De Robertis and Bennet, 1955; Palay, 1956). The "vesicular hypothesis" further suggested that the contents of the vesicle were released into the synaptic cleft by fusion of the vesicle membrane with the presynaptic membrane and extrusion of the vesicle's contents into the postsynaptic cleft (Del Castillo and Katz, 1957). The vesicle hypothesis continues to be a contentious issue which has received considerable discussion in the literature (Heuser, 1978; Whittaker and Zimmerman, 1978; Marchbanks, 1978; 1979; Kelley et al., 1979; Collier, 1979; Israel et al., 1979; Dunant and Israel, 1979). Nevertheless, a number of studies have attempted to correlate the physiologic

activity of a neuron with its morphological appearance in light of these initial observations (for review see Ceccarelli and Hurlbut, 1980).

Although a variety of biological systems have been used for the study of membrane recirculation, as it relates to neurotransmission, retinal photoreceptors are an attractive model system for a number of reasons, the most important of which are the following.

(1) The cells are compact and highly polarized in both morphological and functional aspects. One end of the vertebrate photoreceptor is concerned with light reception, the other with synaptic transmission (Holtzman and Mercurio, 1980).

(2) The cell's activity is temporally polarized to a high degree, receiving light stimulation during the day and releasing a depolarizing neurotransmitter during the night (Dowling, 1974; Kaneko and Shimizaki, 1975).

In 1956 De Robertis and Franchi first described alterations which occured in the synaptic terminals of photoreceptor cells in response to changes in lighting conditions. They reported that when albino rats were kept in the dark for 24 hrs, synaptic vesicles accumulated near the membrane and after 9 days in the dark, the vesicles decreased in size. These results were not exposed to stringent statistical testing and were later refuted by Mountford (1963), who could find no statistically

significant change in the diameter of guinea pig photoreceptor synaptic vesicles following light or dark adaptation. She also observed that the packing of synaptic vesicles in photoreceptor terminals varied from terminal to terminal, but this could not be correlated with the animals' adaptive state. Cragg (1969) also studied the retinal synapses of newborn rats, minutes after their first exposure to daylight, hoping to record the changes which occured in photoreceptor terminals in this more drastic alteration in light experience. He supported Mountford's (1963) findings and could find no changes in the synaptic vesicle diameter in light or dark conditions. However, he did discover that there were more synaptic-vesicle profiles in light than in dark-adapted photoreceptor terminals. In addition, Cragg noted that photoreceptor terminals appeared to decrease in width as soon as 3 min after first exposure to daylight. Because of this finding, he suggested that the total number of synaptic vesicles in photoreceptor terminals does not vary, but instead remains constant during both light and dark conditions; being dispersed in the larger terminals in the dark, and concentrated in the smaller terminals in the light. Alterations in the size of photoreceptor synaptic terminals and changes in the degree of invagination of the postsynaptic elements under different lighting conditions have also been observed in the turtle (Schaeffer and Raviola, 1976), chick (Cooper and McLaughlin, 1977, 1978) and goldfish (Raynauld et al., 1978) retina.

The above morphological findings can be explained in light of the physiological evidence presented by Dowling (1974) and Kaneko and Shimizaki (1975), who showed that photoreceptor cells are partially depolarized in the dark and release a depolarizing neurotransmitter from their terminals. If it is assumed that transmitter release is mediated by the fusion of photoreceptor synaptic vesicles with the terminal plasma membrane (Matsumura et al., 1981), then this fusion process would be maximal in the dark and minimal in the light. Synaptic vesicle fusion in the dark would create an excess of plasma membrane, which would then have to be removed and recycled. It is now widely accepted that membrane recycling does occur by the process of membrane endocytosis, as first proposed for the neuromuscular junction in 1973 by Heuser and Reese.

It has been observed in frog retinal rods, that under longstanding conditions of dark adaptation, an electron-dense material is found to accumulate in the synaptic clefts. This dense material is not present in the light, however a progressive build-up in the numbers of dense-cored vesicles does occur with continuous exposure to light for 9 days (Monaghan and Osborne, 1975; Osborne and Monaghan, 1976). In addition, prolonged exposure to light for 21 days causes a flattening of synaptic vesicles associated with the synaptic lamellae in rods and cones (Osborne and Monaghan, 1976). However, no reduction in either the number of vesicles present in the synaptic

terminal or the appearance of the synapses was observed in these studies. Osborne and Monaghan (1976) proposed that the appearance of dense-cored vesicles after 9 days of continuous exposure to light was due to a build-up in stores of neurotransmitter, and that after 12 days of continuous light, the photoreceptors underwent a metabolic reorganization which ultimately resulted in the cessation of transmitter synthesis. In the dark, the dense-cored vesicles were never observed, but dense material was seen in the synaptic cleft because, as it was suggested, transmitter was being rapidly released into the synaptic cleft by synaptic vesicles.

When an extracellular tracer such as horseradish peroxidase (HRP) is incubated with a dark adapted retina, HRP reaction product accumulates within the synaptic vesicles of photoreceptor terminals (Ripps et al., 1976; Schacher et al., 1976; Schaeffer and Raviola, 1978; Cooper and McLaughlin, 1977). Such activity is not observed in the light, when photoreceptors are physiologically inactive with respect to transmitter release. This then would seem to suggest that the membrane retrieval process occurs concurrently with synaptic vesicle fusion. Schaeffer and Raviola (1976; 1978) have demonstrated that this membrane retrieval process can be slowed down or stopped by lowering tissue temperature to 4^{0} C. Further evidence that a membrane retrieval process in photoreceptors operates in the dark is illustrated by the finding that the number of coated

vesicles and large membrane cisterns, commonly associated with the process of endocytosis, is greater in dark-adapted cone photoreceptors than in light-adapted cones (Cooper and McLaughlin, 1978; 1979; Schaeffer and Raviola, 1978).

Other changes which have been reported to occur in photoreceptor terminals in response to light and dark are fluctuations in the synaptic area formed with bipolar and horizontal cells (Schaeffer and Raviola, 1978), and variations in the number of photoreceptor synaptic lamellae (Spadero et al., 1978).

Schaeffer and Raviola (1978) have shown that the ratio of synaptic to nonsynaptic surface area is higher in the dark than in the light. However, Schaeffer and Raviola's (1978) definition of "synaptic surface area" included the entire vitreal surface of photoreceptors which are invaginated by postsynaptic endings (ie., specialized synaptic membrane and the nonspecialized areas between). Their measurements are therefore not necessarily an indication of the area of physiologically active synaptic membrane.

A number of studies have attempted to correlate changes in the number and length of photoreceptor synaptic lamellae, with a diurnal cycle in both the pineal gland (Vollrath, 1973, Hewing, 1979; McNulty, 1980; 1981) and retina (Wagner, 1973; Spadero et al., 1978; Grün, 1980; Wagner and Ali, 1977). The number of synaptic lamellae in the cones of fish

retina have been reported to be reduced by 1/3 (Wagner, 1973) and 1/2 (Wagner and Ali, 1977) during the night, however, such variations were not observed in the terminals of rod photoreceptors. Similar findings have been reported in the rat for both rods and cones (Spadaro et al., 1978). The synaptic lamellae in these animals displayed peak numbers at 1800 hrs, just prior to "lights on", and then decreased to a low at 0300 hrs. The length of synaptic lamellae in the photoreceptors of goldfish retina have also been reported to be reduced in the dark (Grün, 1980), however in the albino rat retina synaptic lamellae were reported to be shorter in the light (Spadero et al., 1978). Similar studies on the synaptic lamellae in the photoreceptors of pineal gland show that these organelles are fewer and shorter during the light period (Vollrath, 1973; McNulty, 1981). Therefore, there appears to be a difference between retina and pineal gland with respect to the timing of synaptic lamellae fluctuations during the day-night cycle.

These variations in the number and length of synaptic lamellae have prompted considerable speculation concerning the function of these organelles within photoreceptors. Synaptic lamellae have been suggested to serve as guides for synaptic vesicles, transporting them in "conveyor-belt" fashion to the active site (Bunt, 1971); to function as storage sites for neurotransmitter (Osborne and Thornhill, 1972); and also to play a role in the turnover of the

receptor plasma membrane (King and Dougherty, 1980). A decrease in synaptic-lamellar number and length during the night, when the photoreceptors are most active, is most consistent with the latter two hypotheses, although the functional significance of the synaptic lamellae is not yet certain. Most disconcerting however, is the fact that none of these studies on the rhythms of synaptic lamellae took into consideration the possibility that photoreceptor terminals may increase in size in the dark, a concept first proposed by Cragg in 1969 for synaptic vesicle populations.

Circadian rhythms have also been reported to affect the morphology of mitochondria in the outer plexiform layer of the albino rat retina (Spadaro et al., 1977). Spadero et al. (1977) describe a swelling and loss of electron density in mitochondria during the day, but during the night the mitochondria appear contracted, with parallel cristae and electron-dense matrices. It was suggested that this appearance of mitochondria during the night period is due to the great activity (exocytosis-endocytosis cycle) occuring in the vicinity of the synaptic terminals at this time.

Since most of the changes in photoreceptor ultrastructure that are reported to occur during light and dark adaptation, have been described under extreme light or temperature conditions, several questions still remain. The results of this work on the newt retina show that many of the changes previously reported to occur under conditions of

extreme lighting and temperature, in fact occur naturally and in a cyclic pattern. These studies also reveal some interesting findings concerning the timing of vesicle endocytosis, exocytosis and neurotransmitter synthesis during the natural day-night cycle.

MATERIALS AND METHODS

Newts of the species Notophthalmus viridescens were maintained under cycled light conditions (L:D= 12:12) in an aquarium at 13° C. A median light intensity of 12.5 Lux was provided by two 80 Watt cool white fluorescent lamps positioned 1 m above the water surface. An interval timer was set to turn the lights on at 0930 hrs and off at 2130 hrs. All animals were accustomed to this lighting regime for at least 2 weeks prior to the initiation of the following experiment.

1) Fixation. Beginning at 0800 hrs, 2 animals were removed from the aquarium at selected times for 27 hrs. They were immediately decapitated; their eyes removed, pierced behind the corneal-scleral junction and placed in cold (4° C) fixative (788 mOsM) consisting of 2.0% glutaraldehyde (70% concentrated, Ladd Research Inc.) and 1.0% paraformaldehyde (Fisher Chemicals), in 0.15 M sodium cacodylate buffer with 2.5 mmol $CaCl_2$ and 1% sucrose at pH 7.3. Retinas in the dark phase were dissected with the aid of a Schott KL 150B cold light source fitted with a Schott dark-red filter and were fixed in the dark. After 0.5 hr, the cornea and sclera were removed, the retina was quartered and fixed for an additional 2.5 hrs. The retina was washed for 15 min in 0.15 M sodium cacodylate buffer containing 5% sucrose (pH 7.3) and then osmicated for 2 hrs in 1% aqueous osmium tetroxide. Pieces of retina were next washed briefly in distilled water and stained en bloc with saturated

aqueous uranyl acetate for 1 hr. Dehydration was carried out in a graded alcohol series to propylene oxide, or a graded acetone series, and the tissue was embedded in "Taab" low viscosity resin (Taab Laboratories, Reading, England).

2) Morphometrics.

a) Sectioning: Thin sections of at least one randomly selected block from each animal were cut on an LKB Ultrotome III and stained for 1 min in Reynold's lead citrate (Reynolds, 1963). Approximate section thickness was determined with the aid of a Sorvall interference-colour thickness scale. Sections were always cut parallel to the long axis of the photoreceptor as indicated by the position of the photoreceptor outer segments in 0.5 µm semi-thin sections.

b) Photography: All micrographs were taken on a Zeiss EM10A electron microscope at a consistent magnification which allowed the entire photoreceptor terminal to be included (approximately 20,000X). In a given section, photographs were taken of every photoreceptor terminal in which synapses were visible. A total of 847 synaptic terminals were sampled over 15 time periods (minimum number per time period= 34).

c) Morphometric Calculations: The area, perimeter and form factor of the photoreceptor synaptic terminals, and the length and number of synaptic lamellae appearing in each photoreceptor were measured with the aid of a semi-automatic

image analysis computer (Zeiss Videoplan, Zeiss Canada Ltd., Don Mills, Ontario). Form factor (Zeiss Videoplan Handbook, 1980) was used to describe the degree of invagination of the postsynaptic elements into the photoreceptor terminal by measuring the irregularity of the photoreceptor terminal perimeter.

In addition to these three parameters, which were calculated by computer, synaptic vesicle density and the index of infolding were calculated for a smaller sample size (minimum number of photoreceptor terminals sampled per time period= 12) and at 9 selected time periods.

For the determination of synaptic vesicle density, high magnification photographs of synaptic terminals, in which synaptic vesicles were clearly visible (approximate magnification: 40,000X) were taken. A template containing an engraved 1 µm square (calculated on the basis of the photograph magnification) was placed over each photograph. All synaptic vesicles lying completely within the template were counted and the template was repositioned within each photograph as many times as possible without overlapping previously measured fields. The numerical particle density of the synaptic vesicles was calculated from the formula:

$$Nv = Nat/\bar{D}+T$$ where:

Nv= Numerical Particle Density

Nat= Number of Vesicles per Square Micrometer

$\bar{D}$= Mean Particle Diameter

T= Section Thickness

(Abercrombie, 1946; Henning, 1967, as cited in Weibel and Bolender, 1973).

Because any assumptions about the three-dimensional shape of a photoreceptor terminal could only introduce additional error, all data is expressed in terms of two dimensions. Therefore, numerical particle density calculated above was converted to the number of synaptic vesicles/μm^2 using the formula:

$$(\sqrt[3]{Nv})^2 .$$

The average diameter of synaptic vesicles in each time period was determined by the technique of Henning and Elias (1970).

Index of infolding measurements were made on the smaller sample of synaptic terminals to complement the form factor measurements calculated by computer. These measurements were made using a 5mm overlay test grid and the formula:

$$I = p/P \quad \text{where:}$$

I= Index of Infolding

p= Number of Intersections of Plasma Membrane with Grid Lines

P= Number of Intersections of Unfolded Plasma Membrane with Grid Lines

(Elias et al., 1978).

d) **Statistical Analysis:** Statistical analysis of all data was carried out with the aid of the computer-based statistical program, "Minitab II" (Ryan et al., 1976). Analysis of variance was performed on all data, as were multiple Student's t-tests, where necessary (both at alpha = 0.05). For easy visualization of the raw data's significance 95% confidence intervals of the mean are plotted with the data.

RESULTS

1) The Early Light Period. Rod and cone synaptic terminals were examined at regular intervals over a 27 hr period, beginning at 0800 hrs, where lights "on" occured at 0930 hrs and lights "off" at 2130 hours. Synaptic terminals from samples fixed at 0800, 0920 and 0940 hrs were morphologically indistinguishable from receptor terminals which were fixed at 1030 hrs. At these times, the cone synaptic terminals (Fig. 1A) contained large numbers of synaptic vesicles that averaged 45 nm in diameter. The synaptic vesicle population was homogeneously distributed throughout the cytoplasm, at a density of about 130 vesicles/μm^2. Few profiles of smooth endoplasmic reticulum or large clear vesicles were observed in terminals of cone photoreceptors at these times. The postsynaptic elements were seen to invaginate the cone terminal centrally, and synaptic lamellae were found in the vicinity of the invaginating postsynaptic elements.

The rod synaptic terminals at 1030 hrs contained a homogenous population of synaptic vesicles that averaged 54 nm in diameter (Fig. 1B). Large electron-lucent vesicles, possibly profiles of smooth endoplasmic reticulum, measuring up to 90 nm in diameter were more frequently observed in rod terminals than in the cone terminals (Fig. 1B). The postsynaptic elements of rod synaptic terminals invaginated laterally, where synaptic lamellae were usually located. An

electron dense material (Fig. 2) was often seen in the synaptic cleft of rod terminals, but was never observed in the synaptic cleft of the cone photoreceptors.

2) The Late Light Period. Few changes were noticeable in the ultrastructure of the photoreceptor synaptic terminals through to 1930 hrs, prior to the beginning of the night cycle at 2130 hrs. Synaptic vesicle density, as well as photoreceptor terminal area and general morphology remained the same throughout the day. The only observable change to occur during the light period was the increased frequency of dense-cored vesicles which were found in the vicinity of the synaptic lamellae (Fig. 3). These vesicles increased in number as the light period progressed, and were found to be most numerous in the 1930 hr sample. However, dense-cored vesicles were not observed in the synaptic terminals of the 2130 hr sample, which was fixed just minutes after the end of the light period.

There was no observed change in the amount of electron-dense material found in the rod synaptic clefts, nor was there any difference in the number of large clear vesicles from 1030 to 1930 hrs.

3) The Early Dark Period. The morphology of the photoreceptor synaptic terminal changed little from 2130 to 0400 hrs. The synaptic-vesicle density and photoreceptor terminal area did not vary significantly from the previously sampled time periods. However, the dense material which was

observed in rod synaptic clefts during the day (Fig. 3) was observed to increase in the 2130 and 2400 hr samples (Fig. 4); later in the dark period at 0400 hrs, the amount of electron-dense material in the synaptic cleft was reduced to the levels seen during the day.

At 2400 hrs there was a significant increase in the number of synaptic lamellae within the receptor cell terminals (Fig. 12A), together with a decrease in their average length (Fig. 12B). There was no significant variation in either the number synaptic lamellae or their length, at any other time of the day. These quantitative observations reflect the preponderance of multiple arrays of short synaptic lamellae, which could be observed at 2400 hrs (Fig. 5). These arrays of lamellae were located in the middle of the photoreceptor terminal, away from the synaptic site at the base of the terminal where they are usually located.

Although the number of dense-cored vesicles which were seen in the cytoplasm of synaptic terminals throughout the light period was found to decrease as the dark period progressed, large clear vesicles increased in number during the dark phase, from approximately $1/\mu m^2$ at 1930 hrs, to approximately $5/\mu m^2$ at 0400 hrs. These large clear vesicles were always more numerous in the cytoplasm of rod terminals. Profiles of smooth endoplasmic reticulum were also more obvious at 0400 hrs (Fig. 6A and 6B). It is possible that the large clear vesicles represent cross sections through

the tubular network of smooth endoplasmic reticulum which is abundant at this time.

4) The Late Dark Period. The most striking alteration in photoreceptor morphology was seen to occur just prior to the lights coming on, at 0730 hrs (Fig. 7A and 7B). Although the cytoplasm of both rod and cone synaptic terminals appeared to contain fewer synaptic vesicles at this time, when synaptic terminal areas were calculated, a great increase in terminal area was found to have occured as well (compare Figs. 1 and 7).

a) Qualitative Analysis of Membrane Pools. To more appropriately describe the morphology of photoreceptor terminals at 0730 hrs, the total membrane in the two major membrane pools of the synaptic terminals (cell plasma membrane and synaptic vesicle membrane) was measured and compared with similar calculations made at other times of the day. Although it appears from electron micrographs that there are fewer synaptic vesicles in photoreceptor terminals at 0730 hrs (Fig. 7), when the number of synaptic vesicles/μm^2 (Fig. 8A) is multiplied by the area of the photoreceptor terminal (Fig. 8B), the actual number of synaptic vesicles per photoreceptor is not significantly different (98% confidence level) from the number of vesicles present at 0400 hrs (Fig. 8C). Nevertheless, there appears to be a gradual decrease in the mean number of synaptic vesicles per photoreceptor terminal as the day progresses.

Finally, the total amount of membrane in the synaptic-vesicle pool at different times of the day (Fig. 8E) was calculated by multiplying the number of synaptic vesicles in a photoreceptor terminal profile (Fig. 8C) by the average circumference of the synaptic vesicles (Circumference= pi x average diameter of synaptic vesicles; Fig. 8D). Although there was no significant difference (98% confidence level) in the synaptic vesicle membrane pool between any of the times during the 24 hr day, it is apparent that there was less membrane associated with synaptic vesicles during the night than during the day (Fig. 8E).

Total membrane due to the plasma membrane at different times of the 24 hr day was determined directly from Videoplan computer measurements of cell perimeters (Fig. 9). Figure 9 indicates that there is no statistically significant variation (tested by one-way analysis of variance) in the amount of membrane in the plasma membrane pool during the day-night cycle. This result was not expected, considering the slight loss of membrane from the synaptic-vesicle pool during the night (Fig. 8E) and especially the great increase in photoreceptor terminal area observed at 0730 hrs (Fig. 8B).

The total amount of membrane in each photoreceptor synaptic terminal at different times of the day (Fig. 10) was calculated by adding the synaptic-vesicle membrane pool (Fig. 8E) to the plasmalemma membrane pool (Fig. 9). If it

is assumed that exocytosis due to synaptic-vesicle fusion with the plasmalemma in the dark is concomitant with endocytosis, which is required for removal of membrane from the plasma membrane and the recycling of synaptic-vesicle membrane, then there should not be any net loss or gain of membrane by the photoreceptor terminal. If this is true, then the amount which the total membrane varies from the mean at different times of the day is the total error made in the measurements of cell perimeter, synaptic-vesicle diameter, number of synaptic vesicles per unit area, and the area of synaptic-terminal profiles. The error, expressed as percent fluctuation of membrane from the mean, is shown above each data point in Fig. 10. It is interesting to note that there is a progressive decrease in total photoreceptor membrane as the 24 hr cycle progresses, and that this is due almost entirely to the influence of the synaptic-vesicle membrane pool which accounts for about 75% of the total synaptic-terminal membrane. If the synaptic-terminal perimeter does not change significantly throughout the day (Fig. 9), then a twofold increase in synaptic-terminal area must be accounted for in some other way.

Computer calculated form factor measurements (Fig. 11A) indicate that the synaptic terminal shape is "more regular" at 0730 hrs. Index of infolding measurements (Fig. 11B) also indicate that the photoreceptor plasma membrane is significantly less folded at 0730 hrs. This unfolding of the plasma membrane which appears to occur at 0730 hrs could

account for the great increase in terminal area which is seen at this time, and which is not accompanied by a concomitant increase in terminal perimeter.

b) Synaptic Lamellae. Neither the number of synaptic lamellae per photoreceptor terminal (Fig. 12A) nor the average length of synaptic lamellae per photoreceptor terminal (Fig. 12B) were significantly different at 0730 hrs, from any other time of the day.

c) Qualitative Observations at 0730 hrs. Another significant change which occured at 0730 hrs was not in the photoreceptor terminal itself, but in the mitochondria of the Müller cells which are found in close association with the photoreceptor terminals. At most times sampled, the Müller cell mitochondria had an electron-lucent matrix with few dense granules (Fig. 13A). At 0730 hrs however, the glial mitochondria had an electron-dense matrix and the number of granules within the mitochondria was markedly increased (Fig. 13B).

The volume increase in synaptic terminals at 0730 hrs and the broad spacing of the synaptic vesicles within the terminal at this time, permit some unique observations of the synaptic terminal substructure (Fig. 14). The widely spaced synaptic vesicles are suspended in a fibrillar cytoskeleton (for review see Porter, 1978) which is not visible at any other time of the day, possibly because of the dense packing of the synaptic vesicles at other times.

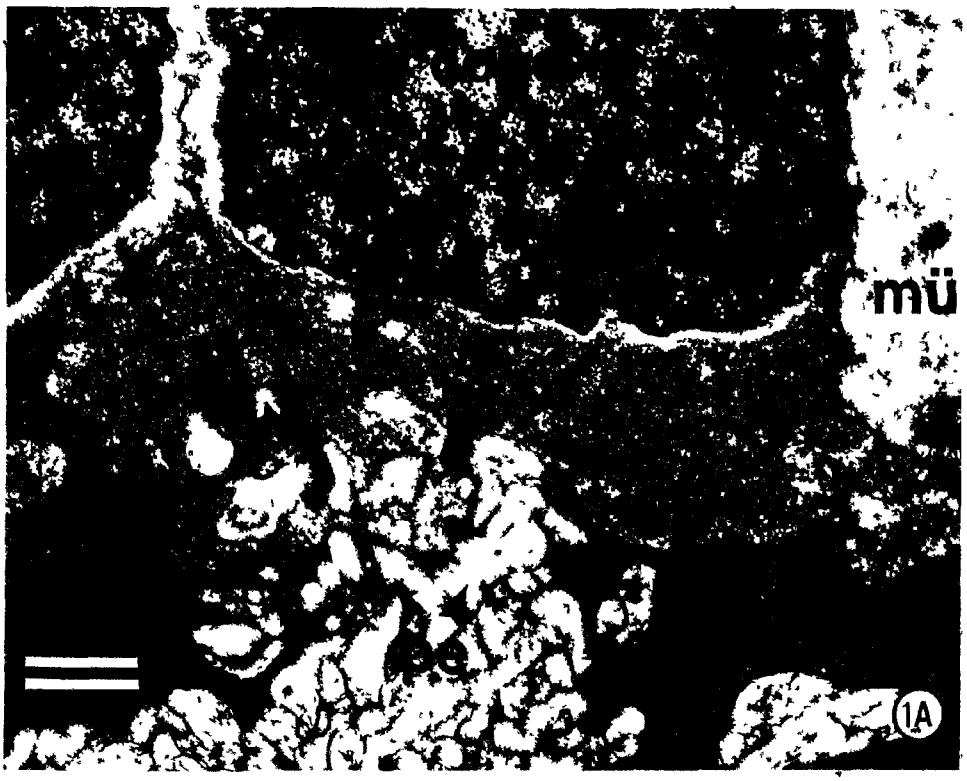
Synaptic vesicles, which were associated with synaptic lamellae, were always densely packed and appeared to be attached to the synaptic lamellae by a fibrillar substance, not unlike the material making up the cytoskeleton. Large clear vesicles, which were seen earlier in the dark period, were also seen in the photoreceptor terminal at 0730 hrs. Finger-like invaginations from adjacent photoreceptors and postsynaptic elements were frequently observed in cross section, deep within the photoreceptor terminal. Coated vesicles were frequently observed budding from the plasma membrane around these invaginations and also from the lateral photoreceptor plasma membrane. Coated vesicles were observed around the entire photoreceptor perimeter at 0730 hrs, and it was not difficult to catch every sequence of endocytosis in one photoreceptor terminal at this time (Fig. 15), indicating that endocytosis was probably occuring at a very rapid rate.

Vesicles were also frequently observed budding from, or fusing with the smooth endoplasmic reticulum in photoreceptor terminals at 0730 hrs (Fig. 16). An electron opaque material of the same density as that in the synaptic vesicles, was seen in the lumen of the smooth endoplasmic reticulum. In addition to endocytosis (as identified by vesicles posessing a clathrin coat; Heuser and Evans, 1980), fusion of synaptic vesicles with the plasma membrane, which are probably exocytotic events, were evident at 0730 hrs as well (Fig. 17).

After the lights were turned on at 0930 hrs, samples taken at 0940 and 1030 appeared morphologically similar to the samples taken at 0940 and 1030 the previous morning. No significant difference was noted in the number of synaptic vesicles per photoreceptor terminal, synaptic terminal perimeter, synaptic terminal area, or in the number and length of synaptic lamellae, from samples taken the previous morning.

FIGURES

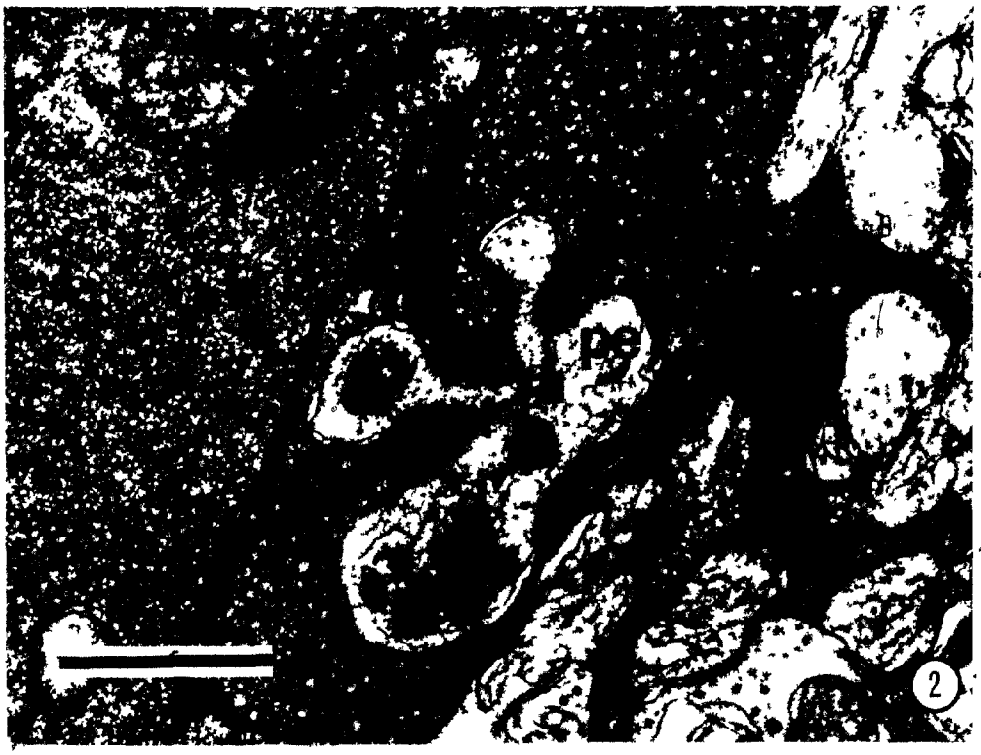
Figure 1. (A and B) Micrographs showing the morphology of cone (A) and rod (B) synaptic terminals at 1030 hrs. Note that the postsynaptic elements (pe) of the cone invaginate into the receptor terminal and synapse centrally, while the postsynaptic elements (pe) of the rod make synaptic contact laterally. The cytoplasm of the rod synaptic terminal is denser than that of the cone and the rod synaptic vesicles are slightly larger when aldehyde fixed. The cytoplasm of an adjacent Müller cell is indicated (Mü). sl= synaptic lamellae, bar= 1.0 µm


mü
1A


1B

Figure 2. Electron micrograph of the synaptic region of a rod photoreceptor sampled at 1030 hrs. Synaptic vesicles measuring 54 nm in diameter completely fill the cytoplasm of the synaptic terminal. Three synaptic lamellae (sl) are shown directed towards the postsynaptic elements (pe). A dense substance (d) is shown in the synaptic clefts lateral to the arciform density (a). bar= 0.5 µm

Figure 3. Electron micrograph of the synaptic region of a rod photoreceptor at the end of the light cycle (1930 hrs). Numerous dense-cored vesicles (arrows) have accumulated near the synaptic lamellae. A small amount of dense substance (d) is located in the cleft between the photoreceptor terminal and the postsynaptic elements (pe). a= arciform density, bar= 0.25 µm


Pe
2

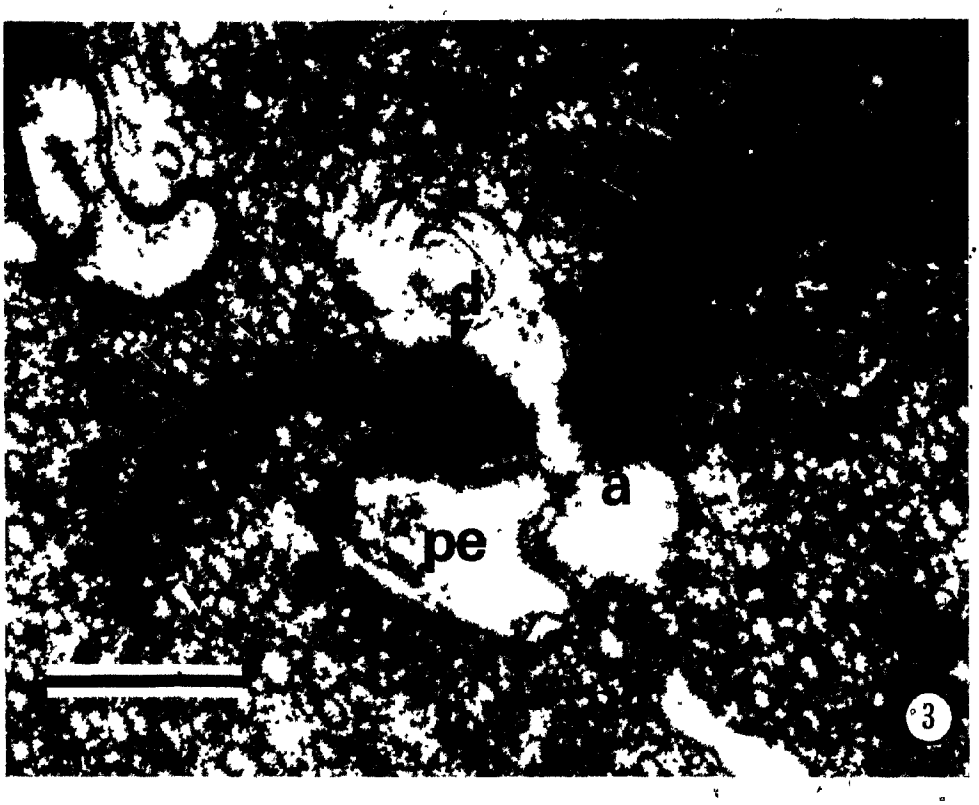

a
pe
3

Figure 4. Electron micrograph showing the synaptic region of a rod photoreceptor at 2400 hrs. The amount of dense material (d) observed in rod synaptic clefts has increased considerably at this time (compare with Figs. 2 and 3). Fusion of synaptic vesicles (v) with the plasma membrane can be frequently observed at this time as well. sl= synaptic lamellae, pse= postsynaptic elements, bar= 0.25 µm

Figure 5. Electron micrograph of a cone synaptic terminal at 2400 hrs showing an array of three short synaptic lamellae (sl). "Ribbon Fields" such as these were frequently observed some distance from the synapse. n= photoreceptor nucleus, bar= 0.5 µm

n²

Figure 6. (A and B) Electron micrographs of the cytoplasm of cone (Fig. 4A) and rod (Fig. 4B) synaptic terminals at 0400 hrs. Large clear vesicles (lcv) were frequently observed in the rod terminal at this time. Profiles of smooth endoplasmic reticulum (ser) were also most prominent at this time. bar= 0.25 μm

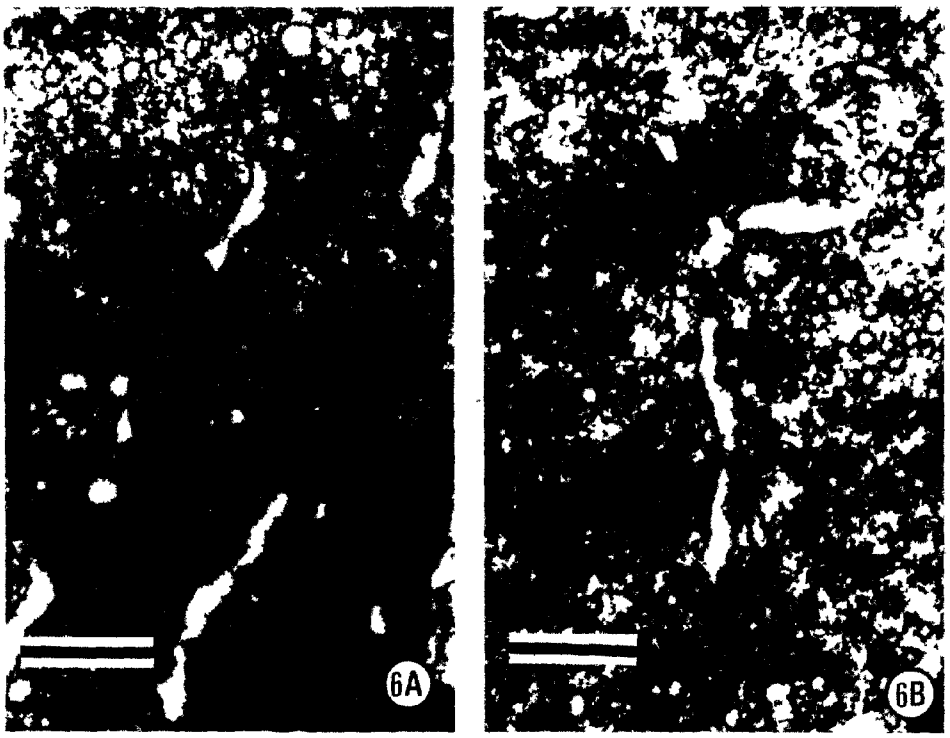
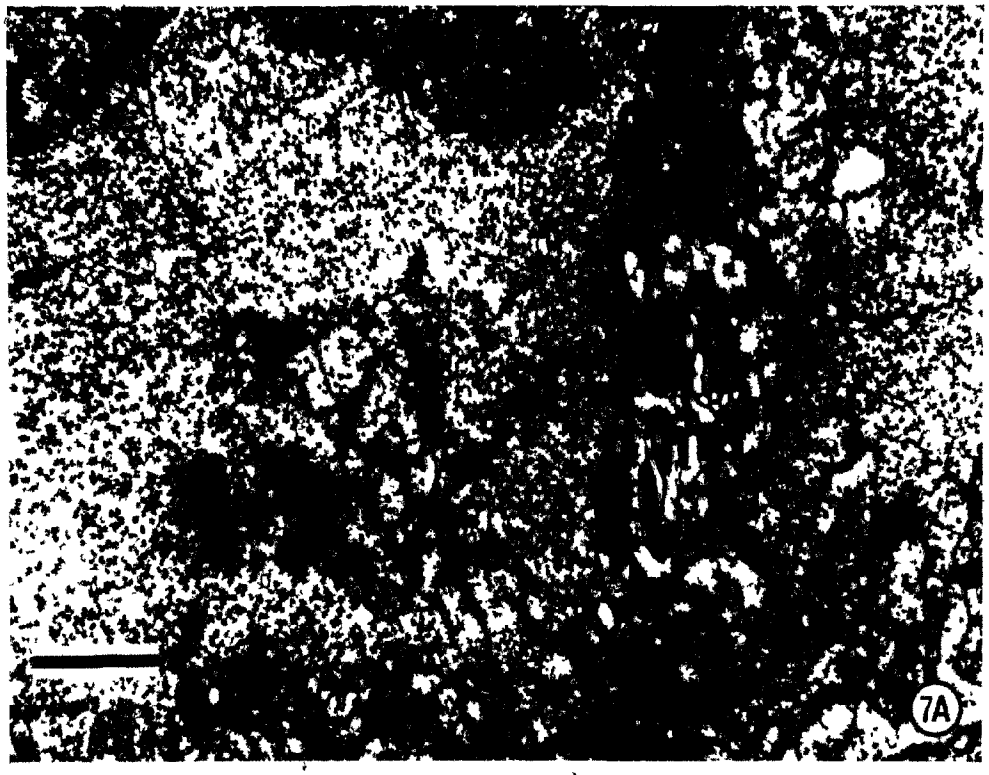
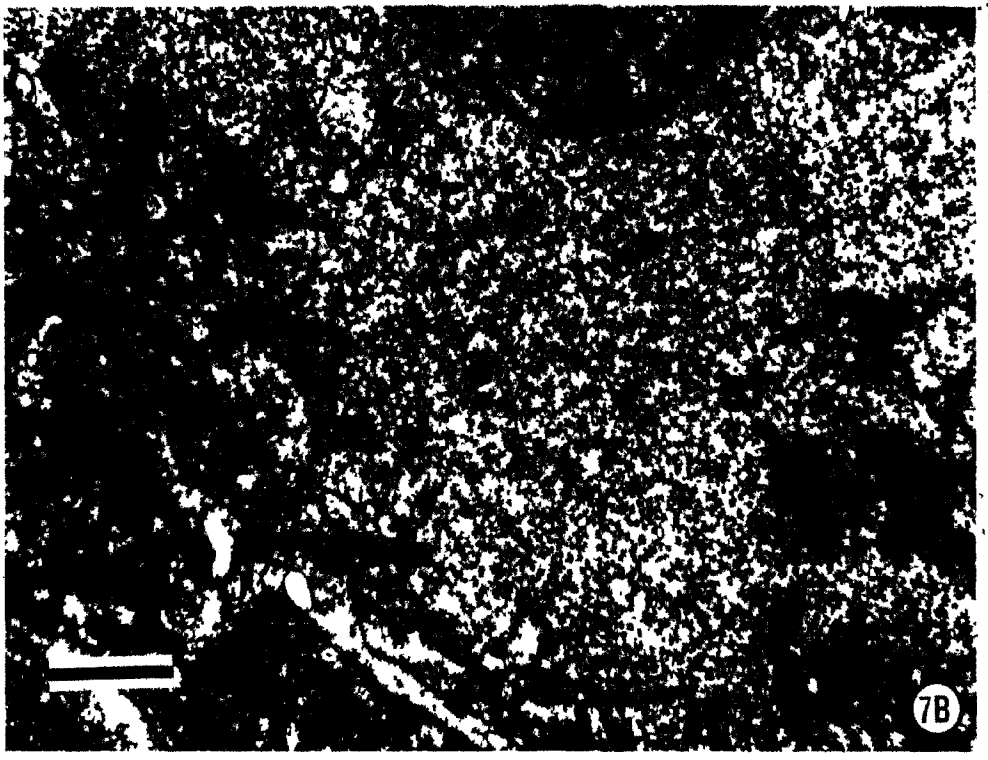

6A
6B

Figure 7. (A and B) Electron micrographs showing the morphology of cone (A) and rod (B) synaptic terminals at 0730 hrs. Note the great increase in synaptic terminal area and the apparent decrease in the number of synaptic vesicles which occurs at this time (compare with Fig. 1A and 1B). The density of the cytoplasm at this time is considerably less than at any other time of the day. sl= synaptic lamellae, pe= postsynaptic elements, i= deep invagination of postsynaptic element, mü= cytoplasm of an adjacent Müller cell, bar= 1.0 µm


7A


7B

density as the day progresses. But this decrease is not nearly as marked as when the numbers of synaptic vesicles per square micrometer are not corrected for synaptic terminal area.

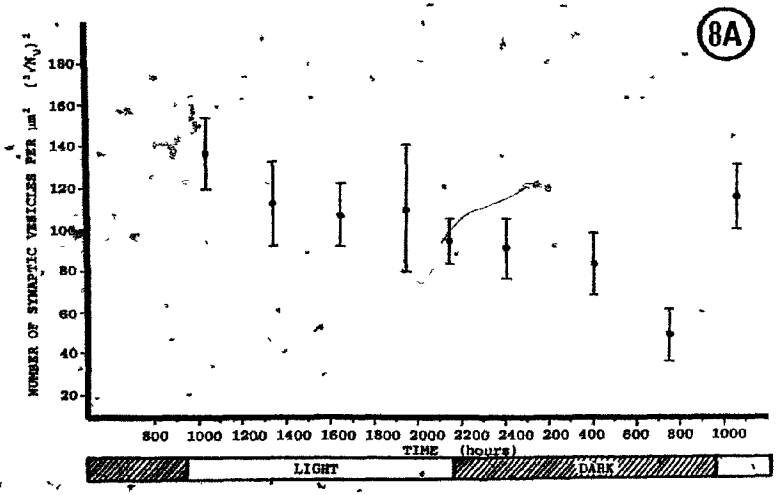
Fig. 8D. This graph shows the variation in the circumference of photoreceptor synaptic vesicles which occured at nine selected time periods over a 27 hr interval. There is no statistically significant difference in the size of synaptic vesicles at any time of the day.

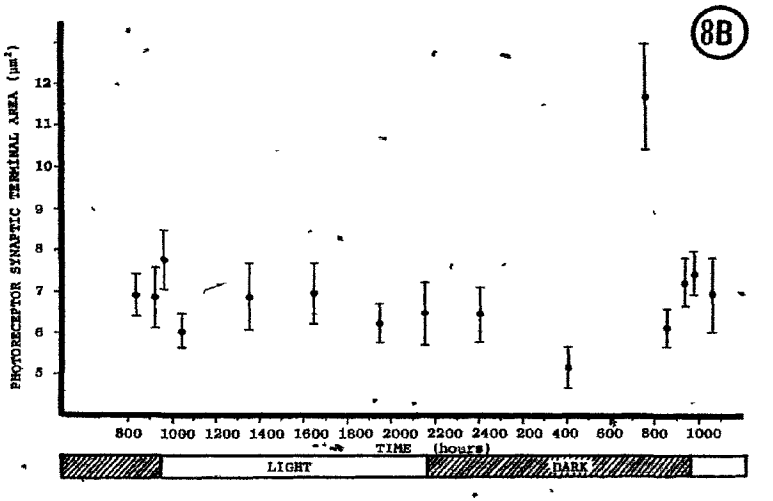
Figure 8. (A to E) This series of graphs quantify the changes which occur in the photoreceptor synaptic vesicle membrane pool over a 27 hr period (bars= 95% confidence interval of the mean).

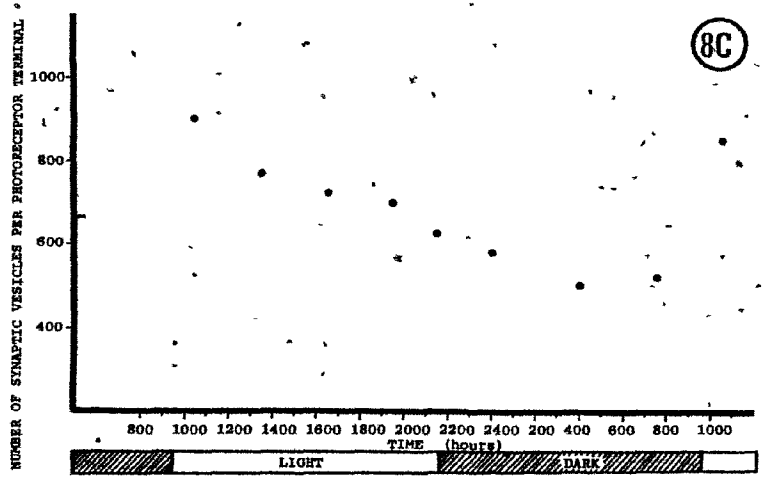
Fig. 8A. This graph shows the variation which occured in synaptic vesicle density at 9 selected time periods over a 27 hr interval. There is no statistically significant difference in the number of synaptic vesicles/μm^2 at any time of the day except at 0730 hrs, at which time there are approximately half as many synaptic vesicles/μm^2 as at any other time of the day.

Fig. 8B. This graph shows the variation which occured in the area of photoreceptor synaptic terminals at 15 selected time periods over a 27 hr interval. There is no statistically significant difference in the area of photoreceptor synaptic terminals, except at 0730 hrs, at which time the synaptic terminal profiles nearly doubled in area.

Fig. 8C. To determine if the number of synaptic vesicles per photoreceptor synaptic terminal varied over a 27 hr period, the number of synaptic vesicles/μm^2 (Fig. 8A) was multiplied by the number of square µm per synaptic terminal (Fig. 8B). Although there is no statistically significant difference (98% confidence level) in the number of synaptic vesicles per photoreceptor terminal at any time of the day, there is a slight decrease in synaptic vesicle


8A
NUMBER OF SYNAPTIC VESICLES PER μm² (¹/₆)²
180
160
140
120
100
80
60
40
20
800 1000 1200 1400 1600 1800 2000 2200 2400 200 400 600 800 1000
TIME (hours)
LIGHT
DARK


8B
PHOTORECEPTOR SYNAPTIC TERMINAL AREA (μm²)
12
11
10
9
8
7
6
5
800 1000 1200 1400 1600 1800 2000 2200 2400 200 400 600 800 1000
TIME (hours)
LIGHT
DARK


8C
NUMBER OF SYNAPTIC VESICLES PER PHOTORECEPTOR TERMINAL
1000
800
600
400
800 1000 1200 1400 1600 1800 2000 2200 2400 200 400 600 800 1000
TIME (hours)
LIGHT
DARK

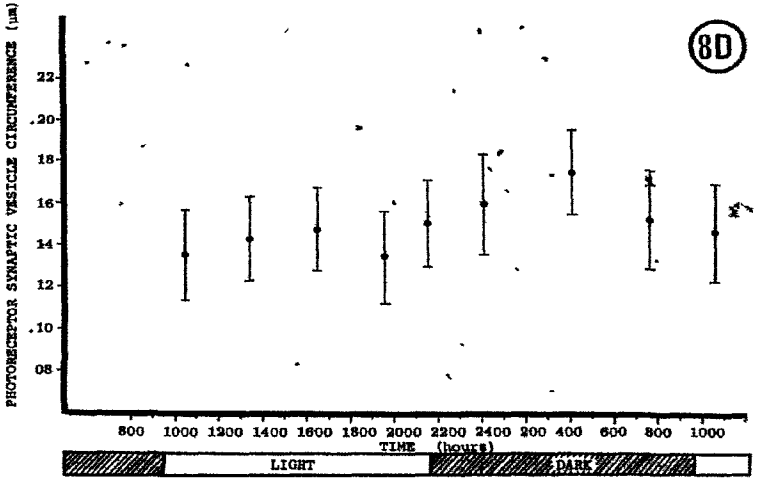
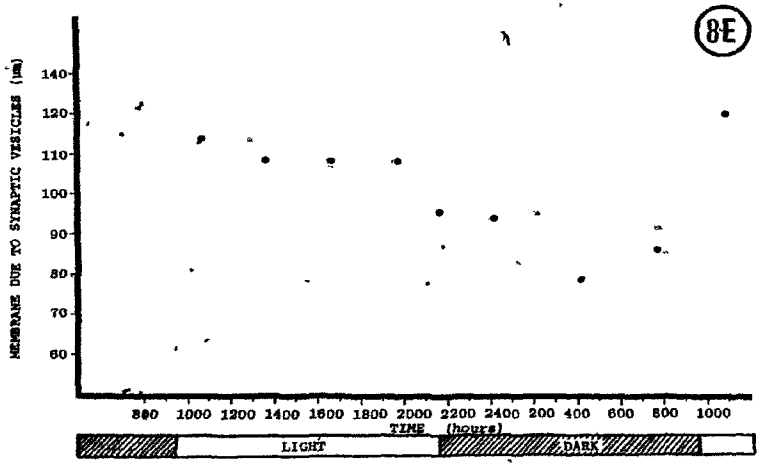
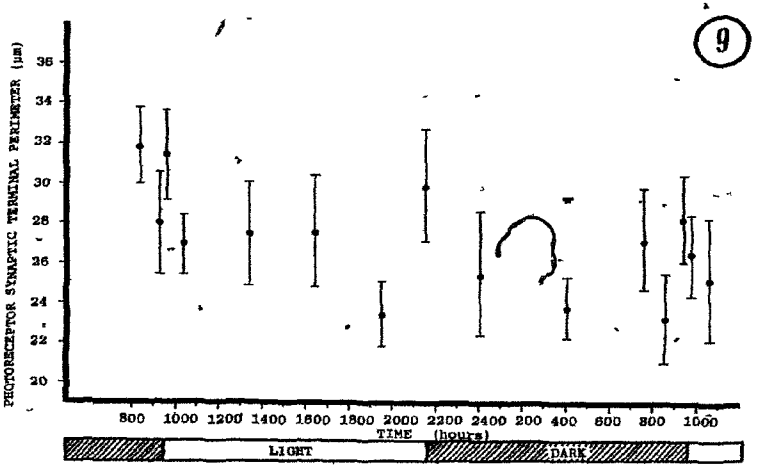

8D
PHOTORECEPTOR SYNAPTIC VESICLE CIRCUMFERENCE (μm)
22
20
18
16
14
12
10
08
800 1000 1200 1400 1600 1800 2000 2200 2400 200 400 600 800 1000
TIME (hours)
LIGHT
DARK

Fig. 8E. To calculate the amount of membrane in the synaptic vesicle pool, the number of synaptic vesicles per photoreceptor terminal profile (Fig. 8C) was multiplied by the circumference of synaptic vesicles (Fig. 8D). This graph shows that there is a slight drop in the amount of membrane due to synaptic vesicles during the night.

Figure 9. This graph shows the variation which occured in the membrane pool due to the plasma membrane (photoreceptor perimeter) at 15 selected time periods over a 27 hr interval. The graph shows that there is no statistically significant variation in the size of photoreceptor synaptic terminal profile perimeters throughout the day. (bars= 95% confidence Interval of the mean)

Figure 10. This graph shows the variation in total photoreceptor synaptic terminal membrane (plasma membrane pool + synaptic vesicle membrane pool; Fig. 8E + Fig. 9) which occurs at 9 selected time periods over a 27 hr period. If it is assumed that the only major exchange of membrane occurs between these two pools, and that the total membrane pool does not change, then the amount by which each point varies from the mean is the error made in the measurements and caculations of total membrane in these two pools.


8E
MEMBRANE DUE TO SYNAPTIC VESICLES (μm)
140
120
110
100
90
80
70
60
800 1000 1200 1400 1600 1800 2000 2200 2400 200 400 600 800 1000
TIME (hours)
LIGHT
DARK


9
PHOTORECEPTOR SYNAPTIC TERMINAL PERIMETER (μm)
36
34
32
30
28
26
24
22
20
800 1000 1200 1400 1600 1800 2000 2200 2400 200 400 600 800 1000
TIME (hours)
LIGHT
DARK

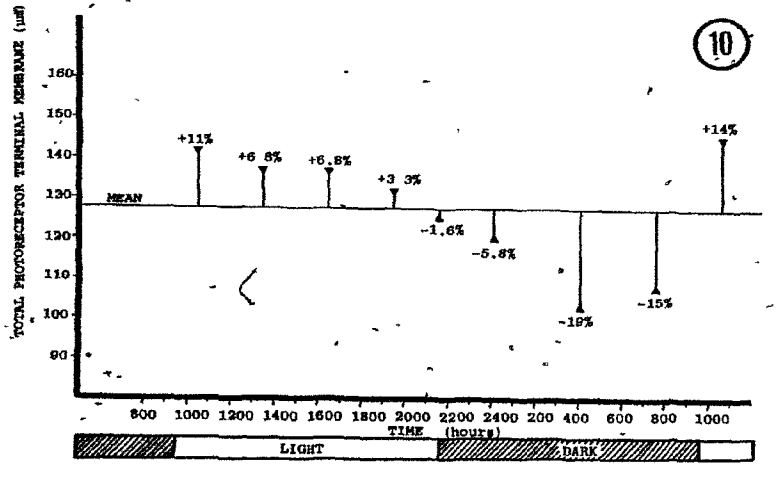
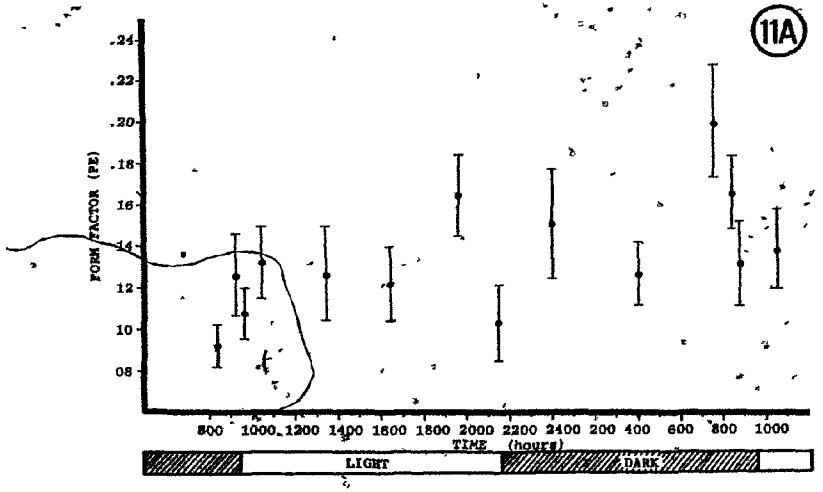
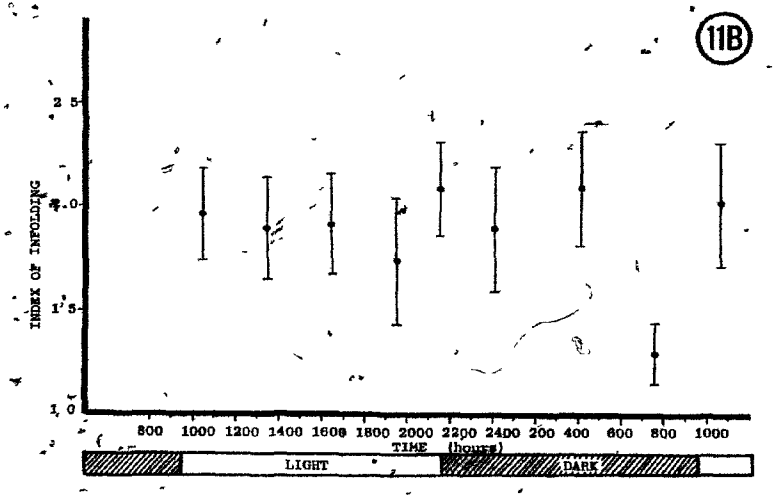
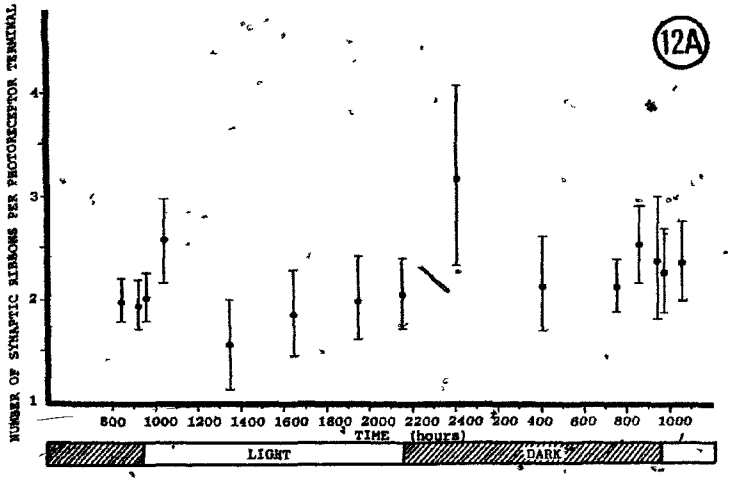

10
TOTAL PHOTORECEPTOR TERMINAL MEMBRANE (μm)
160
150
140
130
120
110
100
90
MEAN
+11%
+6.8%
+6.8%
+3.3%
-1.6%
-5.8%
-19%
-15%
+14%
800 1000 1200 1400 1600 1800 2000 2200 2400 200 400 600 800 1000
TIME (hours)
LIGHT
DARK

Figure 11. (A and B) Since there is no change in the photoreceptor perimeter throughout the day (Fig. 9) addition of membrane to the plasmalemma cannot account for the increase in synaptic terminal area which is observed at 0730 hrs (Fig. 8B). The increase in photoreceptor synaptic terminal area which is observed at 0730 hrs must therefore be due to a decrease in the infolding of the plasma membrane at this time. Fig. 11A is a graph showing how "regular" synaptic terminal profiles are at 15 selected time periods over a 27 hr interval. This graph indicates that synaptic terminal profiles are more regular at 0730 hrs than at any other time of the day. Fig. 11B is a graph showing how "folded" the synaptic terminal profiles are at 9 selected time periods over a 27 hr interval. The graph shows that synaptic terminal profiles are much less infolded at 0730 hrs than at any other time of the day.

Figure 12. (A and B). These graphs show the variation in synaptic-lamellae number (Fig. 12A) and length (Fig. 12B) per photoreceptor synaptic terminal at 15 selected time periods over a 27 hr interval. There is no statistically significant variation in the number or length of synaptic lamellae per photoreceptor terminal, except at 2400 hrs, when they are shorter and more numerous.


11A
FORM FACTOR (FE)
.24
.22
.20
.18
16
14
12
10
08
800 1000 1200 1400 1600 1800 2000 2200 2400 200 400 600 800 1000
TIME (hours)
LIGHT
DARK


11B
INDEX OF INFOLDING
2.5
2.0
1.5
1.0
800 1000 1200 1400 1600 1800 2000 2200 2400 200 400 600 800 1000
TIME (hours)
LIGHT
DARK


12A
NUMBER OF SYNAPTIC RIBBONS PER PHOTORECEPTOR TERMINAL
4
3
2
1
800 1000 1200 1400 1600 1800 2000 2200 2400 200 400 600 800 1000
TIME (hours)
LIGHT
DARK

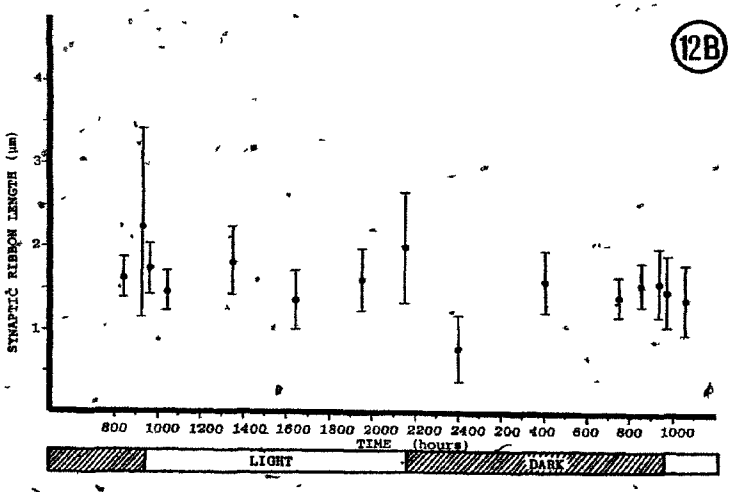

12B
SYNAPTIC RIBBON LENGTH (µm)
4
3
2
1
800 1000 1200 1400 1600 1800 2000 2200 2400 200 400 600 800 1000
TIME (hours)
LIGHT
DARK

Figure 13. (A and B) Electron micrographs of glial (Müller) cell cytoplasm (mu) adjacent to photoreceptor terminals. Fig. 13A shows that the mitochondria (m) of the glia cells are usually in a conventional configuration (light matrix, few granules). Fig. 13B shows the Müller cell mitochondria in a condensed configuration in a 0730 hr sample (dense matrix, increased number of matrix granules) which is indicative of increased energy production by the mitochondria at this time. c= matrix granules, bar= 0.5 µm

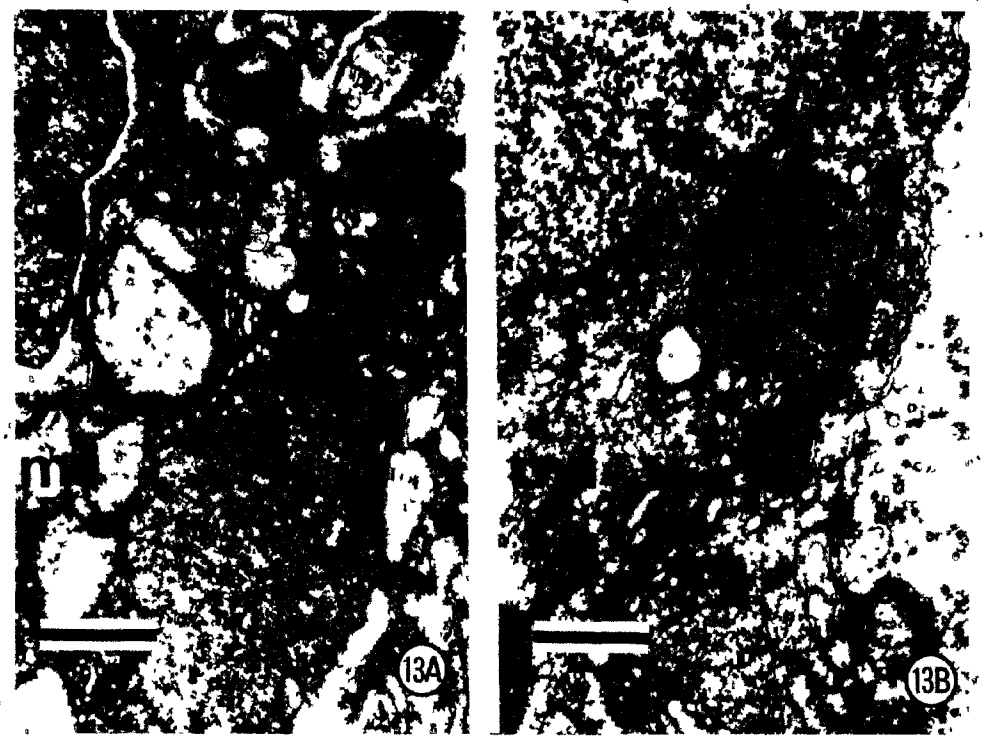

13A
13B

Figure 14. Electron micrograph of a newt photoreceptor synaptic terminal which was fixed at 0730 hrs. It is apparent that there are fewer synaptic vesicles per unit area of photoreceptor terminal. The synaptic vesicles are suspended in a cytoskeleton, as are large clear vesicles (lcv) and profiles of smooth endoplasmic reticulum (ser). Although there are few synaptic vesicles in the surrounding cytoplasm, a full compliment of synaptic vesicles are associated with the synaptic lamellae (sl). Coated vesicles (cv) can be seen in the cytoplasm, budding from the plasma membrane (ppm), and from the deep invaginations of other photoreceptor cells and postsynaptic elements (i).
bar= 0.5 µm

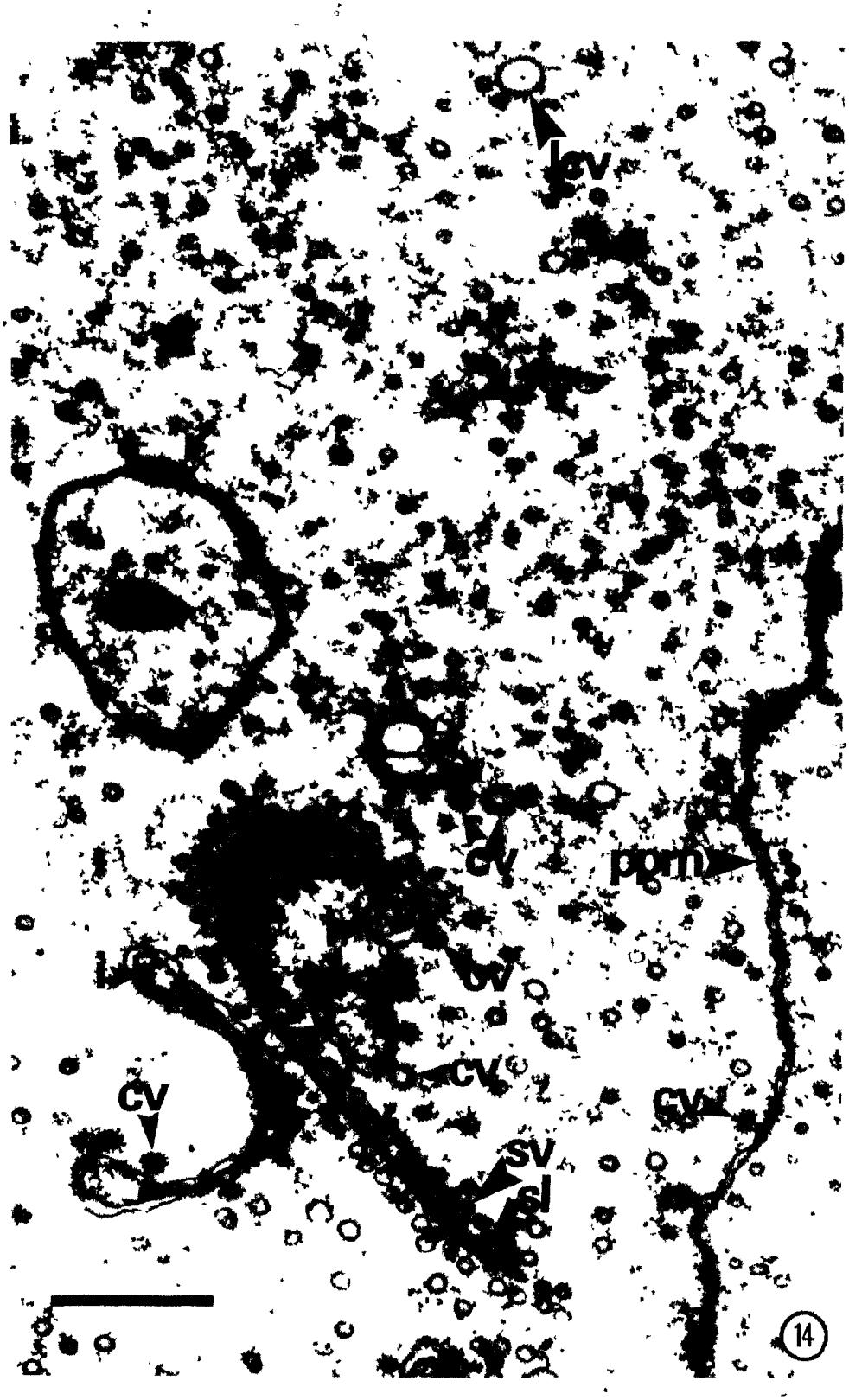

lev
cv
ppm
cv
cv
cv
cv
sv
sl
14

Figure 15. (A to F) A series of high power electron micrographs taken from a photoreceptor synaptic terminal which was fixed at 0730 hrs, showing synaptic vesicle endocytosis. The process begins with a slight invagination of the plasma membrane after the aggregation of clathrin protein molecules (arrows) on the cytoplasmic surface. This invagination continues until a vesicle is pinched off from the plasma membrane (ppm) and a coated vesicle is formed (cv). Such profiles were commonly observed at 0730 hrs, indicating that endocytosis is occuring at a vary rapid rate. bar= 50 nm

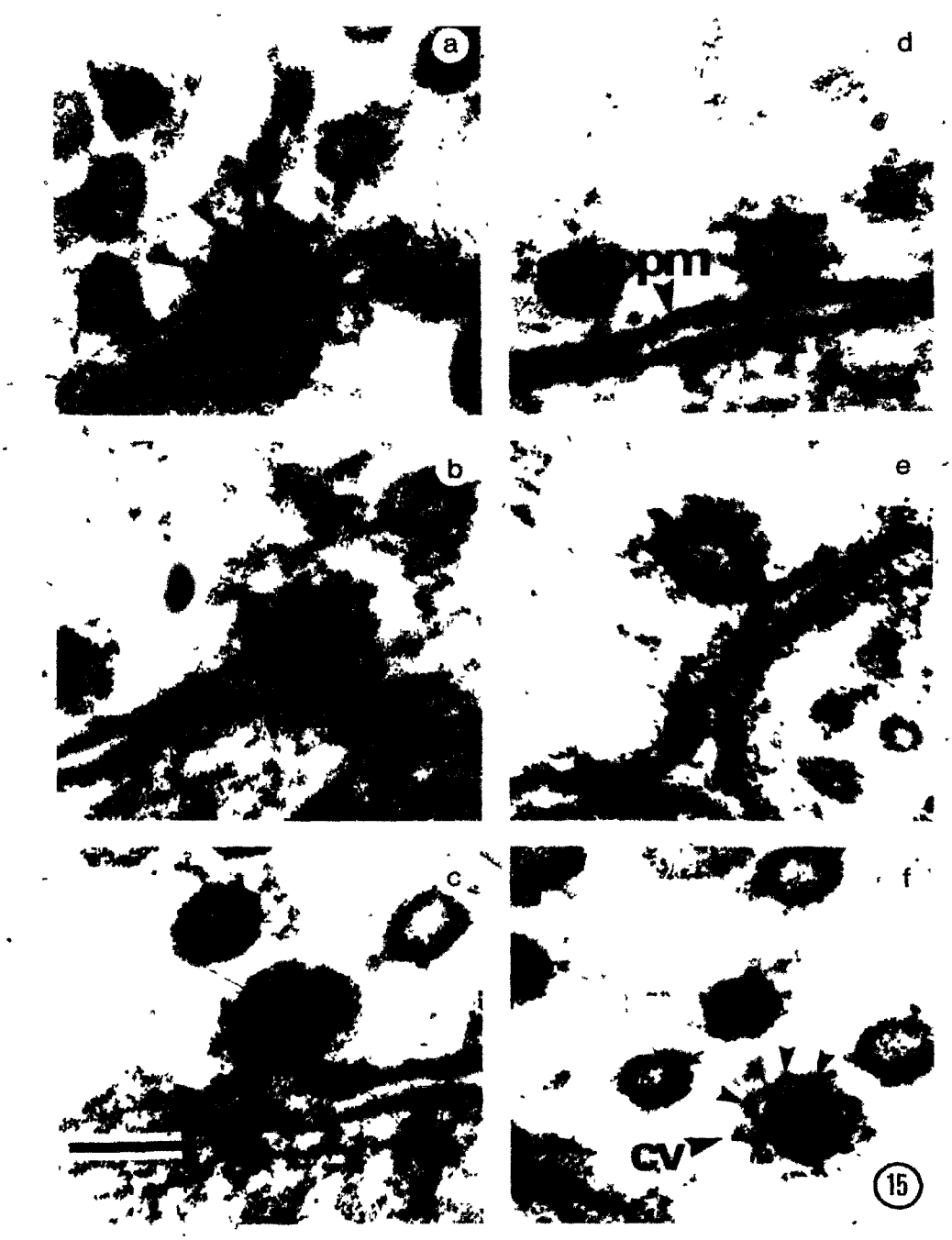
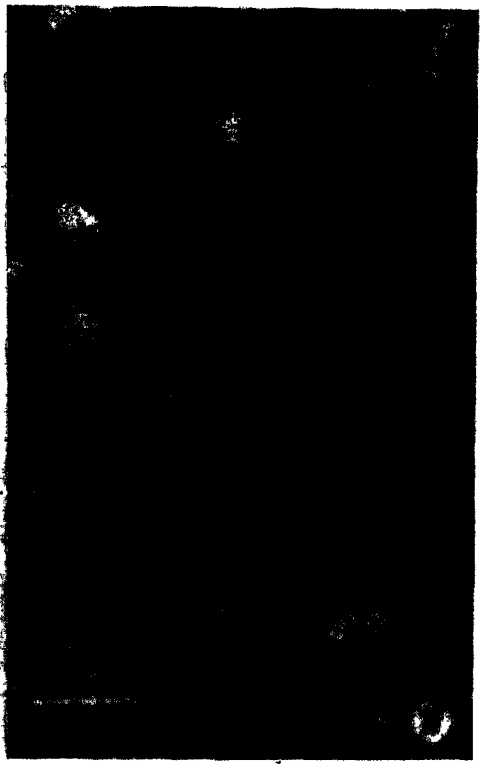
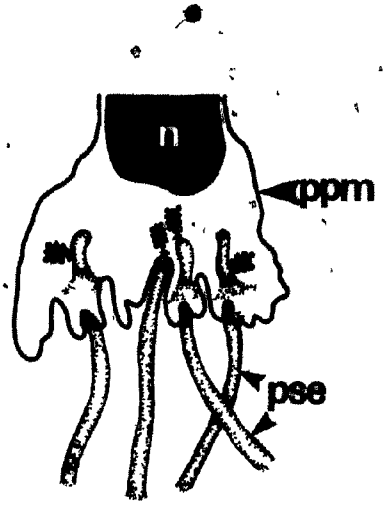

a
d
ppm
b
e
c
f
CV
15

Figure 16. Electron micrograph of photoreceptor synaptic terminal cytoplasm which was fixed at 0730 hrs, showing the fusion of vesicles (v) with, or the budding of vesicles from, a profile of smooth endoplasmic reticulum (ser). Note that a dense material (d), which has the same density as that found in synaptic vesicles (sv), is found inside these vesicles (v) and within the lumen of the smooth endoplasmic reticulum. bar= 0.1 um

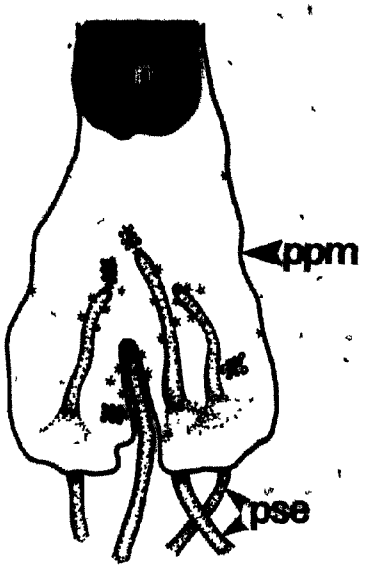
Figure 17. High power electron micrograph taken in the synaptic region of a photoreceptor terminal which was fixed at 0730 hrs. Fusion of a synaptic vesicle (sv) with the plasma membrane (ppm) is shown. Note the irregularity of the plasma membrane which suggests that exocytosis is occuring very rapidly at this site. bar= 50 nm

Figure 18. Schematic drawing of photoreceptor terminals which are representative of photoreceptor synaptic terminals observed at 1030 hrs and 0730 hrs. The drawing which is representative of terminals at 1030 hrs has an area of 1624 mm and a perimeter of 319 mm. If this drawing's perimeter is unfolded, without increasing its length, the area doubles and becomes representative of the appearance of synaptic terminals at 0730 hrs.





TIME = 1030 hours
AREA = 1624 mm^2
PERIMETER = 319 mm
FORM FACTOR = 0.20



TIME = 0730 hours
AREA = 3392 mm^2
PERIMETER = 319 mm
FORM FACTOR = 0.42

DISCUSSION

Any interpretation of synaptic activity from morphological experiments in which chemical fixatives, are used, is fraught with uncertainty. Modern fixatives, which give morphologically pleasing results, almost always contain ions such as sodium and calcium which are known to affect synaptic activity physiologically. It has been recently shown that low concentrations of aldehydes, especially formaldehyde, affect synaptic activity in an unpredictable manner, even more so than certain monovalent and divalent cations (Smith and Reese, 1980). Although rapid freezing of tissue is the prefered preservation technique for the morphological analysis of synaptic activity (Heuser, 1978), only the superficial 10 to 50 µm of tissue is adequately preserved. To date, aldehyde fixatives yield the best preservation of tissues, but it should be realized that these chemicals may impart their own characteristics to cellular ultrastructure, making interpretations about physiological activity from ultrastructural appearance alone, difficult. It is nevertheless valid to discuss these results in the light of previous, similar, studies.

Another possible source of error is the fact that although animals were fixed in the dark, removal of the eyes and dissection was carried out under dim red light. It is known that neural adaptation to light occurs very quickly, within at most, a few minutes (Dowling, 1967). Therefore, adaptation to dim red light could occur before the retinas

were completely fixed. Although the use of dim red light shifts the sensitivity of the rods to that of the relatively insensitive cones, it would be more appropriate to dissect in complete darkness or under invisible infrared light. This was not feasible in the present study.

The results however, demonstrate that photoreceptor synaptic terminals in the newt undergo significant morphological changes throughout a 24 hr cycle. No significant change in the synaptic-vesicle diameter was observed during the light or dark phase, and this finding is in agreement with other, previous studies by Mountford (1963) and Cragg (1969). It was discovered, however, that the synaptic vesicle-density varied inversely with alterations in the synaptic terminal area. But, when synaptic vesicle density was corrected for vesicle size and section thickness and multiplied by the terminal area, no statistically significant difference existed between synaptic vesicle numbers at 0730 hrs and other times of the day or night. These findings provide morphometric and statistical support for the working hypothesis put forward by Cragg in 1969, which proposed a constant and stable synaptic vesicle population in photoreceptors, regardless of the adaptive state of the animal. If one assumes that the vesicular hypothesis for neurotransmitter release (Heuser, 1978) applies to photoreceptor cells as well, and that the membrane which is added to the plasma membrane during vesicle exocytosis is recycled by endocytosis to

reconstitute the vesicle population, there is no reason to suggest that the synaptic vesicle population should change during dark or light conditions. Likewise, there is no reason for the terminal plasma membrane to vary in surface area either. Schaeffer and Raviola (1978) have shown that only when one of these processes (endocytosis) is interfered with, does an imbalance in the two membrane pools occur. However, it is naive to assume that membrane turnover in the synaptic terminal is a closed system. Membrane addition to the plasmalemma from the synaptic-vesicle membrane pool may be taken up by the inner and outer segments of the photoreceptors. If retinomotor movements occur in the newt photoreceptor, as they do in fish cones (Levinson and Burnside, 1981), the plasma membrane in the vicinity of the inner segment must increase to account for as much as a 20% lengthening during the night. Such a redistribution of plasma membrane may account for the slight loss of membrane from the synaptic-vesicle membrane pool during the night, without an increase in the plasma-membrane pool of the synaptic terminal (Fig. 10E).

Because the two pools of membrane in the synaptic terminal (the synaptic-vesicle pool and the plasma membrane pool) do not change significantly when the photoreceptors are inactive (with respect to synaptic transmission) during the day or when they are active during the night, exocytosis could then seem to be occuring at the same rate as endocytosis. Therefore, an increased rate of fusion of

synaptic vesicles with the plasma membrane, without a concomitant increase in recycling of the membrane by endocytosis, cannot account for the apparent increase in volume of newt photoreceptor synaptic terminals, which occurs just prior to the light cycle at 0730 hrs. Index of infolding and form factor measurements of synaptic terminal profiles have, however, shown that a significant reduction in infolding of the receptor plasma membrane can account for the overall changes in surface to volume ratio which occur in synaptic terminals at 0730 hrs. This effect is illustrated in Fig. 18. The drawing, representative of photoreceptor terminals observed at 1030 hrs, has an area of 1624 mm^2 and a perimeter of 319 mm. Using the Videoplan, the perimeter of this drawing was unfolded to represent terminals observed at 0730 hrs. Simply by unfolding the perimeter, the area and the form factor doubled. This model mimics the events occuring in the synaptic terminals of newt photoreceptors at 1030 and 0730 hrs and demonstrates that unfolding of the plasma membrane can account for the doubling of synaptic terminal area observed at 0730 hrs.

Fluctuations in the folding of photoreceptor terminals have also been reported for chick (Cooper and McLaughlin, 1978), turtle (Schaeffer and Raviola, 1976; 1978) and goldfish (Raynauld et al., 1979) retinas. The reason why such significant unfolding of the plasma membrane occurs at 0730 hrs is, however, unclear. Schaeffer and Raviola (1976) surmise that the increase in turtle photoreceptor area,

together with the increased invagination of the postsynaptic elements in the dark, occurs because the surface area of the receptor pedicle increases and the photoreceptor ending accomodates the redundant membrane by flowing along the length of the postsynaptic processes. However, their assumption that the surface area of the pedicle increases has not been verified by measurements of plasma membrane perimeter. In the dark-adapted newt photoreceptor, increased membrane resulting from the fusion of synaptic vesicles with the terminal plasma membrane may flow along the length of the invaginating postsynaptic elements, where it is immediately recycled by endocytosis. Thus, during rapid exocytotic and endocytotic events such as those occuring at 0730 hrs, a change in the shape of the photoreceptor terminal may occur without a change in either the synaptic vesicle population or the photoreceptor perimeter. This change in shape may be either a consequence of, or an adaptation to, such active recycling of membrane.

It has been suggested that cisternae within the photoreceptor terminal may increase surface to volume ratios for membrane retrieval (Holtzman and Mercurio, 1980). Since endocytosis of membrane is almost always associated with the plasma membrane around the deep invaginations of postsynaptic elements, the photoreceptor plasma membrane in this area may be a preferential site of membrane uptake. The appearance of newt photoreceptor terminals at 0730 hrs is not unlike the appearance of dark adapted turtle cone

photoreceptors (Schaeffer and Raviola, 1978). Schaeffer and Raviola noted that, like newt photoreceptors, the postsynaptic elements of dark adapted turtle photoreceptors were deeply invaginated into the receptor terminal.

Endocytosis has also been observed to occur at a rapid rate, as indicated by the uptake of the extracellular tracer, horseradish peroxidase (Schaeffer and Raviola, 1978). The quantitative studies of newt photoreceptor cell synaptic terminals have shown that endocytosis is also occuring at a similar, rapid rate. Coated vesicles and large cisterns of membrane, which are the morphological characteristics of rapid rates of membrane recycling in most cell types (Douglas et al., 1977; Holtzman and Mercurio, 1980), increased in numbers as the night period progressed and were most prominent just before the light period began. Since, as previously suggested, exocytosis always occurs at a rate equal to endocytosis, exocytosis must be occuring at a very rapid rate in newt photoreceptors at 0730 hrs as well. Observations of uncoated vesicles and omega profiles associated with the photoreceptor plasma membrane are indicative that high rates of exocytosis are occuring at 0730 hrs. But why should there be such an increase in the turnover rate of synaptic vesicles in photoreceptors towards the end of the night cycle? This question may be best answered after considering the changes which occured in newt photoreceptor terminals during the light period.

It was found that as the light period progressed, there

was an increase in the number of dense-cored vesicles found in the cytoplasm of newt photoreceptor terminals, especially near the synaptic lamellae. Similar changes have been observed in light adapted frog photoreceptors that have been light adapted for a long period of time (Monaghan and Osborne, 1975; Osborne and Monaghan, 1976). Monaghan and Osborne suggest that light adaptation of photoreceptors reduces the rate of vesicle release, but not neurotransmitter packaging, with the result that many vesicles become "supercharged" with transmitter substance. The higher concentrations of neurotransmitter in the synaptic vesicle then causes the core of the vesicle to become electron-dense. In newt rods, these dense-cored vesicles are preferentially associated with the synaptic lamellae at 1930 hrs. During the early night, the dense-cored vesicles decrease in frequency and the quantitity of the electron-dense substance in the synaptic cleft increases. Thus, neurotransmitter stored in the vesicles during the late day may be released into the synaptic cleft during the early night. Admittedly, the suggestion that the appearance of an electron-dense core in synaptic vesicles and the accumulation of electron-dense material in the synaptic cleft of newt rods is actually neurotransmitter material, is highly speculative. However, if Monaghan and Osborne are correct in interpreting the appearance of dense-cored vesicles during the day as "supercharging" of vesicles with transmitter substance, then it is quite conceivable that the opposite phenomenon occurs

in the dark. If one assumes that neurotransmitter synthesis takes place at a constant rate throughout the light and dark period, then synaptic vesicles would have an opportunity to become "supercharged" with transmitter during the day, but in the night, synaptic vesicles would be released at a greater rate than transmitter could be synthesized and packaged. As a result, the synaptic vesicles would become "undercharged" with transmitter substance. Since the photoreceptor postsynaptic elements must be depolarized throughout the dark period; the photoreceptor cells would then be required to increase exocytosis toward the end of the dark period to maintain sufficient levels of transmitter in the synaptic cleft. The rate of endocytosis and exocytosis would thus gradually increase as the night period progressed, resulting in the morphological differences which are observed late in the night cycle. By early morning, after the lights have come on, exocytosis and endocytosis would be reduced, the photorecéceptors would regain their "normal" morphology, and the synaptic vesicles would become fully charged again.

This proposal poses two problems. First, if the photoreceptor is to keep the postsynaptic elements depolarized, a feedback mechanism must be present, which either informs the photoreceptor as to the amount of transmitter in the synaptic cleft, or how much transmitter is in the synaptic vesicles. This information would be essential for the photoreceptor to effect the appropriate

rates of exocytosis and endocytosis. Whether such a system exists remains to be determined. Secondly, it is well established in the neuromuscular junction, that transmitters are discharged in small bursts, which are very constant in size and are termed "quanta" (Katz, 1971). The vesicular hypothesis for transmitter release states that a quantum of transmitter substance is contained within each synaptic vesicle, and that the small recorded bursts of activity (miniature end plate potentials) correspond to the release of transmitter from single synaptic vesicles (Heuser et al., 1979). In the newt photoreceptor, according to the above proposition, the quanta of transmitter released by synaptic vesicles would vary with time, being greater at the beginning of the night cycle than at the end, because synaptic vesicles in photoreceptors are supercharged at the beginning of the night and undercharged at the end of the night. To determine if the size of the quanta in photoreceptor synaptic vesicles does in fact vary during the day-night cycle, must await further physiological studies on this phenomenon.

In any event, the energy requirements for the rapid turnover of membrane in newt photoreceptors at 0730 hrs must be great, and it is likely that the glia, as well as the photoreceptors, play an active metabolic role in photoreceptor synaptic transmission. As in the dark-adapted rat retina (Spadaro et al., 1977), the mitochondria of the newt Müller cells appear in condensed (electron-dense)

configurations at 0730 hrs. This is indicative of active mitochondrial respiration and phosphorylation (Novikoff and Holtzman, 1976). It is also known that mitochondria are active in the removal of calcium during synaptic transmission (Kelley et al., 1979). The entry of calcium into the presynaptic nerve terminal is a necessary prerequisite for the fusion of synaptic vesicles with the plasma membrane (Llinas and Heuser, 1977). The dark granules in the mitochondrial matrix of newt retinal Müller cells are similar to the granules in the mitochondria of other cells which have been identified as calcium sinks (Peachy, 1964; Lehninger, 1970). If the granules in the Müller cell mitochondria, which increase in number at 0730 hrs, are calcium associated, then the glial mitochondria may be an important calcium-buffering system for the control of calcium ion movements in the vicinity of the photoreceptor synaptic terminal during the night. The observation that smooth endoplasmic reticulum and large clear vesicles increase in frequency during the night, may also represent a cellular effort to sequester calcium (Blaustein et al., 1980).

Although synaptic lamellae have been reported to decrease in number and length during the night in fish cones (Wagner, 1973; Wagner and Ali, 1977) and both the rods and cones of the albino rat (Spadero, 1978) retinas, no significant differences between the day and night were observed in either the number or length of synaptic lamellae

in newt photoreceptors. However, synaptic lamellae in newt photoreceptors were significantly shorter and more numerous at 2400 hrs, than at any other time of the day. This was probably due to the large number of lamellar arrays which were observed in this sample period. These arrays were similar to the "ribbon fields" which have been described in the pineal gland (Vollrath, 1973). The reason why fluctuations in the number and length of of synaptic lamellae over a 24 hr period could not be detected, except at 2400 hrs, is unknown. However, it is possible that the cyclic variations only occur in one photoreceptor type, as they do in the fish retina (Wagner, 1973; Wagner and Ali, 1977). The significance of the large number of "ribbon fields" which were observed in the 2400 hr sample is also unknown. It has been suggested that synaptic lamellar turnover in the pineal gland occurs at 8 (Vollrath, 1973) or 24 hr (McNulty, 1981) intervals. Therefore, it is possible that the "ribbon fields" constitute some part of this synaptic lamellae renewal process in photoreceptors. It is difficult to comment on these findings because of the apparent species variation in the fluctuations of photoreceptor synaptic lamellae and the lack of information concerning the structure and function of this unique organelle.

In conclusion, these findings support the suggestion that transmitter is released from photoreceptor synaptic terminals in the dark. In addition, the observations show

that the morphology of newt photoreceptor synaptic terminals changes dramatically over a natural 24 hr day-night cycle. These changes suggest that neurotransmission in the newt photoreceptor may differ from the neuromuscular junction in that the amount of transmitter in each synaptic vesicle may vary in a cyclic manner over each 24 hr period.

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CHAPTER 2

STUDIES ON THE PHOTORECEPTOR NEUROTRANSMITTER IN THE RETINA

INTRODUCTION

Ultrastructural studies, described in Chapter 1, provide evidence that newt photoreceptor cells release a neurotransmitter in the dark by the process of exocytosis, as has been suggested for other vertebrate retinas. Electrophysiological studies have shown that in other vertebrate retinas, the chemical transmission from photoreceptors to horizontal and bipolar cells is mediated by a depolarizing neurotransmitter (Kaneko and Shimizaki, 1975). The exact identity of the photoreceptor transmitter is, however, unknown.

1) Catecholamines. The accumulation of dense-cored vesicles in the light-adapted rod terminals of the toad was considered to be indicative of possible indoleamine or catecholamine stores in photoreceptors (Monaghan and Osborne, 1975). A similar accumulation of dense-cored vesicles has been observed in newt photoreceptor synaptic terminals in the light (Chapter 1) suggesting the possibility that an amine may be the neurotransmitter of the newt photoreceptors as well. In addition, the total catecholamine content of toad, frog and rabbit retinas is

greater in light than in dark-adapted retinas (Drujan et al., 1965); however, the separate amounts of adrenaline and noradrenaline have been shown to vary amongst species, and they are present in differing concentrations, depending on the adaptive state of the animal (Stell, 1972). The formaldehyde condensation induced fluourescent technique of Falck et al. (1962) has been highly successful in localising neuroactive amines in the retina (Malmfours, 1963; Ehinger, 1966). In most species studied using this technique, dopamine and noradrenaline (as differentiated by microspectrophotometry) have only been localized to cell bodies within the inner portion of the inner nuclear layer (Stell, 1972). In addition, autoradiographic localization of labelled dopamine and its precursor, DOPA, resembles the distribution of catecholamines as observed with the fluorescence techniques (Ehinger and Falck, 1971), and photoreceptors have been shown not to synthesize catecholamines or serotonin in the turtle retina (Lam, 1975). Furthermore, amines have been shown not to have an excitatory effect, but rather a slow inhibitory effect on photoreceptor postsynaptic elements (Van Harreveld, 1976). In light of this evidence then, the photoreceptor transmitter is unlikely to be an amine.

2) Acetylcholine. Numerous studies have also been aimed at demonstrating the slow-acting, excitatory neurotransmitter, acetylcholine, as the photoreceptor transmitter. Although acetylcholine has been known for some

time to be present in the frog retina (Therman, 1938), the location of the degradative enzyme acetylcholinesterase, together with acetylcholine receptor sites, are now used to demonstrate the existance of, and to localise, a cholinergic system. Such studies have demonstrated acetylcholinesterase activity in amacrine cells and in some cases, ganglion cells in a number of species (Nichols and Koelle, 1968; Stell, 1972). In the newt, acetylcholinesterase activity has been observed throughout the outer plexiform layer, although not along the photoreceptor membranes at any distance from the synaptic terminals (Dickson et al., 1971). Only nonspecific acetylcholinesterases could be demonstrated in association with the horizontal cells of pigeon and ground squirrel retinas (Nichols and Koelle, 1968).

It has recently been pointed out, that acetylcholinesterase activity is not necessarily indicative of the presence of a cholinergic or a cholinoceptive neuron (Lehman and Fibiger, 1979). Furthermore, acetylcholinesterase has been found to hydrolyse substance P, and therefore may be responsible for the termination of this neuropeptide's activity (Chubb et al., 1981) if it is a neurotransmiter in the outer plexiform layer.

Much biochemical and pharmacologic evidence also suggests that acetylcholine is utilized as a neurotransmitter in the retina (Val'tsev, 1966; Glow and Rose, 1964; Ames and Pollen, 1969 Neal and Gilroy, 1975; Vogel et al., 1979; Brecelj et al., 1979) and, of several

putative neurotransmitters, only acetylcholine has been shown to be synthesized by turtle photoreceptors (Lam, 1972). However, a later study by Lam (1975) showed that the specific activity of the acetylcholine synthesizing enzyme, choline acetyl transferase, was only 10-30% of the specific activity of that in the whole turtle retina, casting some doubt on his original thesis. Although (3H)-choline is accumulated by the photoreceptor cells of the rabbit retina, it is incorporated into phospholipids, and not into acetylcholine (Masland and Mills, 1979; 1980). The only cells in the rabbit retina which have been shown to synthesize acetylcholine are a population of amacrine cells (Masland and Mills, 1979; 1980). Because there is no difference in the acetylcholine content of light and dark adapted goldfish retinas, and because acetylcholine can only be released by flicker stimulation, it has been suggested that acetylcholine is used as a neurotransmitter by neurons in the inner retina, and not photoreceptors (Vivas and Drujan, 1980).

Recently, muscarinic and nicotinic acetylcholine (ACh) receptor sites have been investigated extensively, and localized in goldfish (Vogel and Nirenberg, 1976; Schwartz and Bok, 1978; Moreno-Yanes and Mahler, 1979a; 1979b), mouse (Pourcho, 1979) and bovine (Moreno-Yanes and Mahler, 1979b) retinas. Using (3H)-quinuclidinyl benzylate ((3H)-QB), muscarinic ACh receptors have been localized in the inner plexiform layer of the chick retina (Sugiyama et al., 1977),

while (125I)-α-bungarotoxin ((125I)-BTX), nicotinic ACh binding sites, have been localized in both plexiform layers of rabbit, chick (Vogel and Nirenberg, 1976), pigeon (Yasulla and Schmidt, 1976; 1977; Yasulla, 1979), turtle and goldfish (Yasulla and Schmidt, 1976; Schwartz and Bok, 1978; Yasulla, 1979) retinas. Electron-microscope autoradiographic localization of (125I)-BTX receptor sites in goldfish retina show that these receptors are on small bipolar cell dendrites and it has been suggested that the best candidate for cholinergic activity in the outer plexiform layer is the photoreceptor (Schwartz and Bok, 1978). However, when chick and pigeon retinas were incubated with nicotine, only the binding of (125I)-BTX in the inner plexiform layer was abolished, indicating that the binding of (125I)-BTX in the outer plexiform layer is probably nonspecific (Yasulla, 1979). Furthermore, elegant autoradiographic experiments conducted by Masland and Mills (1979) indicate that while rabbit photoreceptors and ganglion cells synthesize choline-containing phospholipids, acetylcholine synthesis is restricted to a few cells at both margins of the inner plexiform layer, probably to a small group of amacrine cells (Hayden et al., 1980). In addition, horseradish peroxidase-conjugated α-bungarotoxin (HRP-BTX) has been used to localize acetylcholine receptors, but they have only been demonstrated in the inner plexiform layer of chicken (Vogel et al., 1979) and mouse (Pourcho, 1979) retinas.

Marshall and Werblin (1978) have shown that acetylcholine uncouples horizontal cells in the tiger salamander retina, thus implying that a population of these cells may secrete acetylcholine and could therefore be responsible for the receptor-site labelling which has been observed in the outer plexiform layer of certain species. However, it has recently been found that muscarinic drugs are only able to competitively block turtle cone to L-type horizontal cell synapses when used in high concentrations (Gerschenfeld and Piccolino, 1977); there was apparently no effect on the connection between cone and hyperpolarizing bipolar cells, indicating that these are not cholinergic synaptic connections either (Gerschenfeld and Piccolino, 1977).

The consistent association of a small population of amacrine cells with the neurotransmitter acetylcholine, implies that cholinergic amacrine cells in a number of species probably have a comparable functional role. There is, however, little conclusive evidence supporting acetylcholine's role as a photoreceptor neurotransmitter. A lack of direct electrophysiological evidence on the effects of cholinergic drugs and acetylcholine on bipolar cell activity, necessitates further study on the role of acetylcholine as a neurotransmitter in the retina.

3) Amino Acids. Amino acids too, have been proposed as putative neuroactive substances and recently have

convincingly been implicated as neurotransmitters in the retina. The two excitatory amino acids, glutamate and aspartate, have been suggested as the most likely candidates for the synaptic transmitter substances of photoreceptors (Starr, 1977). Sillman et al. (1969) observed that sodium glutamate or aspartate eliminates most of the electroretinogram when applied to the photoreceptor surface of frog retina, an effect which mimics that of photoreceptor transmitter release in the dark. Furthermore, intracellular recordings from carp and skate bipolar cells indicate that topically applied glutamate and aspartate mimic the effects of the photoreceptor transmitter (Tomita, 1976). Sodium aspartate has also been found to depolarize the horizontal cells of the carp retina (Stell, 1972; Wu and Dowling, 1978; Negishi and Drujan, 1979), and synaptosomal preparations of rabbit photoreceptor synaptic terminals were shown to contain more than 40% of the total aspartate in the retina (Neal and Atterwill, 1974). Kennedy and Voaden (1974b) reported as well, that levels of aspartate and glutamate were twice as high in the photoreceptor layer, as in the rest of the frog retina; similarly that retinal glutamate levels were highest in the photoreceptor layer of the rat retina (Kennedy et al., 1977). However, Sarthy and Lam (1979) and Berger et al. (1977) found no difference in the content of these amino acids in the various layers of either turtle or monkey retinas, respectively. The reasons for such species variations is not clear. Although kinetic analysis reveals that there is a high-affinity uptake system

in the retina for glutamate and aspartate (Starr, 1977), autoradiographic studies have shown that only glial cells accumulate appreciable amounts of either of these two amino acids (Ehinger and Falck, 1971; Ehinger, 1972; 1977; Bruun and Ehinger, 1974; White and Neal, 1976). Difficulty then, in demonstrating that these amino acids are, in fact, the photoreceptor neurotransmitter, stems from the fact that both amino acids play an active role in intermediary metabolism, and it is impossible to distinguish between transmitter molecules and those associated with other metabolic activities.

The uptake of aspartate and glutamate by retinal glia does not necessarily negate the possible use of these amino acids as neurotransmitters by photoreceptors. Although some neurotransmitters are inactivated by enzymes in the synaptic cleft, or by re-uptake by the presynaptic process, retinal Müller cell processes in the newt are so closely associated with photoreceptor terminals (see Chapter 1) that inactivation of photoreceptor neurotransmitters in this species may be by uptake into the Müller cells. In fact, Fagg and Lane (1979) suggest that it is unlikely that amino acids are extensively metabolized extracellularly, and that re-uptake likely represents a more widespread means of amino acid transmitter inactivation. Furthermore, Starr (1977) suggests that glutamate, released by the photoreceptors in the dark, may be taken up by Müller cell processes and formed into glutamine, a metabolite which could then be

returned to the photoreceptors to participate in the synthesis of the fresh transmitter, glutamate. However, some studies (Ehinger, 1977; Sarthy and Lam, 1979) have failed to verify the existence of the glutamate-glutamine-GABA cycle proposed by Starr (1977), although Voaden et al. (1978) have suggested that glutamine may be a major source of GABA in the retina.

Recently, it has been shown that intraocular injections of kainic acid, an analog of glutamate, causes swelling of certain goldfish horizontal cell dendrites. The implication is then, that rods use glutamate as their neurotransmitter; cones on the other hand, are beleived to use aspartate as their neurotransmitter substance (Yazulla and Kleinschmidt, 1980). Wu and Dowling (1978) also suggest that aspartate may be the cone transmitter because it is more effective than glutamate in depolarizing cone horizontal cells, and its antagonist blocked the depolarizing effect of both aspartate and the natural transmitter. Finally, Neal (1976) and Neal et al. (1979) have reported that aspartate, and not glutamate, is released from the retina of the rabbit upon stimulation by light flashes, while Kondo and Toyoda (1980) found no difference in the ability of glutamate or aspartate to act on the subsynaptic membrane. To date, more evidence supports glutamate and aspartate as the photoreceptor neurotransmitters than any other putative neurotransmitter compound.

Taurine, a sulphur containing amino acid, is the

predominant free amino acid in the retina and 75% of the total taurine content in frog retinal tissues has been shown to be associated with photoreceptor cells (Kennedy and Voaden, 1974a). Autoradiography has localized (3H)-taurine within photoreceptor cells and certain other cells, including the amacrine interneurons and the retinal bipolar cells (Lake et al., 1978). Although taurine is one of a number of putative amino acid neurotransmitters, and photoreceptors have been demonstrated to have a high affinity uptake system for taurine (Kennedy and Voaden, 1976), taurine is not widely accepted as a candidate for the photoreceptor neurotransmitter because it has contrasting physiological actions and because light stimulation, rather than darkness, causes an efflux of taurine from the retina (Pasantes-Morales et al., 1974). However, taurine appears to be essential for normal photoreceptor maintenance (Cohen et al., 1973).

Miller and Steinberg (1979) suggest that taurine is involved in an iso-osmotic regulating mechanism that helps preserve osmotic balance during calcium fluxes, which result from light stimulation of the photoreceptor outer segments. Furthermore, taurine is known to stimulate production of melatonin by as much as forty-fold (Wheeler et al., 1979) in the pineal gland. Since melatonin is likely to be synthesized by photoreceptor cells of the retina (Gern and Ralph, 1979), it is possible that taurine may play a role in the regulation of the synthesis of this compound in the

retina as well.

Taurine has also been suggested as having a neuromodulatory role in the central nervous system (Davison and Kaczmarek, 1971; Kuriyama, 1980). Modulators differ from transmitters in that the cellular efflux of these substances and thus the magnitude of their effects, may change, depending of the state of neuronal activity and on extracellular potassium concentrations; modulator-release mechanisms are independent of calcium and probably change more slowly with time; and modulators are not present in synaptic vesicles (Orrego, 1979). Taurine is thought by some, to modulate membrane excitability by decreasing the concentration of intracellular free calcium and thus inhibiting the release of other transmitters (Kuriyama, 1980). If photoreceptors utilize taurine as a neuromodulator, then the implication is that they release a neurotransmitter via synaptic vesicles, and release taurine by way of some other cytoplasmic release mechanism. Such exceptions to Dale's principle (one neuron, one neurotransmitter) are currently being investigated (Burnstock, 1976).

At least two other excitatory amino acids, in addition to L-glutamate and L-aspartate occur in normal CNS tissues. They are L-cysteate and L-cysteine sulphinate. Cysteate is the sulphonic analogue of aspartate and is present, albeit in low concentrations, in the rat brain (Curtis and Johnston, 1974). This amino acid has been shown to

depolarize cat spinal motor neurons, is comparable in potency to aspartate and can be metabolized by decarboxylation to taurine, while cysteine sulphinate can be metabolized by decarboxylation to hypotaurine (Curtis and Johnston, 1974). Furthermore, the enzyme cysteine sulphinate decarboxylase has been found in rat brain synaptosomal preparations (Agrawl et al., 1971). Cysteate and cysteine sulphinate are not yet commercially available as radiolabelled compounds, so uptake and release studies have not yet been performed. However, cysteate is a strong competitive inhibitor of glutamate in rat brain slices and could therefore be a substrate for a glutamate transport system (Curtis and Johnston, 1974).

Sulphur containing amino acids, such as taurine, are further implicated as possible neurotransmitters in photoreceptor cells because the photoreceptor synaptic vesicles of some species stain with prolonged osmium treatment (Pourcho and Burnstein, 1975) and the synaptic vesicles of newt photoreceptors have been shown to stain with the Zinc Iodide Osmium (ZIO) technique (Flight and Van Donnselaar, 1975a). Pellegrino de Iraldi (1975; 1977) has suggested that some of the specificity of the ZIO reactivity is due to the presence of free -SH groups, however no control experiments have yet been done until this work, to see if -SH groups are responsible for the staining observed in newt photoreceptor synaptic vesicles.

The distribution of free amino acids in whole or

microdissected retinas has been previously studied by amino acid analysis in monkey, rat, frog (Kennedy and Voaden, 1974b; Voaden, 1977; Karlsen, 1977; Berger et al., 1977), cat, rabbit (Bauer and Ehinger, 1977; Neal et al., 1979) and mouse (Cohen et al., 1973) retinas, in an attempt to identify potential amino acid neurotransmitters. Because photoreceptors release an excitatory neurotransmitter in the dark, it should be a relatively simple matter to measure and compare the free amino acid content of the retina during light and dark conditions to detect fluxes in putative amino acid transmitters. In this chapter, many of the techniques described above have been employed to study newt photoreceptor transmitter candidates in light, dark and cycled light conditions.

MATERIALS AND METHODS

1) Localization of Muscarinic and Nicotinic Acetylcholine Receptor sites.

a) Preparation of Radiolabel:

To detect acetylcholine binding sites in newt retina, tritiated quinuclidinyl benzylate ((3H)-QB; 29 Ci/mmol) and iodinated α-bungarotoxin ((125I)-BTX; 18 μCi/μg) were purchased from New England Nuclear (Boston, Massachusetts) and the latter was used within one week of its arrival. Samples of 5, 10, 20 and 40 μl of each isotope were evaporated with dry nitrogen in a fume hood at 20 °C, and resuspended in 100 μl of unsupplemented tissue culture media (M-199 with glutamine; GIBCO, Grand Island, New York) in small 200 μl chambers (Final concentrations: (3H)-QB, 7.1 μmol for the 20 μl dilution; (125I)-BTX, 0.0137 μmol for the 20 μl dilution). Newts were killed by decapitation, the eyes were enucleated, the corneas removed and the eyecups were placed in the incubation chambers with radioactive media which was oxygenated with 98% oxygen during incubation. After either 15 or 30 min incubation periods, the eyecups were washed in oxygenated M-199 without label for 5 min. The eyecups were then fixed and processed for electron microscopy (see Chapter 1). The use of this incubation procedure, rather than *in vivo* injections of label into the vitreous, more accurately controls the

concentration of label available to the retina.

b) Autoradiographic Methods:

To shorten autoradiographic exposure times, a scintillator (10% diphenyloxazole; New England Nuclear, Boston, Massachusetts) was added to the embedding media during infiltration and embedding (Fisher et al., 1971; Kopriwa, 1979). Half-micrometer plastic sections of retina were cut on an ultramicrotome and heat fixed, 2 cm from the end of glass slides. The slides were then dipped, in the dark, in undiluted Kodak NTB-2 nuclear tract emulsion (Eastman Kodak Company, Rochester, New York) and dried, in the dark, for 2 hrs in a high humidity (>70%) atmosphere. The slides were then stored at 4 °C in light-tight boxes containing silica gel. After varying exposure times (usually 1-2 weeks), the slides were developed in Kodak D-19 developer for 2 min, briefly washed in distilled water, and fixed in Kodak fixer for 5 min. After washing in distilled water for 10 min, the slides were dried thoroughly in a dust free atmosphere. Some slides were stained through the emulsion prior to photography with a solution of 1% toluidine blue in 1% borax. Ultrathin sections were also cut from blocks containing tissues which were incubated in (125I)-BTX. These were prepared for electron microscope autoradiography as described by Ball et al. (1981).

2) Amino Acid Analysis of Free Retinal Amino Acids during Light and Dark Adaptation. Because the amino acids

aspartate and glutamate are putative photoreceptor transmitter substances, an amino acid analysis was performed on light, dark, and cycle-adapted retinas to compare the fluctuations in amino acid concentration which might occur as they are released in response to different physiological stimuli.

One group of newts was light adapted continuously for 9 days by placing them in a tank illuminated by a 150 Watt flood lamp, placed 10 cm from the water surface. The light intensity in the holding tank was about 700 lux. Heat from the lamp was dissipated with a water-jacket system, so that the temperature of the water in the tank never exceeded 20^{0}C. Other newts were dark adapted for 9 days by placing them in a tank which was contained within a light tight box. The tank water in both light and dark conditions was oxygenated with an air line. The water in the tanks was changed every 2 days and the newts were fed freeze-dried brine shrimp at this time as well. Cycle-adapted newts were kept in an aquarium as described in Chapter 1. Cycle-adapted newts were killed by decapitation during the light phase under normal room light, while the light or dark adapted newts were killed in a similar manner in the light or under dim-red light (Chapter 1), respectively. After the 9-day period, the left eye of 3 newts in each adaptation regimen was processed for amino acid analysis and the right eyes were processed for transmission electron microscopy (see Chapter 1). The right eyes were checked for

morphological changes which might have occured due to the extreme adaptation regimen.

For amino acid analysis, the cornea of each eye was removed, and the retina carefully teased away from the pigment epithelium. The retinas were briefly washed free of vitreous in amphibian ringers (0.11 M NaCl, 0.0019 M KCl, 0.0008 M CaCl , 0.0024 M NaHCO , 0.003 M glucose) and then placed together in 3 ml of 10% trichloroacetic acid (TCA) at 4 ^{0}C. The tissue was homogenized in TCA and sonicated at 4 ^{0}C. The homogenate was then centrifuged at 4,000 RPM for 20 min, and the supernatant was carefully decanted and millipored to remove any precipitated protein, prior to lyophilization and storage at -70.^{0}C. Before analysis by ion exchange chromatography, the samples were reconstituted in 50 µl of tris buffer (pH 2.2). Amino acids were separated, on the basis of their charge and molecular weight, with a high-pressure column packed with ion exchange resin (Hamilton, 1963). After reaction with ninhydrin, the amino acids were eluted from the ion exchange column and detected with an ultraviolet flow photometer at wavelengths of 440 nm and 570 nm, and the results were printed on a two-channel chart recorder. Amino acids were assigned peaks by comparing their position with a run of amino-acid standards and with the aid of a ninhydrin reactive substance chart (Hamilton, 1963). The concentration of each amino acid was determined from the area beneath each peak, as compared with a 5.0 nmol norleucine standard and expressed as µmol/gm wet

wt of tissue.

3) Autoradiographic Localization of Aspartate, Glutamate and Taurine in Newt Retina. To determine the uptake pattern of putative amino acid neurotransmitters which, on the basis of amino acid analysis (Section 2), are suspected of being the photoreceptor neurotransmitter, newt retinas were incubated in media containing (3H)-L-glutamate, (3H)-L-aspartate or (3H)-taurine. Tritiated amino acids were purchased from New England Nuclear (Boston, Massachusetts) with the following specific activities: (3H)-glutamate, 18 Ci/mmol; (3H)-aspartate, 15 Ci/mmol; (3H)-taurine, 23 Ci/mmol. Samples containing 5, 10, 20, and 40 µl quantities of these amino acids were evaporated with dry nitrogen in a fume hood at 20°C and resuspended in 100 µl of unsupplemented tissue culture media (M-199 with glutamine; GIBCO, Grand Island, New York) in a small 200 µl chamber. Final concentrations of amino acids for the 20 µl dilution were: (3H)-glutamate, 10.65 µmol; (3H)-aspartate, 13.20 µmol; (3H)-taurine, 8.63 µmol. Based on the kinetic parameters for taurine uptake by frog retina (Lake et al., 1978), concentrations in the µmol range should be sufficiently low enough to ensure exclusive activation of the high affinity uptake systems for these amino acids, if such uptake systems exist in newt retina. Newt eye-cup preparations were incubated in each of these labelled compounds, as described in Section 1a of this Chapter. Light microscope autoradiography was performed on 0.5 µm

plastic sections, as previously described in this chapter. Electron-microscope autoradiography was only performed on thin sections from tissue that had been incubated in (3H)-taurine.

4) Kainic Acid Lesions of Newt Photoreceptor Postsynaptic Elements. Intraocular injections of kainic acid, a glutamate analog, has been used to determine if glutamate is a neurotransmitter in the retina (Olney et al., 1974; Yazulla and Kleinschmidt, 1980). Kainic acid binds to glutamate receptors if they are present, and will cause swelling of the postsynaptic processes. Intraocular injections of kainic acid into the newt eye were used to determine if kainic acid affected horizontal and bipolar cell dendrites, and therefore if glutamate is a likely photoreceptor neurotransmitter candidate.

Newts were anaesthetized with 3 g/l MS-222 (Tricanemethansulfonate, Syndel Laboratories, Vancouver). The anaesthetized newts were placed on gauze, dampened with MS-222, next to a micromanipulator fitted with a Hamilton 10 µl syringe. The left eye of each animal was pierced near the corneal-scleral junction, and the micropipette was inserted through the incision. Five µl of a 2.5-200 nmol solution of kainic acid (Sigma, St. Louis, Missourri) in amphibian ringers was injected over a 5 min period. The newts were then returned to a tank where they were held until an appropriate survival time of 15, 30, 60, or 120 min had elapsed. Newts were then killed by decapitation and the

eyes enucleated and processed for electron microscopy, as previously described (see Chapter 1).

5) Histochemical Demonstration of -SH Groups in the Outer Plexiform Layer of the Newt Retina: ZIO, MO, and DACM Reactions. In 1975, Flight and Van Donselaar (1975a) applied the Zinc Iodide Osmium (ZIO) technique to the retina of the newt and observed staining of synaptic vesicles in the photoreceptor cells. Pellegrino de Iraldi later suggested (1977) that at least some of the specificity of the ZIO reaction may be due to the presence of free -SH groups. To test this hypothesis, the newt retina was stained with the ZIO technique after the retina had been incubated in highly specific -SH blocking or protecting reagents. The presence of -SH groups was then verified with the Mercury Orange (MO) and DACM fluorescent-probe techniques for light microscopy.

a) Zinc Iodide Osmium Technique. Eyecup preparations were placed in oxygenated amphibian ringers solution containing: a) 5.0 mmol dithioerythrotol (DTE; Sigma, St. Louis, Missourri) which protects -SH groups and reduces S-S bonds (Pellegrino de Iraldi, 1975); b) 0.1 M N-ethyl maleimide (NEM; Sigma, St. Louis, Missourri) which specifically blocks -SH groups (Pellegrino de Iraldi, 1977); or c) amphibian ringers only. The eyecup preparations were preincubated in these solutions at room temperature for 30 min. The eyecups were then fixed with 6.4% glutaraldehyde in 1.5 M phosphate buffer (pH 7.3; 1500 mOsM) at room

temperature for 2 hrs, washed in tris buffer (pH 7.3) for 15 min, and finally impregnated with ZIO solution (as prepared according to Kristic, 1972) for 16 hrs in the dark, at 4°C. The retina was then teased from the pigment epithelium, quartered, washed briefly in 0.1 M cacodylate buffer (pH 7.3) and dehydrated and embedded in TAAB resin (as described in Chapter 1).

b) Mercury Orange. The mercury orange (MO) technique for the demonstration of -SH groups is considered to be one of the most sensitive and specific for the detection of thiols (Pearse, 1968). Retinas were fixed with 6.5% glutaraldehyde in 0.75 M phosphate buffer (pH 7.3, 1200 mOsM), dehydrated in an acetone series, cleared in chloroform and embedded in paraffin. Ten-micrometer sections were cut on a metal knife and mounted on albumenized slides. Sections were then deparaffinized in xylene, and brought to water in a graded series of alcohols. Sections were treated with: a) 5.0 mmol DTE in 0.1 M phosphate buffer; b) 0.1 M NEM in 0.1 M phosphate buffer (pH 7.0); or c) in phosphate buffer alone, by immersion in Coplin jars containing the above solutions, for 4 hrs at room temperature. Sections were next briefly washed in buffer and brought to 100% ethanol in a graded series. Sections were then placed in Coplin jars containing a saturated Mercury Orange (1-(4-chloromercuriphenylazo) -napthol-2; Sigma, St. Louis, Missourri) solution in 80% ethanol (Bennet and Watts, 1958) for 24 hrs at room

temperature. The sections were then washed for 2 min in 2 changes of 100% ethanol, immersed briefly in xylene, and mounted in Depex.

c) DACM. Recently, a new highly specific, fluorescent thiol reagent, N-(7-dimethylamino 4-methyl coumarinyl)-maleimide (DACM) has been synthesized (Ogawa et al., 1979) which is non-fluorescent by itself, but which will react readily with -SH groups to form highly fluorescent products. Newt retinas were fixed with a dialdehyde fixative (see Chapter 1), washed briefly in tris buffer (pH 7.3), dehydrated in an acetone series and embedded in paraffin. Ten-micrometer sections were cut, deparaffinized in xylene and brought to water in a graded alcohol series. The sections were placed in Coplin jars containing solutions of: a) 0.1 M NEM in 5.0 mmol tris acetate buffer (pH 6.8) containing 0.85% NaCl (TAS) for 1 hr at 35 C; b) 5.0 mmol DTE in TAS for 1 hr at 35 °C; or c) TAS only for 1 hr at 35 °C. The sections were then rinsed in TAS, and placed in a solution of alum-haematoxylin for 2 min, to quench nuclear fluorescence. The sections were then coverslipped and allowed to react in a solution of 0.1 mmol DACM (WAKO Chemical Industries, Japan) in TAS and sections were viewed with a Zeiss fluorescence microscope using a BG12 excitation filter (400 nm).

RESULTS

1) Localization of Muscarinic and Nicotinic Acetylcholine Receptor sites in Newt Retina. When newt retinas were incubated in media containing (3H)-quinuclidinyl benzylate ((3H)-QB), muscarinic acetylcholine receptors were observed in both plexiform layers (Fig. 1). Label was also present in the position of the apical and foot processes of the Müller cells, indicating that at least some of the binding in the plexiform layers was due to the Müller cell processes which ramify throughout each of these layers. The labelling pattern of newt retina which was incubated in (125I)-α-Bungarotoxin ((125I)-BTX) was similar to that of (3H)-QB binding. Binding to nicotinic acetylcholine (ACh) receptors was observed in both plexiform layers of newt retina (Fig. 2). As with muscarinic receptor localization, a small number of nicotinic receptors was observed at the outer limiting membrane, indicating that some of the binding was to Müller cells. To determine if any of the nicotinic binding sites were associated with synaptic profiles, newt retinas which had been incubated in (125I)-BTX were prepared for electron microscope autoradiography. Figure 3 shows the distribution of silver grains near a cone synaptic terminal. Although a distinct band of label can be observed in the outer plexiform layer by light microscope autoradiography, (125I)-BTX binding sites could not be localized to a specific subcellular site in this layer by electron microscope autoradiography. On the other hand, silver

grains were readily seen in association with specific synaptic profiles in the inner plexiform layer (Figs. 4 and 5). In Fig. 4, silver grains are associated with a process containing numerous microtubules and a few synaptic vesicles. There appears to be a synapse (arrow) between this process and an amacrine cell process containing numerous small, electron-lucent vesicles. Figure 5 shows silver grains associated with an amacrine cell process. Next to this process is a bipolar cell process (as identified by the presence of numerous, small, synaptic vesicles and dense cytoplasm) which may, or may not, be making a synapse at this point. Despite the fact that many ultrastructural details are obscured by silver grains, it is apparent that the autoradiographic localization of (125I)-BTX binding sites to neuronal structures in the inner plexiform layer is more easily accomplished than to structures in the outer plexiform layer.

2) Amino Acid Analysis of Free Retinal Amino Acids During Light and Dark Adaptation. The results of amino acid analysis of newt retina under light, dark and cycled conditions are shown in Table I. These values are consistent with the free amino-acid content of other vertebrate retinas (Voaden, 1977). Of the amino-acids identified by ion exchange chromatography, six have been reported to have neuroactive effects in the retina. Taurine is the most abundant of the identified neuroactive amino acids, and is found in concentrations which are two or five

TABLE I.

AMINO ACID ANALYSIS OF NEWT RETINA

AMINO ACID	CYCLE		LIGHT		DARK	
Taurine	57.90	*72.08**	36.80	*58.41*	37.50	*57.69*
Serine	3.82	*4.75*	1.20	*1.90*	4.66	*7.17*
Glutamine	5.97	*7.43*	3.17	*5.03*	9.77	*15.03*
Glutamate	10.54	*13.12*	8.62	*13.68*	6.90	*10.62*
Proline	2.70	*3.36*	2.08	*3.30*	1.22	*1.89*
Glycine	4.31	*5.36*	1.40	*2.22*	3.42	*5.26*
Alanine	5.67	*7.06*	1.40	*2.22*	3.63	*5.58*
Valine	1.30		1.01		1.12	
Leucine	0.72		0.31		0.56	
Tyrosine	0.56	*0.69*	0.16	*0.25*	0.32	*0.49*
Phenyl-alanine	0.39		0.57		0.27	
γ-amino-butyrate	4.80	*5.97*	4.52	*7.17*	3.59	*5.52*
Tryptophan	50.19	*62.48*	12.94	*20.54*	13.47	*20.72*
Lysine	2.60	*3.24*	0.66	*1.05*	0.51	*0.78*
Histidine	4.30	*5.35*	1.74	*2.76*	2.37	*3.65*

Results are expressed in μ moles / gm wet weight tissue.

**Numbers in Italics represent μ moles amino acid per gram wet weight tissue divided by the mean values of phehylalanine, valine, & leucine(μ moles/gm wet wt.) for each sample. Since these amino acids should not change in light or dark conditions, this procedure was done to correct amino acid values for variations in the sample size.*

times higher than that of other neuroactive amino acids.

The concentrations of the neuroactive amino acids fluctuated significantly under light and dark conditions. These findings are presented graphically in Fig. 6. Because morphological evidence suggests that the photoreceptor neurotransmitter is stored in the light and released in the dark, it might be expected that the concentration of a putative photoreceptor transmitter would vary under these conditions as well. Of the neuroactive amino acids identified by ion exchange chromotography, glutamate is the most likely candidate for the photoreceptor transmitter. As would be expected of the phtoreceptor transmitter substance, the concentration of glutamate was higher in the long-term light-adapted newt retina than in the long-term dark-adapted newt retina. The inhibitory amino acid GABA, which may be a neurotransmitter in the inner retina, was found in higher concentrations in the light-adapted retina. This was also true for the amino acids taurine, alanine and proline, which also have inhibitory effects on the retina. Glycine was the only amino acid which was found in lower concentrations in the light-adapted newt retina. Glutamine, which has been suggested to be a product of glutamate metabolism and a precursor of the inhibitory amino acid GABA, was found in lower concentrations in the light-adapted retina than in the

dark-adapted retina.

3) Autoradiographic Localization of Aspartate, Glutamate and Taurine in Newt Retina. The uptake pattern of labelled glutamate, aspartate, and taurine by newt retina is shown in the light microscope autoradiographs, Figs. 7 to 10. Figure 7 shows the uptake of (3H)-glutamate by the newt retina. As is evident from this micrograph, labelled glutamate has been taken up by Müller cells since the nuclear region, as well as the apical and foot processes are heavily labelled. No other retinal cell type accumulated significant amounts of glutamate.

Figure 8 shows the uptake of (3H)-aspartate by newt retina. This label too, was taken up preferentially by Müller cells, as is evident from the localization of silver grains over Müller cell nuclei and processes. In addition, there was an accumulation of (3H)-aspartate by some cells in the outer nuclear layer, as is evident from the band of silver grains in this region (Fig. 8). Figure 9 is a high magnification light micrograph of a toluidine-blue stained retina, showing that the (3H)-aspartate labelling pattern in the outer nuclear layer is not due to the apical processes of Müller cells, but rather to the accumulation of (3H)-aspartate by photoreceptor cells.

Figure 10 shows the uptake of (3H)-taurine in the newt retina. Label has been taken up by photoreceptor cells, cells in the position of the amacrine interneurons, and by

some cells in the ganglion cell layer as well. To determine if the (3H)-taurine was associated with photoreceptor cell synaptic organelles, electron microscope autoradiography was also performed. Figures 11A and 11B are low power electron microscope autoradiographs showing that silver grains were not associated with any particular photoreceptor subcellular organelle, nor was taurine specifically localized to photoreceptor synaptic terminals; the distribution of silver grains over photoreceptors was general.

4) Kainate Lesions of Newt Photoreceptor Postsynaptic Elements. Injections of kainic acid, an analog of glutamate, into newt eyes caused swelling of certain retinal neurons. This swelling increased in severity with time and the concentration of the kainic acid solution injected. At concentrations greater than 5 nmol, or survival times longer than 30 min, electron microscope observations showed that extensive damage occured in both plexiform layers. Some processes in the inner and outer plexiform layers showed obvious signs of swelling, while other processes appeared morphologically normal (Figs. 12 and 14). Cell death (as indicated by chromatolytic changes) at higher concentrations and longer survival times occured randomly, without affecting an identifiable subpopulation of neurons.

Figure 12 shows the effect of a 2.5 nmol injection of kainic acid after a 30 min survival time, on synaptic processes of the outer plexiform layer. Several processes show distinct swelling (asterisks) and some of these swollen

processes receive synaptic input (arrows) from rod and cone synaptic terminals. The fact that these elements appear swollen implies that they possess glutamate receptors on their surface. Figure 13 (A and B) are high magnification electron micrographs showing swollen postsynaptic elements (asterisks) near cone and rod synaptic terminals. The cone terminal shown in Fig. 13A forms a ribbon synapse with two swollen postsynaptic elements (P1 and P2). The same cone terminal also forms a ribbon synapse with two postsynaptic elements (P3 and P4) which show no obvious sign of swelling. The rod synaptic terminal shown in Fig. 13B forms a ribbon synapse with two postsynaptic elements (P1 and P2) which are also unaffected by kainic acid. In summary, the terminals of both rods and cones were seen to make synaptic contact with postsynaptic elements which showed obvious signs of swelling and with postsynaptic elements which were seemingly unaffected by kainic acid.

In the inner plexiform layer, cell processes also demonstrated signs of swelling. Figure 14 shows cell processes in the inner plexiform layer which have become swollen (asterisks) following a 2.5 nmol injection with a 30 min survival time. Bipolar cell terminals, possible monoamine containing cells (as indicated by the presence of dense-cored vesicles), and certain amacrine cell processes, were unaffected by the kainic acid injection. To which class of retinal neuron the swollen cells belong is unknown, because the treatment obliterates all cellular features.

Figure 15 is an electron micrograph of an optic nerve fascicle, indicating that the ganglion cell axons do not show early signs of swelling or degeneration following a 5.0 nmol injection of kainic acid.

5) Histochemical Demonstration of -SH groups in the Outer Plexiform Layer of the Newt Retina: ZIO, MO, and DACM Reactions. When newt photoreceptor cells were stained by the zinc iodide osmium (ZIO) technique to demonstrate free -SH groups, a clear and well-defined staining pattern was evident (Fig. 16). Staining of the outer segments of rods, but not cones, the matrix of some mitochondria, and synaptic vesicles within the synaptic terminals of both rods and cones, was observed. Nearly all of the synaptic vesicles were stained by this method (inset, Fig. 16), imparting a density to the photoreceptor synaptic terminals which was evident even at the light microscope level. When newt retina was incubated prior to staining with the ZIO technique in dithioerythrotol (DTE), a substance which protects -SH groups, the staining of synaptic vesicles was intense (Fig. 17A). When the control newt retina was incubated prior to staining with the ZIO technique in N-ethyl maleimide (NEM), a substance which blocks -SH groups, the reactivity of synaptic vesicles in both rods and cones almost completely disappeared (Fig. 17B).

The specificity of the ZIO technique for -SH groups was further tested with the mercury orange (MO) stain for thiols. Figure 18 is a series of light micrographs showing

newt retina which was stained with MO after incubation in buffer (Fig. 18A), DTE (Fig. 18B), and NEM (Fig. 18C). A light mercury-orange stain precipitate is seen over the outer plexiform layer in Fig. 18A. This reaction was enhanced with pretreatment in DTE (Fig. 18B) and abolished with pretreatment in NEM (Fig. 18C).

Similar results were obtained when newt retina was treated with the highly fluorescent thiol probe, DACM. The outer plexiform layer of the newt retina was highly fluorescent when treated with DACM (Fig. 19A). If sections of retina were pretreated with DTE, the outer nuclear layer, as well as the outer plexiform layer fluoresced (Fig. 19B). If sections of retina were treated with NEM prior to DACM treatment, the fluorescence which was induced in the outer plexiform layer is completely abolished.

Although some synaptic vesicles within neuronal processes of the inner plexiform layer stain with the ZIO technique, they are not present in significant concentrations as to be evident with these highly sensitive and specific, light microscope methods for the demonstration of thiols. Furthermore, all of the staining of the vesicles in the inner plexiform layer could not be abolished by blocking -SH groups with NEM. So, it is clear that the outer plexiform layer is unique for both the specificity of the staining of free -SH groups and the concentrations of -SH groups which can be localized there.

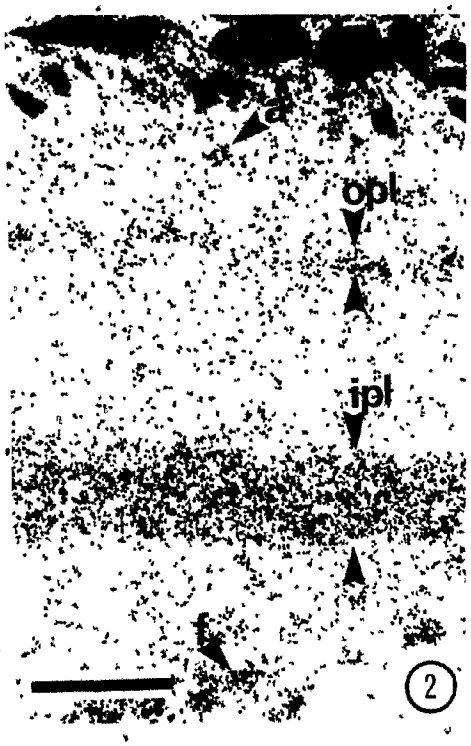
FIGURES

Figure 1. Light microscope autoradiograph showing the binding of (H)-quinuclidinyl benzylate ((H)-QB) to muscarinic acetylcholine receptors in the outer plexiform layer (opl) and inner plexiform layer (ipl) of the newt retina. The binding of (3H)-QB in the plexiform layers implies that these receptors are located on dendrites which are postsynaptic to cholinergic cells. However, at least some of these receptors are localized on Müller cell membranes because the apical processes (a) and foot processes (f) of Müller cells are labelled. bar= 50 µm.

Figure 2. Light microscope autoradiograph showing the binding of (125I)-α-bungarotoxin (125I)-BTX receptors in the outer plexiform layer and inner plexiform layer of the newt retina (arrows). The binding pattern of (125I)-BTX is similar to that of (3H)-QB. In addition to binding in the plexiform layers, the apical processes (a) and foot processes (f) of Müller cells are also labelled. bar= 50 µm.

Figure 3. Electron microscope autoradiograph showing the subcellular distribution of (125I)-BTX binding sites in the outer plexiform layer of the newt retina. Silver grains (s) are generally distributed and no specific localization to a population of postsynaptic processes can be observed. cn= cone nucleus, bar= 1 µm.


a
opl
ipl
1

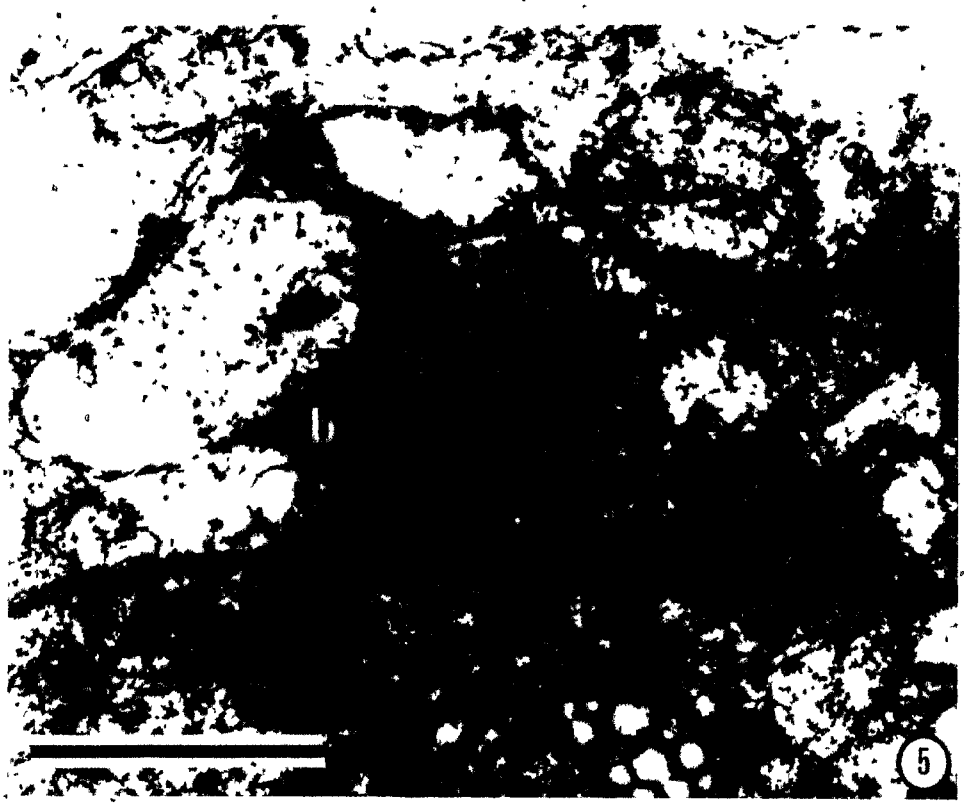

a
opl
ipl
2


3

Figure 4. Electron microscope autoradiograph showing the subcellular distribution of (125I)-BTX binding sites in the inner plexiform layer of the newt retina. Silver grains are associated with an amacrine cell process (a') which appears to be receiving synaptic input (arrow) from another amacrine cell (a) containing numerous, small, clear vesicles. bar= 1 µm.

Figure 5. Electron microscope autoradiograph showing the subcellular distribution of (125I)-BTX binding sites in the inner plexiform layer of newt retina. Silver grains are associated with an amacrine cell process (a) which may be receiving synaptic input from an adjacent bipolar cell (b). bar= 1 µm.

4



5

Figure 6. Graph showing the fluctuation of neuroactive amino acids in the newt retina in light and dark conditions, as identified by ion exchange chromatography. All of the amino acids show fluctuations in their corrected concentration (Table I) as the light condition changes. The corrected concentration of free glutamine, alanine, and glycine is greater in the dark adapted newt retina and the concentration of free taurine, glutamate, GABA, and proline is greater in the light adapted newt retina.

FLUCTUATION OF NEUROACTIVE AMINO ACIDS IN NEWT RETINA

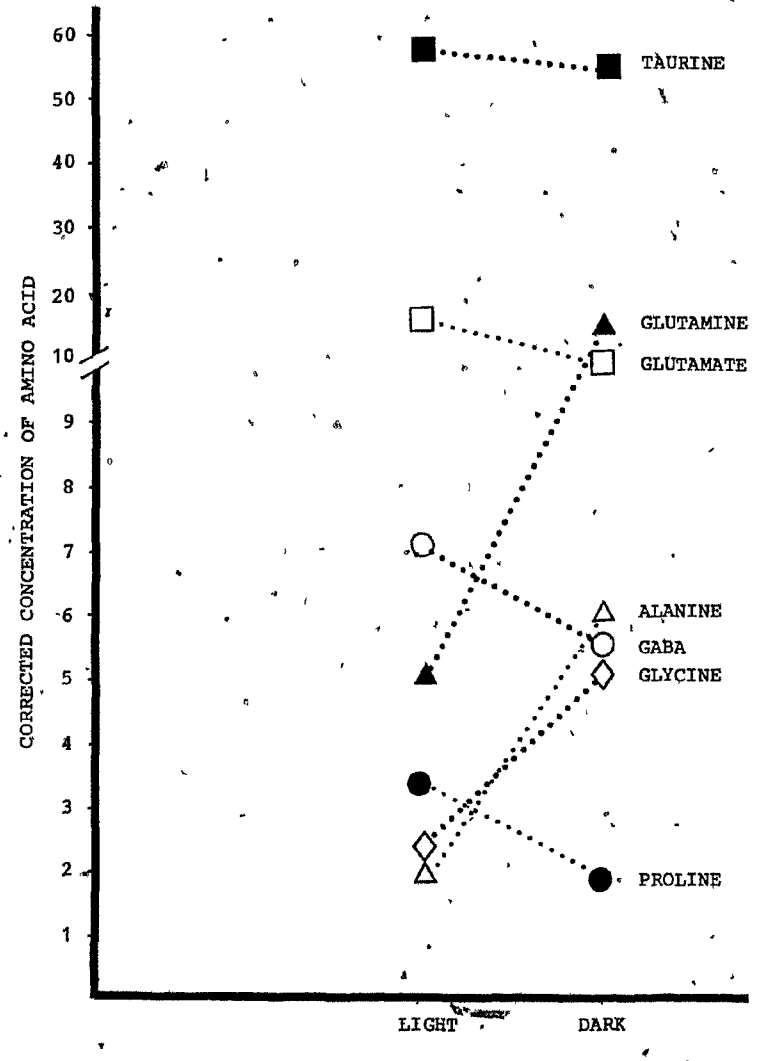


Figure 7. Light microscope autoradiograph showing the uptake of (3H)-glutamate by newt retina. Label is seen over Müller cell apical (a) and foot processes (f), as well as over cell bodies in the position of the Müller cell nuclei (mn). Silver grains were not localized in the plexiform layers. bar= 50 µm.

Figure 8. Light microscope autoradiograph showing the uptake of (3H)-aspartate by newt retina. In addition to the accumulation of silver grains over Müller cell processes (f) and Müller cell nuclei (mn), label has been accumulated by cells in the outer nuclear layer (onl). os= photoreceptor outer segments, bar= 50 µm.

Figure 9. High power light microscope autoradiograph of a toluidine blue-stained section of newt retina which was incubated in (3H)-aspartate. Silver grains are associated with the photoreceptor nuclei (pn) and not the columns of Müller cell cytoplasm which separate the photoreceptor cells. pe= pigment epithelium, opl= outer plexiform layer, bar= 10 µm.

Figure 10. Light microscope autoradiograph showing the uptake of (3H)-taurine by newt retina. There is heavy accumulation of silver grains over photoreceptors (p), a few cells lining the inner border of the inner nuclear layer (inl), and some cells in the ganglion cell layer (gcl). arrows=plexiform layers, bar= 50 µm.

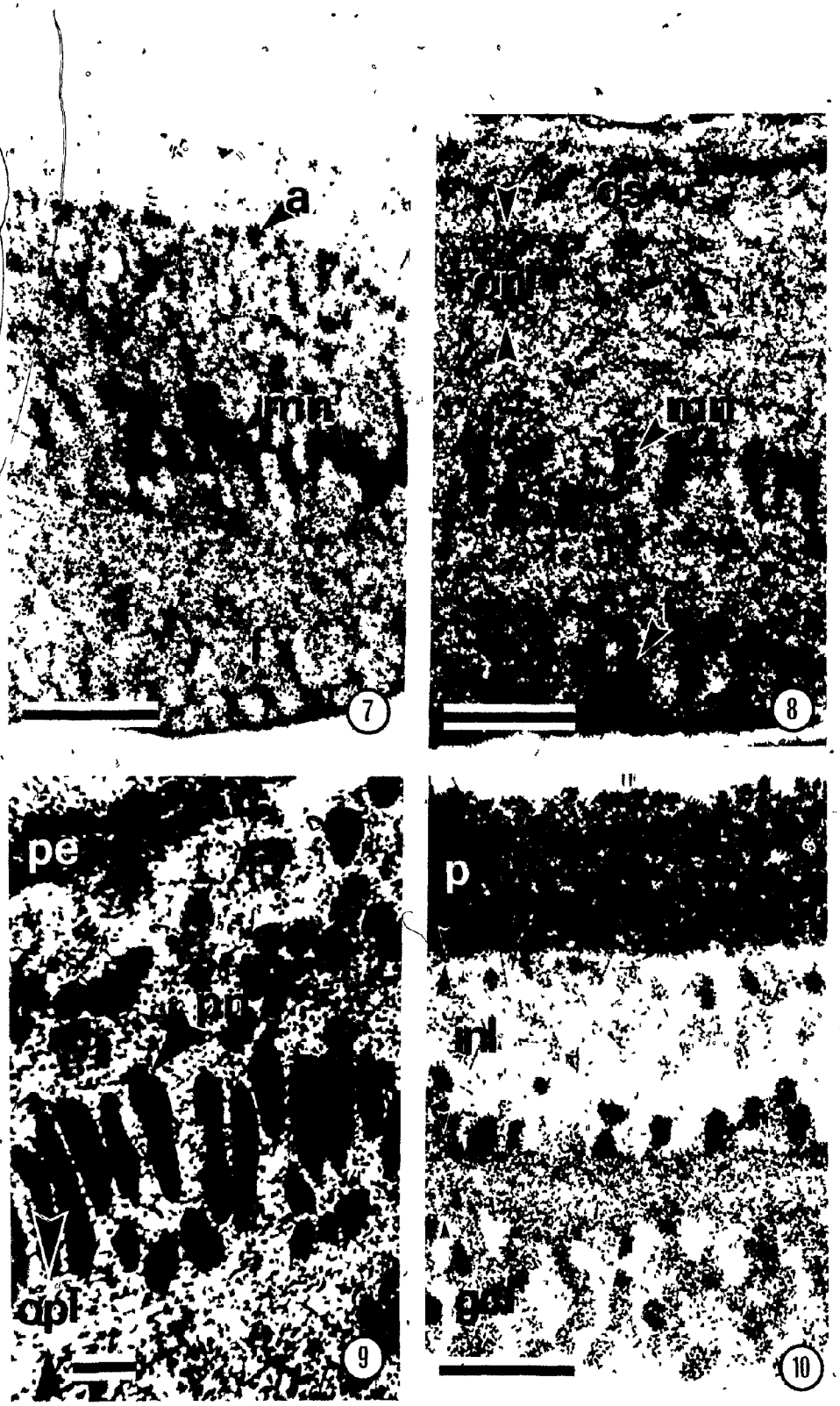

a
mn
7
os
cm
mn
8
pe
pp
apl
9
p
nl
gcl
10

Figure 11. (A and B) Low power electron microscope autoradiographs showing the subcellular distribution of (3H)-taurine uptake by newt photoreceptor cells. Silver grains are generally distributed over all portions of the photoreceptor. os= cone outer segment, e= ellipsoid, p= parabaloid, m= myoid, cn= cone nucleus, rn= rod nucleus, ct= cone synaptic terminal, bars= 5 µm.

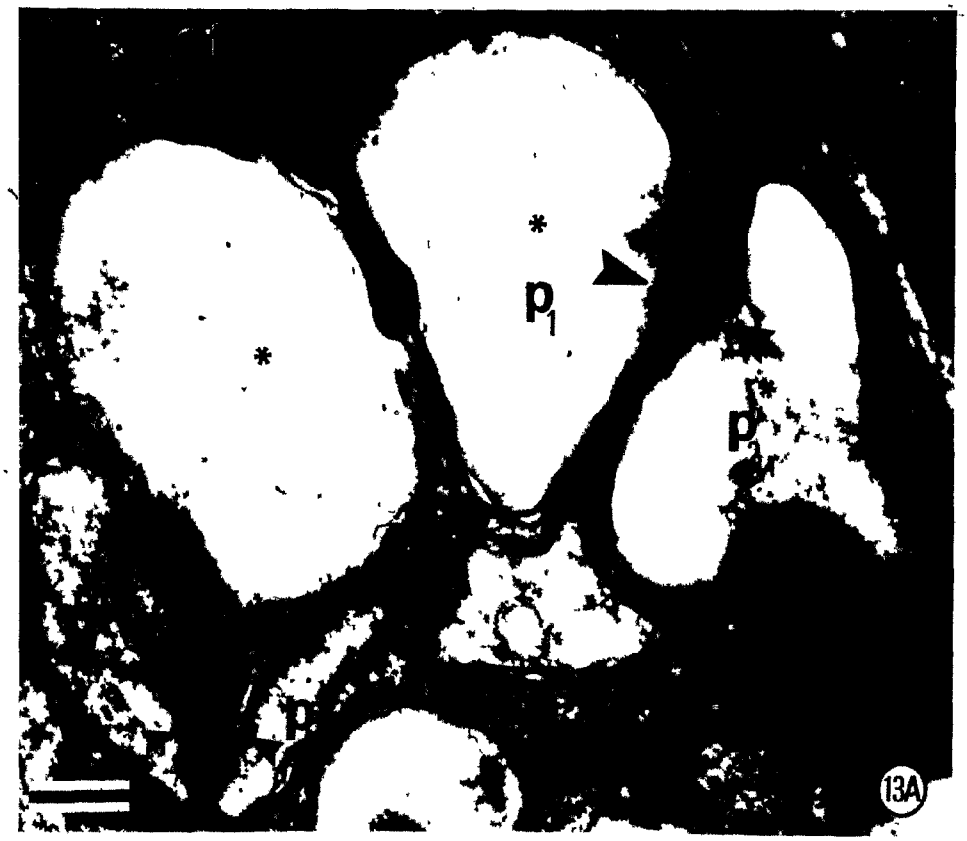

P
ROS
11A


ITT
11B

Figure 12. Low power electron micrograph of the outer plexiform layer of the newt retina showing the effect of an intraocular kainic acid injection on neuronal processes in this layer. A large number of processes show marked signs of swelling (asterisk) while others are not affected at all. Some of the swollen processes are postsynaptic to rod and cone synaptic terminals (arrows). rt= rod terminal, cn= cone nucleus, bar= 1 μm.


12

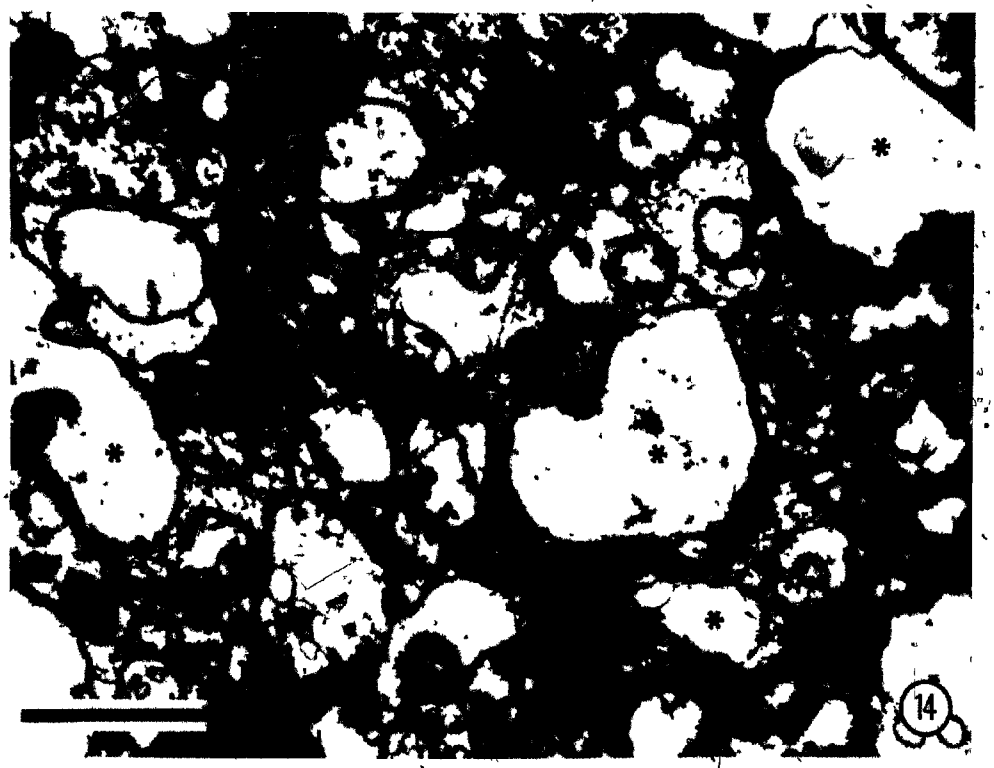
Figure 13. (A and B) High magnification electron micrographs showing the effect of intraocular injection of kainic acid on the postsynaptic elements of newt cone (Fig. 13A) and rod (Fig. 13B) photoreceptors. Some postsynaptic elements which receive synaptic input from photoreceptors (arrows) show distinct signs of swelling (asterisk), while others do not. In Fig. 13A, p1 and p2 swollen cone postsynaptic elements form a ribbon synapse with a cone terminal (ct). Postsynaptic elements p3 and p4 are cone postsynaptic elements which are seemingly unaffected by kainic acid injection, although they form a ribbon synapse with the same terminal as p1 and p2. In Fig. 13B, p1 and p2 are normal rod postsynaptic elements forming a ribbon synapse with a rod terminal (rt). bars= 0.25 µm.


P1
P2
13A

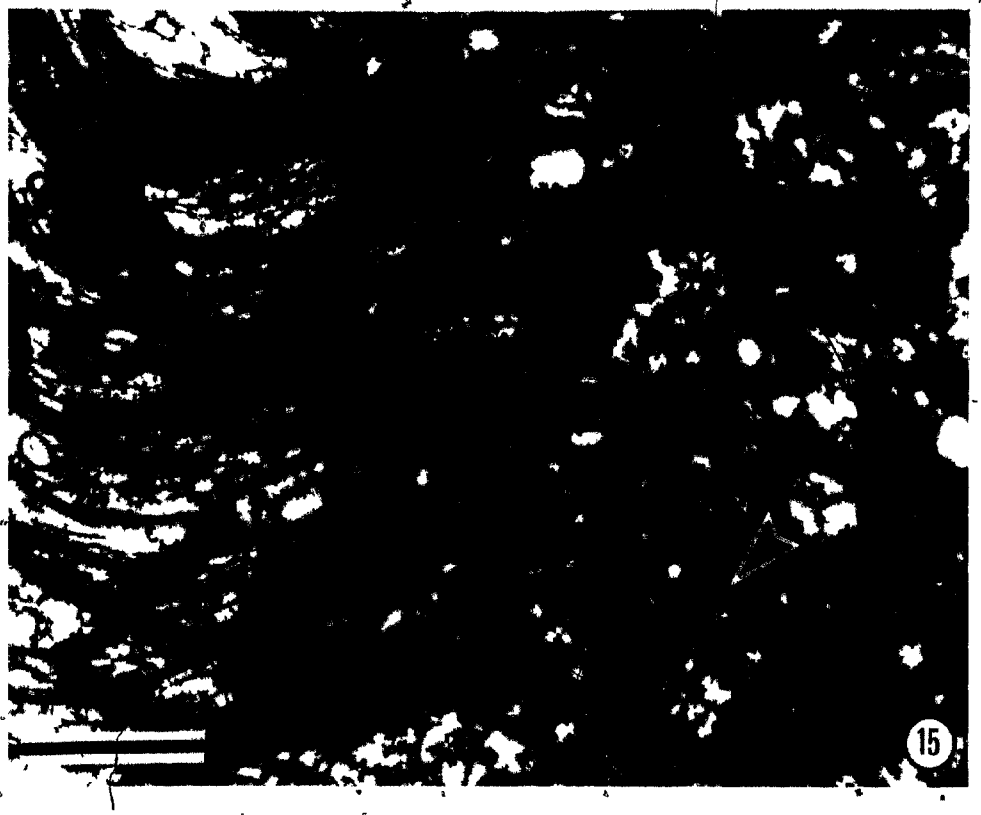

13B

Figure 14. Electron micrograph showing the effect of intraocular injection of kainic acid on neuronal processes in the inner plexiform layer of the newt retina. Several processes show obvious signs of swelling (asterisk) while certain amacrine cells (a), bipolar cells (b) and possible monoamine containing cells (d) appear to be unaffected. bar= 1 µm.

Figure 15. Electron micrograph showing the effect of intraocular kainic acid injection on ganglion cell axons (arrow) as they pass through the ganglion cell layer. The ganglion cell axons show few signs of swelling in response to kainic acid injection. bar= 1 µm.



14

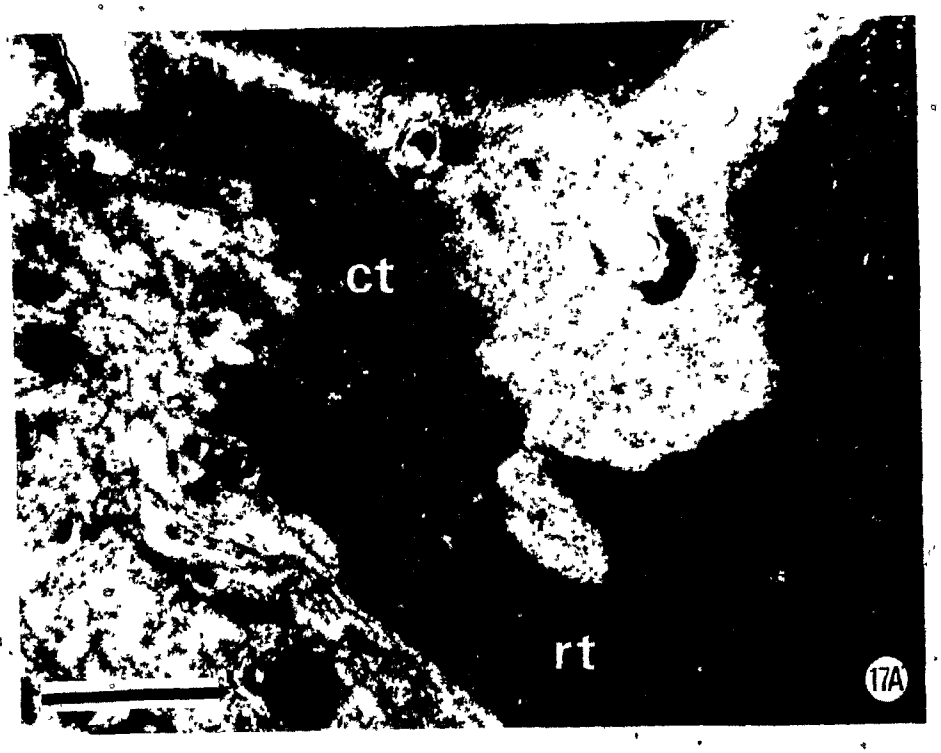


15

Figure 16. Low power electron micrograph of newt photoreceptors showing their reactivity with the Zinc Iodide Osmium (ZIO) technique. The outer segments of rods (ros) show strong reactivity, while the outer segments of cones (cos) show no reactivity to ZIO. Although the synaptic terminals of both rod and cone photoreceptors are strongly reactive, high power electron micrographs of a newt photoreceptor synaptic terminals (inset) show that the ZIO reactivity in the terminal is restricted to synaptic vesicles (sv). n= photoreceptor nucleus, sr= synaptic lamellae, bar= 5.0 µm, INSET bar= 0.2 µm

Figure 17A. Electron micrograph of photoreceptor synaptic terminals which were pretreated with dithioerythrotol (DTE), to protect -SH groups, prior to ZIO treatment. Synaptic vesicles in both rod (rt) and cone (ct) synaptic terminals are as strongly reactive as when pretreatment was in buffer only (Fig. 16). bar= 1 μm.

Figure 17B. Electron micrograph of cone (ct), and rod (rt) synaptic terminals which were pretreated with N-ethyl maleimide (NEM), to block -SH groups, prior to ZIO treatment. Few synaptic vesicles are reactive to ZIO. These specific enhancing (DTE) and blocking (NEM) reactions prove that the ZIO reactivity of photoreceptor synaptic vesicles is due to free -SH groups. bar= 1 μm.


ct
rt
17A


17B

Figure 18A. Light micrograph of newt retina showing the reaction of the outer plexiform layer (arrows) to the -SH group reagent, Mercury Orange (MO). Precipitate can be seen in the outer plexiform layer where photoreceptor synaptic terminals make contact with bipolar and horizontal cells. pn= photoreceptor cell nuclei, opl= outer plexiform layer, bar= 25 µm (for Figs. 18a, b, c)

Figure 18B. Light micrograph showing that if newt retina is pretreated in DTE, to protect -SH groups, the reactivity of the outer plexiform layer with Mercury Orange is enhanced.

Figure 18C. Light micrograph showing that if newt retina is pretreated in NEM, to block -SH groups, the reactivity of the outer plexiform layer with Mercury Orange is completely abolished.

Figure 19A. Fluorescence micrograph showing the reactivity of the outer plexiform layer of newt retina to the thiol directed probe, DACM. Fluorescence can be observed in a band corresponding to the position of the outer plexiform layer. pn= photoreceptor nuclei, opl= outer plexiform layer, bar= 25 µm (for Figs. 19a, b, c)

Figure 19B. Fluorescence micrograph of newt retina showing that if retina is pretreated in DTE to

protect -SH groups, fluorescence is induced in the entire photoreceptor cell layer. The remainder of the retina remains unreactive.

Figure 19C. Fluorescence micrograph of newt retina showing that if retina is pretreated with NEM, to block -SH groups, prior to reaction with DACM, fluorescence induced in the inner plexiform layer is completely abolished.

19A
19B
19C
pd
18A
18B
18C
pd
cm

DISCUSSION

The techniques described in this chapter have been aimed at elucidating the nature of the photoreceptor transmitter in the newt.

1) Localization of Acetylcholine Receptor Sites. Over the years, a wide variety of compounds have been put forth as putative synaptic transmitters in retinal photoreceptors. Acetylcholine is one such putative transmitter, and attempts to localize it within specific retinal cells have been through the demonstration of the degradative enzyme acetylcholinesterase, or more recently, through ligands which specifically label binding sites on neurons which are postsynaptic to acetylcholine-releasing synaptic terminals. Quinuclidinyl benzylate ((3H)-QB) binds muscarinic acetylcholine receptors, while α-bungarotoxin binds to nicotinic acetylcholine receptors.

The results of (3H)-QB binding in the newt retina show that (3H)-QB receptors are localized in both plexiform layers of the newt retina. Although some of the binding in the plexiform layers was to Müller cell processes, the intense labelling of both plexiform layers indicates that (3H)-QB binding sites are located on neuronal processes as well. These results are not in agreement with the findings of Sugiyama et al. (1977), who were only able to localize (3H)-QB binding sites in the inner plexiform layer. This is despite the fact that (3H)-QB binding sites have been

localized to a purified P1 fraction (containing photoreceptor synaptic terminals) of subfractioned bovine retina by Moreno-Yanes and Mahler, (1979a). However, because muscarinic agents, such as atropine and probanthine, block transmission to turtle hoizontal cells (although at high concentrations), and nicotinic agents do not (Gershenfeld and Piccolino, 1977), it is more likely that if a cholinergic system exists in the outer plexiform layer, it is by muscarinic cholinergic transmission.

The results show that (125I)-BTX binding sites have been localized in both plexiform layers of the newt retina. Like the binding of (3H)-QB to receptors in the plexiform layers, some, but probably not all, of this binding is to Müller cell processes. Binding of (125I)-BTX to both plexiform layers has been reported in other species as well (Vogel and Nirenberg, 1976; Yazulla and Schmidt, 1976; Schwartz and Bok, 1978; Yazulla, 1979). Although this study did not employ the use of inhibitors to examine the specificity of (125I)-BTX or (3H)-QB binding for acetylcholine receptors, such studies have been carried out for pigeon and chick retina (Yazulla and Schmidt, 1977; Yazulla, 1979). Yazulla (1979) found that binding of (125I)-BTX in the avian outer plexiform layer could not be inhibited by nicotine, implying that receptor sites located there are not specific. As pointed out by Yazulla (1979), interpretation of (125I)-BTX binding in the retina must await evaluation of the ability of α-bungarotoxin to block

cholinergically-induced retinal activity. Recent evidence suggests that α-bungarotoxin may bind to specific BTX receptors which are distinct from nicotinic acetylcholine receptors, and which have their own unique function in the central nervous system (Oswald and Freeman, 1981). Non-neuronal and non-specific binding of (125I)-BTX to receptors in the outer plexiform layer could account for the difficulty in localizing (125I)-BTX binding sites to specific neuronal processes in the newt outer plexiform layer by electron microscopy, and the inability to localize horseradish peroxidase-conjugated α-bungarotoxin (HRP-BTX) binding sites in the outer plexiform layer of chick (Vogel et al., 1979; Daniels and Vogel, 1980) and mouse (Pourcho, 1979) retinas. Because (125I)-BTX binding sites have been localized to both plexiform layers of the rabbit retina, but only the inner plexiform layer of the rat retina, it cannot be ruled out that there are species differences in the ability of postsynaptic elements in the outer plexiform layer to bind (125I)-BTX (Vogel and Nirenberg, 1976). The binding sites of (125I)-BTX are also difficult to associate with neuronal processes in the outer plexiform layer because of the small size of the bipolar and horizontal cell processes. Yazulla (1979) has pointed out that the ability of electron microscope autoradiography to localize (125I)-BTX binding sites to specific outer plexiform layer neuronal processes is limited by the resolution of the technique.

Although both light and electron microscope autoradiography show that (3H)-QB and (125I)-BTX receptors are located within the outer plexiform layer, to what extent these receptors are localized to Müller cell membranes could not be determined. Until the specificity of (3H)-QB and (125I)-BTX to bind acetylcholine receptors has been determined, and these receptors can be accurately localized, the significance of (3H)-QB and (125I)-BTX binding in the inner plexiform layer of the newt retina cannot be determined.

2) Analysis of Free Retinal Amino Acids. Amino acid analysis of newt retina in both light and dark conditions suggests that the putative amino acid neurotransmitters, which have been identified in other studies, fluctuate significantly, depending on the lighting conditions.

The excitatory amino acid glutamate, was found in higher concentrations in the light-adapted newt retina than in the dark-adapted newt retina. This finding is consistent with the fluctuations of glutamate in the light and dark-adapted mouse retina (Cohen et al., 1973). These results are also consistent with the theory that glutamate may be the photoreceptor transmitter. If the photoreceptor transmitter is stored in the light and released in the dark, then the concentration of neurotransmitter would be higher in the light than in the dark. Furthermore, if the transmitter, which is released into the synaptic cleft in the dark, were swept up by Müller cells and metabolized into

some other compound, in other words, if the glutamate-glutamine-GABA cycle hypothesis (Starr, 1974) is correct, then glutamate which is released in the dark, could be removed and metabolized into glutamine. Amino acid analysis of newt retina showed that the concentration of glutamine was higher in the dark. This finding is consistent with the possibility that glutamate is released from photoreceptors in the dark and metabolized into glutamine. Glutamine has also been suggested to be a precursor for the inhibitory amino acid GABA, in a number of species (Voaden et al., 1978). If glutamine was a source of GABA in the newt retina, it would be expected that when the concentration of glutamine was low, the concentration of GABA would be high. Amino acid analysis of newt retina showed that GABA was found in higher concentrations in the light-adapted newt retina. These results therefore support the hypothesis that a glutamate-glutamine-GABA cycle could exist in the retina, and that glutamate could be the photoreceptor neurotransmitter.

Aspartate is also a candidate for the photoreceptor transmitter. Aspartate has been found in higher concentrations in the light-adapted mouse retina (Cohen et al., 1973) however, aspartate was not identified in the amino acid analysis of newt retina. Why aspartate could not be identified by ion exchange chromatography in these preparations is unknown. It is therefore impossible to comment on the possible role of aspartate as a photoreceptor

transmitter in the newt retina.

Taurine, although not suspected of being a photoreceptor transmitter, is important for normal photoreceptor maintenance. The extremely high concentration of taurine in the newt retina is a characteristic of most vertebrate retinas, where it can constitute as much as 50% of the free amino acid pool (Starr, 1977). Even though there is no doubt that taurine is present in the retina, the absolute levels of taurine, as determined by ion exchange chromatography, may be artifactually high because of possible contamination by glycerophosphoryl ethanolamine (Tachiki and Baxter, 1979). It is also possible that the taurine peak is contaminated by other sulphur-containing amino acids. The fluctuation of taurine in the newt retina may be due to the fluctuations of other -SH containing amino acids such as cysteate or cysteine sulphinate, which co-chromatograph with taurine. Furthermore, the fluctuation of taurine in these whole retinal homogenates may be influenced by taurine's role as a transmitter in the inner retina, and not be related to its function in photoreceptors at all.

It should be stressed that these experiments have been performed on whole retinas, and not just the photoreceptor layer. Futhermore, even though the fluctuation of amino acids in newt retina were similar to those reported for mouse (Cohen et al., 1973) and frog retina (Graham et al., 1970), relationships between these fluctuations and

neurotransmission are difficult to establish because all of the neuroactive amino acids are tightly linked to the tricarboxylic acid cycle. It is therefore hard to determine which amino acid molecules are destined for use in general metabolic activities and which are destined to function as neurotransmitters. This is expecially true if the concentration of amino acids required for neurotransmission is relatively small, compared to that required for metabolism. It is also possible that an amino-acid transmitter may differ in its release response, with different physiological stimuli and in different retinal layers. It is therefore much more useful to compare amino acid fluctuations in similar retinal layers, or individual cell types. It is important to realize that these light and dark adaptation experiments cannot be correlated with a number of studies which demonstrate transmitter release from retinal neurons using high frequency light flashes or potassium (Kennedy and Neal, 1978). These experiments have only attempted to identify a likely amino acid photoreceptor transmitter on the premise that it would be more concentrated in a long-term light-adapted retina (Chapter 1).

3) Autoradiographic Localization of Aspartate, Glutamate and Taurine. Autoradiography has been quite successful in implicating amino acids as neurotransmitters in the newt retina. Although the uptake of glutamate by newt retina was shown to be mainly into Müller cells, this finding is

similar to results reported for rabbit (Ehinger and Falck, 1971), frog (Kennedy et al., 1974) and rat retinas (White and Neal, 1976). Slight uptake of glutamate by rod photoreceptors has been reported for cat, monkey, human (Bruun and Ehinger, 1974) and rat retina (White and Neal, 1976), however neuronal uptake was not observed in the newt retina. As pointed out in Section 2, the uptake of glutamate by Müller cells for general metabolic purposes may mask any possible neuronal uptake of glutamate. Light may also affect the uptake of glutamate by photoreceptors. If glutamate were the transmitter of photoreceptors, it would be expected that the turnover of glutamate would be greater in the dark. This aspect of amino acid uptake was not explored in these experiments. Glutamate has also been reported to be accumulated by a few cells in the position of the amacrine cells and within the ganglion cell layer of the rabbit retina, when incubation in (3H)-glutamate is followed by incubation in a buffer (Redburn, 1981). It is therefore possible that the method of administration may also be an important factor governing the uptake of glutamate by neurons.

Although the autoradiographic localization of the cells which take up glutamate has received thorough attention in the literature, few attempts have been made to localize aspartate in the retina. Ten years ago, Ehinger and Falck (1971) described the uptake of aspartate by rabbit retina as essentially the same as glutamate; that is, into glial

cells. The uptake of aspartate by newt retina reveals that the photoreceptors accumulate exogenously-applied aspartate. The band of silver grains over the outer nuclear layer however, became apparent only after long exposure times (>6 months). This indicates that the uptake of aspartate by photoreceptors, although greater than by any other neuronal population, is much less than that accumulated by the Müller cells. As well, there is no indication from these results that any difference exists in the ability of rods or cones to accumulate aspartate. However, the localization of aspartate in photoreceptor cells substantiates more recent physiological evidence that this amino acid is the neurotransmitter substance (Neal, 1976; Wu and Dowling, 1978; Neal et al., 1979).

The results presented here show that (3H)-taurine is readily accumulated by photoreceptors. The uptake of taurine into the photoreceptor cells of newt retina is consistent with the uptake of taurine by photoreceptors in the cat (Voaden et al., 1977; Pourcho, 1981), rat, guinea pig, rabbit, pigeon (Voaden et al., 1977), and frog retinas (Kennedy and Voaden, 1976; Lake et al., 1977). Because the newt retina contains high concentrations of taurine and because the photoreceptors accumulate (3H)-taurine, it was hoped that the subcellular localization of (3H)-taurine within the photoreceptors would give some clue to the function of taurine in photoreceptor cells. Because the photoreceptor cell is compartmentalized in both functional

and structural aspects, it might be expected that the localization of (3H)-taurine to a specific region of the photoreceptor would shed some light on its function in these cells. For instance, if taurine were a neurotransmitter or neuromodulator of photoreceptor cells, it would be expected that taurine would be preferentially localized to the synaptic terminal. Unfortunately, electron microscope autoradiography has failed to associate (3H)-taurine with any specific photoreceptor region. The general localization of (3H)-taurine is more consistent with taurine's possible role as a regulator of ion movements, or a stabilizer of membranes in photoreceptors.

4) Kainic Acid Lesions. Although the autoradiographic uptake patterns of aspartate and glutamate imply that aspartate is more likely to be the newt photoreceptor transmitter, the effects of kainic acid on retinal neurons in other animals suggests that glutamate may be the photoreceptor transmitter (Olney et al., 1974). It has been postulated that at low concentrations, kainic acid binds to extended glutamate receptors on neurons, exciting these neurons to death. Kainic acid-resistant neurons are therefore suggested to be non-glutamate receptive or glutamatergic. At higher concentrations, however, kainic acid is neurotoxic to all retinal cells (Hampton et al., 1981).

Light microscope observations of chicken retina which have been treated with low concentrations of kainic acid

indicate that only cells in the inner retina are affected (Bizière and Coyle, 1979; Ehrlich and Morgan, 1980). However, in goldfish retina, Yazulla and Kleinschmidt (1980) demonstrated that all postsynaptic elements of rods were affected by kainic acid treatment, but only certain cone postsynaptic elements were affected. This apparent inconsistency was explained by the suggestion that two types of receptors are present on photoreceptor postsynaptic elements and that goldfish rods use glutamate, whereas cones use aspartate as their transmitter (Yazulla and Kleinschmidt, 1980). In the rabbit retina however, the postsynaptic elements which contact cones are sensitive to kainic acid (Hampton et al., 1981). These observations are further complicated by the observations of kainic acid's effect on the postsynaptic elements of newt photoreceptors, where some postsynaptic elements of both rods and cones swell in response to kainic acid, while others, adjacent to the same terminal, do not. If photoreceptors use glutamate exclusively as a neurotransmitter, kainic acid would be expected to bind receptors on every postsynaptic element and elicit a neurotoxic effect. However, the suggestion that kainic acid receptors are selectively linked to a retinal glutamatergic pathway has been inferred from other studies in the central nervous system. There is recent evidence which suggests that kainic acid does not bind glutamate receptors at all (Krammer et al., 1980; Michaelis et al., 1980; Hampton et al., 1981). It is certainly inappropriate then, in light of this new information questioning the

specificity of kainic acid, to suggest that newt photoreceptors are glutamatergic on the basis that kainic acid causes swelling of only some photoreceptor postsynaptic elements.

5) Histochemical Demonstration of -SH Groups. Light microscope histochemical techniques have shown that there are unusually high concentrations of -SH groups in the outer plexiform layer of the newt retina. At the ultrastructural level, free -SH groups have been localized within the synaptic vesicles of photoreceptor synaptic terminals, and these terminals occupy a significant portion of the outer plexiform layer of the newt retina. There are two possible reasons why -SH groups are present in the photoreceptor synaptic vesicles. Firstly, there may be a proteinaceous matrix within synaptic vesicles which is rich in free -SH groups and is involved in the transmitter storage or release mechanism, or secondly, the -SH groups may be due to the presence of the transmitter material itself.

Recent evidence suggests that specific intracellular sulfhydryl compounds, together with calcium, are essential for exocytosis (neurotransmitter release) to occur (Elferink and Riemersma, 1980). X-ray microanalysis of ZIO deposits at subcellular sites in smooth muscle suggest that zinc osmicate is associated with high affinity calcium-binding sites, probably displacing calcium by having a higher affinity for available calcium binding sites (Gilloteaux and Naud, 1979). In addition, Ripps et al. (1979) have

localized calcium deposits to the synaptic vesicles of skate photoreceptors. If the -SH groups located in newt photoreceptor synaptic vesicles represent sites of calcium binding, then this could explain both the staining of newt photoreceptor synaptic vesicles with ZIO and the ability of sulfhydryl blocking agents to inhibit ZIO staining. Staining of newt photoreceptor synaptic vesicles has also been observed with prolonged osmium treatment (Flight and Van Donnselaar, 1975b); this staining, in the case of mouse photoreceptors, has been shown to be increased by light stimulation (Pourcho and Burnstein, 1975). Although there is no evidence to suggest that the mechanisms of the two reactions are the same, they probably both stain synaptic vesicles for the same reason. When considered in light of the results of Chapter 1, which suggests that transmitter is stored within photoreceptor synaptic vesicles in the light, it is also quite possible that ZIO and prolonged osmication are reacting with -SH groups present in the transmitter material itself. By far the most reactive compounds with osmium are the sulphur-containing amino acids (Deetz and Behrman, 1981). It is therefore possible that prolonged osmium and ZIO staining of synaptic vesicles in photoreceptors could be due to the reactivity of these staining agents with a sulphur-containing amino acid, such as cysteic acid or cysteine sulphinate, which may act as a neurotransmitter. It is unlikely, however, that the sulphur-containing amino acid, taurine could be responsible for the presence of -SH groups in the synaptic vesicles of

photoreceptors, because (3H)-taurine could not be specifically localized to the synaptic terminals using electron microscope autoradiography.

As pointed out above, it has been suggested that -SH groups within the synaptic vesicles are responsible for the calcium-sequestering ability of synaptic vesicles (Gilloteaux and Naud, 1979). However, the localization of calcium and -SH groups within synaptic vesicles differs. One of the main differences between calcium localization and the staining of synaptic vesicles with either prolonged osmium treatment or ZIO is that calcium deposits are discretely localized on a small portion of the inner surface of the synaptic vesicle membrane (Ripps et al., 1979; Israel et al., 1980), while osmium and ZIO stains the entire vesicular interior. Furthermore, the staining of -SH groups, responsible for calcium binding, should be a feature of all synaptic vesicles, not just those of a select population of cells. Although the intensity of the prolonged osmium staining may be less in the dark, due to the occupation of the -SH group reactive sites by calcium which flows into the photoreceptor terminal during active neurotransmission, it is also possible that the staining of vesicles is less intense in the dark because there is less neurotransmitter material present. If the prolonged osmium reactivity is due to the presence of free -SH goups in an amino acid neurotransmitter such as cysteate, then reactivity would certainly be expected to be lower in the dark and more

intense in the light, a time when neurotransmitter is stored (see Chapter 1).

Whether the osmium and ZIO reactivity is due to part of the transmitter release mechanism or the neurotransmitter material itself, remains to be determined. However, the demonstration of -SH groups in the synaptic vesicles of photoreceptors, the comparable potency of aspartate and cysteate as neurotransmitters, and the metabolic link between cysteate and taurine, makes cysteate a candidate for the photoreceptor transmitter substance that deserves much more investigation.

In conclusion, these studies on the photoreceptor transmitter show that:

1). Acetylcholine receptor-binding studies are complicated by the binding of muscarinic and nicotinic reagents by newt Müller cell membranes. While there is specificity in the binding of (125I)-BTX to neuronal membranes, postsynaptic to cholinergic processes in the inner plexiform layer, no such specificity was evident in the outer plexiform layer. The ability to localize acetylcholine receptor-binding sites to photoreceptor postsynaptic elements was limited by non-neuronal and non-specific binding of cholinergic ligands, and the resolution of the electron microscope autoradiographic technique.

2) The fluctuation of free retinal amino acids in response to changes in the light condition is probably

complicated by their role in intermediary metabolism, the complex nature of the stimuli expected to release amino acid neurotransmitters, and the possible use of a single amino acid transmitter in two neuronal cell populations, which release transmitter during opposite stimulatory conditions. Nevertheless, the fluctuations of the amino acid, glutamate, in light and dark conditions, are consistent with its functioning as a photoreceptor neurotransmitter.

3) Of the two excitatory putative amino acid neurotransmitters, glutamate and aspartate, aspartate is more likely to be the newt photoreceptor transmitter because an uptake system was identified for aspartate in these cells. Taurine, which is not a photoreceptor transmitter candidate, has a ubiquitous subcellular localization in the newt photoreceptor which implies that it may function as a regulator of ion movement, or a stabilizer of membranes in photoreceptors.

4) The glutamate analog, kainic acid, causes swelling of only some newt photoreceptor postsynaptic elements. This finding, together with recent studies showing that kainic acid does not bind to glutamate receptors, as was once thought, cannot be taken in support of glutamate as the newt photoreceptor transmitter.

5) Histochemical tests show that there are unusually high concentrations of free -SH groups in the synaptic vesicles of newt photoreceptor terminals. Although the

significance of the -SH groups is unknown, their presence may indicate that the photoreceptor transmitter contains sulphur groups, such as the sulphonic amino acids, cysteate and cysteine sulphinate.

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CHAPTER 3

LOCALIZATION OF PUTATIVE INHIBITORY AMINO ACID TRANSMITTERS IN THE INNER RETINAL LAYERS

INTRODUCTION

The outer nuclear layer of the retina contains the perikarya of the photoreceptor cells which probably utilize only one or two transmitter substances. The inner nuclear layer is much more complex by comparison, in that the perikarya of the bipolar cells, horizontal cells, amacrine cells and interplexiform cells are all located there. Furthermore, there are subpopulations of these neurons, each of which probably use one or more neurotransmitters.

1) Horizontal Cells. The horizontal cells, which counteract the direct photoreceptor stimulation of bipolar cells, probably release an inhibitory neurotransmitter (Starr, 1977). Since amine-accumulating cells have been localized only to that area lining the inner margin of the inner nuclear layer, it is likely that the transmitter of the horizontal cells is an inhibitory amino acid (Starr, 1977). Although high-affinity uptake systems for the inhibitory amino acids taurine and glycine have not been demonstrated in the horizontal cells of most species studied so far (Bruun and Ehinger, 1972; Lake et al., 1975; 1977; 1978; Voaden et al., 1977; Kong et al., 1980; Pourcho, 1981; Rayborn et al., 1981), a great deal of evidence supports

"γ-aminobutyric acid" (GABA) as a likely transmitter candidate in horizontal cells (for review see: Neal, 1976; Starr, 1977). However, a high-affinity uptake system, as identified by the autoradiographic uptake of (3H)-GABA has not been demonstrated in all species studied.

Horizontal cells which accumulate (3H)-GABA have been identified in chick (Marshall and Voaden, 1974a; Yazulla and Brecha, 1980), pigeon (Marshall and Voaden, 1974a), goldfish (Marc et al., 1978; Lam et al., 1980; Yazulla and Brecha, 1980), frog (Voaden et al., 1974; Hollyfield et al., 1979), and catfish (Lam et al., 1978) retinas. However, in rabbit (Ehinger, 1970; Ehinger and Falck, 1971; Ehinger, 1972; Bruun and Ehinger, 1974; Ehinger, 1977; Brandon et al., 1979; Pourcho, 1980; Redburn, 1981), cat (Bruun and Ehinger, 1974; Nakamura et al., 1980), guinea pig, rat, monkey, and human (Bruun and Ehinger, 1974) retinas, no uptake of (3H)-GABA by horizontal cells has ever been observed. The uptake of (3H)-GABA by horizontal cells of the goldfish retina has been studied extensively, and it has been determined that GABA uptake is only into H1 horizontal cells, contacting red, green and blue sensitive cones (Marc et al., 1978). The neurotransmitter of other identified goldfish horizontal cell types (H2 and H3) is unknown.

The identity of the neurotransmitter(s) of the horizontal cells in species which do not demonstrate any uptake of GABA is also not known.

2) Bipolar Cells. The dendritic arborization of the bipolar cells enables them to contact and integrate the responses of numerous photoreceptor cells. This information is passed to amacrine cells and ganglion cells at excitatory ribbon synapses within the inner plexiform layer (Starr, 1977). The only likely candidate agreed upon for the bipolar cell transmitter to date, is "acetylcholine".

Acetylcholinesterase has been localized within the inner plexiform layer of pig, sheep, rabbit, rat, guinea pig, chick, minnow (Francis, 1953), frog (Brecelj et al., 1979), and newt (Dickson, personal communication) retinas. In sheep and rabbit retinas, the enzyme can be localized specifically to bipolar cells (Francis, 1953). However, in the rabbit, the only cells which have been shown to synthesize acetylcholine are a population of amacrine cells (Masland and Mills, 1979), and it is likely that the acetylcholinesterase staining of the inner plexiform layer in the rabbit is due to the processes of these cells.

Nicotinic and muscarinic acetylcholine binding sites have been identified in the inner plexiform layer of the newt retina (Chapter 2) and several other species (Yazulla, 1979; Daniels and Vogel, 1980). The inner plexiform layer's acetylcholine receptors have been localized to ribbon (bipolar) synapses and conventional (amacrine) synapses using horseradish peroxidase conjugated α-bungarotoxin (HRP-BTX) in mouse (Pourcho, 1979) and chick (Vogel et al., 1977) retinas.

Although acetylcholine is the only transmitter which has been associated with bipolar cell terminals, it is possible that other putative transmitters may be present in different classes of bipolar cells. Only 14-20% of the bipolar synapses in the inner plexiform layer of the chick retina have been shown to be associated with HRP-BTX (Vogel et al., 1977). Furthermore, bipolar cells in the rabbit retina do not synthesize acetylcholine, implying that they must use some other transmitter substance (Masland and Mills, 1979). Whether or not acetylcholine is the only bipolar cell transmitter must await further studies on acetylcholine's role in the retina.

3) Amacrine Cells. As previously stated, amacrine cells are the most diversified of all the retinal neurons because they form both excitatory and inhibitory synapses in the retina. As a result, a number of transmitter substances have been associated with these cells (Starr, 1977).

a) Acetylcholine: There is little doubt that some of the acetylcholinesterase staining which can be observed within the inner plexiform layer is due to a population of "cholinergic" amacrine cells, as well as some bipolar cells. Binding sites labelled with (125I)-α-bungarotoxin can be specifically localized to amacrine cell processes in newt retina (Chapter 2) and HRP-BTX staining has been shown to be associated with amacrine cell synapses in mouse (Poutcho, 1979) and chick (Vogel et al., 1977) retinas. In the rabbit retina, a small group of amacrine cells (2-5%) has been

shown to synthesize acetylcholine (Masland and Mills, 1979). This small percentage agrees with the percentage of amacrine cell synapses which bind HRP-BTX in mouse (Pourcho, 1979) and chick (Vogel et al., 1977) retinas, implying that these cells may serve a comparable functional role in these species (Pourcho, 1979). The effects of acetylcholine on ganglion cell responses in carp retina suggests that cholinergic amacrine cells make excitatory synapses onto ON-OFF ganglion cells (Glickman and Adolph, 1981).

b) Monoamines: Formaldehyde-induced fluorescence of retinal neurons has clearly demonstrated that "monoamines" are present in cells occupying the position of the amacrine interneurons. In teleost fish and new world monkeys, dopamine fluorescence has been determined to be present in the interplexiform cell (Dowling, 1979). However, catecholamine fluorescence is observed in other cells, namely the amacrine cells (Malmfors, 1963; Adolph et al., 1980). These fluorescent cells account for 5-10% of the total number of cells in the innermost region of the inner plexiform layer (Rodieck, 1973).

Although most of the catecholamine fluorescence in retina is attributable to dopamine, at least some of the fluorescence is due to noradrenaline (Ehinger and Falck, 1969a). Autoradiography of cells accumulating (3H)-L-DOPA or (3H)-dopamine results in labelling of cells in a similar position to the fluorescent perikarya described above, and as well, divides the inner plexiform layer into sublaminae

which corresponds to those seen with formaldehyde induced fluorescence microscopy (Ehinger and Falck, 1971; Sarthy et al., 1981). Furthermore, the uptake of the potent dopaminergic agonist (3H)-ADTN also labels amacrine cells in the rabbit retina, which correspond in position to fluorescent catecholaminergic amacrine cells, and has a unique distribution that cannot be confused with indolamine, cholinergic, GABA-ergic, or glycinergic amacrine cells (Ehinger, 1981).

In addition to catecholamine fluorescence, serotonin (5-hydroxytryptamine) fluorescence is evident within the inner retina as well (Ehinger and Floren, 1976; Fukuda et al., 1979). Depending on the species studied, these indolamine-accumulating neurons exceed in numbers, the dopaminergic neurons by a factor of 2 to 20 (Ehinger and Holmgren, 1979; Adolph et al., 1980). Even though the cells which fluoresce in response to indolaminergic injection are sure to be a different population of neurons from the catecholaminergic neurons, they: 1) cannot be observed without prior injection of indolamines; 2) the putative serotinergic neurons in the retina cannot be selectively destroyed by specific toxins; 3) the serotonin cannot be detected, even though they are 2 to 20 times more numerous than the catecholaminergic amacrine cells, with highly sensitive assay techniques; and 4) there is no detectable amount of tryptophan hydroxylase in the retina (Ehinger and Floren, 1978; Floren, 1979; Floren and Hansson, 1980).

These findings suggest that serotonin is not a transmitter in the retina, but rather it is another, as yet unidentified, indolamine (Floren, 1979). Recently however, Osborne and Richardson (1980) tested the hypothesis that tryptamine may be the indolamine transmitter of the cells which accumulate serotonin, but instead found more evidence for serotonin as a transmitter in bovine retina. Furthermore, bovine retina was demonstrated to possess the enzymes necessary to synthesize serotonin from tryptophan (Osborne, 1980). The only retinas in which indolamine-accumulating neurons have not been found are human, and Cynomogus (old world) monkey retinas (Ehinger and Floren, 1979). The indolamine accumulating cells are also difficult to demonstrate in rat and guinea pig retinas, indicating that there are species differences in the indolamine system of the retina.

Synaptic analysis of the catecholamine-containing amacrine cells suggests that the catecholamine neurons only contact other amacrine cells (Adolph et al., 1980) and project to 1 - 3 sublayers in the inner plexiform layer, depending on the animal species (Ehinger, 1978). In mudpuppy, human, old world monkey, cat, and rat retinas, the catecholamine fluorescent layer is in the region where the OFF-centre cell synapses are located, implying that the catecholaminergic amacrine cells modulate the generation of OFF-centre responses by contacting other amacrine cells (Adolph et al., 1980). The indolamine accumulating amacrine

cell synaptic terminals, on the other hand, 1) are much more numerous than the neurons, 2) are generally distributed in one or more sublayers of the inner plexiform layer, and 3) their terminals contact mainly bipolar cells (Ehinger and Holmgren, 1979; Adolph et al., 1980). This implies that they modify bipolar cell activity by connecting different bipolar cells and through feedback mechanisms onto single bipolar cells, which also have input from other, non-indolamine accumulating neurons (Ehinger and Holmgren, 1979). Because monoaminergic amacrine cells have been identified in every species so far studied, there is little doubt that such a class of cells exists in the retina of the newt. The fact that the inner plexiform layer of the newt retina contains many processes filled with large, dense-cored and pleomorphic vesicles (Chapter 2), such as those associated with monoaminergic terminals (Ehinger and Holmgren, 1979; Adolph et al., 1980; Dowling et al., 1980), supports this hypothesis.

c) Amino Acids: Besides the great deal of information regarding the possible use of monoamines by amacrine cells of the retina, a number of studies have implicated amino acids as the transmitter substances of amacrine cells. Gamma-aminobutyric acid (GABA), taurine and glycine have all been proposed as prospective mediators of feedback and lateral inhibitions in the inner retina as well (Starr, 1977).

GABA. Uptake of (3H)-GABA has been reported by

amacrine cells in the retinas of rabbit (Ehinger and Falck, 1971; Brandon et al., 1979; Redburn, 1981), goldfish (Lam and Steinman, 1971; Marc et al., 1978), frog (Voaden et al., 1974), monkey, guinea pig (Bruun and Ehinger, 1974), chicken, pigeon (Marshall and Voaden, 1974a) and cat (Bruun and Ehinger, 1974; Pourcho, 1980). It has been suggested that the method of administration of the label is an important factor governing the uptake site of (3H)-GABA (Bruun and Ehinger, 1974). Intravitreal injections of (3H)-GABA seems to result in GABA uptake by amacrine cells in most species studied to date, although glial uptake also occurs in monkey and rat retinas. In the isolated rat retina however, (3H)-GABA uptake is mainly into Müller cells (Neal and Iversen, 1972; Bruun and Ehinger, 1974; Marshall and Voaden, 1974b). Similar results have been reported for cat, baboon, guinea pig, goat and rabbit retinas by Marshall and Voaden (1975). The exceptions are guinea pig and cat retinas, where amacrine cell uptake is still observed (Bruun and Ehinger, 1974). It has been suggested that the Müller cells accumulate (3H)-GABA *in vitro* because they utilize GABA in general metabolism by a so-called GABA shunt (Bauer and Ehinger, 1978). The high affinity uptake of GABA by glia frequently masks neuronal uptake of GABA in some species, but this phenomenon can be avoided by using (3H)-DABA as a neuronal tracer in the place of (3H)-GABA in rat, cat and rabbit retinas (Bauer and Ehinger, 1978). Müller cell uptake can also be avoided by employing a postincubation in media without (3H)-GABA, as the glial

turnover of GABA is more rapid than the neuronal turnover (Ehinger, 1977; Redburn, 1981).

In the cat retina, the uptake of (3H)-GABA has been demonstrated by four morphologically distinct cell populations (Pourcho, 1980). Such a localization of (3H)-GABA uptake to specific amacrine cell types and within distinct sublaminae of the inner plexiform layer of the retina may give some clues as to the function of GABA-ergic amacrine cells in the retina. Uptake of (3H)-GABA by amacrine cells has been observed to cause a subdivision of the inner plexiform layer into three sublaminae, with the heaviest band in the innermost portion of the inner plexiform layer (Bruun and Ehinger, 1974; Marc et al., 1978; Yazulla and Brecha, 1980; Pourcho, 1981). Similar lamination patterns have occured when the GABA synthesizing enzyme, L-glutamate decarboxylase, has been localized in the inner plexiform layer of the rabbit retina by immunocytochemistry (Brandon et al., 1979). However, the identification of GABA receptor sites using (3H)-muscimol shows that the GABA receptors are evenly distributed throughout the inner plexiform layer, implying that (3H)-GABA uptake is useful for identifying the cytoplasmic bulk of possible GABA-ergic amacrine cell processes, but receptor binding is more useful for identifying neuronal interactions (Yazulla, 1981). The suggestion that GABA-ergic amacrine cells affect processing in all portions of the inner plexiform layer is supported by the

physiological studies of Glickman and Adolph (1981), which show that GABA inhibits most cell types in the carp retina.

Although receptor binding may be more useful for exploring the functional relationships of the GABA-ergic amacrine cells in the retina, (3H)-GABA uptake has still proved very useful in elucidating the function of GABA-ergic amacrine cells in the goldfish retina. In the goldfish retina, (3H)-GABA is accumulated by "Ab amacrine cells" which have their dendritic arborization deep in the inner plexiform layer (Marc et al., 1978). The axon terminals of centre-depolarizing bipolar cells (type b), the dendrites of ON-centre ganglion cells (Gb), and the processes of depolarizing sustained amacrine cells (Ab) are located in sublamina b (Famiglietti et al., 1977). Because the depolarizing ON-centre amacrine cells (Ab) show maximal uptake of (3H)-GABA in darkness, or exposure to green and blue light, and minimal uptake of (3H)-GABA by exposure to red light, it has been suggested that GABA-ergic amacrine cells in the goldfish retina are specifically involved in the red-colour pathway (Marc et al., 1978). Electrophysiological studies on the action of GABA on neurons of the mudpuppy retina also implies that GABA is an inhibitory transmitter involved in the ON channel (Cunningham and Miller, 1980a).

It is also possible that GABA-ergic amacrine cells modulate the function of the catecholaminergic amacrine cells, which in turn modulate the generation of the

OFF-centre response. In the rat, catecholamine fluorescence is located in sublamina b (Adolph et al, 1980) where the bulk of the processes accumulating GABA are also located (Bauer and Ehinger, 1978). Recent studies on the effects of GABA antagonists and light, on dopamine turnover in rat retina, imply that a subpopulation of GABA-ergic amacrine cells is involved in the activation of the catecholaminergic neurons by light, while another subpopulation of GABA-ergic amacrine cells produces tonic inhibition of the catecholaminergic neurons in darkness (Kamp and Morgan, 1981; Marshburn and Iuvone, 1981). Such studies on the significance of the GABA-ergic amacrine cells has just begun and in the years to come, will no doubt elucidate the role of these neurons in the processing of visual information by the retina.

Glycine. The inhibitory amino acid, glycine, is present in rat, frog, chicken, goldfish (Neal, 1976) and newt (Chapter 2) retina. The major concentration of glycine in monkey retina has been localized to the region occupied by amacrine cells (Berger et al., 1977). Autoradiographic studies have shown that glycine is accumulated by a population of amacrine cells and some ganglion cells in rat (Bruun and Ehinger, 1974; Marshall and Voaden, 1974b), guinea pig (Bruun and Ehinger, 1974), cat (Bruun and Ehinger, 1974; Pourcho, 1980), monkey (Bruun and Ehinger, 1974), rabbit (Ehinger and Falck, 1971; Kong et al., 1980), and frog (Voaden et al., 1974; Rayborn et al., 1981)

retinas.

Unlike the uptake of (3H)-GABA into retinal neurons, the uptake of (3H)-glycine is not affected by the method of administration, implying that glial uptake of glycine by Müller cells is minimal (Bruun and Ehinger, 1974). It has been suggested that there may be a population of amacrine cells which indiscriminately accumulate amino acids, and that the cells which accumulate GABA belong to the same population which accumulate glycine and taurine (Starr, 1977). However, the pattern of (3H)-glycine uptake by retina differs significantly from the uptake of (3H)-GABA.

In addition to the accumulation of (3H)-glycine by neurons lining the inner margin of the inner nuclear layer, glycine is accumulated by certain cells whose processes are irregularly scattered throughout the inner plexiform layer (Bruun and Ehinger, 1974; Ehinger and Falck, 1971). As a result, uptake of (3H)-glycine by retinal neurons generally does not cause a sublayering of the inner plexiform layer (Bruun and Ehinger, 1974), as does (3H)-GABA uptake. Although sublayering of the inner plexiform layer by (3H)-glycine uptake is rare, when it is apparent it is localized to a wide band near the outer portion of the inner plexiform layer (Pourcho, 1980).

In the cat retina, three subpopulations of glycine-labelled amacrine cells have been differentiated from four subclasses of GABA cells on a morphological basis

(Pourcho, 1980). Furthermore, half of the ON-OFF ganglion cells have inhibitory postsynaptic potentials which are blocked by strychnine (a glycine antagonist), while the remainder are blocked by picrotoxin or biccuculline (GABA antagonists). This implies that there are separate GABA and glycine releasing amacrine cells in the retina (Shefner and Levine, 1977). The glycinergic amacrine cells have been suggested to belong to a class of diffuse amacrine cells, similar to the narrow-field bistratified (type II) amacrine cells of the cat retina (Kong et al., 1980) because: 1) the terminals of the glycinergic amacrine cells are diffusely distributed throughout the inner plexiform layer; 2) the (3H)-glycine accumulating cells located in the middle portion of the inner nuclear layer are cone bipolar cells which likely receive the (3H)-glycine label through gap junctions between these cells and diffuse glycinergic amacrine cells (Kolb and Famiglietti, 1974; Famiglietti and Kolb, 1975); and 3) the glycinergic amacrine cells which are antagonized by strychnine make local feedback synapses which are attributable to diffuse amacrine cells (Caldwell and Daw, 1978).

Reports of (3H)-glycine uptake by vertebrate retina never describe uptake of (3H)-glycine by processes in the outer plexiform layer. Recently, however, (3H)-glycine has been reported to be accumulated by a population of cells with terminals in both the inner and outer plexiform layers of toad retina (Rayborn et al., 1981). The uptake of

(3H)-glycine by toad retina seems to be exclusively into a population of interplexiform cells and not into amacrine cells. The reason for this unique species difference is not clear, but it is possible that in some previous studies describing uptake of (3H)-glycine into a presumptive population of amacrine cells, uptake may have actually been into a population of interplexiform cells. Further study is clearly necessary to establish glycine as an interplexiform cell transmitter in other species.

Taurine. The predominant amino acid in the retinas of most vertebrate species, including newt (Chapter 2), is taurine. Initial studies on the uptake of taurine into cat, rat, rabbit, pigeon, and guinea pig retinas showed that uptake was mainly into glia and photoreceptor cells (Voaden et al., 1977). However, a repeat of these experiments using low enough concentrations of (3H)-taurine to ensure activation of the high affinity uptake systems in rat, frog, mouse, cat, pigeon, guinea pig, and baboon retinas, has localized (3H)-taurine to photoreceptors, and a species-variable uptake of (3H)-taurine by perikarya located within the inner nuclear layer has also been demonstrated (Lake et al., 1978).

In the frog retina, presumptive bipolar cells accumulate (3H)-taurine, but in many other species studied, certain amacrine, horizontal, and ganglion cells accumulate (3H)-taurine (Lake et al., 1978). Although there is some dispute as to whether taurine is a true neurotransmitter in

the central nervous system (Kuriyama, 1980), there is a great deal of evidence which suggests that taurine may function in this capacity in the retina (Neal, 1976; Starr, 1977). However, the uptake of (3H)-taurine by bipolar cells in frog (Lake et al., 1978) and cat retinas (Pourcho, 1981) is not due to the use of taurine as a neurotransmitter in these cells, because bipolar cells release an excitatory transmitter (Starr, 1977). Nevertheless, the similar morphology of bipolar and photoreceptor cell synaptic terminals suggests that some bipolar cells utilize the same excitatory transmitter as photoreceptor cells and therefore accumulate (3H)-taurine for the same, as yet undetermined, reason (see Chapter 2 for discussion). It is also possible that the light labelling of rod bipolar cells by (3H)-taurine in the cat retina (Pourcho, 1981) may be due to the diffusion of label into bipolar cells through gap junctions formed between these cells and putative taurinergic amacrine cells; a similar process has been suggested for the labelling of bipolar cells by (3H)-glycine in the rabbit retina (Kong et al., 1980).

It is doubtful if the amacrine cells which accumulate (3H)-taurine and those which accumulate (3H)-glycine, are in actuality the same cell, accumulating both putative transmitter substances. This is because: 1) the processes which accumulate (3H)-glycine are generally distributed throughout the inner plexiform layer, while the processes which accumulate (3H)-taurine are stratified in pigeon (Lake

et al., 1978) and cat retina (Pourcho, 1981); and 2) the cells which label with (3H)-glycine in the cat retina (Pourcho, 1980) differ from those cells which label with (3H)-taurine in number, size, nuclear appearance and dendritic pattern (Pourcho, 1981).

In the cat retina, (3H)-taurine has been localized to both plexiform layers (Pourcho, 1981), suggesting that it may be taken up by a population of interplexiform cells as well. This hypothesis will need verification by the identification of the terminals which accumulate (3H)-taurine in both plexiform layers by electron microscope autoradiography. There is no doubt that at least some of the label which is present in the outer plexiform layer, is due to the accumulation of (3H)-taurine by photoreceptor cell terminals. But, because so little is known about the uptake patterns and physiology of taurine in the retina, it is difficult to speculate on the function of the cells which accumulate (3H)-taurine in the inner retina. Electrophysiological studies have been hampered because both glycine and taurine are antagonized by strychnine and it is probable that they both act on the same synaptic receptor (Cunningham and Miller, 1980a; 1980b). These studies further show that while GABA is selectively involved in ON-channel activity in the mudpuppy inner plexiform layer, taurine and/or glycine subserve an equivalent role for the OFF channel (Cunningham and Miller, 1980b). Although this implies that taurinergic cells should send most of their

processes to sublamina a of the inner plexiform layer, the heaviest band of silver grains overlies sublamina b in pigeon (Lake et al., 1978) and cat (Pourcho, 1981) retinas.

Glutamate. Although (3H)-glutamate is not accumulated by any particular retinal cell type (White and Neal, 1976), the kainic acid-induced lesions of rabbit (Hampton et al., 1981), goldfish (Yazulla and Kleinschmidt, 1980), chick (Ehrlich and Morgan, 1980) and newt (Chapter 2) inner plexiform layer neuronal processes, implies that glutamate may be an amacrine cell transmitter as well. However, no other evidence substantiates the notion that glutamate is a transmitter substance in the inner retina (Neal, 1976). Furthermore, as pointed out in Chapter 2, the ability of kainic acid to specifically lesion cells which are exclusively postsynaptic to glutamatergic neurons has very recently been questioned.

How the amacrine cells, which utilize the inhibitory amino acids as neurotransmitters, regulate information processing in the retina, will continue to receive much attention in the literature as clues to their function and distribution become apparent.

d) **Neuropeptides:** In addition to the "conventional" transmitters which have just been discussed, it has become apparent very recently, that a growing number of neuroactive peptides function as neurotransmitters in amacrine cells of the retina. These include substance P (Eskay et al., 1980;

1981; Karten and Brecha, 1980; Glickman and Adolph, 1981), somatostatin (Krisch and Leonhardt, 1979; Eskay et al., 1980; Yamada et al., 1980; Brecha et al., 1981; Buckerfield et al., 1981), opioid peptides such as enkephalins and endorphins (Brecha et al., 1979; Humbert et al., 1979; Howells et al., 1980; Djamgoz et al., 1981), α-melanocyte stimulating hormone (Bauer et al., 1980), neurotensin (Brecha et al., 1981), vasoactive intestinal polypeptide (Loren et al., 1980) and thyrotropin releasing hormone (Eskay et al., 1980).

Substance P. This neuropeptide has been found to excite about 50% of responding ON-type ganglion cells (Glickman and Adolph, 1981); and immunocytochemistry has demonstrated that substance P immunoreactive processes are confined to a very narrow band in the middle of the inner plexiform layer (Karten and Brecha, 1980) or the outer portion of sublamina b (Eskay et al., 1981).

Somatostatin. In goldfish retina, there is more than one type of amacrine cell which demonstrates somatostatin-like immunoreactivity (Yamada et al., 1980) and immunoreactive processes are found in both sublamina a and sublamina b of the inner plexiform layer, implying that these cells modulate the activity of both ON and OFF ganglion cell activity (Krisch and Leonhardt, 1979; Yamada et al., 1980; Buckerfield et al., 1981)

Opioid Peptides. Djamgoz et al. (1981) have

recently identified an opiate system in goldfish retina, which is involved in the pathway to ON-centre ganglion cells. These new findings suggest interesting interpretations of the functional organization of the vertebrate retina and indicate that the processing of visual information by amacrine cells in the inner plexiform layer is much more complex than previously thought.

4) Interplexiform Cells. Over the past decade, it has become more evident that all vertebrate retinas contain a sixth type of neuron, the interplexiform cell. The perikarya of these cells are located in the inner portion of the inner nuclear layer, amongst the amacrine cell bodies, and send processes to both plexiform layers. They were first discovered in teleost fish and new world monkey retinas (Ehinger and Falck, 1969b; Ehinger et al., 1969). Because these cells probably utilize dopamine as a neurotransmitter in these species, they are easily visualized by fluorescence microscopy. Though they are particularly refractory to metal impregnation techniques, they have recently been visualized occasionally in Golgi preparations of some species (Dowling, 1979).

The input to interplexiform cells is from amacrine cells and the interplexiform cells synapse with other amacrine cells in the inner plexiform layer, but never contact ganglion cells (Dowling, 1979). In the outer plexiform layer, the interplexiform cells contact horizontal cell and bipolar cell dendrites, but never contact the

photoreceptor terminals (Dowling, 1979). In the fish retina, electrophysiological and histochemical findings prompted the proposal that cholinergic amacrine cells stimulate dopaminergic interplexiform cells to release the inhibitory catecholamine around horizontal cells in the outer plexiform layer (Negishi and Drujan, 1979; Hayashi, 1980). These, and other findings, suggest that the interplexiform cells of the fish retina suppress inhibitory actions of the horizontal cells and at the same time polarize the bipolar cells, to enhance their responsiveness to central illumination (Dowling, 1979).

Although at least one of the neurotransmitter substances of the interplexiform cell in teleost fish and new world monkeys has been confirmed, little is known about the transmitter substances or the function of interplexiform cells in other species. Nakamura et al. (1980) have determined by serial-section electron microscope autoradiography, that 2% of the cells in the cat retina which label heavily with (3H)-GABA are interplexiform cells. Pourcho (1980) however, has not been able to confirm the suggestion by Nakamura et al. (1980) that one of the four types of cells in the cat retina which label with (3H)-GABA is an interplexiform cell.

Yazulla and Schmidt (1976) have suggested that one possible reason that (125I)-α-bungarotoxin receptors are present in the outer plexiform layer of the retina is that a population of interplexiform cells is cholinergic. A

cholinergic interplexiform cell could account for the electron microscope localization of (125I)-α-bungarotoxin receptors on bipolar cell dendrites in goldfish retina (Schwartz and Bok, 1978) and also the presence of (125I)-α-bungarotoxin and (3H)-quinuclidinyl benzylate receptors in the outer plexiform layer of the newt retina (Chapter 2). The evidence for a cholinergic interplexiform cell is very weak however, and will require further verification.

Besides GABA, taurine and glycine (Rayborn et al., 1981) have been implicated as interplexiform cell transmitters, but the evidence for the use of these amino acids as neurotransmitter substances by interplexiform cells is still weak. The presence of silver grains over both the outer plexiform and inner plexiform layers after autoradiographic detection of tritiated amino acid uptake, will indicate the presence of a possible interplexiform cell only if horizontal and bipolar cells are known not to accumulate the amino acid, and the interplexiform cell processes in both plexiform layers have been identified by electron microscopy.

5) Ganglion Cells. A great deal of evidence suggests that retinotectal transmission in goldfish (Oswald and Freeman, 1980; Schwartz et al., 1980; Schmidt et al., 1980) and frog (Oswald and Freeman, 1979), is mediated by way of cholinergic neurons, and some evidence suggests that the same is true for birds and reptiles (Oswald and Freeman,

1980). In the newt retina, acetylcholinesterase is associated with some cells in the ganglion cell layer and optic nerve fibres (Dickson, personal communication). However, it has been suggested that the uptake of (3H)-choline by ganglion cells in the rabbit retina is due to the incorporation of choline into phospholipids and not acetylcholine (Masland and Mills, 1979).

Glutamate has also been suggested as a transmitter in ganglion cells, but the studies supporting glutamate as a ganglion cell transmitter are open to other interpretations (Oswald and Freeman, 1980).

Taurine has been reported to be taken up by ganglion cells and transported along the optic nerve axons in young animals. However, it is believed that taurine functions in the young optic nerve to stabilized electrical properties of optic nerve axons, prior to their becoming functional (Politis and Ingoglia, 1979).

Ganglion cells have been reported to accumulate most amino acids. However, neurons with long axons, such as retinal ganglion cells, may accumulate amino acids more rapidly because of higher protein requirements (Ehinger, 1972). Uptake of amino acids by ganglion cells therefore does not necessarily imply that ganglion cells utilize them as neurotransmitter substances.

Studies on the uptake of (3H)-taurine into neurons of

newt retina has indicated that taurine is accumulated not only by photoreceptors, but by cells in the inner retina as well (Chapter 2). The purpose of this chapter, is to further explore the possible use of taurine as a neurotransmitter in the inner retina of the newt. Although the general accumulation of taurine has been demonstrated by cells of the inner nuclear layer in other species, no study has been undertaken to localize (3H)-taurine to specific processes within the inner nuclear layer. Therefore, by localizing taurine to specific presynaptic processes, it is hoped that taurine can be implicated as a true neurotransmitter and not just as a general modulator of synaptic function. This will also verify if the stratification of the inner plexiform layer by (3H)-taurine uptake represents nerve terminal uptake (Voaden et al., 1977). In addition to taurine, the uptake of the inhibitory amino acid transmitters, glycine and GABA, by newt retina will be studied using autoradiograhic techniques. Amino acid analysis of newt retina (Chapter 2) indicates that these substances may be utilized as inhibitory neurotransmitter substances in the retina as well.

MATERIALS AND METHODS

To determine the uptake pattern of amino acids which are suspected of being neurotransmitters in the inner retina of the newt, retinas were incubated as in Chapter 2, in media containing (3H)-taurine, (3H)-L-glycine, and (3H)-γ-aminobutyric acid ((3H)-GABA). Tritiated amino acids were purchased from New England Nuclear (Boston, Massachusetts) with the following specific activities: (3H)-taurine: 23 Ci/mmol; (3H)-glycine: 8.6 Ci/mmol; and (3H)-GABA: 28.2 Ci/mmol. Five, 10, 20, and 40 µl quantitities of these amino acids were evaporated with dry nitrogen in a fume hood at 20 ^{0}C and resuspended in 100 µl of unsupplemented tissue culture media (M-199 with glutamine; GIBCO, Grand Island, New York) in a small 200 µl chamber. Final concentrations of the amino acids for the 20 µl dilution were: (3H)-taurine, 8.63 µmol; (3H)-glycine, 23.2 µmol; (3H)-GABA, 7.22 µmol. These concentrations were chosen since they would allow equal amounts of radioactivity (20 µCi) to be available for uptake, while at the same time, providing sufficiently low enough concentrations to ensure the activation of high affinity transport systems for each of these amino acids (Lake et al., 1978).

Following decapitation, newt eyes were enucleated, the corneas removed, and the eyecups placed in the incubation chambers. The media was oxygenated with 98% oxygen during incubation. After 15 and 30 min incubation periods, the eyecups were rinsed in tissue culture media without label

for 5 min. The eyecups were then fixed in a cacodylate-buffered glutaraldehyde-formaldehyde fixative (Chapter 1) and processed for electron microscopy as previously described (Chapter 1). Light microscopic autoradiography was performed on 0.5 µm plastic sections as previously described (Chapter 2). Electron microscopic autoradiography was performed on thin sections cut from blocks containing tissue which was incubated in (3H)-taurine and (3H)-GABA. The procedure was carried out as described by Ball et al. (1981).

RESULTS

1) Glycine. When newt retina was incubated in media containing (3H)-glycine, two patterns of labelling were observed; 1) a generalized uptake over the whole retina and, 2) specific labelling of selected neuronal cell bodies and their processes. A number of neurons in the inner half of the inner nuclear layer accumulated glycine (Fig. 1). Although their identity cannot be assured from this light microscope autoradiograph, they likely belong to a class of amacrine cell. The cells which accumulate (3H)-glycine in the middle of the inner nuclear layer are probably a class of bipolar cell. This uptake of (3H)-glycine by a population of bipolar cell is verified by the labelling of Landolt's clubs (apical processes of bipolar cells) near the outer limiting membrane. The outer plexiform layer was also heavily labelled with (3H)-glycine (Fig. 1, arrows) as was the inner plexiform layer; however, the density of label in the outer plexiform layer was much heavier.

Because there was a generalized uptake of label by the whole retina, which terminates at the outer limiting membrane, it is likely that Müller cells accumulate (3H)-glycine as well. The presence of dense label over the region of the retina occupied by the foot processes of the Müller cells, and the lightly labelled elliptical nuclei near the middle of the inner nuclear layer, further suggests that (3H)-glycine has been accumulated by Müller cells. There were no cells in the ganglion cell layer which

accumulated (3H)-glycine, and the inner plexiform layer was not divided into sublaminae by the processes of the labelled amacrine and bipolar cells.

2) Taurine. When newt retina was incubated in media containing (3H)-taurine, photoreceptors, a few cells in the inner nuclear layer, and cells in the ganglion cell layer accumulated (3H)-taurine (Fig. 2).

The uptake of (3H)-taurine by photoreceptors has been completely described in Chapter 2. The labelled cells at the outer and inner margins of the inner nuclear layer are probably populations of horizontal cells and amacrine cells respectively. The perikarya in the middle of the inner nuclear layer which were lightly labelled with (3H)-taurine (Fig. 2, arrows) are likely those of Müller cells, since the foot processes of the Müller cells at the inner limiting membrane were also labelled. The cells in the ganglion cell layer which accumulated (3H)-taurine were more numerous in the outer half of the ganglion cell layer. A large number of cells in the inner half of the ganglion cell layer did not accumulate (3H)-taurine at all.

The (3H)-taurine accumulating cells lining the inner margin of the inner nuclear layer and the outer margin of the ganglion cell layer appear to give rise to processes which course laterally along the outer and inner margins of the inner plexiform layer. The labelling of these amacrine and ganglion cell processes divides the inner plexiform

layer into three layers, the middle layer being wider and less densely labelled than the other two layers (Fig. 2). The bulk of the processes from the amacrine cells appear to be confined to sublamina a of the inner plexiform layer, while the bulk of the processes from the ganglion cells are confined to sublamina b.

The cells in the position of the amacrine and ganglion cells which accumulated (3H)-taurine, could be identified by electron microscope autoradiography. In Fig. 3, an amacrine cell adjacent to the outer margin of the inner plexiform layer is labelled, while two nearby amacrine perikarya and a Müller cell are unlabelled. In Fig. 4, a cell body in the outer margin of the ganglion cell layer is heavily labelled, while two adjacent ganglion cells did not accumulate (3H)-taurine. Figs. 3 and 4 demonstrate the effectiveness of the electron microscope autoradiographic technique for identifying the perikarya and processes which accumulate (3H)-taurine in the newt retina.

Some of the processes from the taurine-labelled cells in the inner nuclear layer and the ganglion cell layer, which ramify throughout the inner plexiform layer, have been identified by electron microscope autoradiography; they are shown in Figs. 5 to 12. In Fig. 5, an amacrine cell process in the inner plexiform layer is clearly labelled. This terminal contains numerous, small electron-lucent vesicles measuring an average of 46 nm in diameter. Profiles of smooth endoplasmic reticulum and microtubules are present in

the terminal as well. In Fig. 6, a labelled amacrine cell process forms a junction with an adjacent, unlabelled amacrine cell (Fig. 6, arrow). The labelled terminal contains numerous synaptic vesicles and two large, dense-cored vesicles which measure 96 nm in diameter. A small, dense mitochondrion is located near the centre of the terminal. In Fig. 7, a labelled amacrine cell terminal, containing numerous small vesicles, a single large dense-cored vesicle, and numerous microtubules, is seen to be postsynaptic to an adjacent unlabelled amacrine cell process (Fig. 7, arrow). In Fig. 8, a labelled amacrine cell process forms a junction with an adjacent, unlabelled amacrine cell process (Fig. 8, arrow): The labelled terminal contains numerous small vesicles measuring an average of 45 nm in diameter.

Although synaptic connections between labelled amacrine cells and other, unlabelled amacrine cells were very common, only rarely were labelled amacrine cell processes observed making synaptic contact with ganglion cell processes. Ganglion cell dendrites were identified as such if they were small (ie. < 0.75 μm in diameter), and did not contain organelles, such as synaptic vesicles (Dubin, 1970). In Fig. 9, a labelled amacrine cell process is seen forming a junction with a process which is presumed to be a ganglion cell dendrite (arrow).

Amacrine cell processes which accumulated (3H)-taurine were observed receiving synaptic input from bipolar cells at

ribbon synapses (Figs. 10 and 11) and they were also observed making feedback synapses onto bipolar cells (Fig. 12). In Fig. 10 a bipolar cell forms a ribbon synapse with two amacrine cell processes (arrows), one which is labelled, and one which is not labelled. The labelled process contains scattered electron-lucent vesicles averaging 46 nm in diameter. A similar configuration can be observed in Fig. 11. The labelled amacrine cell process which is postsynaptic to the bipolar cell terminal (arrows) contains numerous small, electron-lucent synaptic vesicles and a single, large dense-cored vesicle. In Fig. 12 a labelled amacrine cell process containing numerous small electron-lucent vesicles and a single dense-cored vesicle, makes synaptic contact with a bipolar cell terminal (single arrow), which in turn makes synaptic contact with two unlabelled amacrine cells at a ribbon synapse (two arrows).

3) GABA. When newt retina was incubated in media containing (3H)-GABA, a number of neurons in the position of the horizontal, bipolar, amacrine cells accumulated, and nearly all ganglion cells accumulated (3H)-GABA (Fig. 13).

The uptake of (3H)-GABA by horizontal and bipolar cell processes resulted in heavy labelling of the outer plexiform layer as well. Because there was little identifiable uptake of (3H)-GABA by Müller cells, the labelled nuclei in the middle of the inner nuclear layer are presumed to be bipolar cells. This is further corroborated by the labelling of Landolt's clubs near the outer limiting membrane. When

thick, 10 μm sections of GABA-labelled newt retina were examined at the light microscope level, processes extending from the bipolar perikarya to the inner and outer plexiform layers, and also to Landolt's clubs could be observed (Fig. 14).

The uptake of (3H)-GABA into ganglion cells was often variable and in some preparations, labelling of ganglion cells was minimal. Under these conditions, the labelling attributed to amacrine, bipolar, and a small population of ganglion cells, divided the inner plexiform layer into distinct sublaminae (Fig. 15). The labelled amacrine and ganglion cells appear to give rise to processes which are densely labelled at the outer and inner margins of the inner plexiform layer respectively (Fig. 15). It is not possible to determine the origin of the processes giving rise to the band of label in the middle of the inner plexiform layer. When (3H)-GABA was accumulated by most ganglion cells, sublamination of the inner plexiform layer was not apparent (Fig. 13).

The horizontal cell bodies which accumulate (3H)-GABA can also be identified by electron microscope autoradiography. In Fig. 16, a horizontal cell at the outer margin of the inner nuclear layer is labelled, while adjacent horizontal cells are unlabelled. The processes of the labelled horizontal cell can be seen to pass laterally along the inner margin of the outer plexiform layer (Fig. 16, arrows). Labelled horizontal cell processes were

rarely observed in close proximity to photoreceptor terminals. When labelled processes could be positively identified within the photoreceptor terminals, they were always associated with cone terminals (Figs. 17A and 17B). In Fig. 17A, the lateral element of a cone triad, which is probably a horizontal cell process, is labelled. In Fig. 17B, a process not involved in a ribbon synapse, receives synaptic input (arrow) from a cone terminal. Silver grains are also associated with small (< 0.4 μm in diameter) processes which do not invaginate deeply within the photoreceptor terminal (Fig. 17B, asterisks). The identification of labelled postsynaptic elements in the outer plexiform layer is hampered by the small size of these processes. It is further complicated by the fact that the processes of both bipolar and horizontal cells accumulate (3H)-GABA in the newt retina. However, because the labelled processes in Fig. 17A occupy a lateral position in the ribbon synapse triad, it is likely that it is a horizontal cell process.

The perikarya of the labelled bipolar cells which are located in the middle of the inner nuclear layer (Figs. 13, 14 and 15) can also be identified by electron microscope autoradiography. In Fig. 18A, two bipolar cell bodies are shown to have accumulated (3H)-GABA and as a result, are labelled with silver grains. These cells are surrounded by other neurons and Müller cell cytoplasm, which has not accumulated (3H)-GABA. The Landolt's club processes of

these bipolar cells can clearly be identified by electron microscope autoradiography as well. In Fig. 18B, a labelled Landolt's club is situated between photoreceptor cells and the apical terminations of the Müller cells, at the outer limiting membrane.

The cells in the ganglion cell layer which accumulate (3H)-GABA can be identified by electron microscope autoradiography as well. In Fig. 19A, a neuron in the ganglion cell layer near the inner limiting membrane is labelled, while an adjacent cell is unlabelled. Some silver grains are located over a nearby fascicle of ganglion cell axons. At higher magnification, it is evident that these silver grains are associated with only some of the axons, presumably the axons of the ganglion cells that have accumulated (3H)-GABA (Fig. 19B).

Cells in the position of the amacrine cells, which were demonstrated in Figs. 13 and 15 to have accumulated (3H)-GABA are identifiable at the electron microscope level as amacrine cells. In Fig. 20, two amacrine cells at the inner border of the inner plexiform layers are labelled, while two adjacent amacrine cells, and a Müller cell have not accumulated appreciable amounts of (3H)-GABA and are therefore unlabelled.

Because the perikarya of amacrine cells (Fig. 20), bipolar cells (Fig. 18A), and ganglion cells (Fig. 19A) were identified by electron microscope autoradiography, the

labelled processes of these cells within the inner plexiform layer were expected to be labelled as well. Despite the fact that the bipolar cells and ganglion cells accumulate (3H)-GABA, silver grains were rarely associated with bipolar cell synaptic terminals or ganglion cell dendrites. On the other hand, labelled amacrine cell processes were easily identified (Figs. 21 to 26). In Fig. 21, an amacrine cell process from a cell which has accumulated (3H)-GABA is clearly labelled by silver grains. This amacrine cell process contains numerous electron-lucent vesicles measuring an average of 44 nm in diameter, mitochondria and smooth endoplasmic reticulum. Labelled amacrine cell terminals frequently contained large, dense-cored vesicles measuring an average of 79 nm in diameter (Fig. 22). The terminals of cells which accumulated (3H)-GABA were observed to make frequent synaptic contact with other amacrine cells, and also with ganglion cell dendrites. In Fig. 23 three amacrine cell terminals are located next to a ganglion cell dendrite. Two of the amacrine cell processes arise from neurons which have accumulated (3H)-GABA because silver grains are located over the terminal. Two of the three amacrine cell processes, only one of which is labelled, make synaptic contact (Fig. 23, arrows) with the ganglion cell dendrite.

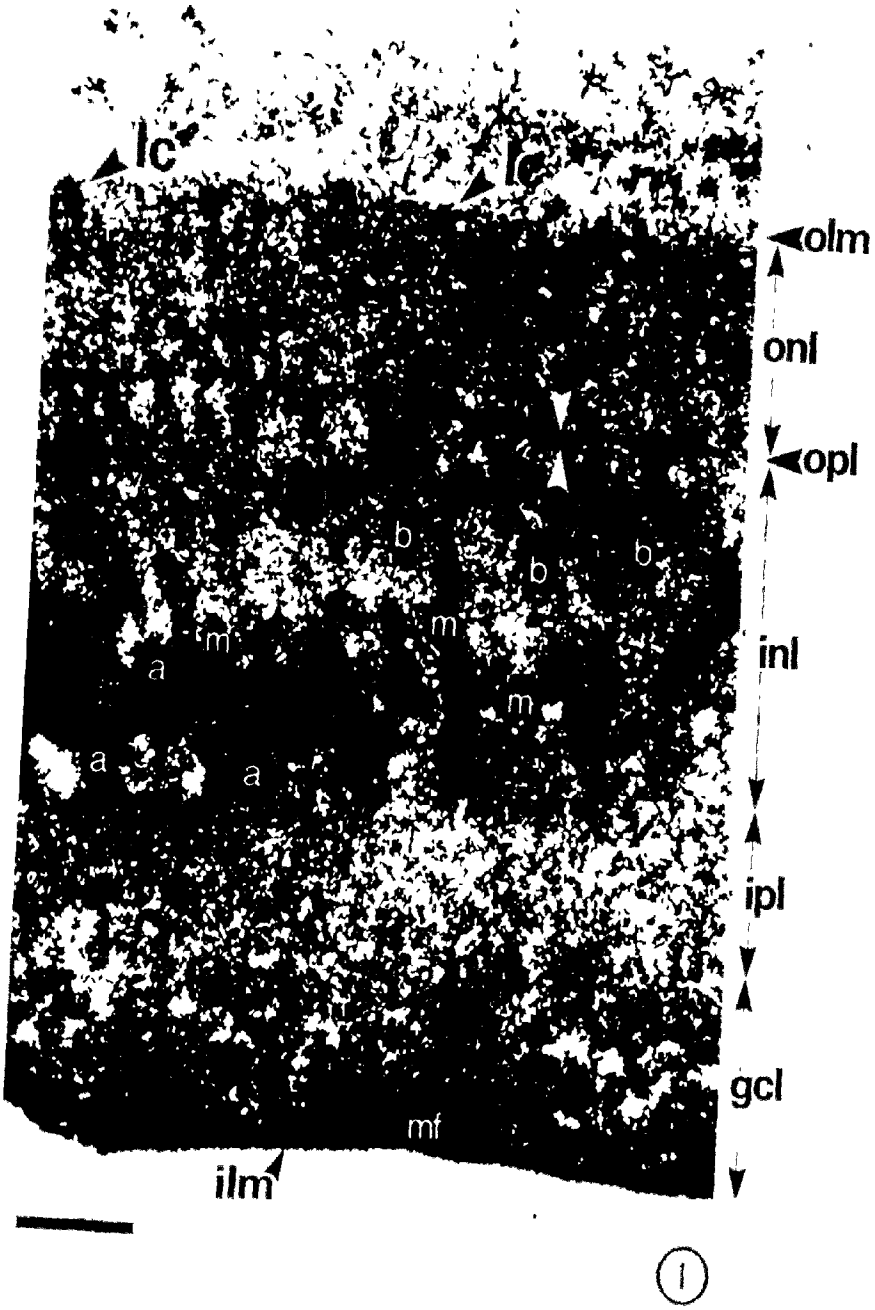
Fig. 24 (A and B) is a serial section autoradiograph showing an amacrine cell process containing small, electron-lucent synaptic vesicles, a single dense-cored

vesicle, and a mitochondrion. Three ganglion cell dendrites are adjacent to the labelled amacrine cell process (Fig. 24A) and one of these dendrites forms a junction (arrow) with the labelled amacrine cell in a subsequent section (Fig. 24B). In Fig. 25 a labelled amacrine cell process containing numerous small electron-lucent vesicles and dense-cored vesicles (small arrows) forms a junction with a nearby, unlabelled amacrine cell process (large arrow).

A further indication that complex synaptic relations occur between different types of amacrine cells is illustrated in Fig. 26. In Fig. 26 a labelled amacrine cell process receives synaptic input from an adjacent, unlabelled amacrine cell which in turn, receives synaptic input from another amacrine cell. Synaptic relationships between amacrine cell processes which accumulate (3H)-GABA and bipolar cells were never observed, nor were silver grains associated with bipolar cell terminals and ganglion cell dendrites. This is surprising, considering that the parikarya of these cell types were labelled in both light and electron microscope autoradiographic preparations.

FIGURES

Figure 1. Light microscope autoradiograph (bright field, unstained 0.5 µm section) showing the uptake pattern of (3H)-glycine by the newt retina. There is a small amount of label present in the region of the foot processes (mf) and nuclei of the Müller cells near the middle of the inner nuclear layer (m), together with a general low level of uptake extending from the inner limiting membrane (ilm) to the outer limiting membrane (olm), indicative of the distribution of Müller cell cytoplasmic processes within the retina. Silver grains are densest over the nuclei in the inner nuclear layer (inl). These include the perikarya of some bipolar cells (b) and their extensions in the form of Landolt's clubs (lc) at the outer limiting membrane (olm). The outer plexiform layer (white arrows) is heavily labelled as well. A large number of cells in the position of the amacrine interneurons (a) are also labelled. onl= outer nuclear layer, opl= outer plexiform layer, gcl= ganglion cell layer, bar= 25 µm



1

Figure 2. Light microscope autoradiograph (bright field, unstained 0.5 µm section) showing the uptake of (3H)-taurine by newt retina. Photoreceptor cells (p) and a small population of cells in the position of the horizontal cells (h) are heavily labelled. There is a small amount of label associated with the foot processes of the Müller cells (mf) and their nuclei near the middle of the inner nuclear layer (inl, arrows). Amacrine cells (a) which line the outer margin of the inner plexiform layer, and some cells in the ganglion cell layer (g) are heavily labelled. The uptake of (3H)-taurine by the processes of these amacrine (a) and ganglion (g) cells causes a subdivision of the inner plexiform layer (ipl) into sublaminae. Two heavy, narrow bands of label (ipl, arrows) are located at the inner and outer margins of the inner plexiform layer. A wide band of lighter label is located between these two heavy bands. olm= outer limiting membrane, onl= outer nuclear layer, opl= outer plexiform layer, gcl= ganglion cell layer, bar= 25 µm

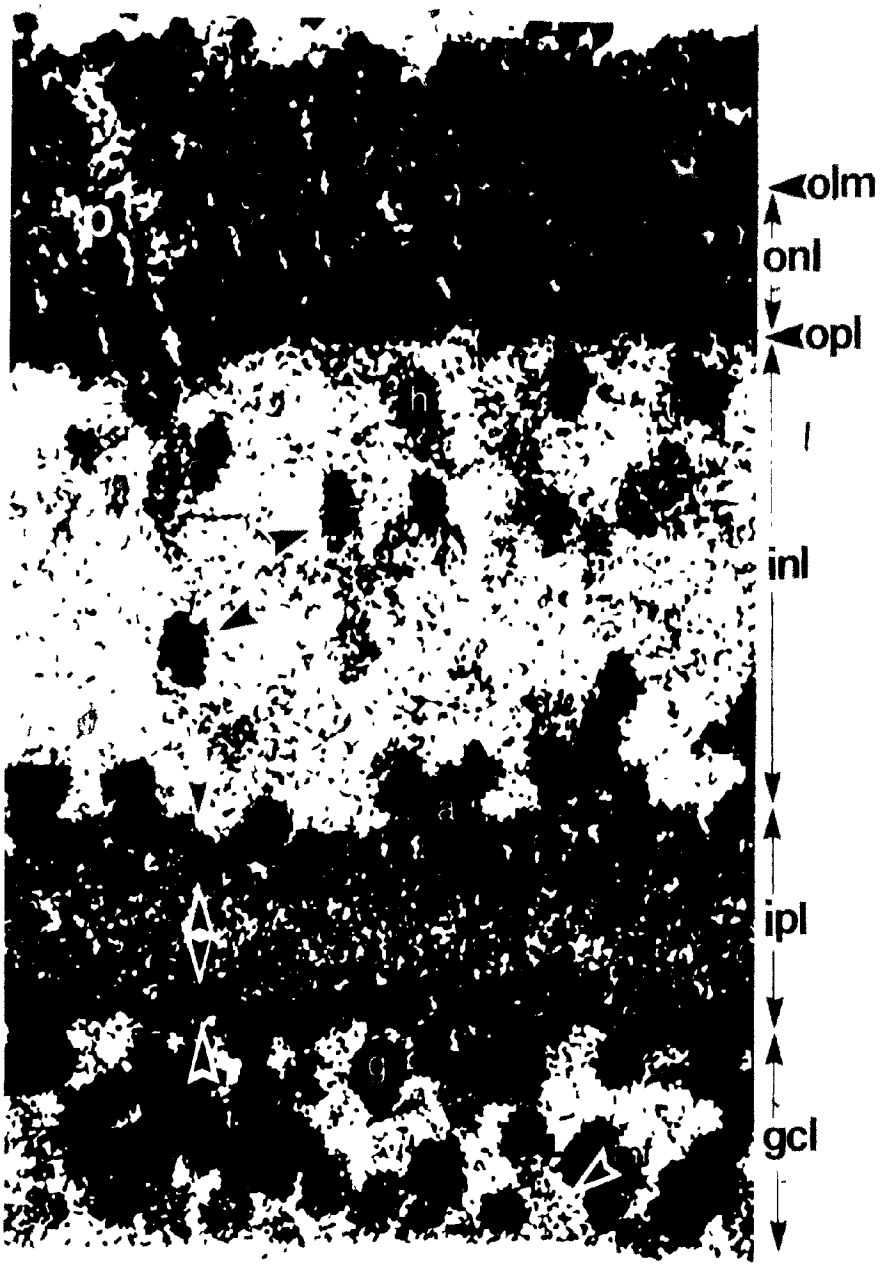


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Figure 3. Low power electron microscope autoradiograph showing the uptake of (3H)-taurine by an amacrine cell (a) near the outer margin of the inner plexiform layer (ipl) of the newt retina. Two adjacent amacrine cells (a) and a Müller cell (Mü) are unlabelled. bar= 2.0 µm

Figure 4. Low power electron microscope autoradiograph showing the uptake of (3H)-taurine by a ganglion cell (g) near the inner margin of the inner plexiform layer (ipl) of the newt retina. Two nearby ganglion cells (g) are unlabelled. bar= 2.0 µm


a
a
a
3


g
g
g
4

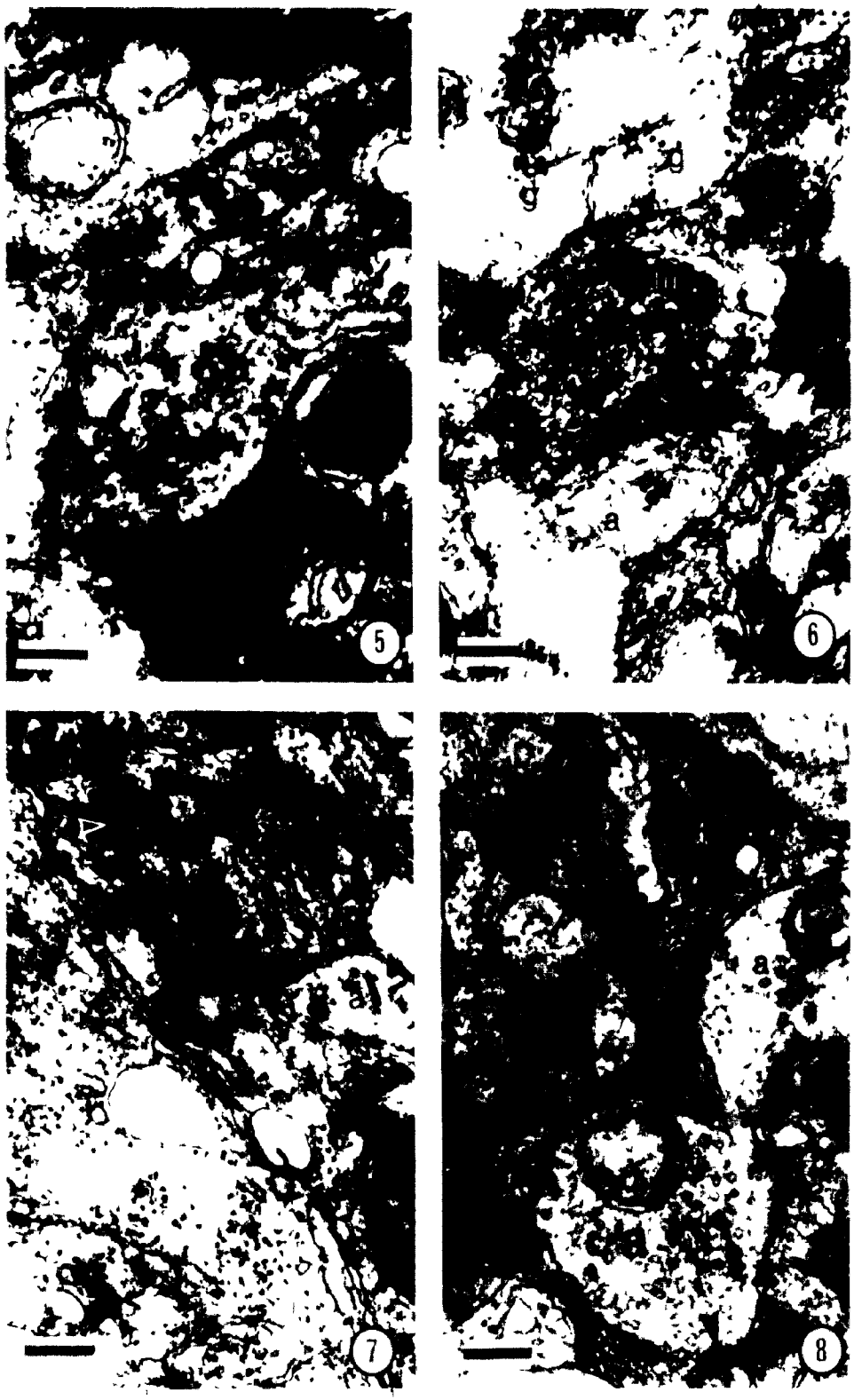
Figures 5 to 8. High power electron microscope autoradiographs showing processes in the inner plexiform layer of the newt retina which have accumulated (3H)-taurine.

Figure 5. A labelled amacrine cell process (a) contains numerous small, electron-lucent vesicles (46 nm in diameter). A profile of smooth endoplasmic reticulum and microtubules are present in the labelled terminal as well. bar= 0.25 µm

Figure 6. The labelled terminal (a'), which contains numerous, small, electron-lucent vesicles and two large, dense-cored vesicles, forms a junction (arrow) with, and is presynaptic to an unlabelled amacrine cell terminal (a). g= ganglion cell dendrites, m= mitochondrion, bar= 0.25 µm

Figure 7. The labelled terminal (a'), which is morphologically similar to the labelled terminals in Figs. 5 and 6, forms a junction (arrow) with an adjacent, unlabelled amacrine cell (a). The labelled terminal is postsynaptic to the unlabelled amacrine cell terminal. bar= 0.25 µm

Figure 8. The labelled amacrine cell terminal (a) forms a junction (arrow) with an adjacent, unlabelled amacrine cell process (a). bar= 0.25 µm


g
g
a
5
6
7
8

Figures 9 to 12. Electron microscope autoradiographs showing labelled amacrine cell terminals in the inner plexiform layer of a newt retina which has been incubated in (3H)-taurine.

Figure 9. The labelled terminal (a) forms a junction (arrow) with a process which is most likely that of a ganglion cell dendrite (g). bar= 0.25 µm

Figure 10. A bipolar cell terminal forms a ribbon synapse (arrows) with two amacrine cell processes in the inner plexiform layer of the newt retina. One of the postsynaptic amacrine cell processes is labelled, indicating that it has accumulated (3H)-taurine. bar= 0.25 µm

Figure 11. A bipolar cell terminal (b) forms a ribbon synapse (arrows) with two amacrine cell processes (a). The labelled amacrine cell process which is postsynaptic to the bipolar cell terminal is morphologically similar to the labelled terminals shown in Figs. 5 to 8. bar= 0.25 µm

Figure 12. The labelled amacrine cell terminal (a) is presynaptic to a bipolar cell terminal (b) which forms a ribbon synapse with two unlabelled amacrine cell processes (a). g= ganglion cell processes, bar= 0.25 µm

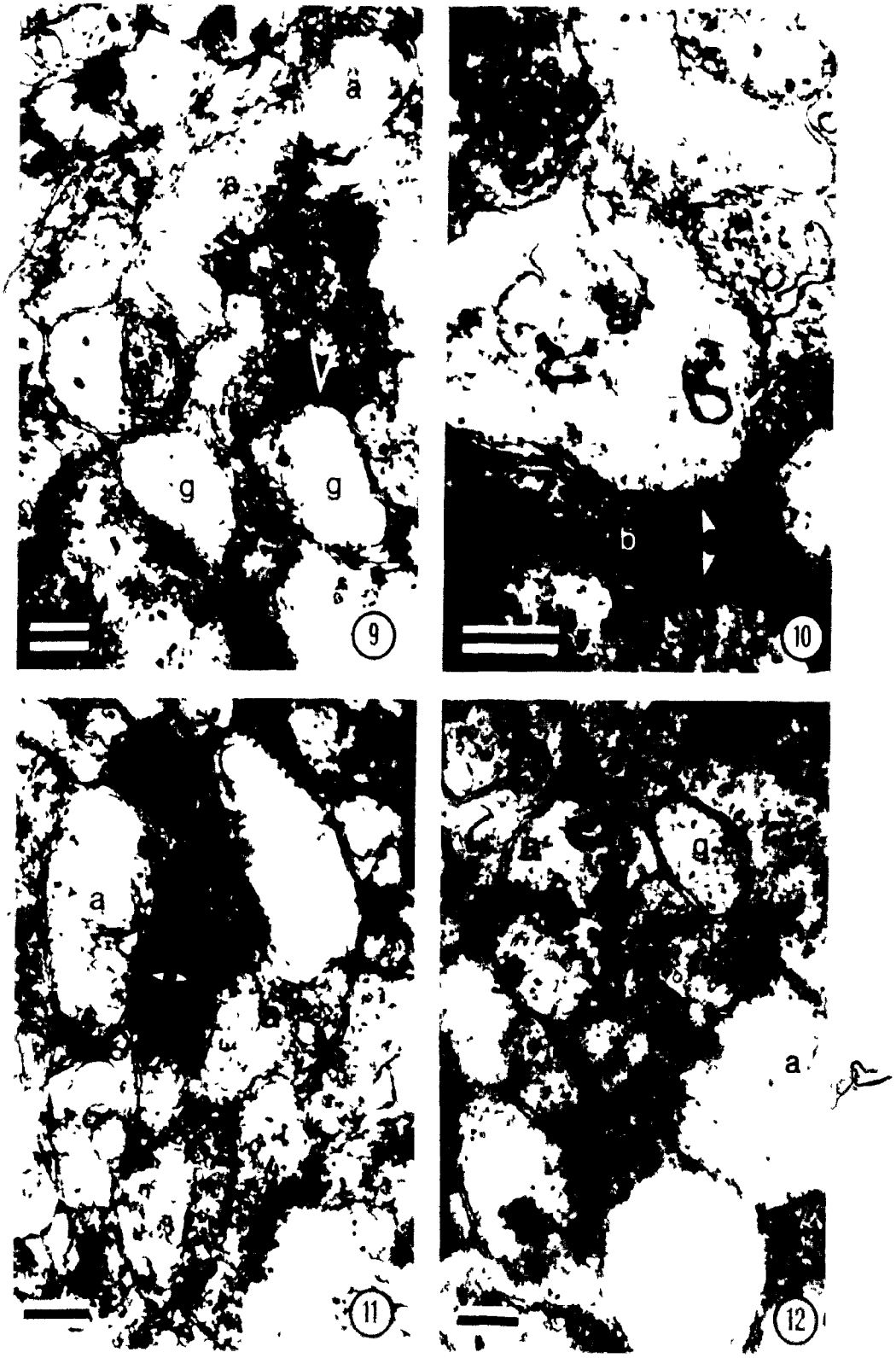
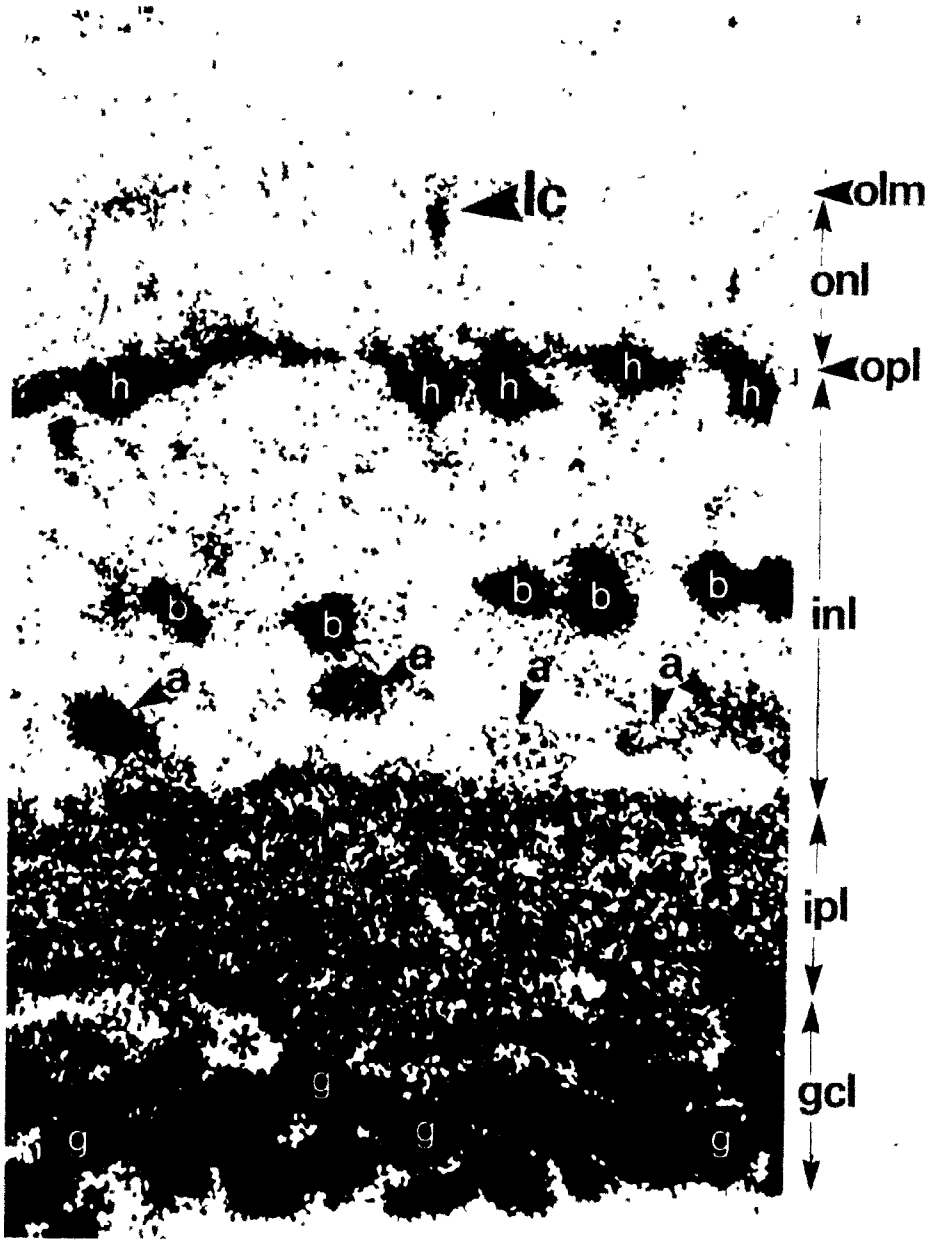

a
g
g
9
b
10
a
11
g
a
12

Figure 13. Light microscope autoradiograph (bright field, unstained 0.5 µm section) from a newt retina incubated in (3H)-GABA. A population of horizontal cells (h), bipolar cells (b) and their Landolt's club processes (lc), amacrine cells (a), and a large number of ganglion cells (g) are labelled. A small population of cells near the inner border of the inner plexiform layer (ipl) do not accumulate (3H)-GABA (asterisks). Labelled cell processes are evident in both the outer (opl) and inner (ipl) plexiform layers. The inner plexiform layer is evenly labelled throughout. olm= outer limiting membrane, onl= outer nuclear layer, inl= inner nuclear layer, gcl= ganglion cell layer, bar= 25 µm

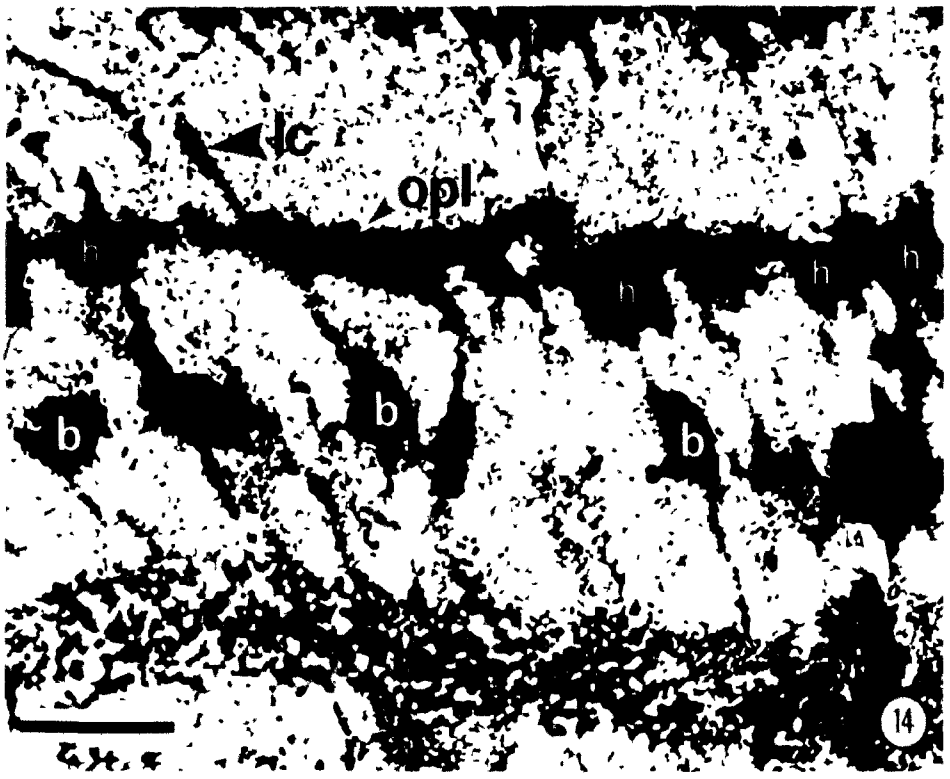


13

Figures 14 and 15. Light microscope autoradiographs (bright field, unstained, 10 µm section) from a newt retina incubated in (3H)-GABA.

Figure 14. Processes of the bipolar cells (b) are heavily labelled and extend from the inner plexiform layer (ipl) to the outer plexiform layer (opl), terminating at the outer limiting membrane as Landolt's clubs (lc). The labelling which occurs within the outer plexiform layer (opl) is due to the lateral expansion of horizontal cell (h) and bipolar cell (b) processes within this layer. bar= 25 µm

Figure 15. The labelled processes in the inner plexiform layer (ipl) are derived from bipolar cells (b) and amacrine cells (a) in the inner nuclear layer (inl), and a few heavily labelled cells (g) at the outer border of the ganglion cell layer (gcl). The uptake of (3H)-GABA by ganglion cells in this autoradiograph is much reduced when compared with Fig. 13. Under these conditions, a sublamination of the inner plexiform layer (ipl) becomes apparent which is due to the labelled processes from amacrine cells, bipolar cells, and labelled cells in the ganglion cell layer. Two densely labelled bands are apparent at the outer and inner margins of the inner plexiform layer with a third, less densely labelled band near the middle of the inner plexiform layer. bar= 25 µm


lc
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b
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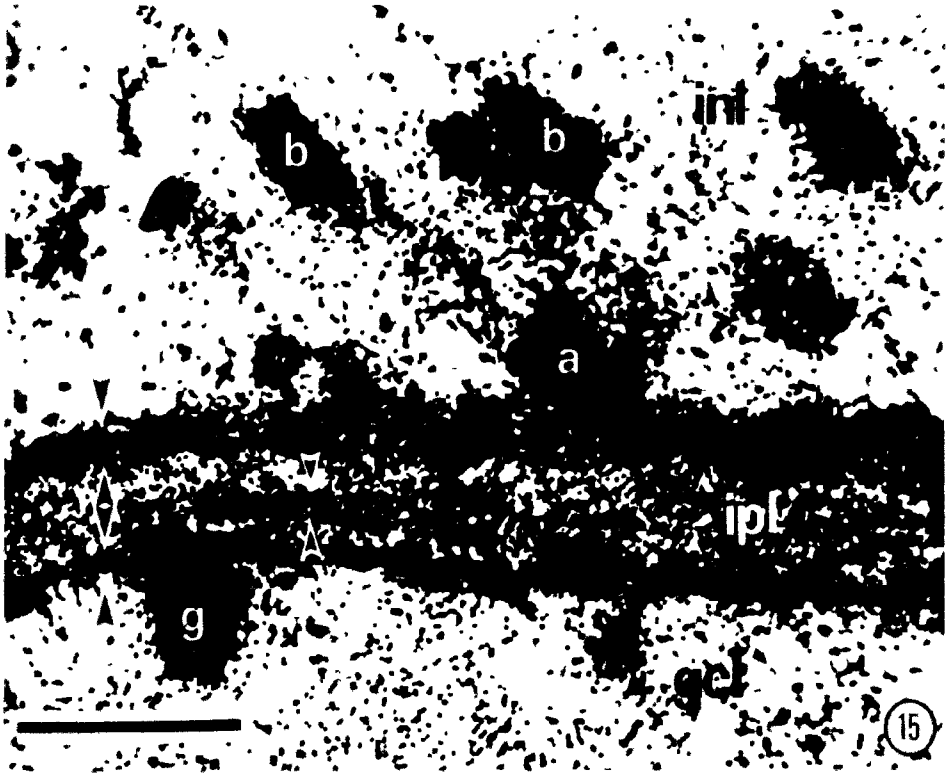

inl
b
b
a
ipl
g
gcl
15

Figure 16. Low power electron microscope autoradiograph showing the outer plexiform layer of the newt retina which has been incubated in and accumulated (3H)-GABA. A labelled horizontal cell (h) sends labelled processes laterally along the inner margin of the outer plexiform layer (arrows). Photoreceptor cells (p) and other horizontal cells (h) are unlabelled. bar= 2.0 μm

Figure 17 (A and B). High power electron microscope autoradiographs showing labelled processes invaginating deeply into cone terminals (ct). In Fig. 17A a labelled process (arrow) is part of a triad and occupies a lateral position in the ribbon synapse. In Fig. 17B a labelled process (d) invaginates deeply into a cone terminal (ct) where synaptic contact is established with the photoreceptor (arrow). Other processes (asterisks) within the photoreceptor terminal are labelled as well. sr= synaptic lamellae, bars= 0.5 μm


16

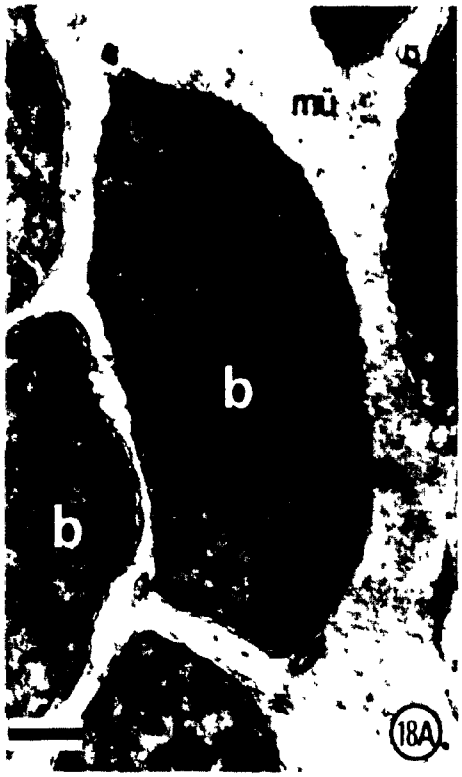

17A


17B

Figure 18 (A and B). Low power electron microscope autoradiographs showing the uptake of (3H)-GABA by bipolar cells in the newt retina. In Fig. 18A, two bipolar neurons (b) in the middle of the inner nuclear layer are labelled. Adjacent cells and Müller cell cytoplasm (Mü) have not accumulated (3H)-GABA. The apical extensions of these bipolar cells, the Landolt's clubs (lc), can be identified at the outer limiting membrane between the apical processes of the Müller cells (Mü) and photoreceptor nuclei (p) (Fig. 18B). ov= optic ventricle, bars= 2.0 µm

Figure 19 (A and B). Electron microscope autoradiographs showing the uptake of (3H)-GABA by ganglion cells (g) and their axons (a) in the newt retina. In Fig. 19A a labelled ganglion cell (g) is situated near the inner limiting membrane (ilm), beside an unlabelled ganglion cell. Silver grains are present over an adjacent fascicle of optic nerve fibres (onf) as well. (bar= 2.0 µm)

Fig. 19B is a high magnification electron microscope autoradiograph of an optic nerve fascicle showing axons of ganglion cells which accumulate (3H)-GABA. bar= 0.5 µm


mu
b
b
18A


OV
mu
18B


g
m
lm
19A


a
a
a
19B

Figure 20. Low power electron microscope autoradiograph of newt retina incubated in (3H)-GABA. Two labelled amacrine cells (a) are seen near the outer border of the inner plexiform layer (ipl). These two amacrine cells have accumulated (3H)-GABA, while two adjacent amacrine cells (a) and a Müller cell (Mü) have not. bar= 5.0 µm

Figures 21 to 23. Electron microscope autoradiographs showing labelled amacrine cell processes in the inner plexiform layer of a newt retina which has been incubated in (3H)-GABA.

Figure 21. The labelled process contains numerous, small, electron-lucent vesicles (44 nm in diameter). The terminal also contains two mitochondria (m) and a profile of smooth endoplasmic reticulum (ser). bar= 0.5 µm

Figure 22. The labelled process is surrounded by unlabelled amacrine cell processes with similar morphology. Not only does this terminal contain numerous, small, electron-lucent vesicles but also a single, large, dense-cored vesicle (arrow). bar= 0.5 µm

Figure 23. Three labelled amacrine cell processes (a) are located next to a ganglion cell dendrite (g). Two of these amacrine cell processes contain (3H)-GABA. A labelled amacrine cell terminal and an unlabelled amacrine cell terminal make synaptic contact (arrows) with the

ganglion cell dendrite. The labelled terminals are morphologically similar to other labelled amacrine cell processes (Figs. 21 and 22). b= bipolar cell, bar= 0.5 µm


mü
a
a
a
a
20


ser
21


a
22


a
23

Figure 24 (A and B). Electron microscope autoradiograph showing a labelled amacrine cell process in two serial sections. In Fig. 24A three ganglion cell dendrites (g) are adjacent to a labelled amacrine cell process (a) which has accumulated (3H)-GABA. In Fig. 24B the labelled amacrine cell terminal (a) forms a junction (arrow) with one of the ganglion cell dendrites. bar= 0.5 µm

Figure 25. Electron microscope autoradiograph of the inner plexiform layer of a newt retina which has been incubated in (3H)-GABA. A labelled amacrine cell process (a) containing numerous, small, electron-lucent vesicles and large, electron-dense vesicles (small arrows) forms a junction (arrow) with an adjacent amacrine cell. bar= 0.5 µm

Figure 26. Electron microscope autoradiograph of the inner plexiform layer of a newt retina which has been incubated in (3H)-GABA. A labelled amacrine cell process (a) receives synaptic input (arrow) from an adjacent, unlabelled amacrine cell which in turn, receives synaptic input (arrow) from a third amacrine cell. bar= 0.5 µm


24A


24B


25


26

DISCUSSION

The results of the uptake studies using (3H)-glycine, (3H)-taurine, and (3H)-GABA have shown that each of these inhibitory amino acids could be utilized as a neurotransmitter substance in the inner retina of the newt.

1) Glycine. The uptake of (3H)-glycine by newt retina differs from that reported for other animals (Bruun and Ehinger, 1974) in that here, (3H)-glycine is accumulated by Müller cells. The reason for Müller cell uptake of (3H)-glycine in the newt retina is probably not due to a species difference or to the method of administration, but rather due to the high concentration of (3H)-glycine used in the incubation medium. Therefore, concentrations of more than 20 µmol appear to activate low affinity uptake systems in the newt retina. However, such nonspecific uptake of (3H)-glycine by the newt retina makes this tissue unsuitable for examination by electron microscope autoradiography. This high uptake also makes the identification of retinal neurons which accumulate (3H)-glycine more difficult at the light microscope level.

Because the horizontal cells of the newt retina did not accumulate (3H)-glycine, the labelling observed within the outer plexiform layer must be due to the accumulation of (3H)-glycine by cells in the inner half of the inner nuclear layer. Labelling of Landolt's clubs near the outer limiting membrane suggests that the labelled neurons near the middle

of the inner plexiform layer are probably bipolar cells which give rise to labelled processes in the outer plexiform layer. In most of the animals studied to date, glycine labelling has not been observed within the outer plexiform layer. In the toad retina however, labelling of the outer plexiform layer has been observed and it is believed to be due to the ramification throughout the outer plexiform layer of processes derived from a population of glycinergic interplexiform cells (Rayborn et al., 1981). In the newt retina however, bipolar cells have been shown to accumulate (3H)-GABA, and because bipolar cell dendrites ramify within the outer plexiform layer, it is likely that these same bipolar cell processes are responsible for most of the labelling which occurs within the outer plexiform layer. The existence of a population of glycinergic interplexiform cells on the other hand, with labelled cell bodies located amongst the amacrine cells and processes ramifying throughout both plexiform layers, is difficult to demonstrate in the newt retina because of the simultaneous uptake of (3H)-glycine by both bipolar and amacrine cells. Although the uptake of (3H)-glycine by cat bipolar cells has recently been reported (Kong et al., 1980; Pourcho, 1980), Kong et al. (1980) suggest that glycine is unlikely to be a neurotransmitter substance of these cells. Rather, it has been suggested that bipolar cells pick up glycine from diffuse amacrine cells, through gap junctions which have been identified between these neurons. This could explain the labelling of bipolar cells in the newt retina as well.

If the above hypothesis is correct, then at least one cell type, if not all of the labelled cells in the inner half of the inner nuclear layer of the newt retina, belong to a population of diffuse amacrine cell. Silver impregnation studies of diffuse amacrine cells in amphibian retina (Cajal, 1893) showed that the perikarya of these cells are located near the middle of the inner nuclear layer. The labelled cells in the newt retina which are located just scleral to a row of lightly labelled Müller cell nuclei, probably correspond to the diffuse amacrine cells of Cajal. The second population of labelled amacrine cells located at the inner margin of the inner nuclear layer may also belong to this class of diffuse amacrine cell, but because of the position of their cell bodies, they are more likely to be a type of polystratified amacrine cell described by Cajal (1893). The uptake of (3H)-glycine by amacrine cells and their processes, never seems to result in a division of the inner plexiform layer into sublaminae, as would be expected if a population of stratified amacrine cells were involved. It could be argued however, that the uptake of (3H)-glycine by glial processes in the inner plexiform layer masks any subtle stratification patterns that might exist. This is unlikely though, because there is little, if any reported stratification of the inner plexiform layer caused by (3H)-glycine uptake in other animals (Bruun and Ehinger, 1974; Pourcho, 1980). Because the processes of the glycinergic neurons are distributed evenly throughout the inner plexiform layer of the newt, it

is likely then that they are involved in the modulation of both ON and OFF-channel activity.

2) Taurine. The pattern of (3H)-taurine uptake by newt retina differs significantly from the uptake of (3H)-glycine by newt retina. The uptake of (3H)-taurine by a very small population of cells in the position of the horizontal cells has been reported in the retinas of other amphibian species, including frog (Lake et al., 1978). However, electron microscope observations of the outer plexiform layer of the newt have not provided evidence supporting the existence of (3H)-taurine labelled horizontal cell processes. This does not necessarily imply that they do not exist, however, because: 1) both the number of cells and labelled processes is small compared to the total number of horizontal cells and processes in the newt retina; and 2) the size of the labelled processes in the outer plexiform layer is so small (<0.75 μm in diameter) that they are not easily detected by an autoradiographic technique with a total error of about 1.0 μm.

The cells in the middle of the inner plexiform layer of the newt retina which are lightly labelled with (3H)-taurine are probably Müller cells, since a similar low level of labelling is present over the foot processes of these cells, at the inner limiting membrane. Although some taurinergic neurons in the middle of the inner nuclear layer of other species have been identified as bipolar cells (Lake et al., 1978; Pourcho, 1981), whether some of the cells which

accumulate (3H)-taurine in the middle of the inner nuclear layer of the newt retina belong to a population of bipolar cells is unknown. If in fact there is a population of bipolar cells in the newt retina which accumulate (3H)-taurine, then they accumulate taurine for some other purpose than its use as a neurotransmitter substance. It is possible that these bipolar cells receive the label by diffusion through gap junctions formed between these cells and certain amacrine cells which accumulate (3H)-taurine.

In the newt retina, there is significant uptake of (3H)-taurine by a population of cells in the position of the amacrine interneurons. Since the perikarya of interplexiform cells are located among those of the amacrine cells, it is possible that some of the cells lining the inner margin of the inner plexiform layer which accumulate (3H)-taurine, are interplexiform cells as well. The existence of a population of taurinergic interplexiform cells is difficult to demonstrate by present methods however, because of the uptake of (3H)-taurine by both horizontal and bipolar cells in the newt retina. It is certain though, that some amacrine cells accumulate (3H)-taurine in the newt retina, as they do in most other species (Lake et al., 1978; Pourcho, 1981). Electron microscope autoradiography of the taurine-accumulating amacrine cells has not permitted classification of these cells into a morphologically distinct population of cells, as has been possible in the retina of the cat (Pourcho,

1981). This is because the neurons of the newt retina, with the exception of the photoreceptor cells, are morphologically indistinguishable.

Electron microscope autoradiography has, however, been successful in localizing (3H)-taurine to the processes of those amacrine cells which divide the inner plexiform layer into three sublaminae. Sublamination of the inner plexiform layer after the uptake of (3H)-taurine has also been observed in the retinas of pigeon (Lake et al., 1978) and cat (Pourcho, 1981). In the pigeon retina, three bands of dense label are observed, the widest band being located in the inner half of the inner plexiform layer (Lake et al., 1978). In the cat retina, a single wide band of dense labelling is located in the inner half of the inner plexiform layer (Pourcho, 1981). In the newt retina, two heavy bands of label are observed at the inner and outer margins of the inner plexiform layer. At the light microscope level, the outer band, in sublamina a (Famiglietti et al., 1977), appears to be due to the processes which arise from the amacrine cells and course laterally along the outer margin of the inner plexiform layer. The inner band, in sublamina b, appears to be due to the processes which arise from the ganglion cells and course laterally along the inner margin of the inner plexiform layer. This implies that these heavy bands are due to label in amacrine and ganglion cell processes, which course laterally along the margins of the inner plexiform

layer, and is not necessarily due to label which is restricted to the synapses formed in these regions. In fact, these banding patterns may have little to do with the synaptic organization of these labelled cells in the inner plexiform layer (Yazulla, 1981). The uptake pattern of (3H)-taurine by the newt retina, therefore does not necessarily imply that the processes arising from the labelled amacrine cells synapse exclusively in sublamina a and that the processes arising from the labelled ganglion cells synapse exclusively in sublamina b. Synaptic binding or electrophysiological studies would seem to be more appropriate techniques to determine the function of taurinergic cells in the newt retina. However, the position of the labelled amacrine cell nuclei does confirm that (3H)-taurine is taken up by a population of stratified amacrine cells.

Electron microscope autoradiography is useful for demonstrating synaptic relationships which the labelled amacrine cell processes make with other cells in the inner plexiform layer. The results show that the processes of the amacrine cells which accumulate (3H)-taurine are postsynaptic to amacrine cells and bipolar cells, and are presynaptic to other amacrine cells, bipolar cells, and possibly ganglion cells. Whether or not these synapses are involved exclusively in the OFF channel as has been suggested for the taurinergic amacrine cells of the mudpuppy retina (Cunningham and Miller, 1980b), is unknown. However,

if the bands of label observed in sublamina a and b of the newt inner plexiform layer are both due to processes of labelled amacrine cells, it would appear that taurinergic amacrine cells in the newt retina are involved in both the ON and OFF channels. In addition, (3H)-taurine labelling of the inner plexiform layer was always associated with amacrine cell terminals containing small, clear synaptic vesicles and a few large dense-cored vesicles, indicating that (3H)-taurine uptake is probably restricted to a single amacrine cell population, and further implicating this amino acid as a true retinal neurotransmitter.

Uptake of (3H)-taurine by ganglion cells has been previously reported in other species (Lake et al., 1978; Pourcho, 1981) where it has been presumed to be of a nonspecific nature. However, the pattern of (3H)-taurine uptake by ganglion cells in the newt retina differs from other species in that there are two levels of (3H)-taurine uptake. One population of ganglion cells lining the inner margin of the inner plexiform layer exhibits high levels of (3H)-taurine uptake, which is equivalent to the uptake of (3H)-taurine by the amacrine cells lining the outer margin of the inner plexiform layer. This subpopulation of ganglion cells also resembles the labelled amacrine cells in the inner nuclear layer, in the lateral spread of their processes along the margins of the inner plexiform layer. Other cells in the ganglion cell layer, as well as the fascicles of optic nerve axons, demonstrate minimal uptake

of (3H)-taurine in the newt retina. These observations suggest that the cells in the ganglion cell layer which exhibit high levels of (3H)-taurine uptake are not ganglion cells, but rather are displaced amacrine cells. This suggestion is further substantiated by the finding that no labelled ganglion cell dendrites were observed in the inner plexiform layer of the newt retina in electron microscope autoradiographs. The possibility that such a large population of displaced taurinergic amacrine cells exists in the ganglion cell layer of the newt retina is further explored in Chapter 4.

3) GABA. The results of (3H)-GABA labelling show that there is specific uptake of this amino acid by a population of horizontal, bipolar, and amacrine cells, and by nearly all ganglion cells of the newt retina. Uptake of (3H)-GABA by a population of retinal horizontal cells has been previously reported for other lower vertebrates such as frog (Voaden et al., 1974; Hollyfield et al., 1979), catfish (Lam et al., 1978), and goldfish (Marc et al., 1978; Lam et al., 1980). The perikarya of the labelled horizontal cells could be easily identified by electron microscope autoradiography, but the processes were less easily identified because of their small size. However, when labelled horizontal cell processes which penetrated deep into the photoreceptor terminals could be identified, they were always associated with the lateral elements of cone triads. This implies that the horizontal cells labelled by (3H)-GABA in the newt

retina may be equivalent to the H1 horizontal cells of the goldfish retina (Marc et al., 1978). If this is true, then GABA-ergic horizontal cells in the newt may subserve an equivalent role in the colour pathway in these two species. Verification of this speculation must await identification of photoreceptor and horizontal cell subtypes in the newt retina and their role in the colour pathway.

The uptake of (3H)-GABA by bipolar perikarya and their Landolt's club processes has been demonstrated in the newt retina, by both light and electron microscope autoradiography. Why such a population of bipolar cells accumulates (3H)-GABA is unknown but it may be for the same reasons that bipolar cells of the newt retina accumulate (3H)-glycine. If the labelling of bipolar cells is due to diffusion of label into these cells through gap junctions formed with amacrine cells which have a high affinity uptake system for these amino acids, then the presence of gap junctions between bipolar cells and amacrine cells must be more widespread than was previously thought. This is not likely since the only cells which form extensive gap junctions with each other in the cat retina are cone bipolar cells and diffuse amacrine cells (Kolb and Famiglietti, 1974; Famiglietti and Kolb, 1975). It seems more likely that there is a population of bipolar cells in the newt retina which is capable of accumulating a number of amino acids for some reason, other than the use of these amino acids as neurotransmitter substances. The suggestion that bipolar

and amacrine cell uptake of (3H)-GABA is an independent process, is substantiated by the finding that many more bipolar cells accumulate (3H)-GABA than amacrine cells. However, it cannot be ruled out that the bipolar cells receive the label through gap junctions formed between bipolar cells and the horizontal cells which accumulate (3H)-GABA.

Light and electron microscope autoradiography has also localized (3H)-GABA uptake to a population of amacrine cells in the newt retina. Uptake of (3H)-GABA by amacrine cells has been reported in most species studied thus far. Problems associated with Müller cell uptake, masking the uptake of (3H)-GABA by neurons, was not encountered with (3H)-GABA localization in the newt retina, presumably because of the brief postincubation in media not containing label. However, the uptake of (3H)-GABA by all ganglion cells masked inner plexiform layer sublamination. Only when ganglion cell labelling was minimal, did a division of the inner plexiform layer into sublaminae become apparent. The heaviest bands, at the inner and outer margins of the inner plexiform layer, are similar to the bands observed with (3H)-taurine uptake. These two bands probably represent the extension of GABA-ergic amacrine cell processes which course laterally along the inner and outer margins of the inner plexiform layer. Unlike the pattern of (3H)-taurine uptake, a third band, near the middle of the inner plexiform layer, is observed in autoradiographs of (3H)-GABA uptake by newt

retina. It is impossible to determine which of the labelled neuronal types gives rise to this band. If it is the labelled amacrine cells, then this pattern is similar to the lamination pattern in goldfish retina (Marc et al., 1978) in which depolarizing (ON) amacrine cells terminating in sublamina b are probably GABA-ergic and are involved in the red colour pathway.

The pattern of inner plexiform layer sublamination by (3H)-glycine, (3H)-taurine and (3H)-GABA uptake indicate that the neurons which accumulate these amino acids belong to different classes of amacrine cells. Although the morphology of the amacrine cell terminals which accumulate (3H)-taurine and (3H)-GABA in the newt retina is similar, the types of synaptic connections that they make with other processes in the inner plexiform layer are different. Unlike the terminals of (3H)-taurine accumulating cells, the terminals of the (3H)-GABA accumulating cells in the newt retina were never observed to be either pre- or postsynaptic to bipolar cells in electron microscope autoradiographs. The terminals of (3H)-GABA accumulating amacrine cells were most frequently observed to be postsynaptic to other amacrine cell processes and presynaptic to the dendrites of ganglion cells. These results provide evidence for a difference in the function of amacrine cells which accumulate (3H)-taurine and (3H)-GABA. A complete statistical analysis is necessary to determine the frequency with which silver grains are associated with synaptic

profiles in sublamina a and b of the inner plexiform layer before any valid comments about the possible function of the taurinergic and GABA-ergic amacrine cells in the newt retina can be made.

The results show that the uptake of (3H)-GABA by ganglion cells of the newt retina is generally uniform and involves all cells, except a small number of neurons lining the outer border of the ganglion cell layer. It is possible that these unlabelled neurons may be the suspected displaced amacrine cells which accumulate (3H)-taurine. The reason why, in some preparations, the uptake of (3H)-GABA by ganglion cells was minimal is unknown, but it may be due to longer postincubation times in media not containing label. When most ganglion cells do not accumulate appreciable amounts of (3H)-GABA, a very small population of cells lining the inner margin of the inner plexiform layer label as intensively as the amacrine cells which accumulate (3H)-GABA. The processes of these cells appear to course laterally along the inner margin of the inner plexiform layer, in a manner similar to that observed for the cells in the ganglion cell layer which accumulate (3H)-taurine. It is possible that these cells belong to a population of displaced GABA-ergic amacrine cells, a possiblity which is further considered in Chapter 4.

In conclusion, these results show that the newt retina possesses a high affinity uptake system for the putative inhibitory amino acid neurotransmitters glycine, taurine,

and GABA. All three transmitter candidates are accumulated by amacrine cells which, on the basis of uptake patterns, belong to different subpopulations of this cell type. Electron microscope autoradiography has localized (3H)-taurine and (3H)-GABA to specific amacrine cell processes, further implicating both of these compounds as neurotransmitters in the vertebrate retin

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CHAPTER 4

DISPLACED CELLS IN THE NEWT RETINA

INTRODUCTION

It is generally considered that the inner nuclear layer of the retina contains the perikarya of the horizontal cells, bipolar cells, interplexiform cells, and the amacrine cells; and that the ganglion cell layer contains the perikarya of the ganglion cells. In some instances, during the formation of the inner and outer plexiform layers, the perikarya of these cell types become displaced to layers in which they are not normally found (Rodieck, 1973). Although the perikarya of these displaced cells change position, the kinds of synaptic contacts which they make with other cells do not (Rodieck, 1973).

Displaced bipolar cells have been occasionally found in the outer nuclear layer in all vertebrate classes except birds (Cajal, 1893 as cited in Rodieck, 1973) and teleost fishes (Munk, 1968).

Retinal ganglion cells which are displaced to the inner portion of the inner nuclear layer were first described by Dogiel (1891) and were considered to be few in number (Rodieck, 1973). They have subsequently been described in most vertebrate species studied, including frog (Cajal, 1892 as cited in Rodieck, 1973), lizard (Cajal, 1892 as cited in

Rodieck, 1973), turtle (Reiner, 1981), dogfish (Stell and Witkovsky, 1973), fish (Finger and Karten, 1977), bird (Heaton et al., 1979; Reiner et al., 1979; Fite et al., 1981), mouse (Dräger and Olsen, 1981), rat (Bunt et al., 1974), rabbit (Honrubia and Grijalbo, 1967), and primate (Boycott and Dowling, 1969; Bunt and Minkler, 1977). In the mouse, displaced ganglion cells constitute about 2% of the ganglion cells in the retina (Dräger and Olsen, 1981). Notwithstanding the fact that the displaced ganglion cells are most distinctive in birds because of their large size (Reiner et al., 1979; Fite et al., 1981), they have only been estimated to constitute about 0.125% of the total number of ganglion cells in the pigeon retina (Binggeli and Paule, 1969).

Although displaced ganglion cells were first identified by silver impregnation techniques, a great deal of new information concerning these cells has arisen from studies employing the retrograde transport of horseradish peroxidase (HRP) along retinal afferents. These studies show that displaced ganglion cells are concentrated around the optic disc of the primate (Bunt and Minkler, 1977) and rat retinas (Bunt et al., 1974), but are located mainly at the periphery of the pigeon (Karten et al., 1977; Fite et al., 1981), chicken (Reiner et al., 1979) and mouse (Dräger and Olsen, 1981) retinas. Although the designation "displaced ganglion cells" implies that these cells differ from other ganglion cells only in position, they differ functionally from

"true"* ganglion cells as well (Stell and Witkovsky, 1973). Tracer studies have shown that most, if not all, of the displaced ganglion cells in pigeon (Fite et al., 1981), chicken (Reiner et al., 1979) and monkey (Bunt and Minkler, 1977) retinas project to accessory optic nuclei which are involved in optomotor reflexes such as visual field stabilization (Reiner, 1981). However, in the turtle retina only 14% of the cells which labelled with HRP after injection of the accessory optic nuclei were displaced ganglion cells (Reiner, 1981), and none of the cells which labelled with HRP after injection of a portion of the accessory optic nucleus in the rabbit were displaced ganglion cells (Oyster et al., 1980). The reason for such species differences is not yet clear.

The displaced ganglion cells are thought to be the "transient" neurons of the inner plexiform layer that generate spike activity in physiological studies (Fite et al., 1981). The dendrites of the displaced ganglion cells have been observed in sublamina a of the inner plexiform layer (Boycott and Dowling, 1969), indicating that displaced ganglion cells would be characterized by OFF-centre responses. It has further been suggested that some of the myelinated fibres observed radiating from the optic nerve and running horizontally near the amacrine cell layer in

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* "true" ganglion cells are those classically described cells whose perikarya lie in the ganglion cell layer and whose axons project to the brain.

goldfish, carp and dogfish retina, are the axons of displaced ganglion cells. These studies show that the displaced ganglion cells are not ganglion cells which find themselves in the inner nuclear layer by some developmental accident, but rather are a subset of specialized neurons in the vertebrate retina which have a unique physiological role in the visual pathway.

Cajal (as cited in Rodieck, 1973) found that in all vertebrate classes studied, the monostratified amacrine cells whose dendrites ramified in the innermost portion of the inner plexiform layer, had their perikarya displaced to the ganglion cell layer. These cells were first identified in Golgi preparations, but have recently received more attention since studies now suggest that there are many more of these displaced amacrine cells in the ganglion cell layer than was previously thought. After comparing the number of cells in the ganglion cell layer to the number of axons in the optic nerve, Bingelli and Paule (1969) determined that 12.5% of the cells in the ganglion cell layer of the pigeon retina were glial cells, 50% were "true" ganglion cells, and 37% were attributed to displaced amacrine cells. A major problem in determining the "true" ganglion cell distribution in the retinas of higher vertebrates is in determining which cells are ganglion cells, which are glia, and which are displaced amacrine cells (Dräger and Olsen, 1981). Hughes (1975) has estimated that the number of neurons (excluding glia, which could be characterized morphologically) in the

cat retina is between 217,000 and 260,000; but there appears to be only 128,000 to 180,000 axons in the cat optic nerve (Stone, 1978; Hughes and Wässle, 1976). This implies that between 31% and 41% of the cells in the ganglion cell layer of the cat retina are either glia or displaced amacrine cells. However, when the neurotoxin kainic acid is applied to cat retina, some of the small cells in the ganglion cell layer, which were presumed to be glia, were lesioned (Hughes and Wieniawa-Narkeiwicz, 1980). These studies now estimate the number of cells in the ganglion cell layer at 10,000,000 and suggest that the displaced amacrine cells of the cat retina may account for up to 86% of the total number of cells in the ganglion cell layer (Hughes and Wieniawa-Narkiewicz, 1980).

Hughes (1977) has also compared the number of ganglion cells in the rat ganglion cell layer to the number of axons in the optic nerve and found that 37% of the neurons in the rat ganglion cell layer do not send an axon to the optic nerve. These neurons survive optic nerve transection and do not transport HRP in the severed optic nerve (Cowey and Perry, 1979). It was once thought that the smallest cells in the rat ganglion cell layer were a type of microglia, however immunofluorescent techniques (Bignami and Dahl, 1979) and Golgi studies (Perry and Walker, 1980) indicate that these cells are displaced amacrine cells, and that the only neuroglia present in the rat retina are the Müller cells. Although rarely impregnated with silver, these

displaced amacrine cells have been successfully visualized in Golgi preparations of rat retina by Perry (1979) and Perry and Walker (1980). Six morphologically distinct types of displaced amacrine cells have been identified in the rat retina, including a type of diffuse amacrine cell and five types of unistratified cells (Perry and Walker, 1980). A number of cells in the ganglion cell layer of the rat retina are immunoreactive for somatostatin-like activity. Since the optic nerve is devoid of immunoreactive material, it is highly probable that these immunoreactive cells belong to a population of displaced amacrine cells (Krisch and Leonhardt, 1979).

When horseradish peroxidase was injected into the macerated lateral geniculate nucleus of the mouse, only 69% of the cells in the ganglion cell layer transported HRP (Dräger and Olsen, 1981). It was considered that the remaining 31% of the cells in the ganglion cell layer which did not transport HRP were displaced amacrine cells (Dräger and Olsen, 1981).

In 1979 Masland and Mills found that the only cells in the rabbit retina which synthesized acetylcholine comprised two small groups of morphologically similar cells lining both margins of the inner plexiform layer. These acetylcholine synthesizing cells were shown to make up more than 20% of the total number of cells in the ganglion cell layer (Masland and Mills, 1979), and their ability to synthesize acetylcholine was not affected by severing the

optic nerve (Hayden et al., 1980a). Furthermore, when "true" rabbit ganglion cells were labelled with a fluorescent dye, which they transported from the lateral geniculate nucleus, a population of cells corresponding in position to the acetylcholine synthesizing cells were not labelled (Hayden et al., 1980). Vaney (1980a) has suggested that these acetylcholine-synthesizing cells, in the ganglion cell layer of the rabbit retina, correspond to that morphologically distinct population of cells known as "coronate" cells. Coronate cells appear to be the same size and have the same distribution as the cells which synthesize acetylcholine in Masland and Mill's (1979) experiments (Vaney, 1980a). Furthermore, the coronate cells do not transport HRP from the distal end of a severed optic nerve and are lesioned by low concentrations of kainic acid (Hughes and Vaney, 1980). Vaney and Hughes (1976) have estimated the number of ganglion cells in the rabbit retina (including the number of coronate cells, but not glia) at 547,000 and the number of axons in the optic nerve at 394,000. This implies that 28% of the neurons in the rabbit ganglion cell layer are displaced amacrine cells. Later estimates by Hughes and Vaney (1980) have now determined that the number of ganglion cells in the rabbit retina (excluding the number of coronate cells and glia) is about 373,500 cells, and show that only 56% of the cells in the peripheral rabbit ganglion cell layer are ganglion cells, 15% are glia, and 29% are coronate or unclassified cells (Hughes and Vaney, 1980; Vaney, 1980b).

In addition to Bingelli and Paule's (1969) determination that 37.5% of the cells in the ganglion cell layer of the pigeon retina were displaced amacrine cells, similar numbers of displaced amacrine cells have been shown to exist in other avian retinas as well. When the optic nerve of the Japanese quail was severed, 20-30% of the cells in the ganglion cell layer did not degenerate (Muchnick and Hibbard, 1980). When the axons of chick retinal ganglion cells were lesioned, a population of neurons which accounted for 30-35% of the cells in the ganglion cell layer, remained intact (Ehrlich and Morgan, 1980). These cells, which were resistant to axotomy, could also be destroyed by intraocular injection of kainic acid, while ganglion cells and glia were unaffected by this neurotoxin (Ehrlich and Morgan, 1980).

Recent studies have consistently shown that the actual number of ganglion cells in the retinas of higher vertebrates is only about 60% of the total number of cells in the ganglion cell layer. Few studies, however, have shown that such large numbers of displaced amacrine cells are present in lower vertebrate retinas. The purpose of the work outlined in this chapter was to determine if such populations of displaced cells existed in the retina of the newt. In Chapter 3, it was pointed out that the ganglion cells which accumulated (3H)-taurine in the newt retina were located near the outer border of the ganglion cell layer and their dendritic processes extended laterally along the inner border of the inner plexiform layer. The pattern of

(3H)-taurine uptake by these cells was strikingly similar to the uptake of (3H)-taurine by amacrine cells in the inner nuclear layer. Furthermore, the results of Chapter 3 have shown that a small population of cells, also lining the outer margin of the ganglion cell layer in the newt retina, accumulate (3H)-GABA, even when most ganglion cells and the optic nerve axons do not. These taurine and GABA-labelled cells correspond in position to the cells in the newt ganglion cell layer which do not respond to optic nerve lesions (Turner et al, 1978), suggesting that 50-60% of the cells in the ganglion cell layer are displaced amacrine cells. Through the use of cell and axon counts, horseradish peroxidase transport experiments, and the uptake of radioactive putative neurotransmitters, this chapter attempts to determine if displaced amacrine and ganglion cells exist in the retina of the newt.

MATERIALS AND METHODS

1) Amino Acid Uptake by Ganglion Cells. Tritiated taurine and (3H)-γ-amino butyric acid ((3H)-GABA) were purchased from New England Nuclear (Boston, Massachusetts) with the following specific activities; (3H)-taurine: 23 Ci/μmol and (3H)-GABA: 28.2 Ci/μmol. Twenty microlitre quantities of these amino acids were evaporated with dry nitrogen in a fume hood at 20°C and resuspended in 100 μl of unsupplemented media (M-199 with glutamine, GIBCO, Grand Island, New York) in a small 200 μl chamber. Final concentrations of amino acids in the incubation media were: (3H)-taurine, 8.63 μmol and (3H)-GABA, 7.22 μmol.

Newt eyes were enucleated, the anterior eye segments removed, and the resulting eyecups were placed in the 200 μl chambers containing media, and either (3H)-taurine or (3H)-GABA. The media was oxygenated with 98% oxygen during incubation. After 20 min of incubation, the eyecups were washed in media without label for 5 min. The retinas were then fixed in a cacodylate buffered glutaraldehyde-formaldehyde fixative and dehydrated as described in Chapter 1. Tissues were either embedded in plastic or paraffin prior to sectioning, and processed for light microscope autoradiography as previously described (Chapter 2).

Counting of Labelled Cells. To determine the percentage of cells in the newt ganglion cell layer which accumulated each of these amino acids, the total number of cells in the

ganglion cell layer and the number of cells which accumulated either (3H)-taurine or (3H)-GABA were counted on autoradiographs of 0.5 µm retinal sections stained with toluidine blue. Approximately 1800 ganglion cells were counted in about 20 sections taken from 10 randomly sectioned blocks of tissue from 5 retinas labelled with (3H)-taurine and 5 retinas labelled with (3H)-GABA.

2) Horseradish Peroxidase Transport by Ganglion Cells. Newts were anaesthetized in a solution of 0.5% (wt/vol) MS-222 (Tricanemethansulfonate, Syndel Laboratories, Vancouver, B.C.). The mandible was removed and major vessels were cauterized immediately. The palatine epithelium and the connective tissue covering the right orbit was incised and the extraocular muscles reflected to reveal the optic nerve. The optic nerve was severed with fine spring scissors, taking care not to damage the ciliary arteries entering the globe. Ten microlitres of a 30-50% solution of horseradish peroxidase (HRP Grade I, Boehringer Mannheim, Dorval, Quebec) in 0.9% NaCl was injected over a 5 min period into the orbit around the severed optic nerve with a Hamilton microlitre syringe, mounted in a micromanipulator. The animals were then returned to a small aquarium containing distilled water. Animals were sacrificed after periods of 12, 24 and 48 hours after axotomy and the right eye was fixed in a cacodylate-buffered glutaraldehyde-formaldehyde fixative, as previously described (Chapter 1). After fixation, the corneas and

lenses were removed and the resulting eyecups were washed for 15 min in 0.05 M tris (trishydroxyaminomethane) buffer (pH 7.6) containing 5% sucrose and then incubated in 0.05% diaminobenzidine tetrahydrochloride (DAB, St. Louis, Missourri) in .05 M tris buffer (pH 7.6) for 30 min at 20 °C. Hydrogen peroxide (30%, BDH Chemicals, Poole, England) was then added (final concentration, 0.01%) and the tissue was further incubated for 30 min at 20 °C. After incubation, each eyecup was washed in tris buffer, the sclera removed, and the retina was dehydrated and embedded in TAAB resin as previously described (Chapter 1), or embedded in paraffin (Chapter 2). Ten micrometer sections of paraffin-embedded tissue were counterstained with eriochrome cyanin (Chapman, 1977).

Counting of Labelled Cells. To determine the percentage of cells in the newt ganglion cell layer which transported HRP, the total number of cells in the ganglion cell layer and the number of cells which transported HRP, were counted on slides containing 0.5 µm sections which were counterstained with toluidine blue. Approximately 1000 ganglion cells were counted from sections obtained from 15 randomly selected blocks of tissue from 17 HRP labelled retinas.

3) Double Labelling of Cells in the Ganglion Cell Layer. To determine if some of the cells which accumulate (3H)-taurine are the same cells which were

labelled with HRP, the optic nerve of the right eye of five animals was severed and flooded with HRP as previously described. Forty-eight hours after axotomy, the right eye of these animals was enucleated, the cornea removed, and the resulting eyecups were placed in a 200 µl chamber containing (3H)-taurine in unsupplemented media, as previously described. After 20 min of incubation, the eyecups were washed in media without label for 5 min and then fixed in a cacodylate-buffered glutaraldehyde-formaldehyde fixative, as previously described (Chapter 1). After fixation, the eyecups were washed in 0.05 M tris buffer (pH 7.6) containing 5% sucrose for 15 min. The tissue was processed for horseradish peroxidase histochemistry with DAB, in the presence of hydrogen peroxide, as previously described. The tissue was then dehydrated, embedded in TAAB resin or embedded in paraffin and processed for light microscope autoradiography as previously described (Chapter 2).

4) Determination of the Number of Cells in the Newt Ganglion Cell Layer. To determine the actual number of cells in the ganglion cell layer of the newt retina, the eyes of 6 animals were enucleated and pierced behind the corneal-scleral junction. Two hundred microlitres of a cacodylate-buffered glutaraldehyde-formaldehyde fixative (Chapter 1) was injected over a 5 min period into each globe with a tuberculin syringe, fitted with a 30-gauge needle. The eyes were then placed in fresh fixative for 8 hours. After fixation, the lens was removed through an incision in

the cornea and the eyes were dehydrated in an acetone series. The eyes were then double embedded in celloidin and paraffin (Peterfi, 1921 as cited in Steedman, 1960) and serially sectioned at 10 μm on a Jung rotary microtome. The number of ganglion cells in one of these eyes was determined by direct counting with the aid of a Zeiss microscope fitted with a drawing tube.

a) Determination of the Volume of the Ganglion Cell Layer: The total volume of the ganglion cell layer was determined in each of 10 newt eyes prepared as described above. This was done by measuring the area of the ganglion cell layer in each serial section of all 10 eyes, with the aid of a semiautomatic image analysis computer (Zeiss Videoplan, Zeiss Canada Ltd., Don Mills, Ontario) and then by multiplying the total area of each section by the section thickness.

b) Determination of the Number of Cells per Cubic Micrometer in the Ganglion Cell Layer: Diameters of 817 cells in the ganglion cell layer, together with the total area occupied by these cells, was calculated with the aid of a Zeiss Videoplan, from randomly selected 10 μm serial sections of newt retina. The corrected diameter of cells in the ganglion cell layer (D) was calculated using the method of Henning and Elias (1970). The corrected number of ganglion cells was determined using the formula:

$$Nv = Nat/D+T$$ where:

Nv= number of cells per cubic micrometer

Nat= number of cells counted per square micrometer

$\bar{D}$= mean diameter of cells

T= section thickness

(Abercrombie, 1946; Henning, 1967 as cited in Weibel and Bolender, 1973).

c) Determination of the Total Number of Cells in the Ganglion Cell Layer: The average number of cells in the ganglion cell layer was then calculated by multiplying the average number of cells/μm^3 by the average volume of the ganglion cell layer.

5) Determination of the Number of Axons in the Newt Optic Nerve. To determine the number of axons in the optic nerve of the newt, the right orbit of 6 anaesthetized animals was injected with 200 µl of a cacodylate-buffered glutaraldehyde-formaldehyde fixative (Chapter 1) using a tuberculin syringe fitted with a 30-gauge needle. The optic nerves were then exposed and excised at the sclera and the optic foramen using fine spring scissors and each optic nerve was removed with a pipette and placed in fresh fixative for 2 hrs. The tissues were then processed for electron microscopy as previously described (Chapter 1). Semi-thin (0.5 µm) cross sections of each optic nerve were stained with toluidine blue for light microscope observations, and thin sections (80 nm) were examined by electron microscopy. A large photomontage of electron

micrographs from one optic nerve was prepared, and the photograph was divided into six pie shaped segments. All of the axons in three of these segments were counted (about half of the number in the optic nerve), and the area of the segments, as well as the area of the entire optic nerve, was measured using a Zeiss Videoplan. The number of axons per square micrometer in the three segments was calculated and multiplied by the area of the optic nerve to determine the total number of axons in this optic nerve.

The cross-sectional areas of the remaining five optic nerves (excluding the area occupied by the central glia) was determined from 0.5 μm semi-thin sections with the aid of a Zeiss Videoplan. The average number of axons/μm^2 in each of these nerves was determined by counting the number of axons within a specific area in random transmission electron micrograph samples taken from these 5 optic nerves. The average number of axons in each of these optic nerves was then estimated by multiplying the average number of optic nerve axons per square micrometer by the average area of each optic nerve.

RESULTS

1) Amino Acid Uptake by Ganglion Cells. When newt retinas were incubated in (3H)-taurine, a number of cells lining the inner margin of the inner nuclear layer accumulated (3H)-taurine (Fig. 1). These cells accounted for 65% of the cells at the inner margin of the inner nuclear layer.

All ganglion cells accumulated (3H)-taurine to some extent, however, certain cells lining the outer margin of the ganglion cell layer labelled as heavily as the amacrine cells in the inner nuclear layer (Fig. 1). These heavily labelled cells made up 24% of the cells in the ganglion cell layer and resembled the labelled amacrine cells, not only in their uptake of (3H)-taurine, but also in the proximity of their perikarya to the inner plexiform layer and the distribution of their processes within the inner plexiform layer. In addition, these cells appeared to occupy the same position as the cells which Turner et al. (1978) reported do not undergo chromatolytic changes in response to optic nerve axotomy.

When newt retina was incubated in (3H)-GABA, a small number of amacrine cells lining the inner margin of the inner nuclear layer accumulated large quantities of (3H)-GABA (Fig. 2). These labelled cells accounted for 29% of the cells at the inner margin of the inner nuclear layer.

A large number of cells in the inner portion of the ganglion cell layer also accumulated (3H)-GABA. These labelled cells accounted for 73% of the cells in the ganglion cell layer. The remaining 27% of the cells, which were unlabelled (Fig. 2, large arrows), were located in the same position as the (3H)-taurine-accumulating cells in Fig. 1. Because the optic nerve fibres were also labelled with (3H)-GABA, it is likely that the cells which accumulate (3H)-GABA are "true" ganglion cells. The dendrites of the labelled ganglion cells (Fig. 2, arrowheads) can be seen passing between the perikarya of the unlabelled cells and projecting into the inner plexiform layer.

In some experiments, the labelling of the ganglion cells was minimal (Fig. 3). In such cases, a small population of cells lining the outer margin of the ganglion cell layer labelled almost as heavily as the amacrine cells lining the inner margin of the inner nuclear layer. In these instances, the labelled cells in the ganglion cell layer accounted for approximately 15% of the cells in the ganglion cell layer. They resembled the labelled amacrine cells both in numbers and in the proximity of their perikarya and processes, relative to the inner plexiform layer.

2) Horseradish Peroxidase Transport by Ganglion Cells.

When the optic nerves of anaesthetized newts were severed and exposed to HRP, ganglion cells transported the HRP back

to their cell bodies through their severed axon (Fig. 4). Forty-eight hours after axotomy, 19% of the ganglion cells transported HRP. There was no statistically-significant difference between the number of ganglion cells which transported HRP 48 hrs, as compared with 24 hrs after axotomy; however, 12 hrs after axotomy, only 11% of the ganglion cells had transported HRP. It was considered that a survival time of at least 24 hrs was necessary for maximal labelling of ganglion cells with HRP. The homogeneous colouration of ganglion cell perikarya and their processes, resulting in Golgi-like impregnations, is due to the intraxonal bidirectional diffusion of the enzyme taken up from the severed optic nerve (Colman et al., 1976).

The dendrites of the ganglion cells (Fig. 4, arrowheads) could be observed extending from the ganglion cell perikarya upwards between the perikarya of unlabelled cells and into the inner plexiform layer. In some cases, the dendrites of the ganglion cells could be traced to various sublaminae of the inner plexiform layer. In Fig. 5, a labelled ganglion cell near the outer margin of the ganglion cell layer sends a thick dendrite through the inner plexiform layer; it bifurcates and travels horizontally along the outer margin of the inner plexiform layer. The labelling of ganglion cell dendrites results in a subdivision of the inner plexiform layer into five sublaminae. Three dense bands of labelling are evident at the inner and outer margins of the inner plexiform layer and in the

middle of the inner plexiform layer. The sublamina nearest the inner nuclear layer usually contained the densest concentration of label (Fig. 5, arrows).

Horseradish peroxidase-labelled cells could also be observed within the inner portion of the inner nuclear layer (Fig. 6), but theiroccurrence was rare, except near the periphery of the newt retina (Fig. 7). The dendrites of these displaced ganglion cells likely contribute to the dense band of label lining the outer margin of the inner plexiform layer. Since they number about 8 cells per retinal section, it is estimated that there are about 2000 of these displaced ganglion cells in the newt retina.

3) Double Labelling of Cells in the Ganglion Cell Layer. The uptake of (3H)-taurine by cells in the ganglion cell layer (Fig. 1) and the labelling of ganglion cells with HRP (Fig. 4) implies that there are two distinct populations of cells in the ganglion cell layer of the newt retina; "true" ganglion cells and displaced amacrine cells. To determine if the unlabelled cells in Fig. 4 belong to the same population of cells which accumulated (3H)-taurine (Fig. 1), both labelling techniques were performed on the same tissue. Figure 8 is a light microscope autoradiograph (unstained) showing the (3H)-taurine accumulating cells (labelled with silver grains) and the "true" ganglion cells (homogeneously filled with HRP) in the same tissue. It is evident that the cells which accumulate (3H)-taurine are not

the same cells which transport HRP. They are therefore displaced amacrine cells. Although some uptake of (3H)-taurine by all cells in the ganglion cells can be observed, appreciable amounts of (3H)-taurine were accumulated by only 4% of the cells which also transported HRP.

4) Determination of the Number of Cells in the Newt Ganglion Cell Layer. HRP transport studies showed only 19% of the cells in the ganglion cell layer to be "true" ganglion cells and therefore suggests that 81% of the cells in the ganglion cell layer are displaced amacrine cells. To verify if such unusually large numbers of displaced amacrine cells exist in the newt retina, the total number of cells in the ganglion cell layer of the newt retina was estimated and compared to an estimation of the number of axons in the newt optic nerve. Each of these estimations was carried out by two different methods so that results of each method would verify those of the other.

When one newt eye was serially sectioned at 10 μm and all of the cells in the ganglion cell layer were counted, it was discovered that there were 169,590 cells. The cost of counting in this way is prohibitive. However, to account for individual variation in retinal size amongst animals, the volume of the ganglion cell layer was estimated and multiplied by an estimation of the number of ganglion cells per cubic micrometer in the retina. The volume of the

ganglion cell layer was determined from 10 μm serial sections (Fig. 9) using a Zeiss Videoplan. The total volume of the ganglion cell layers from 10 eyes, of 5 animals, was calculated to be 136,292,226 ± 18,863,163* μm^3. The number of ganglion cells per cubic micrometer measured on randomly selected 10 μm retinal sections from these same 10 eyes was determined to be 0.0009673 ± 0.0001833* cells/μm^3. These measurements show that on the average, there are 131,835 cells in the ganglion cell layer of the newt retina, a value which is not significantly different from the direct ganglion cell count.

5) Determination of the Number of Axons in the Newt Optic Nerve. Figure 10 is a photomontage of 25 low-power electron micrographs of the newt optic nerve as it passes through the sclera (actual working size: 45 x 55 cm). The area of this optic nerve is 7,511 μm^2. The sum of area a, b, and c was calculated to be 3,775 μm^2 and contained 36,972 axons. The entire optic nerve therefore can be estimated to contain 73,562 axons. The cost of sampling such large areas of optic nerve prohibits the sampling of many nerves in this manner.

To account for possible variations amongst individual animals, another sampling approach was used. The areas of five optic nerves from five animals were determined to be

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* 95% Confidence Interval of the Mean.

11,627 ± 1,581* μm^2. Figure 11 is a light micrograph of a 0.5 μm toluidine blue-stained cross section through one of the 5 newt optic nerves. The area occupied by the central glia (Fig. 11), which makes up as much as 20% of the total area of the optic nerve area, was not included in the optic nerve area measurements because few optic nerve axons are located in this region. Electron microscope samples, from which the number of axons per square micrometer was calculated, were not taken from this region either. Figure 12 is an electron micrograph which is typical of the samples taken from the five optic nerves in which the number of axons/μm were calculated. It was estimated that there were 6.95 ± 1.3* axons/μm^2 in the five optic nerves which were sampled. Of these axons, 2.4% were large and possessed a myelin sheath. These estimates then show that there are about 80,844 axons in the newt optic nerve, a value which is not significantly different from the number of axons counted directly from the electron microscope photomontage.

Although there are about 1940 axons in the optic nerve which posses a myelin sheath, no myelinated axons were observed in samples of the optic nerve taken at the optic nerve head (Fig. 10). It must be assumed then, that these axons aquire their myelin sheath after leaving the globe (Figs. 11 and 12). It was also noted that the nuclei of the ependymoglia in the region of the optic nerve head were

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* 95% Confidence Interval of the Mean.

scattered randomly throughout the optic nerve (Fig. 11), but within the orbit, the ependymoglia were located centrally in the nerve (Fig. 12).

When ganglion cell counts are compared with the optic nerve axon counts, it is apparent that 61% of the cells in the ganglion cell layer send an axon into the optic nerve. The remaining 39% of the cells in the ganglion cell layer of the newt retina are displaced amacrine cells. This finding corroborates the observation that 40-50% of the ganglion cells in the newt retina do not degenerate following severance of the optic nerve (Turner et al., 1978).

FIGURES

Figure 1. Light microscope autoradiograph (bright field, 0.5 µm unstained section of a newt retina which has been incubated in 8.63 µmol (3H)-taurine. Certain cells in the inner nuclear layer (inl), in the position of the amacrine cells (a), as well as many cells (da and g) in the ganglion cell layer (gcl) accumulate (3H)-taurine. Some cells (da) are labelled as heavily as the amacrine cells (a). These heavily labelled cells make up 24% of the cells in the ganglion cell layer of the newt retina. ipl= inner plexiform layer, da= putative displaced amacrine cells, g= "true" ganglion cells, bar= 25 µm

Figure 2. Light microscope autoradiograph (bright field, 10 µm unstained section) of a newt retina which was incubated in 7.22 µmol (3H)-GABA. A small number of cells in the position of the amacrine cells (a) have accumulated the label. Many cells (g) in the ganglion cell layer (gcl), and the nerve fibre layer (nfl), accumulate (3H)-GABA as well. Of the cells in the ganglion cell layer, 27.4% are unlabelled (arrows). These cells line the outer margin of the ganglion cell layer (gcl), and are in the same position as the cells which accumulate (3H)-taurine (Fig. 1). The dendrites of the ganglion cells (g) pass upwards towards the inner plexiform layer (ipl) between the perikarya of the unlabelled cells in the ganglion cell layer (arrowheads). bar= 25 µm

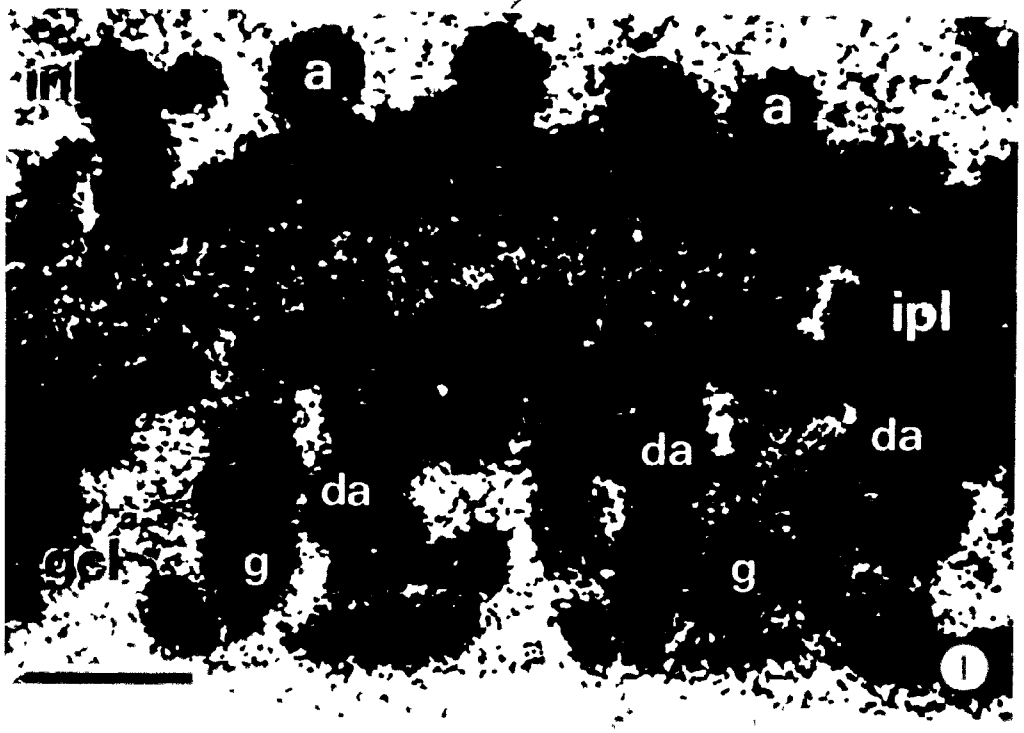

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Figure 3. Light microscope autoradiograph (bright field, 10 µm unstained section) of a newt retina which was incubated in 7.22 µmol (3H)-GABA. In this experiment there was minimal uptake of (3H)-GABA by cells in the ganglion cell layer (gcl), but cells which did accumulate (3H)-GABA (da) were located along the outer margin of the ganglion cell layer. These cells were similar in numbers to cells at the inner margin of the inner nuclear layer (inl) which also accumulate (3H)-GABA (a), and are probably displaced amacrine cells. The labelled cells (da) make up 15% of the cells in the ganglion cell layer of the newt retina. a= amacrine cells, da= putative displaced amacrine cells, bar= 25 µm

Figure 4. Light micrograph (10 µm section, eriochrome cyanin counterstain) showing retinal ganglion cells (g) which have transported HRP through their severed axons. Only 19% of the cells in the ganglion cell layer transported HRP. Most labelled cells were located in the lower portion of the ganglion cell layer (gcl). The axons of the labelled ganglion cells are seen within the nerve fibre layer (nfl) while their labelled dendrites (arrowheads) terminate within the inner plexiform layer (ipl). This labelling pattern is similar to the labelling of ganglion cells by (3H)-GABA (Fig. 2). inl= inner nuclear layer, bar= 25 µm

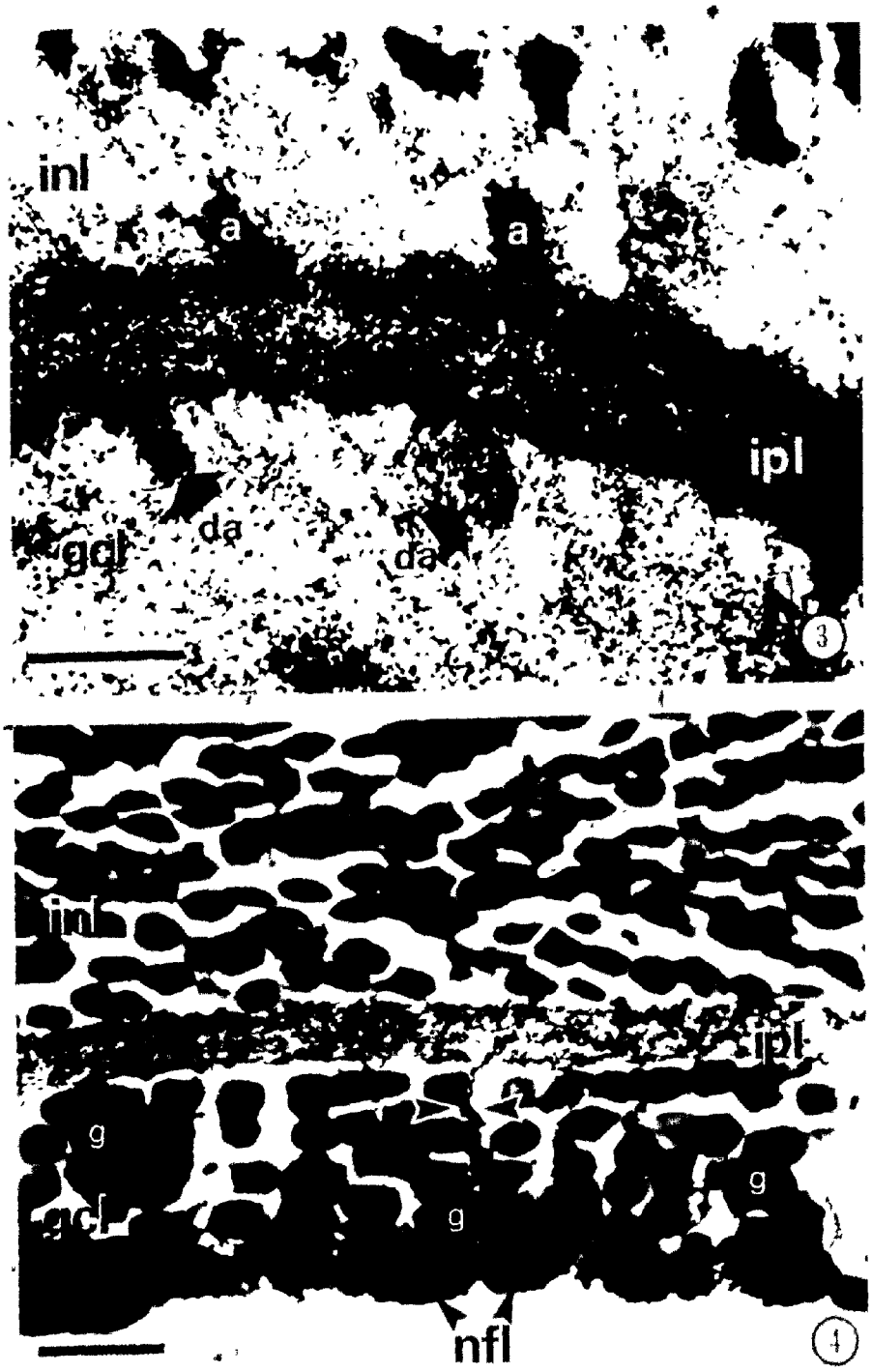
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Figure 5. Light micrograph (10 µm section, eriochrome cyanin counterstain) showing HRP labelled ganglion cells in the newt retina. The dendrites of one ganglion cell (g) can be observed passing through the inner plexiform layer (ipl) where it bifurcates (arrowheads) to pass laterally along the outer margin of the inner plexiform layer. The labelling of ganglion cell (g) dendrites by HRP divides the inner plexiform layer into 5 sublaminae. Dense bands, due to the concentration of labelled dendrites, are located at the inner and outer margins and near the middle of the inner plexiform layer. The densest band was usually located near the outer margin of the inner plexiform layer (large arrows). gcl= ganglion cell layer, bar= 25 µm

Figure 6. Light micrograph (10 µm section, eriochrome cyanin counterstain) showing HRP labelled ganglion cells in the retina of the newt. The labelled cell (dg) in the amacrine cell region of the inner nuclear layer (inl) is a displaced ganglion cell (Dogiel cell). This cell appears to send its dendrites along the densely labelled band (arrows) near the outer margin of the inner plexiform layer (ipl). Displaced ganglion cells were not frequently observed in the central retina. g= ganglion cells, dg= displaced ganglion cell , ipl= inner plexiform layer, gcl= ganglion cell layer, bar= 25 µm


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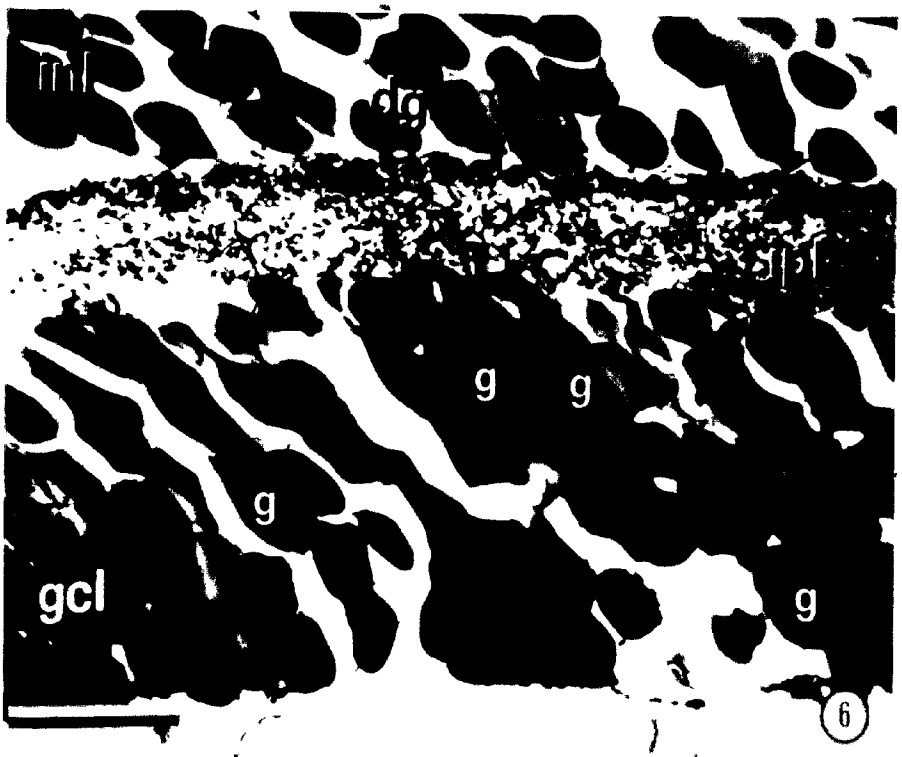
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Figure 7. Light micrograph (10 µm section, eriochrome cyanin counterstain) of peripheral newt retina showing HRP-labelled displaced ganglion cells (dg) in the inner plexiform layer (ipl). Displaced ganglion cells were more frequently observed near the periphery of the retina (arrows indicate narrowing of inner plexiform layer). Since there are about 8 displaced ganglion cells observed per retinal section, it is estimated that they number about 2000 cells, or 2.5% of the total number of "true" ganglion cells in the retina. dg= displaced ganglion cells, g= "true" ganglion cells, bar= 25 µm

Figure 8. Light microscope autoradiograph (bright field, 0.5 µm unstained section) of a newt retina which was incubated in 8.63 µmol (3H)-taurine prior to backfilling of ganglion cells with HRP through their severed axon. The population of cells in the ganglion cell layer (gcl) which accumulate large quantities of (3H)-taurine rarely transport HRP, and so are likely displaced amacrine cells (da). Some "true", HRP-transporting, ganglion cells also label lightly with (3H)-taurine (g'). g= "true" ganglion cells labelled with HRP, da= displaced amacrine cells which have accumulated (3H)-taurine, inl= inner nuclear layer, bar= 25 µm

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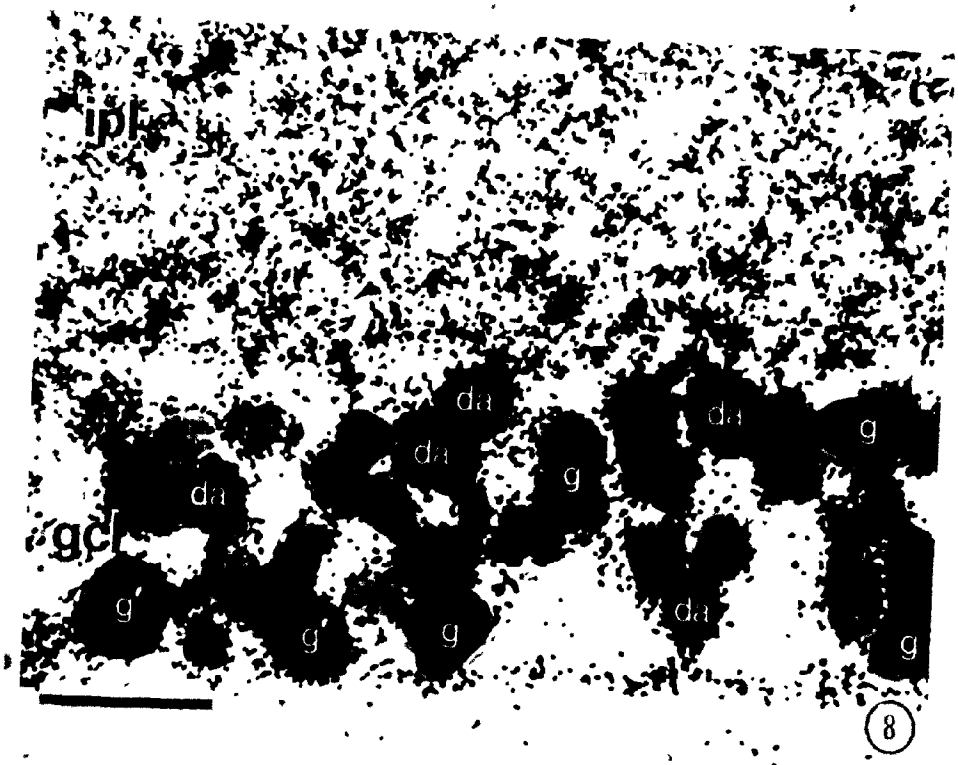

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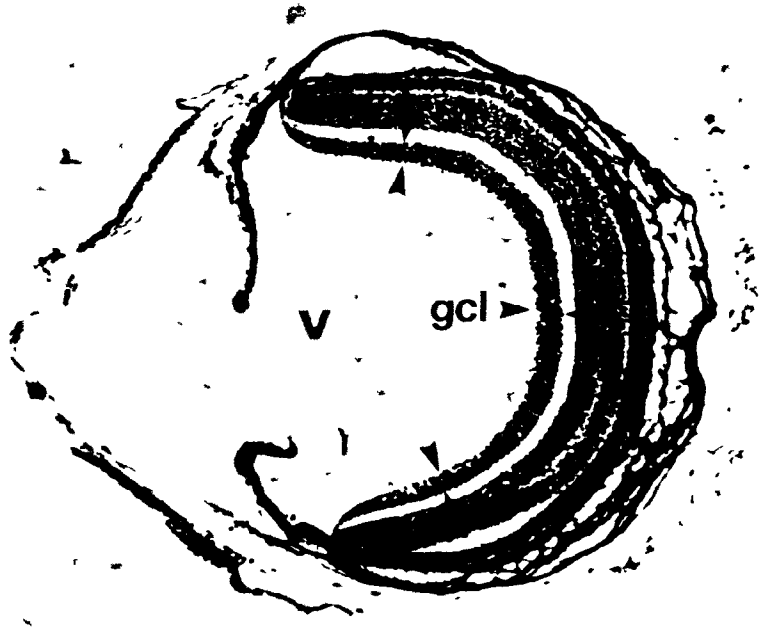
Figure 9. Low power light micrograph (10 µm section, eriochrome cyanin) of a celloidin-paraffin embedded newt eye. Area mesurements of the ganglion cell layer (gcl) and the number of retinal ganglion cells/μm^3 were made on serial sections such as this. It was estimated that there are about 131,835 cells in the ganglion cell layer of the newt retina. v= vitreous body, bar= 0.5 mm

Figure 10. Reproduction of a photomontage of electron micrographs (actual working size 45 x 55 cm) of a newt optic nerve on which direct counts of optic nerve axons were made. The optic nerve was divided into six segments, and all of the axons in three of these areas (cross hatched regions) were counted. The area of segments a, b, and c was calculated and the number of axons/μm^2 determined. The number of axons/μm^2 was then multiplied by the area of the optic nerve. e= ependymoglial cell, bar= 20 µm



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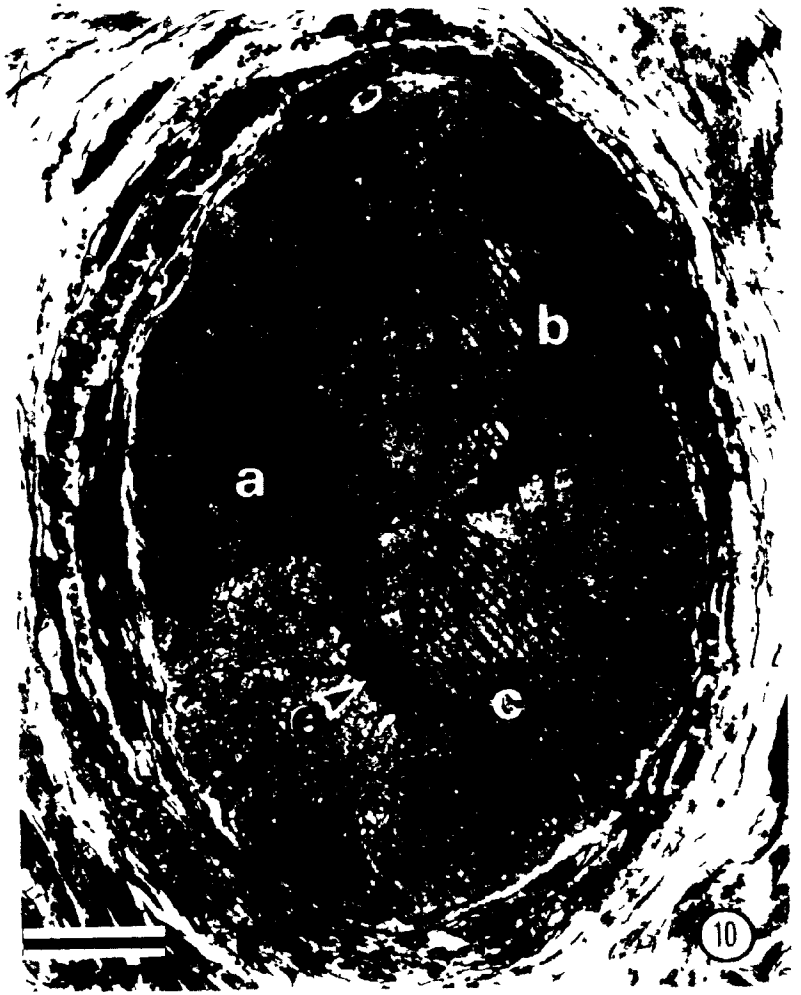

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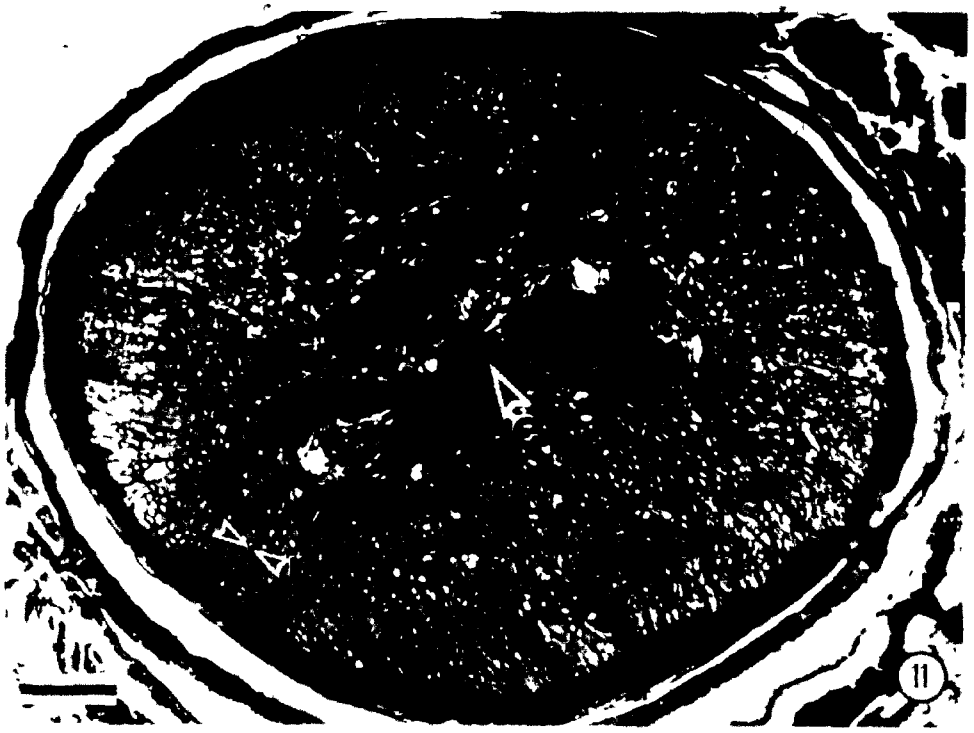
Figure 11. Light micrograph (0.5 µm section, toluidine blue) of a cross section through the optic nerve of the newt retina. The average area of the newt optic nerve was determined on sections such as this. Because electron microscope samples for the determination of the number of axons/μm^2 were chosen from the periphery of the optic nerve, the central area containing the ependymoglia (cross hatched area) and the few optic nerve axons contained within this area, were excluded from these measurements. e= ependymoglia cells, r= radial processes of ependymoglial cells, bar= 10 µm

Figure 12. Electron micrograph of a cross section through the optic nerve, similar to samples from which the number of axons/µm' were calculated. Both myelinated (ma) and unmyelinated axons (a) are present in the optic nerve. The myelinated axons made up 2.4% of the total number of axons in the newt optic nerve. r= radial processes of ependymoglia cell, bar= 1.0 µm



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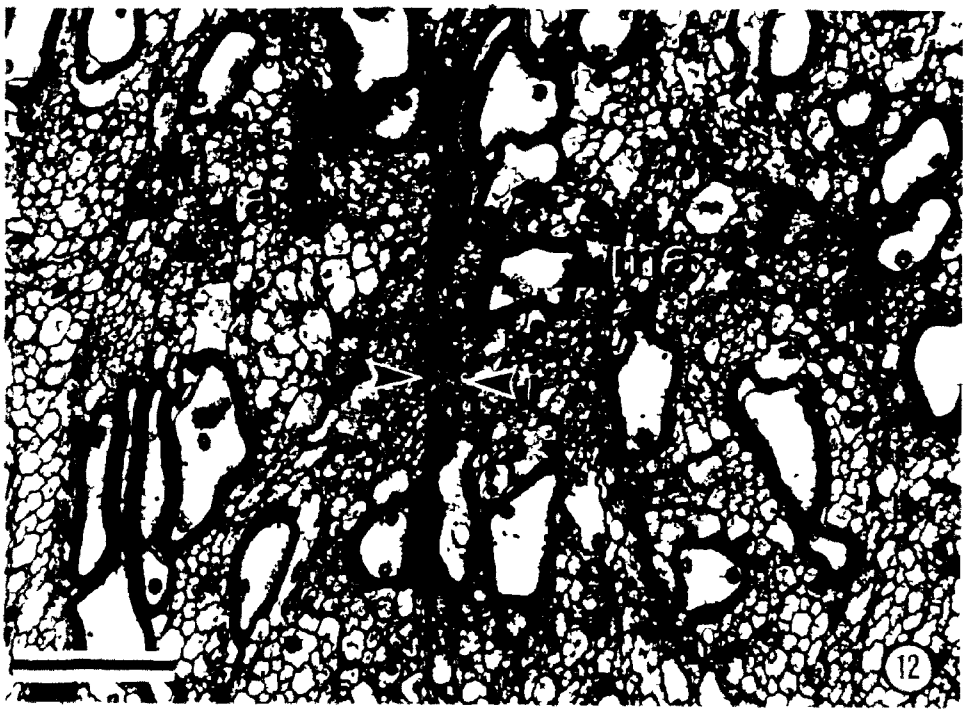

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DISCUSSION

Double labelling of cells in the newt ganglion cell layer with (3H)-taurine and HRP has shown that the cells which line the outer margin of the ganglion cell layer are not true ganglion cells, but rather are displaced amacrine cells. If the displaced cells use taurine as a neurotransmitter substance, they probably have a similar function to the taurinergic amacrine cells in the inner nuclear layer.

Amphibian retinas are characterized by large numbers of amacrine cell synapses which are formed with other cell types and with each other, within the inner plexiform layer (Dubin, 1970). To account for this complexity, the amphibian retina must possess large numbers of amacrine cells with extensive dendritic arborizations. The numerous amacrine cells must be accomodated in the retina so that their processes effectively ramify throughout both sublamina a and sublamina b (Famiglietti et al., 1977). It makes perfect sense that the perikarya of the putative taurinergic amacrine cells, which modulate the OFF response, should be located in the inner nuclear layer, and that the taurinergic amacrine cells, which modulate the ON response, should be located in the ganglion cell layer. Such an organization permits the processes of the amacrine cells in the inner nuclear layer to pass laterally along the outer margin of the inner plexiform layer, in sublamina a and the processes of the amacrine cells in the ganglion cell layer to pass

laterally along the inner margin of the inner plexiform layer, in sublamina b. This hypothesis is verified by the labelling pattern of inner plexiform processes in retinas which were incubated in (3H)-taurine. The number of (3H)-taurine accumulating cells in the ganglion cell layer is similar to the number of (3H)-taurine accumulating cells in the inner nuclear layer, implying that the ON and OFF channels have similar signal processing requirements.

The (3H)-taurine accumulating, displaced amacrine cells make up 23.9% of the cells in the ganglion cell layer, and account for 61% of the displaced amacrine cell population. The remaining, unidentified population of displaced amacrine cells may belong to the population of cells which accumulate (3H)-GABA. Of the cells in the ganglion cell layer, 72.6% accumulate (3H)-GABA, while ganglion cell versus optic nerve axon counts indicate that only 61% of the cells in the ganglion cell layer are "true" ganglion cells. This implies that 11.6% of the (3H)-GABA accumulating cells are not true ganglion cells at all, but rather displaced amacrine cells. In fact, in experiments when the uptake of (3H)-GABA is minimal, a small population of cells making up 15% of the cells in the ganglion cell layer, label just about as heavily as amacrine cells in the inner nuclear layer. If 61% of the cells in the ganglion cell layer are "true" ganglion cells, 23.9% of the cells are taurinergic amacrine cells, and 15% are GABAergic amacrine cells, then 99% of the cells in the ganglion cell layer of the newt

retina can be accounted for.

Although the displaced amacrine cells which accumulate (3H)-GABA are fewer in number than the cells which accumulate (3H)-taurine, they are organized in a similar manner. Like the (3H)-taurine acccumulating amacrine cells, (3H)-GABA accumulating amacrine cells are similarly disposed along both margins of the inner plexiform layer. The processes of the (3H)-GABA accumulating amacrine cells in the inner nuclear layer pass laterally along the outer margin of the inner plexiform layer and the processes of the (3H)-GABA accumulating amacrine cells in the ganglion cell layer pass laterally along the inner margin of the inner plexiform layer. The putative GABAergic amacrine cells in the inner nuclear layer probably modulate the OFF channel, by sending processes to sublamina a and the GABAergic amacrine cells in the ganglion cell layer probably modulate the ON channel, by sending processes to sublamina b.

It is surprising that the cells which accumulate (3H)-taurine and (3H)-GABA would account for the entire population of displaced amacrine cells in the ganglion cell layer of the newt retina. If displaced amacrine cells use taurine and GABA as neurotransmitters, this implies that other displaced amacrine cell neurotransmitter systems play a minor role in information processing in the inner retina. Although (3H)-taurine and (3H)-GABA accumulating cells in the inner nuclear layer also account for 94% of the cells lining the inner margin of the inner nuclear layer, at least

some of the unidentified cells have been demonstrated to belong to a population of displaced ganglion cells. Although most of the amacrine cells which accumulate (3H)-glycine (Chapter 2) are located near the middle of the inner nuclear layer, it is likely that some of the unidentified amacrine cells which are located along the inner margin of the inner nuclear layer also belong to this population of glycinergic amacrine cells. Nevertheless, like the amacrine cells in the ganglion cell layer which accumulate (3H)-taurine and (3H)-GABA, most of the amacrine cells in the inner portion of the inner nuclear layer of the newt retina also accumulate (3H)-taurine and (3H)-GABA. The extent to which other neurotransmitter systems are involved in information processing by amacrine cells in the newt retina remains to be determined. If GABA and taurine are actually used as neurotransmitter substances by amacrine cells, it would appear that other neurotransmitters play a minor role in the processing of visual information within the inner plexiform layer of the newt retina.

The possiblity that centrifugal fibres (fibres carrying impulses from the brain to the retina) exist in the optic nerve of the newt could result in an over estimation of the number of "true" ganglion cells in the retina when the number of "true" ganglion cells are estimated from optic nerve axon counts. The centrifugal fibre system of birds has been extensively studied, and it has been determined that only 0.4% of the axons in the optic nerve are

centrifugal (Rodieck, 1973). Although most species are considered to have a similar system, centrifugal fibres have not been identified in the frog or toad retina (Scalia and Teitelbaum, 1978). However, Fritzsch and Himstedt (1981) have identified an average of 28 labelled cells in the midbrain of three species of salamanders which send efferents to the retina. There are therefore, only about 12 centrifugal axons in the salamander optic nerve. Because these fibres would only make up 0.015% of the axons in the newt optic nerve, they contribute a negligible error to the estimation of "true" ganglion cells in the newt retina.

Ganglion cell versus optic nerve axon counts corroborate degeneration studies (Turner et al., 1978) which suggest that 61% of the cells in the ganglion cell layer of the newt retina are "true" ganglion cells. However, HRP was transported by only 19% of the cells in the ganglion cell layer. The reason for such a discrepancy between the number of cells which transport HRP and the actual number of ganglion cells is probably due to the degeneration of newt optic nerve axons immediatly after axotomy so that they are incapable of transporting HRP. Turner and Singer (1975) have shown that the rate of progress of Wallerian degeneration following optic nerve section in the newt, is one of the swiftest among vertebrate central nervous system tissues. Significant degeneration of newt unmyelinated optic nerve axons occurs as soon as 6 hrs after axotomy and is complete within 48 hrs (Turner and Glaze, 1977).

Nevertheless, HRP transport has proved useful for distinguishing between populations of "true" ganglion cells and displaced amacrine cells in the newt retina. Because of the Golgi-like impregnations achieved with HRP transport by a severed axon, the dendritic arborizations of ganglion cells can be clearly observed as well. The reason why the bulk of ganglion cell dendrites extend laterally along the outer margin of the inner plexiform layer is unclear. However, the three dense bands of label due to the concentration of ganglion cell dendrites at the margins and middle of the inner plexiform layer may represent the respective dendritic arborizations of ON, OFF and ON-OFF ganglion cells in these regions.

If HRP transport only labels a portion of the "true" ganglion cell population in the ganglion cell layer, does this then mean that the number of displaced ganglion cells demonstrated by HRP transport is underestimated as well? HRP labels about 2000 displaced ganglion, which is about 2.5% of the total number of "true" ganglion cells in the newt retina. This number is similar to the number of displaced ganglion cells in the mouse retina (Dräger and Olsen, 1981). It is unlikely that a higher percentage of displaced ganglion cells could be present in the retina of the newt. Boycott and Dowling (1969) have suggested that the myelinated axons in the fish optic nerve correspond to the axons of the displaced ganglion cells. The newt retina contains about 1940 myelinated axons, or about 2.4% of the

total number of axons in the newt optic nerve. The number of myelinated axons in the optic nerve corresponds closely to the number of displaced ganglion cells in the newt retina, implying that the myelinated axons may be the axons of the displaced ganglion cells. Since fibre myelination begins at the optic nerve head, it is impossible to make a definite correlation between the myelinated axons and displaced ganglion cells. However, if the myelinated axons are the axons of the displaced ganglion cells, it helps to support the thesis that HRP fills all of the displaced ganglion cells and only a portion of the "true" ganglion cells in the ganglion cell layer. Since it has been shown that the myelinated axons of the newt retina degenerate much later than the unmyelinated axons (Turner and Singer, 1975), it is reasonable to suggest that myelinated axons remain intact longer, for transport of HRP by displaced ganglion cells. It can therefore be concluded that HRP labels nearly all of the displaced ganglion cells of the newt retina and does not underestimate their numbers.

In conclusion, these studies show that, like the retinas of certain higher vertebrates, in the newt retina, only 61% of the cells in the ganglion cell layer are "true" ganglion cells. The remaining cells are displaced amacrine cells which may use taurine or GABA as their neurotransmitter substances. The newt retina, like the retinas of other vertebrates, also contains a small number of displaced ganglion cells. These cells are more numerous

near the periphery of the retina, and their axons may correspond to the myelinated axons of the optic nerve. Whether these cells project exclusively to accessory optic nuclei or not, remains to be determined.

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CONCLUSION

For nearly 100 years since the extensive studies of the vertebrate retina by Cajal, few studies provided much insight into the structure and function of the retina. However, in the past 15 years, basic research in this area has blossomed. These studies have shown that the vertebrate retina is much more complex than previously thought, and have raised new questions concerning the processing of visual information by the cells of the retina. This thesis has attempted to explore some of the aspects of neurotransmission in the retina of the newt.

Changes in newt synaptic terminal morphology have been observed over a 24 hr (LD 12:12) cycle. Dense-cored vesicles were found to increase in number as the light phase progressed, supporting the hypothesis that synaptic vesicles may become "supercharged" with transmitter substance when photoreceptors are inactive with respect to transmitter release. During the dark phase, the dense-cored vesicles decreased in number, while profiles of smooth endoplasmic reticulum increased in frequency. Although synaptic terminal volume was shown to increase near the end of the dark phase, no difference in the total number of synaptic vesicles per photoreceptor terminal was observed throughout the day-night cycle. Morphometric analysis and qualitative observations showed that endocytosis and exocytosis were occuring at a very rapid rate toward the end of the dark

phase. Taken together, these observations suggest that when photoreceptors are actively releasing neurotransmitter substance during the dark phase, transmitter packaging cannot keep pace with transmitter release. As a result, synaptic vesicles become "undercharged" with transmitter substance toward the end of the dark phase, requiring increased rates of exocytosis and endocytosis. The rapid exocytosis and endocytosis rates resulted in considerable changes in synaptic terminal morphology toward the end of the dark phase. These studies imply that the "quanta" of transmitter contained within the synaptic vesicles varies over a natural day-night cycle.

This thesis has also attempted to identify possible candidates for the neurotransmitter substance of photoreceptor cells. Although acetylcholine has been suggested as the photoreceptor transmitter, nicotinic and muscarinic acetylcholine receptor sites could not be unequivocally localized to photoreceptor postsynaptic membranes. Amino acid analysis of long-term light and dark-adapted newt retinas suggested that glutamate, or certain sulphur-containing amino acids are more likely candidates for the photoreceptor transmitter. However, autoradiographic studies showed that glutamate is not accumulated by photoreceptors. Photoreceptors do accumulate labelled aspartate, indicating that aspartate may be the photoreceptor transmitter. But because histochemical studies localized high concentrations of -SH groups in

photoreceptor synaptic vesicles, the sulphur containing amino acids cysteate and cysteine sulphinate should be considered as likely candidates as well.

Light and electron microscope autoradiographic localization of glycine, taurine and GABA within cells in the inner retina indicates that these inhibitory amino acids may be used as neurotransmitters by distinct populations of amacrine and ganglion cells in the inner retina of the newt.

These studies also showed that the inner nuclear layer and ganglion cell layer of the newt retina contain taurine and GABA accumulating cells which are located immediately adjacent to the inner plexiform layer. The bulk of the processes from the labelled cells in the inner nuclear layer were confined to sublamina a of the inner plexiform layer, while the bulk of the processes from the labelled cells in the ganglion cell layer were confined to sublamina b of the inner plexiform layer. These observations suggest that these two populations of labelled cells have equivalent functional roles in the ON and OFF channels of the newt retina.

Autoradiography combined with HRP-transport studies, have indicated that the taurine-accumulating cells in the ganglion cell layer of the newt retina are not "true" ganglion cells, but are actually displaced amacrine cells. This finding is corroborated by optic nerve axon versus ganglion cell counts, which showed that only 50-60% of the

cells in the ganglion cell layer of the newt retina were truly ganglion cells.