STUDIES ON MONOAMINE-CONTAINING NEURONS BY
IMMUNOENZYME-HISTOCYTOCHEMISTRY AND
IMMUNOHISTOCYTOCHEMISTRY WITH
SPECIAL REFERENCE TO GOLD-
FISH BRAIN

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In the goldfish brain, eleven groups of tyrosine hydroxylase (TH)-positive (catecholaminergic) neurons and four groups of serotonin (5-HT)-positive (serotonergic) neurons are described as follows by immunofluorescence and light microscopic level of PAP technique: 1) The olfactory bulb (TH); 2) The area ventralis telencephali (TH); 3) The nucleus preopticus (TH); 4) The nucleus dorsolateralis thalami (TH); a) The pretectal area (5-HT); 5) The nucleus ventromedialis thalami (TH); 6) The nucleus posterioris periventricularis (PVO-accompanying cells) (TH); b) The nucleus recessus lateralis (NRL) (5-HT); 7) The rostro-medial part of the nucleus recessus lateralis (NRL) or the paraventricular organ (PVO) pars anterior (TH); b) PVO pars anterior (5-HT); 8) The nucleus posterior tuberis (NPT)(TH); c) The nucleus recessus posterioris (NRP)(5-HT); 9) The lateral isthmal region (TH); d) The raphe nuclei of the brainstem (5-HT); 10) The reticular formation of the medulla oblongata (TH); and 11) The post-obocular region (TH).

By immunoelectron microscopy, two kinds of cerebrospinal fluid (CSF)-contacting neurons were confirmed in the PVO pars anterior. One contained TH-positive granules (80-120 nm) and the other 5-HT-immunoreactive granules (100-200 nm in diameter). In the telencephalon, preoptic nucleus (PON) and nucleus posterior tuberis (NPT), TH-positive non-CSF-contacting neurons showed similar ultrastructure such as well developed rough surfaced endoplasmic reticulum, and immunoreactive granules (106-110 nm).

In 1972, we started to demonstrate noradrenaline (NA)-synthesizing enzyme, dopamine- β -hydroxylase (DBH), in ligated sciatic nerves by biochemical (12) and immunofluorescence (9) techniques. When comparison was made between the proximal accumulation rate of DBH and that of NA (2), the accumulation rate of NA was much higher than that of DBH, indicating that NA can be synthesized by peripheral DBH in the sciatic nerves. Not only distal accumulation of NA (2), but also retrograde axoplasmic transport of inactive DBH was observed in the sciatic nerves for the first time (14).

Two decades have passed since the establishment of the histofluorescent method and most monoaminergic maps of the vertebrate brains have been completed by

this method. For the last 10 years, immunohistochemistry using antibodies towards catecholamine (CA)-synthesizing enzymes have enabled us to demonstrate brain dopaminergic, noradrenergic or adrenergic neuron systems. Nearly all results obtained by immunohistochemistry were similar to and confirmed the results obtained by histofluorescence.

In the last 3 years, immunohistochemistry using antibody to serotonin (3, 15, 16), NA or adrenaline (A) (17, 18) has been newly introduced, and the antibody directed towards dopamine (DA) coupled to bovine serum albumin (BSA) by glutaraldehyde (4) appears to be another new specific tool for the study of neurons and paraneurons.

The time is now ripe to compare the results obtained by the histofluorescence technique, immunohistochemistry using antisera to amines themselves, and immunoenzyme-histochemistry using antibodies against monoamine-synthesizing enzymes (8).

MATERIALS AND METHODS

Highly specific antiserum against bovine adrenal tyrosine hydroxylase (TH) (in dilution of 1/6,000) (13) and antiserum to serotonin (5-HT)-BSA conjugate (in dilution of 1/3,000) (19) were produced in rabbits in our laboratory. Specificity of localization was tested on an adjacent section, with the section exposed to the immunochemicals, except that the primary antibody was pretreated for 12 hr with an excess of the antigen prior to incubation on the tissue section (8, 21). Controls were taken with antiserum from non-immunized rabbits for TH, or antiserum against BSA for 5-HT.

For immunofluorescence study, nine goldfish (*Carassius auratus*, 5–10 g body weight) were perfused with 10 ml of Zamboni's solution (2% paraformaldehyde-0.2% picric acid in 0.1 M phosphate buffer, pH 7.2) for 5 min. Brains were removed and fixed in the same fixative for an additional 5–17 hr and washed with phosphate-buffered saline containing 10% sucrose. For immunoelectron microscopy, ten goldfish were perfused with 3% paraformaldehyde-0.05% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2. Brains were subsequently removed and fixed in 3% paraformaldehyde for 5–17 hr, and washed with phosphate-buffered saline for 24 hr.

Indirect immunofluorescence and peroxidase-antiperoxidase (PAP) techniques were used to demonstrate the localization of TH and 5-HT in goldfish brain at the light microscopic (13, 19) and electron microscopic levels (6, 10).

RESULTS

The localization of monoaminergic neurons of the goldfish brain is summarized in Table 1 and Fig. 1. TH-positive (catecholaminergic) neurons had 11 groups, and 5-HT-positive (serotonergic) neurons had 4 groups. A short communication concerning this has already been published (19).

In one of the circumventricular organs (CVOs), the paraventricular organ (PVO) especially pars anterior or the rostro-medial part of the nucleus recessus lateralis (NRL) of the goldfish brain, cerebrospinal fluid (CSF)-contacting neurons

TABLE 1. *Monoaminergic neurons in the goldfish brain*

TH	5-HT
1. The olfactory bulb	
2. The area ventralis telencephali pars dorsalis (n. olfactorius medialis, basal nuclei, paleostriatum)	
3. The nucleus preopticus (parvocellular preoptic nucleus)	
4. The nucleus dorsolateralis thalami (the area lateral to the SCO)	a. The pretectal area
5. The nucleus ventromedialis thalami	
6. The nucleus posterioris periventricularis (PVO-accompanying cells)	b. NRL
7. <u>The rostro-medial part of the nucleus recessus lateralis (NRL) or the paraventricular organ (PVO) pars anterior</u>	b. PVO pars anterior
8. The nucleus posterior tubercis (NPT)	c. The nucleus recessus posterioris (NRP)
9. The lateral isthmal region (isthmus tegmentum)	d. The raphe nuclei of the brainstem
10. The reticular formation of the medulla oblongata	
11. <u>The post-obelcular region</u>	

Underline: Cerebrospinal fluid-contacting neurons

FIG. 1. Drawing of sagittal and frontal sections of the brain of goldfish. CC: central canal; FLM: fasciculus longitudinalis medialis; NPO: nucleus preopticus; NPT: nucleus posterior tubercis; NRL: nucleus recessus lateralis; NRP: nucleus recessus posterioris; OC: optic chiasma; Olf: olfactory bulb; PC: posterior commissure; SCO: subcommissural organ; Tec: tectum; Tel: telencephalon

are composed of TH-positive and 5-HT-positive neurons. Immunoelectron microscopic studies confirmed that TH-positive reaction products were distributed in both cytoplasmic matrix and cored vesicles (80–120 nm in diameter, Fig. 2a) (6), and 5-HT-positive ones were also in both cytosol and cored vesicles (100–200 nm) as shown in Fig. 3a. In the nucleus recessus posterioris (NRP) of the goldfish, only 5-HT-positive and TH-negative CSF-contacting neurons were detected. 5-HT-immunoreactive granules were also observed in the NRP. These CSF-contacting neurons (10–13 μm in size) had slight invaginated nuclei (Figs. 2a, 3a) and were situated in close proximity to the blood vessels as shown in Fig. 2a. Their processes containing immunoreactive granules surrounded non-fenestrated capillaries (Fig. 2b). In contrast, TH-positive, 5-HT-negative PVO-accompanying cells contained larger TH-positive granules (200–250 nm) in the cytoplasm and processes (Fig. 3b). PVO-accompanying cells (25–45 μm in size) were surrounded by glial cells even until their processes (Fig. 3b).

In the telencephalon, TH-positive, 5-HT-negative neurons were observed and extended their nerve fibers to the olfactory bulb. These neurons were oval in shape and the size of the perikaryon was about 8–15 μm . They had TH-positive granules (106–110 nm) between well developed rough surfaced endoplasmic reticulum (Fig. 4a). These ultrastructures were similar to those of the preoptic nucleus (PON) (Fig. 4b) and nucleus posterior tuberis (NPT) (Fig. 4c).

DISCUSSION

There were some discrepancies when comparison was made between formaldehyde-induced fluorescence (FIF) and immunohistochemical techniques in the neurons of the CVOs of the frog as reported previously (11, 20, 21). In the goldfish, we observed only 5-HT-positive and TH-negative CSF-contacting neurons in the NRP. This finding was different from the FIF results. By the FIF technique, catecholamine (CA) fluorescence was observed in the goldfish NRP (1, 7).

Fig. 2. Electron micrographs of the CSF-contacting neuron (a) and its processes (b) in the rostro-medial part of the NRL (or PVO) after incubation with TH-antiserum by PAP method. a. TH-positive reaction products are observed in the cytoplasmic matrix and granules (arrows). A large number of endoplasmic reticulum (er) and mitochondria (m) are seen. Near the CSF-contacting neurons, non-fenestrated capillary (Cap) is observed. Synaptic formation is shown by 2 arrows in the square. The adjacent cell with Golgi apparatus (G) and non-immunoreactive granule (arrow head) seems to be a non-catecholaminergic CSF-contacting neuron. b. TH-positive nerve processes containing positive granules surround a capillary. a, $\times 12,000$; b, $\times 7,000$

Fig. 3. Electron micrographs of the 5-HT-immunoreactive liquor-contacting neuron of the PVO (a) and TH-positive PVO-accompanying cell (b). a. 5-HT-positive granules (arrows) and reaction products are seen in the cytoplasmic matrix. An invaginated nucleus is observed. b. A PVO-accompanying cell contains rather bigger TH-positive granules (arrows) and is surrounded by glial cells (*). a, $\times 9,500$; b, $\times 6,300$

Fig. 4. Electron micrographs of neurons in the telencephalon (a), PON (b) and NPT (c). All these cells are non-CSF-contacting neurons and show TH-positive immunoreaction in the cytoplasm and granules. Well developed endoplasmic reticulum (er) are observed. Capillary (Cap) seems to be non-fenestrated type. Mitochondria (m) show no immunoreaction. a, $\times 6,300$; b, $\times 6,500$; c, $\times 7,000$

TABLE 2. Discrepancy between immunoenzyme-histocytochemistry (IEHC) and immunohistocytochemistry (IHC) or formaldehyde-induced fluorescence (FIF)

	IEHC	IHC $\neq$ FIF
1) The catecholamine (CA)-positive histofluorescence with tyrosine hydroxylase (TH)-positive reaction of cells indicates that the cells are capable of synthesizing and storing the CA.	+	+
2) The CA-positive histofluorescence with the TH-negative reaction of cells may indicate that those cells do not synthesize the CA but take up and store it.	—	+
3) The CA-negative histofluorescence cells which display TH-positive reaction indicate that the cells are capable of synthesizing the CA but do not store it.	+	—

Kotrschal and Adam (7) described that the telencephalon of *Blennius incognitus* (Teleostei) was devoid of fluorescent perikarya by means of FIF. Two diencephalic nuclei, the NRL and NRP displayed fluorescent perikarya by FIF. They claimed that both NRL and NRP contained DA.

In contrast, our previous report (19) showed TH-positive immunofluorescent neurons in the telencephalon (basal nuclei or paleostriatum), and in this report we confirmed TH-positive perikaryon (about 8–15 μ m in size) containing TH-positive granules by immunoelectron microscopy. Moreover, in the NRP and NRL except its rostro-medial part, we confirmed only 5-HT-immunoreactive, TH-negative CSF-contacting neurons with 5-HT-positive granules. Our independent results concerning 5-HT-immunoreactive perikarya confirmed the report of Kah and Chambolle (4), an immunocytochemical study on serotonin in the brain of the goldfish. They also found 5-HT immunoreactive CSF-contacting neurons in the NRL and NRP of the hypothalamus. Geffard *et al.* (5) revealed that DA was present in the NRL and NRP of the goldfish. In our study TH-positive CSF-contacting neurons were observed in the rostro-medial part of the NRL (corresponding to nucleus posterior periventricularis by Kah and Chambolle) (3) intermingled with 5-HT-containing CSF-contacting neurons. In the NRP, no TH-positive reaction was observed. Without TH, CSF-contacting neurons may have the power to take up DA. The coexistence of DA with/without 5-HT is a future problem.

To date, antibody to DA (4), NA or A (17, 18) can be utilized to solve this problem, but there still remains functional differences in localization between amines and amine-synthesizing enzymes. Finally, to our previous proposal to use the term of immunoenzyme-histocytochemistry (8) we add functional aspects and summarize as shown in Table 2.

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