Histochemical Localization of γ-Aminobutyric Acid Transaminase Activity in the Carp Retina

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Although numerous histochemical studies on central and peripheral nervous tissues have been conducted, a little informations are available for neurotransmitters in the carp retina, which has been used widely for electrophysiological experiments[1)-3)]. The present study is one of our attempts to classify retinal cells histochemically in the carp[4)-7)].

The localization and endogenous concentration of γ-aminobutyric acid (GABA), GABA-transaminase (GABA-T) and GABA-synthesizing enzyme (GAD) were extensively investigated biochemically in the invertebrate and vertebrate retinas[8)9)]. To study the localization of GABA-ergic cells, an uptake method applying exogenous [^{3}H]-GABA was employed[10)-14)]. These earlier experiments showed that the radioactive substance was taken up by all classes of retinal cells in rats and rabbits, and possibly accumulated predominantly in Müller cells. In the goldfish retina, however, [^{3}H]-GABA was found to be loaded in particular cells[15)]. These included external and internal horizontal cells, a few cells at the vitreal side of the inner nuclear layer (perhaps, amacrine cells), and some ganglion cells. Recently, Marc *et al.*[16)], from their findings in the uptake experiments with the goldfish retina, concluded that pyriform amacrine and external horizontal cells appear to be GABA-ergic.

A histochemical method to investigate the localization of GABA-T in central nervous tissues was developed by van Gelder[17)18)]. However, using a method further modified from the original technique of van Gelder, Hyde and Robinson[19)] could observe the GABA-T activity only in Müller and photoreceptor cells in the rat retina. Therefore, no one has demonstrated histochemically the GABA-T activity in retinal cells other than in Müller and photoreceptor cells.

Materials and methods

The retinas were isolated from carp (body weight, about 700 g), which had been dark-adapted for 1 hr, and divided into 2-4 pieces under dim light. To localize the GABA-T activity, the pieces were dealt with in a procedure similar to that of van Gelder[17)18)]. A Ringer solution (high Mg^{2+} and low CA^{2+}) was used, consisting of (g/l): NaCl (6.132), KCl (0.268), $MgSO_4$ (4.436), $CaCl_2$ (0.029), $NaHCO_3$ (1.899), NaH_2PO_4 (0.012), and Na_2HPO_4 (0.057). For the standard incubation medium, nicotinamide-adenine dinucleotide (NAD; 8 mg), α-ketoglutarate (30 mg) and GABA (400 mg) were added to the Ringer (10 ml), while nitro blue-tetrazolium (NBT; 8 mg) was first dissolved in dimethyl sulphoxide (0.8 ml) and then added to the Ringer (9.2 ml). If GABA-T exists in cells,

鯉網膜におけるギァバァトランスアミナーゼ (GABA-T) 活性の組織化学的検索：金沢大学医学部付属神経情報研究施設情報伝達研究部門　林　俊治，根岸晃六.

it degrades GABA taken up by the cells from the incubation medium. An intermediate metabolite, succinic acid semialdehyde reduces NAD to NADH. The latter further reacts with NBT, forming formazan deposits, which could be detected as blue granules (formazan reaction) in the cells under light microscopy.

The retinal pieces were incubated in different media under different conditions depending upon experimental purposes. Most of them were incubated in a medium containing all the components described above (the standard medium) for 30 min at 37°C and pH 6.6-6.9 under dim light. For control studies, however, GABA was omitted from the medium. To inhibit the GABA-T activity, amino oxyacetic acid (AOAA; 400 mg/10 ml) was added to the standard incubation medium.

After incubation, the retinal pieces were fixed in ethylalcohol (95 %) for about 3 hr, and embedded in paraffin. The paraffin blocks were sectioned radially to 15 or 20 μm thickness. Paraffin was washed out from the sections with xylene, and then the sections were mounted with Entellan. Some preparations were counter-stained with carmalaum or methyl green.

Results

Formazan reaction was dependent on medium pH, temperature and GABA concentration. Below pH 6.0 no formazan deposit was seen even when the temperature was adequate and the GABA concentration was sufficient (see below). Above pH 7.2, on the other hand, formazan deposits appeared exclusively in photoreceptor cells and at the outer limiting membrane. The optimal pH was assumed to be a range of 6.6-6.9 to get the present findings (see below). When the retina was incubated at room temperature (22-25°C) with sufficient GABA at the optimal pH, no formazan-positive cells were observed. Neither did any reaction occur in the retinal pieces pre-heated at 100°C for 15 min. In the present series of experiments, the retinal tissues were incubated in the standard medium (see Methods) at 37°C and pH 6.6-6.9.

Very faint formazan-deposits were detected in retinal pieces which were incubated in a medium containing of all the compounds (listed in the Methods) except for GABA (control preparations). When GABA was added to the medium (100-300 mg/10 ml), the formazan reaction was seen mainly in most photoreceptor and Müller cells (including the outer and inner limiting membranes), and in some cells in the amacrine and ganglion cell layers. The size of formazan-granules was larger in Müller cells than in the others. As the GABA concentration was increased (higher than 400 mg/10 ml), the formazan reaction was enhanced in intensity and additional classes of cells (external horizontal and bipolar cells) became positive (Fig. 1). In these preparations, the external horizontal cell layer was diffusely positive, containing some heavily stained cells, but the reaction seen in bipolar cells was always weak and varied from preparation to preparation. Amacrine cells whose processes showed the reaction could be divided at least into two types; pear- and spindle-shaped (Fig. 1A and B). The former has a single process extending into the proximal half part of the inner plexiform layer (IPL, stratum b), while the latter has bilateral process seemingly branching diffusely in the IPL (strata a and b). The ratio of cell numbers between the pear-shaped and spindle-shaped amacrine cells was approximately 5:1, estimated from their cell counts in radial sections. Furthermore, about 10-20% of so-called amacrine cells, were formazan-positive from estimation in radial and counter-stained sections; the variation in the ratio seemingly depends upon the area calculated. Exceptionally, large cells located in the middle part of the IPL became formazan-positive. Their bilateral processes were usually long (more than 300 μm).

Retinal tissues, incubated previously in a Ringer solution containing AOAA (400 mg/10 ml) for 0.5-1 hr, showed only a faint formazan-reaction after the standard incubation, indicating that AOAA largely inhibited the GABA-T activity. However, the retinal tissues, previously treated with AOAA, showed a strong reaction

when they were incubated in a medium containing succinate (400 mg/10 ml) instead of GABA. This indicates that AOAA did not interfere with formazan-formation from succinate in the retina.

Discussion

The present results obtained in the carp retina are common in rats as far as the GABA-T activity is positive in photoreceptor and Müller cells[19]. However, the GABA-T activity was found in all classes of retinal cells in carp, and [^{3}H]-GABA was taken up by all cells in frogs[13)20]. Therefore, the presence of the GABA-T activity does not mean these cells to be GABA-ergic. Nevertheless, it might be significant that some cells in certain classes of cells were found to be GABA-T positive in the present study. Formazan-deposits observed were stronger in photoreceptor, Müller and amacrine cells than in bipolar, external horizontal and ganglion cells. Although the axonal terminals of external horizontal cells were shown to take up [^{3}H]-GABA[16], formazan-deposits could not be detected in our preparations, suggesting that the GABA-T activity might be weak in the terminals.

Photoreceptor cells were found to exhibit the GABA-T activity in carp. Voaden *et al.*[20] demonstrated that [^{3}H]-GABA was taken up diffusely by the cell bodies at the receptor cell layer in frogs after 30-min incubation, although other investigators[10)13] did not describe such a phenomenon even after longer incubations (1-4 hr). Kuriyama *et al.*[8] and Graham[9] showed in rabbits and frogs, respectively, that the endogenous GABA concentration in the photoreceptor cell layer was much lower than that of the other retinal layers. All the above results may indicate that GABA is more rapidly degraded in photoreceptors than in the other cells. The reason for the presence of GABA-T in photoreceptor cells (probably, cones) may be due to the fact that these cells appear to be postsynaptic to external horizontal cells, which were found to take up [^{3}H]-GABA[16] and assumed to function as a feedback system in turtles[21] and carp[22]. Alternately, GABA may be used simply as an energy source in photoreceptors.

The present finding of formazan-deposits in some bipolar cells is contradictory, because these cells are generally considered to link in a direct excitatory pathway to ganglion cells[23)24]. However, there exists a possibility that some bipolar cells are postsynaptic to GABA-ergic pyriform amacrine cells[16].

Based on the present and earlier studies[6], the ratio of the numbers of GABA-T positive, acetylcholinesterase (AchE-) positive and dopaminergic cells can be estimated in radial section; these cells are aligned at the innermost border of the inner nuclear layer. AchE-positive amacrine cells are most abundant and dopaminergic cells are smallest in number. The population ratio of AchE-positive cells: GABA-T positive cells: dopaminergic cells is approximately 35:15:1.

A class of amacrine cells morphologically similar to the formazan-positive pear-shaped cell was described as "pyriform" amacrine cell in Golgi-preparations by Parthe[25]. According to Famiglietti *et al.*[24] and Marc *et al.*[16], this class of amacrine cells functionally belongs to ON-center type and takes up [^{3}H]-GABA. On the other hand, some formazan-positive amacrine cells had a spindle-shaped soma and bilateral proximal processes, arborizing diffusely in the IPL. This shape appears to be similar to that of ON-OFF type of amacrine cells marked with an intracellularly injected dye by Famiglietti *et al.*[24]. Miller *et al.*[26] suggesting from their electrophysiological observation in the *Necturus* retina that there exist at least two populations of amacrine cells; one releases GABA and the other glycine as inhibitory neurotransmitters.

In the present histochemical study, the GABA-T activity was found in all classes of retinal cells. However, our findings of formazan-deposits in external horizontal cells and in some cells at the amacrine and ganglion cell layers appear to be in harmony with earlier electrophysiologic[15)22)26] and autoradiographic[15] re-

sults, suggesting that these cells are GABA-ergic. To further ascertain this assumption, immunohistochemical localization of GABA-synthesizing enzyme (GAD)[27] would be valid.

Conclusion

Histochemical studies were conducted on the carp (*Cyprinus carpio*) retina to reveal the cellular localization of the gamma-aminobutyric acid transaminase (GABA-T) activity in radial sections. Formazan-deposits were found in all classes of retinal cells; they were relatively heavier in photoreceptor cells (probably, cones), Müller cells and some amacrine cells than in external horizontal cells, bipolar cells and a few cells in the ganglion cell layer. Morphologically, amacrine cells showing the GABA-T activity appeared to belong to 2 subsets; pyriform and fusiform amacrine cells. The former has a single process extending into the proximal half part of the inner plexiform layer, while the latter showed bilateral processes seemingly arborizing diffusely in the inner plexiform layer. The population ratio of GABA-T positive pyriform to fusiform amacrine cells was approximately 5:1. On the basis of earlier findings by us and others in retinal studies, some functional aspects of GABA-T positive cells were discussed, and the external horizontal cells and some cells in the amacrine and ganglion cell layers were assumed to be GABA-ergic.

Key words: carp retina, GABA-T activity, histochemistry

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Figure legends

Fig. 1. Localization of the GABA-T activity. Photomicrographs of radial sections from a retina incubated with GABA (400 mg/10 ml). A: Counterstained with calmalaum. Nuclei of cells are stained pink in color. Photoreceptor, bipolar and amacrine cells are formazan-positive (arrows). Two amacrine cells are seen in the center. An amacrine cell is pear-shaped at the left (p) and the other is spindle-shaped at the right (s). Upper and lower asterisks indicate the outer plexiform layer (OPL) and inner plexiform layer (IPL), respectively. B: Two amacrine cells are positive. An amacrine cell is pear-shaped (left) and the other spindle-shaped (right). The former extends a proximal process to stratum a, while the latter has bilateral processes; one seems to extend in stratum a (arrow with a) and the other reaches stratum b (arrow with b). The outer and inner limiting membranes are also formazan-positive; the levels are marked with solid circles. C: The external horizontal cell layer is diffusely formazan-positive (solid square). Müller cells (arrow with M) are positive with relatively large granules. One bipolar cell and two amacrine cells are also detectable. D: One cell located in the ganglion cell layer is intensely positive (arrow with G). This is an exceptional case, because most cells at this level are weakly positive. A, B, C, D: ×650.

鯉網膜におけるギァバァトランスアミナーゼ (GABA-T) 活性の組織化学的検索：金沢大学医学部付属神経情報研究施設情報伝達研究部門　林　俊治，根岸晃六．金沢市，920．金沢大学十全医学会雑誌，第90巻，501－507，(昭和56年)．

抄　録　鯉網膜における GABA-T 活性の局在を，縦断切片（厚さ15－20μm）標本を用い，組織化学的に検索した．GABA-T 活性は光受容器細胞（恐らく錐体）と Müller 細胞の大部分，アマクリンおよび節細胞層に存在する一群の細胞に見出された．また，外水平細胞層および少数の双極細胞に軽度な活性が認められた．GABA-T 活性を示すアマクリン細胞は，形態的に2種に区別できる．1つは西洋梨状の細胞体を呈し，その細胞体より垂直に1本の突起を出して内網状層内半側部のみに分枝分布するが，他は紡錘状細胞体を呈し，それから横方向に2本の突起を伸ばして内網状層全域に亘って分枝分布している．GABA-T 陽性の梨状および紡錘状アマクリン細胞の密度比は約5：1であった．これまでの私たちおよび他研究者らの知見に基づき，GABA-T 活性を示す網膜細胞の機能を考察し，外水平細胞とアマクリンおよび節細胞層に存在する一群の細胞が GABA を伝達物質として用いていると推定した．

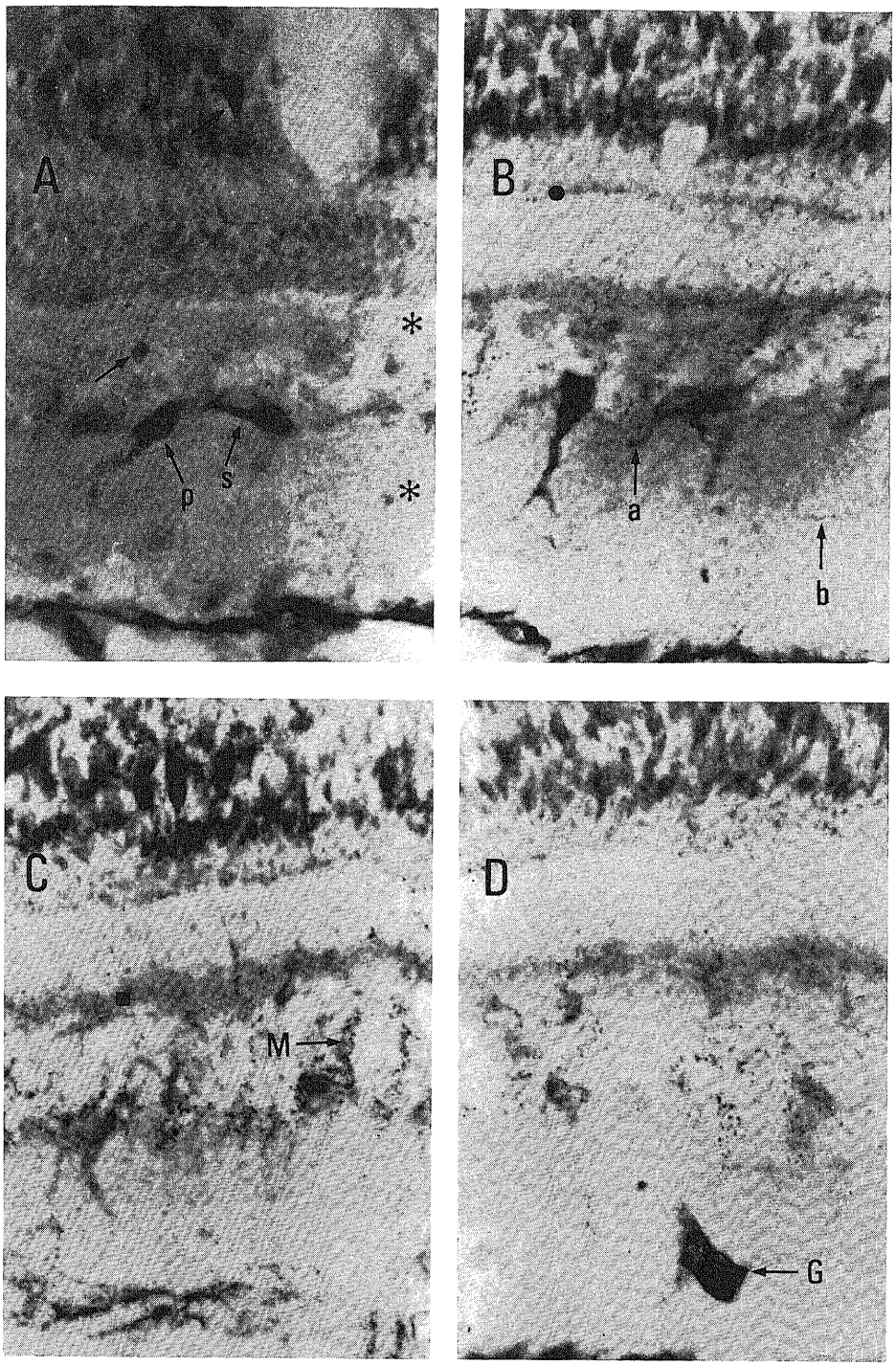

Fig. 1. Histochemical Localization of GABA-T activity