HISTOCHEMICAL LOCALIZATION OF DOPAMINE AND ACETYLCHOLINESTERASE ACTIVITY IN THE CARP RETINA

TOSHIHARU HAYASHI*

*Department of Neurophysiology, Neuroinformation Research Institute,
University of Kanazawa School of Medicine, Kanazawa 920*

Received for publication January 9, 1980 and in revised form March 15, 1980

Histochemical studies were conducted on the carp (*Cyprinus carpio*) retina to reveal the cellular localization of catecholamines (CA) and acetylcholinesterase (AChE) activity. CA was found almost exclusively in interplexiform cells, which extend processes towards both the outer and inner plexiform layers, forming dense networks at the inner nuclear and plexiform layers. It was demonstrated that the endogenous CA is dopamine (DA) and that CA-cells are dopaminergic in nature. The AChE activity was seen in a certain group of amacrine cells and in some ganglion cells, and diffusely in the inner plexiform layer and weakly in external horizontal cells. Among cells located at the innermost row of the inner nuclear layer in radial sections counterstained with methyl green, approximately 1 and 35% were estimated to be DA-containing interplexiform cells and AChE positive amacrine cells, respectively. Simultaneous visualization of DA-fluorescence and AChE activity showed that AChE-positive processes are in contact with DA-fluorescent cell bodies.

Numerous histochemical studies on central and peripheral nervous tissues have been conducted. However, there has been little information available on the carp retina, which has been used widely for electrophysiological experiments. The present series of studies is a histochemical attempt to classify retinal cells in the carp by localizing catecholamines (CA) and by the activity of acetylcholinesterase (AChE).

Since Falck (12) established a fluorescence method for the cellular localization of monoamines, this method was frequently applied to studying the retina (3, 5, 6, 7, 11). According to their extensive work using the fluorescence method with the Golgi-EM technique, retinal CA-containing cells were found commonly among amacrine cells in all species examined; fish, toads, frogs, birds, and mammals. However, these cells in goldfish and *Cebus* monkeys extend their processes towards both the outer (OPL) and inner plexiform layers (IPL), reciprocally contact amacrine cells in the IPL and, in addition, innervate bipolar and external horizontal cells in the inner nuclear layer (INL). Thus, the CA-cells form a feedback loop from the IPL to OPL, and for this reason are referred to as "interplexiform cells" (8).

The cellular localization of AChE and the ACh metabolism in the retina have

* In partial fulfilment towards a degree of Doctor of Medical Sciences.

been also extensively investigated (4, 9, 29, 31). It has been commonly described that AChE activity is predominantly positive in amacrine and ganglion cells and at the IPL. Several workers attempted to investigate the histological relationship between AChE positive and CA-containing cells in the central nervous system. For this attempt, one way was the application of the formaldehyde-fluorescence method and the Koelle method for AChE to alternate cryostat sections (20). Another way was to use the AChE method for a given section which had been examined under a fluorescence microscope (18). We attempted to detect the CA-fluorescence and AChE activity in the same section of the carp retina at the same time.

The present study confirms the presence of DA-containing cells and AChE-positive cells in the carp retina, and demonstrates for the first time histological interrelations between AChE-positive cells and dopaminergic cells in the retina.

MATERIALS AND METHODS

Demonstration of CA-fluorescence

A histofluorescence (FGS) method, modified by Nakamura (21) from the Faglu method of Furness *et al.* (14), was used to investigate catecholamine (CA)-containing cells in the carp (*Cyprinus carpio*) retina. The solution for tissue fixation (FGS solution) consisted of 25 ml of distilled water at 60°C, 2 g of paraformaldehyde, 2 drops of 1 N NaOH, 24 ml of 0.2 M phosphate buffer (pH 7.4), 1.25 ml of 20% glutaraldehyde, and 17.5 g of sucrose. Isolated retinas were fixed in this solution at 4°C for 24 hrs. The retinas thus fixed were freeze-sectioned radially or tangentially to 18–20 μ m thickness in a cryostat. The sections were dried over Zeolite in a desiccator for 1–2 days, mounted with Entellan, and examined under a fluorescent epiillumination system (Olympus BR-RFL). The filters used consisted of a BG-3/IF-405 for emission and a Y-475 for absorption. Some preparations were counterstained with methyl green (22). Other procedures used for particular purposes will be described in the appropriate sections.

Demonstration of AChE activity

A new technique developed by Nakamura and Torigoe (23) was used for the localization of AChE. Freeze-sectioned and dried preparations were incubated in an Ach substrate solution (see below) at 4°C for 30 min. After brief washing with a 55% sucrose solution, the sections were further incubated in an enhancement-medium (see below) at 4°C for 30 min. Then, they were dried in a desiccator for 2–3 hr, and mounted with Entellan. The Ach substrate solution consisted of 6.0 ml of 0.2 M phosphate buffer (pH 5.8), 4.5 g of fructose, 1.0 ml of 2.94% sodium citrate, 1.0 ml of 0.75% copper sulfate, 1.0 ml of 0.169 %potassium ferricyanide, 1.0 ml of 2×10^{-4} M iso-OMPA, and 10 mg of acetylthiocholine iodide. The enhancement-medium used for the second step of incubation consisted of 10.0 ml of 10% sodium acetate, 0.5 ml of 0.1% rubeanic acid, and 1.5 g of sucrose. For the simultaneous visualization of AChE activity and CA-fluorescence, the retinal sections prepared with the FGS method were subsequently incubated in the above solutions. Even after these treatments, the CA-fluorescence was still preserved in the preparations. Refer to Nakamura and Torigoe (23) for the detailed technique and procedure of the simultaneous visualization of AChE activity and CA-fluorescence.

RESULTS

Localization of DA-containing cells

In other than control retinas, the carp were treated in several ways. Some of them were treated with an intramuscular injection of reserpine (3 mg/kg body weight) 20 hr prior to enucleation, intravitreal injection of noradrenaline (NA; 10 μ g in 10 μ l Ringer) to one eye 3 hr prior to enucleation, or with a combination of both the injections. The intramuscular injection of reserpine completely depleted CA from the retina, while an intravitreal injection of NA accentuated the fluorescence and increased the number of visible cells (27).

Most of the fluorescent cell bodies appeared sparsely distributed among amacrine cells in the innermost cellular row of the INL and occasionally in the distal half part of the IPL; only in exceptional cases were they found among ganglion cells. A large number of CA-containing cells were spindle-shaped and the rest were pear-shaped. Approximately 1% of so-called amacrine cells were fluorescence-positive when cells were counted in radial sections counterstained with methyl green.

CA-containing cells, sparsely distributed among amacrine cells and occasionally located in the IPL, send their processes towards both the OPL and IPL. The distal processes could not be traced because they are usually poor in fluorescence, and because their fine fibers form dense networks in the INL, especially at the external horizontal cell layer (Fig. 1). It was possible to follow proximal processes relatively well, as drawn in Fig. 2; the branches originating from a given soma extend and distribute widely throughout the IPL. Small varicosities were seen abundantly along the course of the processes.

Fig. 2 is a collection of representative CA-containing cells seen in radial (rows A, B and C) and tangential (D and E) sections of control retinas. All the cells shown here are those with relatively long processes which were traceable in a single section (20 μ m thickness). In rows A–C, the upper broken line corresponds to the innermost level of the INL, the lowest line to the ganglion cell layer, and the middle line divides the IPL to strata a and b. Therefore, these show the three classes of CA-containing cells at different retinal layers as previously described.

In the tangential section at the innermost level of the INL, 6 cells (Fig. 2D) were drawn from one and the same plane of section, and 2 cells (E) were from another section; the processes of the latter 2 cells were traceable for the longest distance from the somata among the cells observed in the present series of control preparations.

It was hard to differentiate dopamine (DA) from NA in the FGS preparations, because both the amines were seen as green fluorophores. Björklund, Ehinger and Falck (2) showed that both DA and NA could be differentiated spectrophotometrically after a HCl-gas treatment, which separately shifted the excitation peaks of both the amines; NA became invisible with acidification. This treatment was applied to the carp retina and the rat salivary gland (Fig. 3). The latter tissue has been shown to possess NA-containing nerve terminals (13, 30). The fluorescence micrographs of both the tissues at the upper row (A and B) are the controls, which were routinely fixed in the FGS solution. Different sections obtained from the same samples were exposed to a gas phase of 6 N HCl for 10 min. As seen in the micrographs, the fluorescent fibers in the salivary gland were decayed (D), while the cells in the retina remained little affected (C). The results indicate that the CA contained

FIG. 1. Demonstration of the cellular localization of CA. Fluorescence micrographs of radial but somewhat oblique sections of the carp retina, which had been treated with intramuscular injection of reserpine and intravitreal injection of noradrenaline. Magnifications are different in A ($\times 345$) and B ($\times 685$). Fluorescent cell bodies are seen at the innermost level of the INL (open circle) and in the IPL (asterisk). Their processes with many small varicosities (arrows) form dense fiber-networks at the INL and IPL.

FIG. 2. Schematic drawings of representative CA-containing cells in the carp retina. See Text for explanation.

in the retinal cells is DA.

A subsequent question arose as to whether the retinal CA-cells are dopaminergic or noradrenergic. In Fig. 4, the retina was isolated from the carp which had been pretreated with an intramuscular injection of reserpine 20 hrs prior to enucleation, and divided into 3 pieces. Each of them was incubated in a different medium commonly containing nialamide (10^{-4} M) and NA (10^{-6} M). One was a solution containing only nialamide and NA (A), another had a benztropine solution (10^{-5} M) added (B), and the third was a desmethylinipramine (5×10^{-6} M)-added solution (C). After a 30 min incubation, the pieces of tissue were fixed in the FGS solution. It has been known that benztropine prevents the amine-uptake of DA-ergic cells while desmethylinipramine interferes with that of NA-ergic cells (1). Since the carp had been reserpinized, all CA-cells were invisible in the original sections. If certain cells took up NA in the incubating medium, the cells would become fluorescent as seen in micrographs A and C. Therefore, it can be stated that the presence of benztropine prevented the NA-uptake by these cells (B), and thus that the fluorescent cells found in the carp retina are dopaminergic.

Localization of AchE activity

AchE activity was found markedly in amacrine and ganglion cell bodies and weakly in their processes (Fig. 5A and C). It was diffusely positive in the IPL (A, asterisk), indicating that abundant fiber terminals of amacrine and ganglion cells appear to contain AchE. When the IPL showed strong AchE activity in some

FIG. 3. Fluorescence micrographs. Demonstration of the endogenous DA in retinal cells. A: Fluorescent cells in the carp retina. B: Fluorescent nerve fibers in the rat salivary gland. C and D: After exposure of both preparations to a gas phase of 6 N HCl. Fluorescent cells in the carp retina remained little affected while fluorescent fibers in the rat salivary gland disappeared. See

Text for further explanation. A and C $\times 345$; B and D $\times 210$

preparations, the external horizontal cell layer became weakly positive (Fig. 5B, arrow with He), indicating that this class of horizontal cells contains AchE. Approximately 35% of so-called amacrine cells were estimated to be AchE-positive in radial and counterstained sections. An AchE-positive cell body located in the IPL was found to extend an ascending process (Fig. 5C, arrow), which bifurcates with some varicosities in the rectangular directions immediately below the amacrine cell layer (D, arrow). However, a branch extending to the right side is out of focus. The section was fixed in the FGS solution and then treated for the localization of AchE activity; this particular cell was fluorescence-negative.

Simultaneous observation of AchE-positive cells with DA-fluorescent cells

Several pairs of cells consisting of one DA-fluorescent cell and one AchE-positive cell were observed; their intercellular distances were variable (0–100 μm). In Fig. 6, micrographs A, C and F were taken under the illumination of ultraviolet and red

FIG. 4.

FIG. 5. Demonstration of AchE activity in the carp retina. Microphotographs of radial sections. A: One amacrine cell and two ganglion cells are AchE-positive. The IPL is diffusely AchE-positive (asterisk). B: Two AchE-positive amacrine cells and one AchE-positive ganglion cell are seen. In these cases, the AchE-positive sites appear only at the cell boundary, thus being seen as black rings. When the IPL is intensively AchE-positive, the external horizontal cell layer (arrow with He) becomes weakly positive. C and D: From a radial section treated for DA-fluorescence and AchE activity. An AchE-positive cell located in the IPL extends an ascending process (arrow in C) very close to the amacrine cell layer, and the process bifurcates in the rectangular directions (arrow in D) with some varicosities. C and D were taken at different focus levels under illumination of tungsten light. A $\times 490$; B $\times 1200$; C and D $\times 650$.

FIG. 4. Fluorescence micrographs. Demonstration of dopaminergic cells in the retina. The retina was isolated from a reserpinized carp, divided into 3 pieces, incubated in different media for 30 min, and then fixed in the FGS solution. A: Incubated in a medium containing only NA (10^{-6} M) and nialamide (10^{-4} M). B: In a solution containing NA, nialamide and benztropine (10^{-5} M). C: In a medium containing NA, nialamide and desmethylinipramine (5×10^{-6} M). Fluorescent cells became visible after incubations in the A and C media. See Text for further explanation. A-C $\times 345$.

FIG. 6

lights, while the others were taken under the illumination of tungsten light. The cell bodies of the two classes of cell in A and B (marked by arrows with d and a, respectively) contact each other. The DA-cell body seems to contain AchE-positive sites (B, arrow with d). This is an exceptional case, however. AchE-positive sites were usually found at the boundary of fluorescent cell bodies. Two pairs of DA- and AchE-positive cells with short intercellular distances (50–70 μm) are shown in micrographs C–E and F–H, respectively. In these cases, the AchE-positive sites along the cell boundary (arrow with d in D and G) are obviously the extension of a process from the AchE-positive cell bodies (a in E and H). The AchE-positive process appears to intimately contact the fluorescent cell body. In turn, the AchE-positive sites (d) in micrograph B might be a part of the terminal from an AchE-positive cell on the surface of the fluorescent cell body.

The IPL was diffusely positive in AchE activity (Figs. 5 and 6), indicating that fine terminals containing AchE are abundant. Fluorescent processes of DA-cells make networks also in the IPL (Fig. 1). Therefore, terminals from both classes of cells are assumed to interconnect with each other in the IPL, although this could not be demonstrated.

DISCUSSION

The present histofluorescence study confirmed earlier findings (3, 10, 11). Ehinger (10) classified CA-containing cells into three classes, based on their localization. These are junctional, eremite and alloganglion cells. The junctional cells surpassed the other classes in number and were sparsely distributed among amacrine cells. The eremite cells were found occasionally in the IPL, and the alloganglion cells were seen exceptionally in the ganglion cell layer. The representative cells schematically illustrated in Fig. 2 may correspond, respectively, to the three classes of CA-cells described by Ehinger (10). It was also demonstrated in the carp retina that the endogenous CA in dopamine (DA; Fig. 3), and that the CA-cells are dopaminergic in nature (Fig. 4), as already shown in the retina of other vertebrates (6, 7, 15, 17, 19, 28).

The cellular localization of AchE activity in the carp retina was consistent with earlier findings (4, 9, 29, 31). AchE activity was found in a certain group of amacrine cells and in some ganglion cells, diffusely in the IPL and weakly in the external horizontal cell layer. According to a cytochemical localization (9), AchE activity was positive in the horizontal cells at the postsynaptic region and at the lateral contact between horizontal cells in a given layer.

With the histochemical technique for AchE activity, the shape of amacrine cell

FIG. 6. Simultaneous demonstration of DA-fluorescence and AchE activity. Micrographs A and B: Pairs consisting of one DA-fluorescent cell (d) and one AchE-positive cell (a); their cell bodies contact each other. A was taken under mixed illumination of ultraviolet and red lights, while B was taken under illumination of tungsten light. Fluorescent fibers are seen at the INL (open circle in A). C–E: Three micrographs show the same region. C was taken under mixed illumination of ultraviolet and red lights, while the others are under tungsten light at different focus levels. The series of these pictures show that an AchE-positive amacrine cell (arrow with a) extends a process, contacting a DA-cell (arrow with d). F–H: Another example of the cellular contact of an AchE-positive cell with a DA-cell. Markings are the same as in C–E. A–E $\times 1200$

bodies was mainly outlined; their processes were always weakly positive and difficult to trace. On the other hand, the histofluorescence method well revealed both the shape of DA-containing cell bodies and their proximal processes. Therefore, it was difficult to compare exactly the classes of cells, which became visible in both methods. However, AchE-positive cells were variable in shape and size, while most DA-cells were found to have a spindle-shaped soma and bilateral processes extending widely throughout the IPL. Furthermore, DA-cells had ascending (distal) processes towards the OPL, almost certainly belonging to interplexiform cells (8).

The ratio of the numbers of AchE-positive cells and DA-fluorescent cells were estimated among so-called amacrine cells, which are aligned at the innermost border of the INL. AchE-positive amacrine cells number much more than DA-fluorescent cells. Approximately 35% and 1% of cells located at the innermost row of the INL appear to be AchE- and fluorescence-positive, respectively.

Unique effects of DA on horizontal and bipolar cells have been demonstrated electrophysiologically in the fish retina, and assumed to simulate an aspect of the function of DA-containing interplexiform cells (8, 16, 24, 25). According to the above results, interplexiform cells play an important role in the lateral spread of an S-potential (light-induced response being recordable from horizontal cells) along the horizontal cell layer. The dense fluorescent fiber-networks observed at the INL may be involved in this electrical phenomenon.

The simultaneous visualization of DA-fluorescence and AchE activity revealed that AchE-positive processes of amacrine cells are in contact with the cell body of DA-containing interplexiform cells. Recent electrophysiological studies suggested that interplexiform cells appear to be activated by the cholinergic system at the IPL in the fish retina (26). Therefore, these histochemical and electrophysiological findings together indicate that DA-containing interplexiform cells are probably cholinceptive. In addition, although only one AchE-positive and DA-negative cell in the IPL was shown to have an ascending process (Fig. 5C-D), this suggests the presence of a class of cells which appears to contact other amacrine cells and horizontal cells located most proximally.

ACKNOWLEDGEMENTS

I wish to thank Drs. K. Negishi and T. Nakamura for their valuable suggestions and discussions in the course of the experiments and in preparing the manuscript. I also thank Mrs. Tami Urano for her secretarial assistance. Some of the drugs used were generously supplied by Ciba-Geigy (Japan) Co. and Nippon Merck-Banyu Co. This work was supported in part by funds from the Ministry of Education of Japan (No. 387043), the Japan Society for the Promotion of Science, and from the Takaoka-Johnan Hospital (Japan) to Dr. K. Negishi (principal investigator).

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