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CHOLINERGIC NEUROTRANSMISSION IN THE MAMMALIAN RETINA

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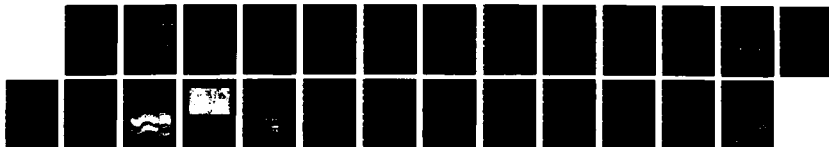
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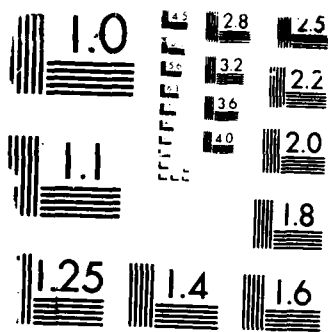
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CHOLINERGIC NEUROTRANSMISSION IN THE
MAMMALIAN RETINA

ANNUAL SUMMARY REPORT

Roberta G. Pourcho

November 30, 1985

Supported by
U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

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Department of Anatomy
Wayne State University
Detroit, MI 48202

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SUMMARY

This study is directed toward the cellular localization of acetylcholine (ACh) and acetylcholinesterase (AChE) in the cat retina, elucidation of the synaptic relationships and biochemical interactions of the neurons containing these substances, and the effects of organophosphorus anticholinesterase compounds on the normal function of the cholinergic system.

During the past year, we have conducted cytochemical studies using a monoclonal antibody to choline acetyltransferase (ChAT) to identify the cholinergic cells in the cat retina as a subpopulation of amacrine and displaced amacrine cells. These neurons have a distinctive starburst-like morphology not unlike that of the more highly branched starburst cells of rabbit cells which are also known to be cholinergic. The amacrine cells (type A14) ramify at a 20% depth level in the inner plexiform layer (IPL) depth while the displaced amacrines (types dA14) ramify at a 50% level. These studies confirm previous evidence from our laboratory showing that amacrine and displaced amacrine cells in the cat are able to synthesize ACh from (³H)choline.

Cytochemical localization of AChE has shown that this enzyme is localized not only in A14 and dA14 amacrines but also in additional amacrine cells and in ganglion cells. AChE was found throughout the IPL with heavy accumulations at 0-6% and 64-78% depth levels, very different from the strata at which cholinergic cells ramify.

Biochemical studies have centered on the development of appropriate high performance liquid chromatography (HPLC) methodologies for quantitative assay of the amino acids and ACh released from the retina in response to application of various neuroactive substances. In order to achieve sensitivity in the picomolar range, amino acids are being determined by means of precolumn derivitization with o-phthalaldehyde and fluorescence detection. ACh is being detected with an electrochemical detector after passing samples through enzyme-loaded columns.

FOREWORD

In conducting the research described in this report, the investigator adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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TABLE OF CONTENTS

Summary	3
Foreward	5
Statement of Problem	9
Background	9
Approach	11
Cytochemical Studies	11
Biochemical Studies	15
Conclusions	17
Recommendations	17
Literature Cited	19
Glossary	21
Distribution List	23

Figures

1. ChAT immunoreactivity in cat retina	10
2. Camera lucida drawing of ChAT-immunoreactive amacrine cell	12
3. Cytochemical localization of AChE in cat retina, light microscopy	14
4. Electron micrograph of cat retina showing AChE in amacrine cells	15
5. Diagram showing lamination of ChAT and AChE in cat retina	16

STATEMENT OF PROBLEM:

Physiological studies indicate that acetylcholine (ACh) serves as a neurotransmitter in the retina as well as at other central nervous system sites and at neuromuscular junctions. This study is directed toward the identification of cholinergic cells in the cat retina and the elucidation of interactions between ACh, acetylcholinesterase (AChE), anticholinesterase organophosphorus compounds, and other neurotransmitter systems.

Last year's report (1) described the identification of a population of amacrine and displaced amacrine cells in the cat retina which exhibit a starburst morphology, similar to that of cholinergic cells in other species. The displaced cells with this morphology are identical to the A14 amacrine cells of cat retina described by Kolb et al. (4). We have called these cells dA14 to reflect their displacement to the ganglion cell layer (GCL) of the retina and used A14 to designate their amacrine layer counterparts. Using computer rotations, we have shown that the A14 ramifies at a 20% depth level in the IPL, while the dA14 ramifies at a 50% depth level. Thus the A14 provides input to sublamina a, while the dA14 inputs to sublamina b.

Autoradiographic studies (1) showed that cells in the position of these amacrine label with newly synthesized (^3H)ACh, supporting the possibility that A14 and dA14 cells are cholinergic. The most definitive test for the identification of cholinergic neurons is the localization of the ACh-synthesizing enzyme, choline acetyltransferase (ChAT) and results of this localization are included in the present report.

The enzyme responsible for hydrolysis of ACh is AChE. This enzyme has also been used historically for identification of putative cholinergic neurons. However, there has been an increasing volume of evidence to indicate that AChE is present not only in cholinergic and cholinceptive neurons but also in other sites which are non-cholinergic. The present report describes the localization of AChE in the cat retina, comparing this distribution with that of ChAT.

To provide additional information regarding neurotransmitter interactions, biochemical studies are being developed to assay amounts of amino acids and other transmitters released from the retina in response to specific neurochemical stimulation.

BACKGROUND:

The biochemical degradation of ACh is mediated by AChE, an enzyme which is inactivated by organophosphorus compounds, especially by the highly potent nerve agents. The retina is an easily accessible portion of the central nervous system with a variety of neurotransmitter systems. Since the neurochemical composition of the retina parallels that of the brain, studies of the retina provide a useful model for understanding functional interactions within the brain. Among mammalian retinas, the cat retina is particularly useful because of the large

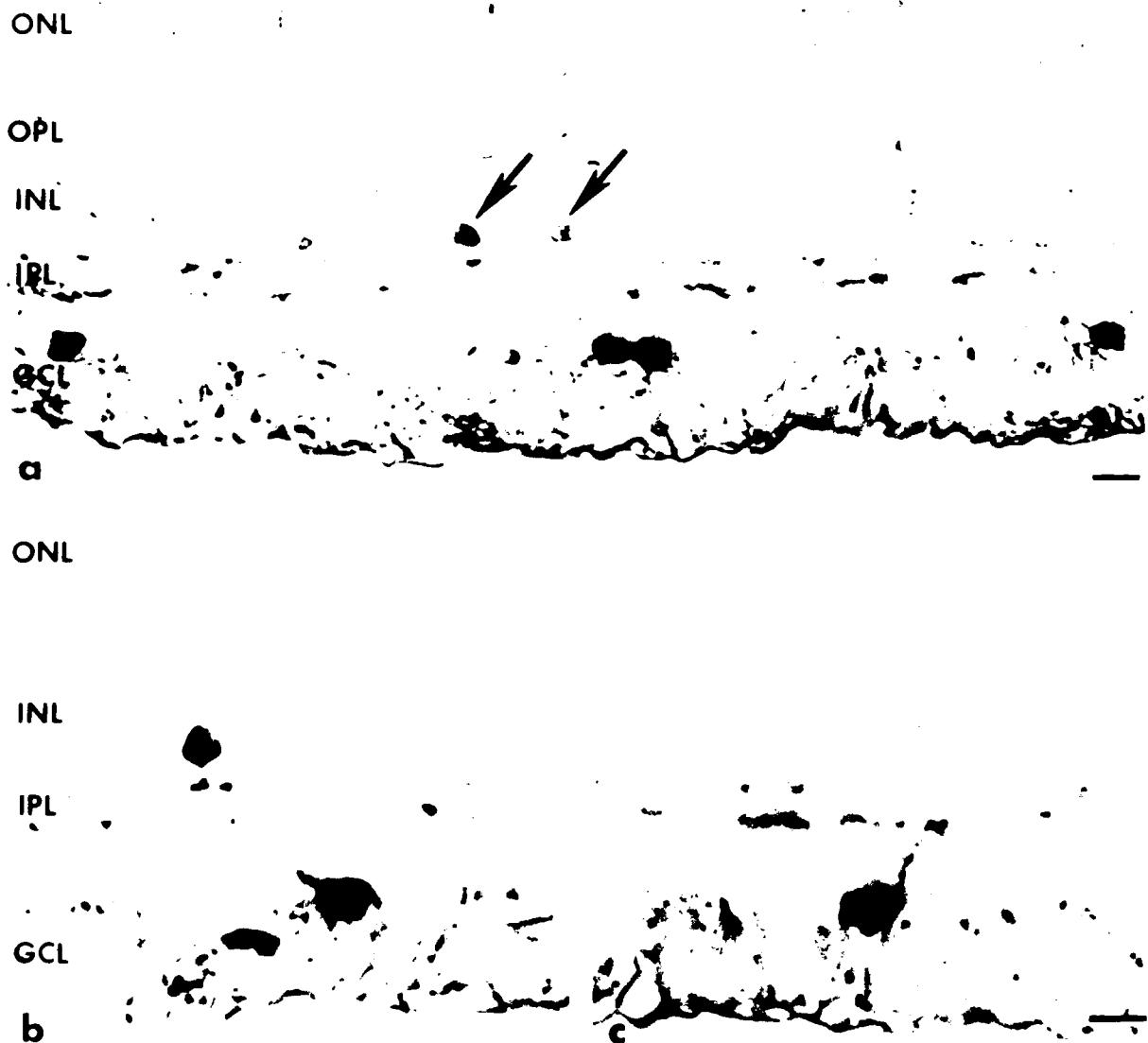


Fig. 1. ChAT immunoreactivity in cat retina. (a). Reaction product is seen in amacrine cells (arrows) in the INL and in displaced amacrine cells in the IPL. The processes of the cells are seen to ramify in two narrow bands within the IPL. (b). ChAT-containing amacrine cells ramify in the inner part of the INL. (c). ChAT-containing displaced amacrine cells send processes to ramify near the middle of the IPL. Bar 10 μ m.

volume of data already available regarding its anatomy, neurochemistry, physiology, and pharmacology. The goal of these studies is to advance our understanding of the cholinergic system in a well-defined location in order to provide a basis for reducing the effect of exposure to anticholinesterase drugs.

APPROACH:

These studies involve two lines of investigation, each related to the cholinergic system in a mammalian (cat) retina. Cytochemical methods are being used to provide morphological identification of cholinergic neurons and AChE-containing neurons, while biochemical methods are being developed to elucidate the relationships of cholinergic neurons with those cells to which they are pre- or post synaptic. The cytochemical studies involve both light and electron microscopy and will provide the opportunity for comparison of data with on going studies of other neurotransmitters in the cat retina. The biochemical studies employ high performance liquid chromatography (HPLC) to assay the release of transmitter substances by a superfused retina in response to challenge by other neurotransmitters.

CYTOCHEMICAL STUDIES:

1. Immunocytochemical identification of cholinergic cells in cat retina, using an antiserum to ChAT.

Materials and Methods. Cat retinas were fixed for 30 minutes in 4% paraformaldehyde in 0.1 M phosphate buffer, rinsed in phosphate-buffered saline, (PBS) and sunk in 30% sucrose. The tissue was preincubated for 1 hour in PBS containing 0.3% Triton X-100 and 1% bovine serum albumin. Retinas were incubated overnight in the cold in 4 g/ml rat monoclonal antiserum against ChAT (Boehringer Mannheim, West Germany). This antiserum has been well characterized (5). Samples were then reacted successively with rabbit anti-rat IgG (1:20 dilution), rat peroxidase antiperoxidase (1:100 dilution), and diaminobenzidine. Some samples were embedded in Epon-Araldite and sectioned for light microscopy while other pieces were flat mounted with Depex (Bio/medical Specialties, Santa Monica, CA).

Results: Immunoreactivity was observed in both amacrine and displaced amacrine cells and in processes ramifying within the IPL at 20 and 50% depth levels (Fig. 1). These are the same levels at which A14 and dA14 cells ramify. Camera lucida drawings of ChAT immunoreactive cells in flat mount preparations showed the same morphological characteristics as the A14/dA14 cells, (Fig. 2).

Discussion: These experiments support the previous evidence from autoradiographic localization of newly synthesized (^3H)ACh (1) and confirm that starburst amacrine cells in the cat retina are cholinergic. The identification of these cells provides the basis for our ongoing studies of the cholinergic system in cat retina.

We previously reported that cat retinal ganglion cells have the capacity to accumulate (^3H) choline under conditions designed to allow visualization of (^3H)ACh in preference to other choline containing compounds (1). However, we have not been able to localize ChAT in ganglion cell bodies or in their projections to the superior colliculus or lateral geniculate nucleus. The mechanism by which ganglion cells accumulate (^3H)choline remains obscure and there is no evidence to indicate that this accumulation is indicative of ACh synthesis.

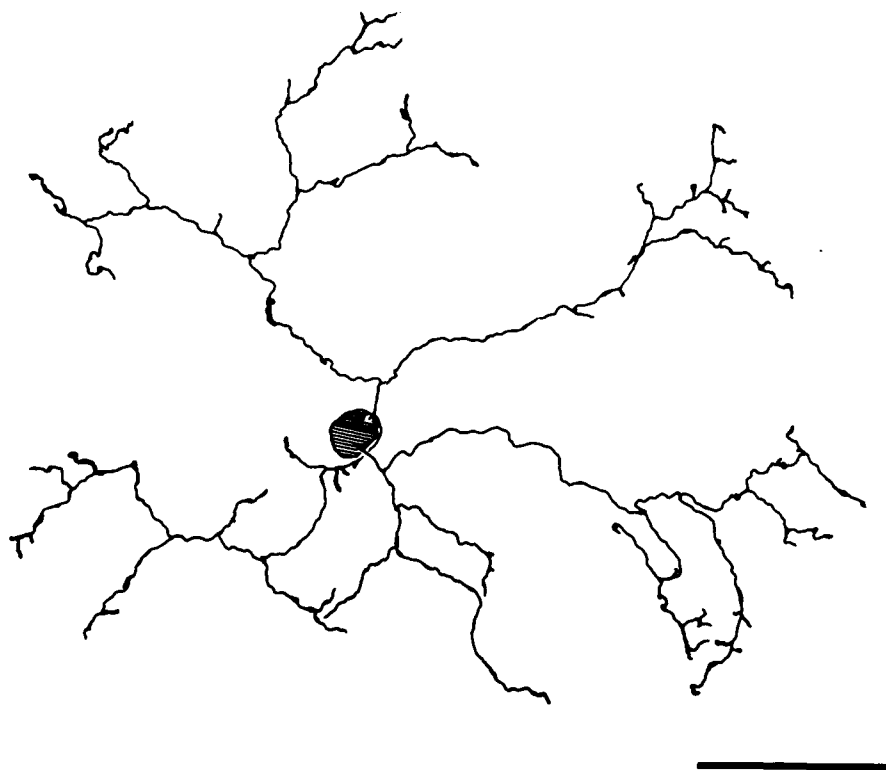


Fig. 2. Camera lucida drawing of a ChAT-immunoreactive displaced amacrine cell from cat retina. Although their branching pattern is relatively sparse, these cells exhibit the regular, dichotomous branching and narrow stratification which characterizes starburst amacrine cells in the rabbit. Bar 50 μm .

2. Cytochemical localization of AChE and comparison with ChAT.

Materials and Methods: Cat retinas were fixed in 4% paraformaldehyde and 0.25% glutaraldehyde in 0.1M phosphate buffer for 4 hr and then washed overnight in buffer. Acetylcholinesterase was visualized by a modification of the Koelle-Friedenwald technique as described by VanOoteghem and Shipley (6). Briefly, this method employs an incubation medium containing 2mM copper sulfate, 10mM glycine, 40 mM sodium acetate, and 4.2mM acetylthiocholine iodide. Incubations were carried out at 37°C for 45-90 minutes with gentle agitation. In control samples, BW 284c51 (1-5mM) was used as an inhibitor of AChE. After incubation, the tissue was stained with freshly prepared 1% sodium sulfide for 1 min. then washed in distilled water followed by a wash in 1% silver nitrate. The tissue was washed again with water and with 1% sodium thiosulfate prior to dehydration. Retinas were processed for flat-mount preparations and covered with Depex or sliced and embedded in Epon-Araldite. One μ m sections of plastic-embedded material were used for light microscopy and were viewed unstained or after counterstaining with Richardson's stain. Thin sections for electron microscopy were stained with uranyl acetate and lead citrate.

Results: Reaction product indicative of AChE activity was concentrated in the IPL and formed two dense bands, a narrow band at the outer margin of the IPL and a wide band in the inner half of the IPL (Fig. 3a). Reactivity was also seen in a large number of amacrine cells and in essentially all of the cells in the GCL. Retinas processed in the presence of 1.5 mM BW 284c51 showed no reaction product (Fig. 3b), indicating that all of the activity is due to specific AChE rather than to non-specific cholinesterase.

Electron microscopy showed that AChE is present, not only in the small ChAT-immunoreactive cells but also in larger amacrine cells. Representative examples are shown in Fig. 4. The percentage of amacrine cells staining for AChE was consistently greater than that seen with ChAT staining.

Stratification of ChAT and AChE staining was determined by measuring the total thickness of the IPL and then assessing the width and location of bands of preferential staining. Locations were expressed in terms of percentage depth in the IPL with 0% being adjacent to the inner nuclear layer and 100% adjacent to the GCL.

Stratification levels within the IPL of ChAT and AChE are summarized in Fig. 5. AChE was distributed throughout the IPL with the greatest activity at 0-6% and 64-78%. These bands do not coincide with the stratification of ChAT but border those bands on both sides.

Discussion: The presence of AChE in so many neurons indicates that its location is not limited to cholinergic cells. It appears to be distributed more widely than the cholinceptive cells, since the major stratification differs from that of the ChAT-immunoreactive cells. Biochemical assays by Ross et al. (7) have shown a similar disparity between ChAT and AChE distribution in the rat retina.

3. Localization of ACh receptors.

We have begun experiments directed at the localization of ACh receptors in cat retina, using propylbenzyl choline mustard (PrBCM) for muscarinic receptors and alpha-bungarotoxin (alpha-BTX) for nicotinic receptors. Cat retinas were incubated in balanced salt solution containing (^3H) PrBCM, with and without atropine (10). The tissue was dehydrated and embedded in Epon Araldite. One micron sections were prepared for light microscopic autoradiography. Control retinas incubated in the presence of atropine showed rather uniform, nonspecific labeling through all the layers of the retina, whereas experimental retinas showed a higher level of labeling in the IPL than in the other layers. Confirming experiments are in progress.

In order to localize nicotinic receptors, we prepared horseradish-conjugated alpha-BTX as previously described (11) and incubated retinas with this reagent. This approach is hindered by the paucity of nicotinic sites and limits the visualization to electron microscopy. We have also incubated retinas with alpha-BTX and processed the tissue for light microscopic autoradiography. These experiments are still in progress.

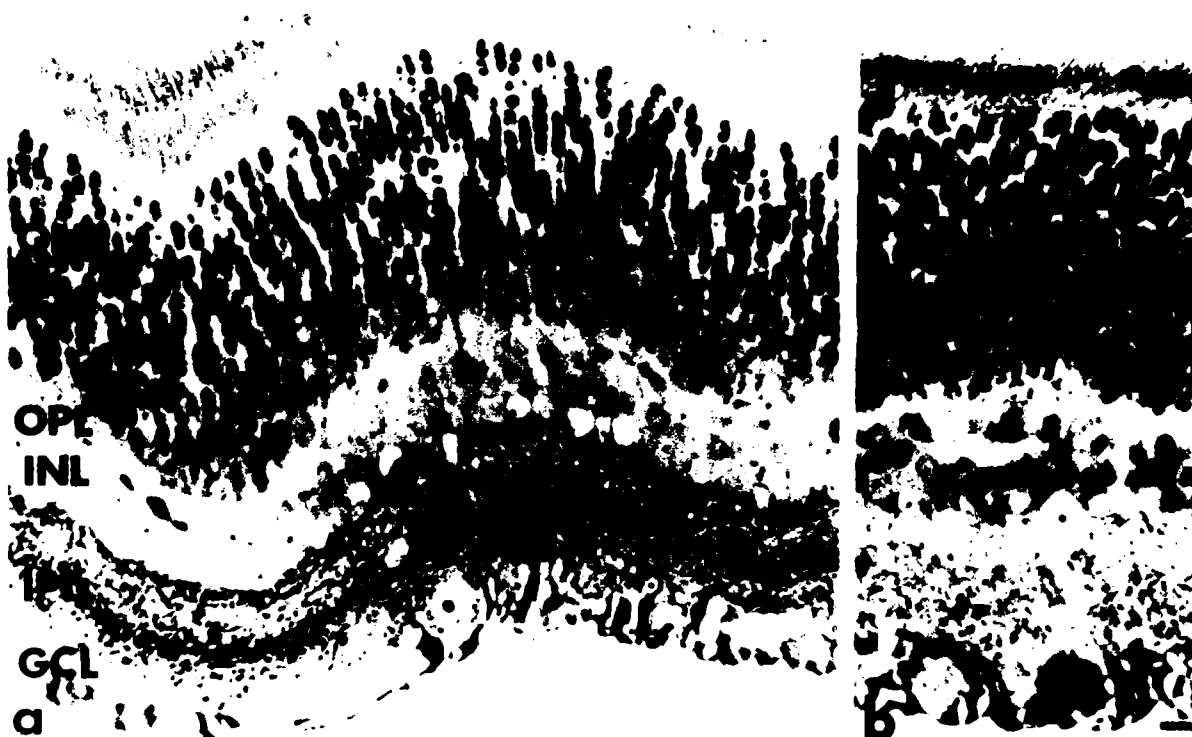


Fig. 3. Cat retina, reacted for AChE. (a). AChE is distributed throughout the IPL with a wide, dense band near the middle of sublamina b and a narrow band at the outer margin of sublamina a. (b). A control retina, reacted in the presence of the specific AChE inhibitor, BW 284c51, shows no reactivity. Bar 10 μm .

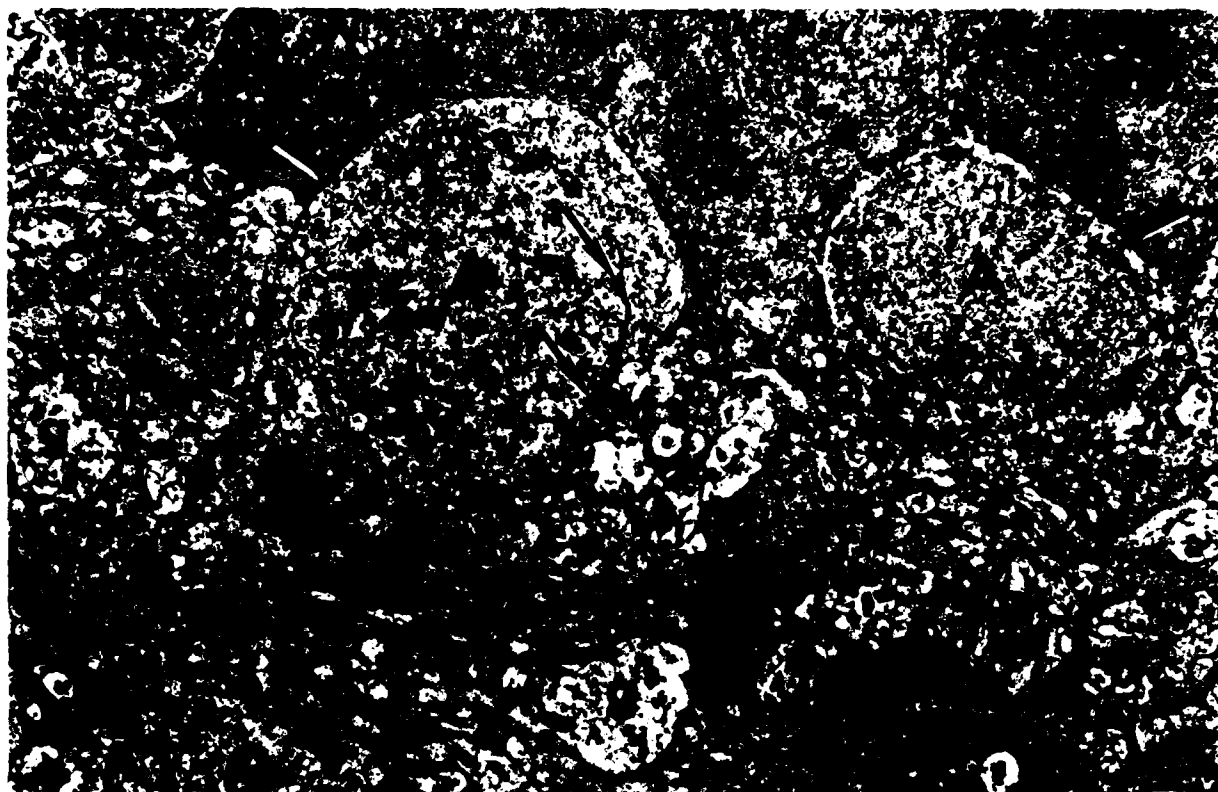


Fig. 4. Electron micrograph of cat retina, reacted for AChE. Reaction product is seen in two types of amacrine cell (A). The smaller cell at the right is similar in size and appearance to the ChAT-immunoreactive cells. The larger cell (left) belongs to a different subpopulation of amacrine cells. Reaction product can be seen along the plasma membrane (arrowheads) and in the nuclear envelope (arrows). Ganglion cell dendrites (g) also exhibit reactivity. Bar 1 um.

BIOCHEMICAL STUDIES:

1. Incubation techniques and amino acid detection.

A major consideration in the application of HPLC in biological systems is the development of appropriate procedures for conducting the experiments. We are now developing a protocol for determination of the effect of ACh on release of amino acid transmitter candidates from the cat retina. Preliminary experiments utilized a superfusion system with samples being taken at 2 minute intervals over a 50 minute period. This has been modified in order to reduce the number of samples and thus the

time required to obtain data points. Freshly dissected retinas are allowed to stabilize in an oxygenated balanced salt solution for 15 minutes. Then this medium is withdrawn by a pipette and replaced by 2 ml of fresh medium. At subsequent 3 minutes intervals, the medium is replaced with fresh medium. In half of the samples, 1 mM ACh is added to the replacement medium in the third change. All samples are frozen immediately and stored at -70°C until they can be assayed by HPLC. The HPLC assay is carried out using precolumn derivitization with o-phthalaldehyde and visualization of amino acids with a fluorescence detector. We have been conducting some of the preliminary experiments using rabbit retinas, which are readily available and provide a useful point of reference for our cat experiments.

2. Electrochemical detection of ACh.

One of the goals of this project is to determine the levels of ACh release from cat retina in response to other transmitters and under varying conditions. We are developing an electrochemical assay for ACh, employing the immobilized enzyme procedure developed by Neff and colleagues (12, 13). This procedure was designed for use with a Bioanalytic System detector which makes amperometric measurements. We have modified the protocol in order to carry out the detection on an Environmental Services Associates (Bedford, MA) instrument which makes

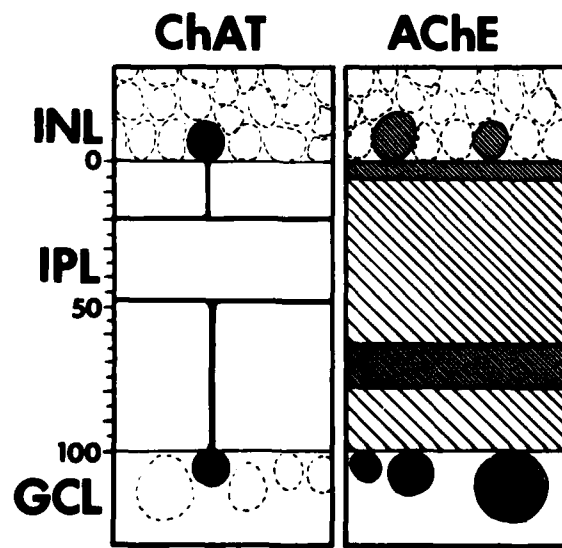


Fig. 5. A comparison of the lamination of ChAT and AChE in the cat retina. ChAT is found in a subpopulation of amacrine and displaced amacrine cells which ramify at 20 and 50% depth levels within IPL. AChE is present in at least two types of amacrine cell and in essentially all of the cells in the GCL. Reaction product is distributed throughout the IPL with heavy concentrations at 0-6% and 64-78% depth levels.

potentiometric measurements. Our procedure utilizes a conventional glassy carbon electrode, eliminating the additional care and polishing required to maintain the Bioanalytic platinum electrode. The combined use of a retained enzyme column and a carbon detection electrode should facilitate the ACh assays.

CONCLUSIONS:

We have identified the cholinergic cells in the cat retina as A14 and dA14 amacrine cells, ramifying, respectively, at 20 and 50% depth levels within IPL. Knowledge of the distribution of cholinergic terminals in the retina forms the foundation for further study of the cholinergic system. We have also localized AChE in the cat retina, finding it concentrated in the inner half of the IPL, in A14 and other amacrine cells, in dA14 cells which are displaced to the GCL, and in essentially all of the other cells in the GCL. HPLC techniques are being developed which should allow us to investigate the interactions of ACh, AChE, and other neurotransmitter systems in the cat retina.

RECOMMENDATIONS:

Studies should be continued to localize both muscarinic and nicotinic receptors in the cat retina. The synaptic connections of the cholinergic ChAT-immunoreactive cells should be determined in order to better understand the interaction of these cells with other retinal neurons, including the AChE-containing cells. Autoradiographic localization of (³H)soman should be carried out and compared to previous findings.

HPLC studies should be continued in order to characterize the biochemical correlates of the synaptic connectivity of the cholinergic amacrine cells. These studies should then be extended to investigate how the normal patterns are changed in the presence of organophosphorus anticholinesterases.

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GLOSSARY

A14	Type of amacrine cell
ACh	Acetylcholine
AChE	Acetylcholinesterase
Alpha -BTX	Alpha-bungarotoxin
ChAT	Choline acetyltransferase
dA14	Type of displaced amacrine cell
(³ H)ACh	Tritiated acetylcholine
GCL	Ganglion cell layer of retina
HPLC	High performance liquid chromatography
IgG	Immunoglobulin type G
IPL	Inner plexiform layer of retina
PBS	Phosphate buffered saline
PrBCM	Propylbenzilyl choline mustard
Sublamina <u>a</u>	Outer third of IPL
Sublamina <u>b</u>	Inner two-thirds of IPL

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