

Induction of glutamine synthetase in embryonic neural retina: Localization in Müller fibers and dependence on cell interactions

(immunofluorescence/retinoglia/cell contact/enzyme induction)

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ABSTRACT The cellular localization of glutamine synthetase [GSase; L-glutamate:ammonia ligase(ADP-forming), EC 6.3.1.2] induced by cortisol in the neural retina of chicken embryos was investigated by immunostaining with GSase-specific antiserum and indirect immunofluorescence. In organ cultures of retina tissue, and in the retina *in vivo*, hormone-induced GSase was found to be confined only to the Müller fibers (retinoglia). Also, in mature chicken retina, which contains a very high level of GSase, the enzyme was detected solely in Müller fibers. In short-term monolayer cultures of dispersed embryonic retina cells, there was no GSase induction and no immunodetectable increase in enzyme level. However, when the dispersed cells were reaggregated and they restituted retinotypic cell associations, GSase could be induced and it was localized in Müller fibers. The results suggest that, in addition to the hormonal stimulus, contact-dependent interactions between Müller glia cells and retina neurons are involved in the mechanism of GSase induction in the retina.

The induction of glutamine synthetase [GSase; L-glutamate:ammonia ligase(ADP-forming), EC 6.3.1.2] in embryonic neural retina by adrenal corticosteroids represents a characteristic developmental feature of this tissue and provides a versatile experimental system for studying neural cell differentiation and hormonal control of gene expression (1). In the chicken embryo retina, GSase begins to rise sharply on the 16th day of development, after elevation of systemic corticosteroids (1, 2); it increases 100-fold in a few days and remains thereafter at this high level. However, what makes this system especially interesting is that GSase can be induced precociously long before its normal rise in the embryo. This can be accomplished *in vivo* by injecting cortisol into eggs and *in vitro* in organ cultures of retina tissue from 9- to 15-day embryos by adding cortisol to the culture medium (3, 4). Studies on organ cultures demonstrated that the hormone promptly elicits accumulation of mRNA for GSase, resulting in rapid increase in the rate of enzyme synthesis and in the enzyme level (5-8).

Other experiments raised the possibility that, in addition to the hormonal stimulus, still another control mechanism may be involved in GSase induction in the retina. They showed that in dissociated retina cells dispersed in monolayer cultures, GSase was not inducible (9); however, if such cells were reaggregated and reconstructed tissue architecture, GSase could be induced (9, 10). These results suggested that histotypic associations and interactions among cells may be required for GSase induction and they raised the question of whether this enzyme was induced and localized in a particular type of cell in the retina. The report that in mature rat retina GSase is confined to Müller fibers (11) added further interest to this question.

In the present study, immunostaining with an antiserum specific for GSase was used to investigate the cellular distribution

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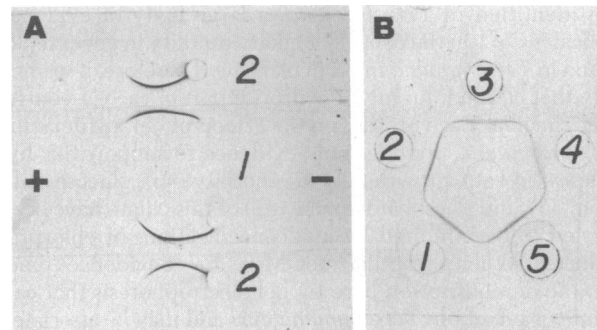


FIG. 1. Characterization of anti-GSase antiserum. (A) Immunoelectrophoresis of purified GSase (well 1) and of GSase-containing extract from mature retina (well 2) against anti-GSase antiserum. The extract was the supernatant from the homogenate of mature retina tissue (in 5 mM phosphate buffer, pH 7.2) after centrifugation at $100,000 \times g$ for 20 min. (B) Double immunodiffusion of anti-GSase antiserum (center well) against extracts from mature retina (wells 1, 3, and 5) and from cortisol-induced embryonic retina (wells 2 and 4). Same amounts of enzyme activity were applied to all wells. Gels were stained with 0.5% Coomassie blue. Antigenic identity is indicated by fusion of the single precipitin lines.

of GSase in (i) mature chicken retina, (ii) hormone-induced embryonic retina, (iii) monolayer cultures, and (iv) aggregates of embryonic retina cells.

MATERIALS AND METHODS

GSase. The enzyme was purified from retinas of adult chicken as described (12). The specific enzyme activity of GSase was determined in sonified preparations of retina tissue and cells by the colorimetric assay used before (13) with some modifications that increased its sensitivity.

Antiserum. Albino rabbits were immunized by two weekly subcutaneous injections of 50 μ g of purified GSase in 5 mM phosphate buffer, pH 7.2, emulsified in Freund's complete adjuvant (1:1, vol/vol). Serum was collected 7 days after the second injection and at weekly intervals thereafter. Each serum batch was assayed for immunoprecipitation of GSase (14). The antiserum was characterized by Ouchterlony double immunodiffusion and by immunoelectrophoresis (15).

Culture Methods. For organ cultures of retina tissue, retinas were aseptically dissected from chicken embryos, bisected, and each half was placed in 3 ml of culture medium in 25-ml erlenmeyer flask. Flasks were gassed with 5% CO₂/95% air mixture and incubated on a shaker (70 rpm) at 37°C. All cultures were in medium 199 (with Hanks' salts) plus 1% glutamine, 1% penicillin-streptomycin mixture, and 10% fetal bovine serum. Cell suspensions were prepared from embryonic

Abbreviations: GSase, glutamine synthetase; FITC-GAR, fluorescein isothiocyanate-conjugated goat anti-rabbit gamma globulin.

retinas by trypsinization according to published procedures (16, 17). For monolayer cultures, freshly dissociated retina cells were plated in 60-mm Falcon dishes in 5 ml of culture medium at a density of 5×10^5 cells per cm^2 and were maintained in a 5% $\text{CO}_2/95\%$ air atmosphere. Cell aggregates were obtained from cell suspensions by the "rotation procedure" (16, 17) in 25-ml erlenmeyer flasks, using approximately 1.5×10^7 cells per 3 ml of culture medium.

Induction of GSase. Induction of GSase in cultures of retina tissue or cells was by addition of cortisol (hydrocortisone-free base) to the medium to a final concentration of $0.33 \mu\text{g}/\text{ml}$ (13). For *in vivo* induction, 5 mg of cortisol (hydrocortisone phosphate) in 0.2 ml of sterile Tyrode's solution was injected onto the chorioallantoic membrane of chicken embryos through a shell window.

Immunostaining with GSase Antiserum. Immunostaining was by the indirect immunofluorescence method. Retina tissue and cell aggregates were fixed for 30 min in 4% paraformaldehyde (in 0.1 M cacodylate buffer, pH 7.2) at room temperature, followed by Carnoy's mixture for 1.5 hr at 4°C . Paraffin sections ($3 \mu\text{m}$) were rehydrated with distilled water. Cryostat sections of paraformaldehyde-fixed material (11) yielded similar results. Monolayer cell cultures grown on glass coverslips were fixed in a mixture of 95% ethanol/5% acetic acid for 20 min at -20°C and then rehydrated with distilled water at room temperature (18). Tissue sections and cell cultures were exposed

to diluted anti-GSase antiserum for 1 hr at 37°C in a humidified chamber, washed twice in Tyrode's solution, and then treated with fluorescein isothiocyanate-conjugated goat anti-rabbit gamma globulin (FITC-GAR) for 1 hr as above. After washing, the preparations were examined for fluorescence by illumination through a 390-nm excitation filter and a 500-nm barrier filter. Controls were treated with equivalent dilutions of non-immune rabbit serum, followed by FITC-GAR. To decrease nonspecific staining, all dilutions of serum and antiserum contained equivalent dilutions of normal goat serum.

RESULTS

The specificity of the anti-GSase antiserum (Fig. 1) was established by immunoelectrophoresis against crude extracts from mature chicken retina and against purified GSase. Formation of a single precipitin line demonstrated that the antiserum contained antibodies specific for GSase (Fig. 1A). Ouchterlony immunodiffusion tests indicated complete antigenic identity between GSase from mature retina and from induced embryonic retina (Fig. 1B); this is consistent with previous evidence that the subunit molecular weight is the same in both cases (19). Thus, by these criteria, the antiserum was specific for GSase from mature retina and induced embryonic retina and, therefore, suitable for use in detection of this enzyme in both situations.

By using anti-GSase antiserum we examined by indirect

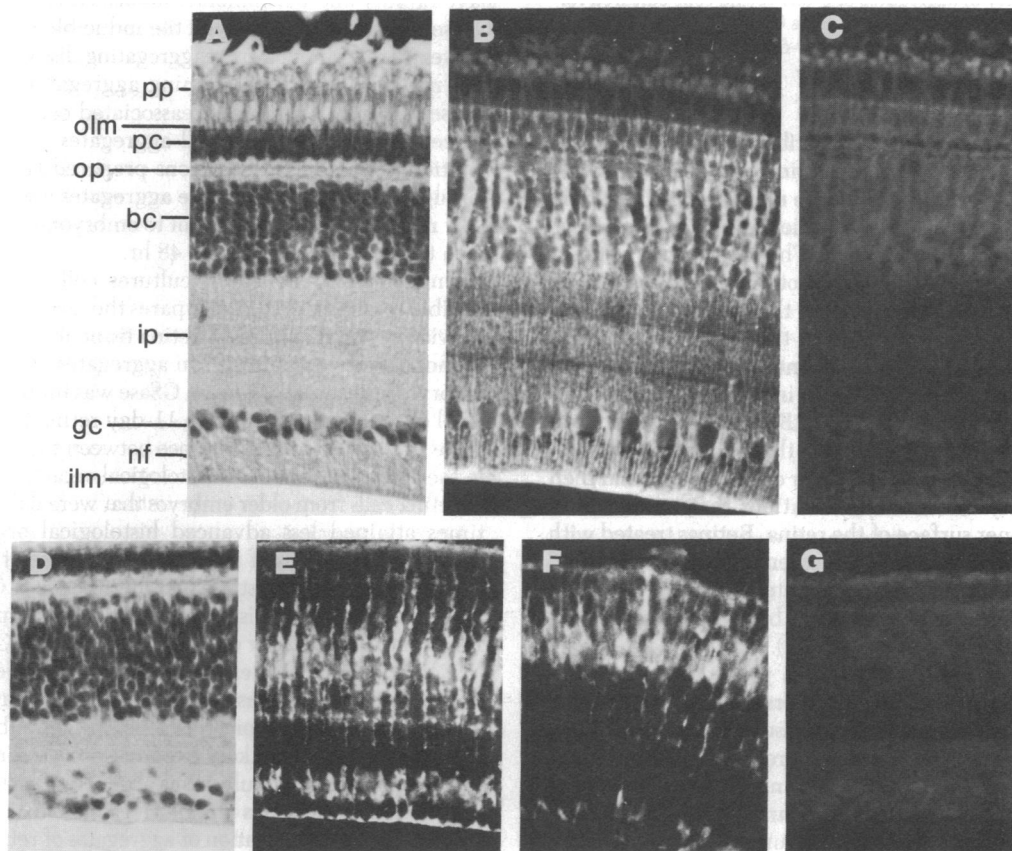


FIG. 2. Cellular localization of GSase in mature chicken retina (A-C) and in cortisol-induced embryonic chicken retina (D-G) by immunostaining and indirect immunofluorescence. (A) Section of mature retina (6-week-old chicken) stained with hematoxylin and eosin. pp, Photoreceptor processes; olm, outer limiting membrane; pc, photoreceptor cell layer; op, outer plexiform layer; bc, bipolar cell layer; ip, inner plexiform layer; gc, ganglion cell layer; nf, nerve fiber layer; ilm, inner limiting membrane. (B) Section similar to that in A, immunostained with anti-GSase antiserum (diluted 1:50) and FITC-GAR. Light areas represent immunofluorescence in Müller fibers. (C) Control section treated with nonimmune rabbit serum and FITC-GAR. (D) Section of 13-day embryo retina stained with hematoxylin and eosin. (E) Thirteen-day retina induced *in vivo* with cortisol. Section was treated with anti-GSase antiserum (diluted 1:20) and FITC-GAR. Fluorescence is localized to Müller fibers. (F) Section of 13-day retina induced in organ culture with cortisol; immunofluorescence shows localization of induced GSase in Müller fibers. (G) Section of control, noninduced 13-day retina treated as in E and F; no immunofluorescence. All magnifications are $\times 350$.

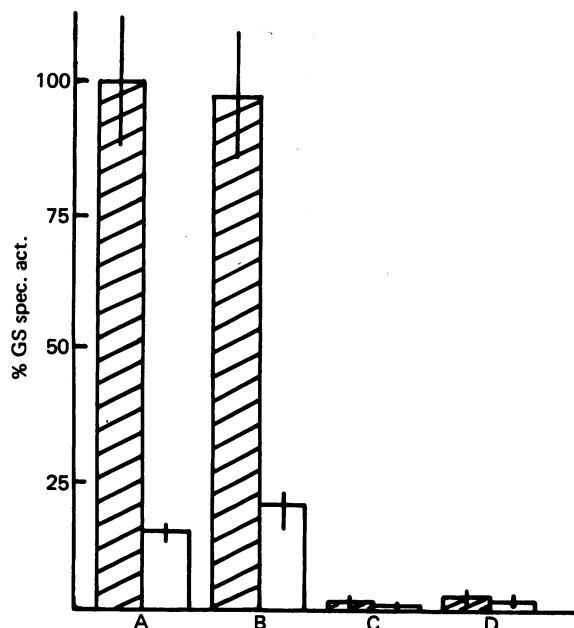


FIG. 3. Specific activity of GSase (expressed as percentage of bars A) induced by cortisol in: organ cultures of embryonic retina tissue (bars A); cell aggregates (bars B); monolayer cell cultures (bars C and D). Hatched bars, cortisol present; open bars, cortisol absent. Bars A, GSase activity in retina from 11-day embryo cultured for 2 days. Bars B, GSase activity in aggregates of 6-day retina cells cultured for 5 days and then induced with cortisol. Bars C and D, monolayer cultures of retina cells from 6-day (C) and 10-day (D) embryos.

immunofluorescence the cellular distribution of GSase in histological sections of mature chicken retina (Fig. 2A), which has a high level of GSase activity. Specific reaction with the antiserum was detected only in cells identified as Müller fibers (Fig. 2B)—i.e., the neuroglia of the retina. The morphology of these cells clearly distinguishes them from others in the retina (20, 21). They are the only ones that span the whole width of the retina; their perikarya are located in the bipolar layer; they extend numerous fine arborizations into the inner plexiform and ganglion layers, which terminate in end-feet anchored in the inner limiting membrane. Fig. 2B shows that immunostaining for GSase is confined to fibers that widen in the region corresponding to the location of Müller cell perikarya and then branch extensively into thin arborizations that terminate in end-feet at the inner surface of the retina. Retinas treated with nonimmune serum showed no fluorescence above background level (Fig. 2C). GSase was not detected in retina neurons; however, its presence there in amounts below the sensitivity of this procedure cannot be ruled out (22).

To examine if in embryonic retina precociously induced GSase was also confined to Müller fibers, retinas from 11-day embryos were cultured for 48 hr in cortisol-containing medium; this elicited a 10-fold (or greater) increase in GSase specific activity (13). GSase was also induced in retinas *in vivo* by injecting cortisol into 11-day eggs (4). Immunostaining of histological sections with anti-GSase antiserum revealed that GSase was present again only in Müller fibers (Fig. 2D–F). Noninduced retinas showed no staining or very weak diffuse staining (Fig. 2G). Retinas of several embryonic ages were similarly studied, and there was a consistent correspondence between the level of GSase induction (assayed biochemically) and immunostaining of Müller fibers. Therefore, the cortisol-induced accumulation of GSase in chicken embryo retina occurs in Müller glia cells.

Next, immunostaining with anti-GSase antiserum was applied to monolayer cultures of dispersed embryonic retina cells; as mentioned earlier, in such cultures there is no biochemically detectable GSase induction. According to previous work (23, 24), Müller fibers assume in these cultures the shape of large epithelioid cells referred to as LER cells, whereas neuronal cells appear smaller and round or bipolar with axonlike processes. Retinas from 6- through 14-day chicken embryos were dissociated by trypsinization into cell suspensions and the cells were plated at low densities, which favored their remaining completely dispersed. Cortisol was added at plating time. After 1–7 days in culture the cells were immunostained for detection of GSase. No staining differences were found between cortisol-treated and untreated cultures at any of the embryonic ages or culture times examined, and none of the cells in these cultures reacted with the antiserum above background level. Assays for release of the enzyme from cells into the culture medium were negative. These results agree with previous biochemical evidence that GSase is not inducible in dissociated, dispersed embryonic retina cells (6). Occasionally, small cell clumps were noted that contained juxtaposed LER and neuronal cells; in the presence of cortisol, LER cells within these composite clumps immunostained for GSase, whereas adjacent but isolated LER cells showed no staining.

The above results raised the possibility that neural–glial cell associations are required for the hormonal induction of GSase. Alternatively, disruption of the tissue into single cells might have rapidly and permanently abolished cell competence for GSase induction or destroyed the inducible cells. These alternatives were examined by reaggregating dissociated retina cells into multicellular tissue-forming aggregates to determine if GSase was inducible in the reassociated cells and if it was localized in Müller fibers. Cell aggregates were produced by rotation (16) of cell suspensions prepared from retinas of 6- through 11-day embryos. The aggregates were cultured until they reached an age equivalent to embryonic day 11 and were then treated with cortisol for 48 hr.

Unlike cells in monolayer cultures, cell aggregates were inducible for GSase. Fig. 3 compares the levels of GSase specific activity in organ cultures of retina tissue in cell aggregates and in monolayer cell cultures. In aggregates of cells from 6-day embryos, cultured for 5 days, GSase was inducible to the same level of specific activity as in 11-day retina tissue. In general, there was a close correspondence between the level of induction in the aggregates and their histological organization. Aggregates of retina cells from older embryos that were cultured for shorter times attained less advanced histological organization and showed lower levels of induction than those of younger cells.

These results demonstrate that the absence of GSase induction in the monolayers of dispersed retina cells was not due to rapid loss of inducible cells or of cell competence for GSase induction. We find that even after 3–4 days in monolayer culture, cells from 6- to 8-day retinas are inducible after reaggregation and restoration of histotypic relationships (unpublished results). Therefore, the most likely explanation for noninducibility of GSase in monolayer cultures is absence of histotypic juxtapositions and associations among the cells.

Histological examination of aggregates of retina cells cultured for 7 days showed them to contain characteristic concentric retinal rosettes (Fig. 4A), consisting of layers of photoreceptor, bipolar, and ganglion cells. Staining with anti-GSase antiserum of sections of cortisol-induced aggregates resulted in immunofluorescence in cells identified as Müller fibers located within the rosettes (Fig. 4B). There was some staining also in areas between rosettes, possibly due to clusters of Müller cell arborizations and end-feet. Aggregates not treated with cortisol (Fig.

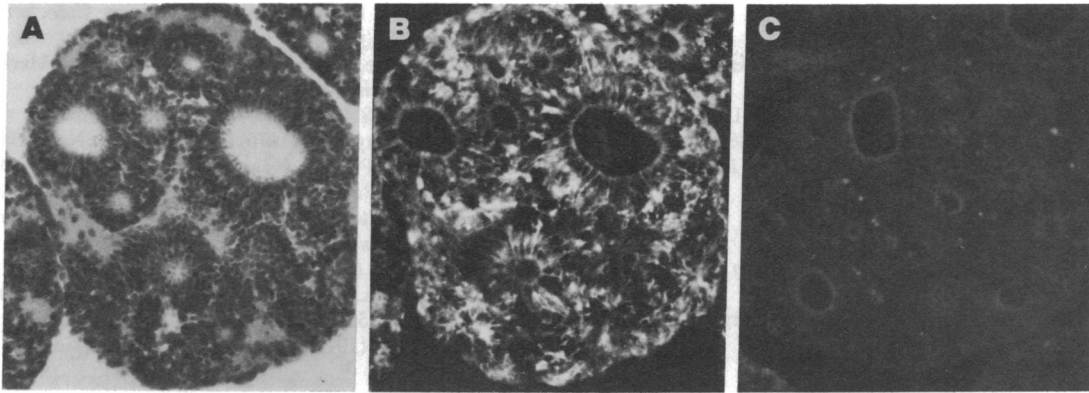


FIG. 4. Cellular localization of GSase induced in retina cell aggregates (see text). (A) Section stained with hematoxylin and eosin showing retinal rosettes. (B) Section similar to A immunostained for GSase; immunofluorescence shows localization of induced GSase in cells identified as Müller fibers. (C) Section of noninduced aggregate immunostained for GSase. All magnifications are $\times 225$.

4C) and control aggregates treated with nonimmune serum showed no specific staining.

DISCUSSION

The findings that in mature chicken retina GSase is localized in Müller fibers and that the cortisol-induced accumulation of GSase in the embryonic chicken retina takes place in these glia cells agree with the reported confinement of this enzyme to Müller fibers also in the rat retina (11, 25); they support the suggestion that GSase is a characteristic marker for certain types of glia cells (26, 27).

The evidence that the induction of GSase in embryonic retina requires histotypic cell associations raises the possibility that contact-dependent interactions between Müller glia cells and retina neurons are involved in the mechanism of this induction. In this connection, it is of interest that the synthesis of the putatively glia-specific protein S-100 in embryonic quail spinal ganglia also requires neural-glial contact interactions,* and in clonal rat glia cells the synthesis of this protein also is cell-contact dependent (28). Interaction between glia and neurons have been postulated to play important roles in various aspects of neurodifferentiation (21, 29); however, their causal significance remains to be clarified. In the case of GSase induction in the retina, several possibilities suggest themselves for future exploration.

Histotypic contacts between Müller cells and retina neurons could result in a particular molecular organization of the cell surface or in interactions between specific cell-cell ligands that may be prerequisite for cell responsiveness to the hormonal inducer (10). There is much evidence from various systems that signals generated by conditions at the cell surface are relayed to within the cells and can affect gene expression. Because GSase induction involves hormone receptors and differential gene expression, its occurrence could depend on such signals and, thus, would be prevented by cell surface changes resulting from disassociation of the cells and their maintenance in a monolayer. Restitution of histotypic cell juxtapositions by cell reaggregation would restore cell surface conditions prerequisite for GSase induction by the hormone.

Another possibility is that cell contacts are necessary for transfer of metabolites and that these may participate in the regulation of GSase induction. In this context, the localization of GSase in Müller fibers is of interest because these cells are apparently also the site of the "small glutamate compartment"

(30-32). There is evidence that metabolites of neuronally released neurotransmitter substances, including glutamate, are transferred into this compartment where conversion to glutamine takes place, presumably by the action of GSase (33, 34). It is conceivable that such metabolite transfer plays a regulatory role in the mechanism of the hormonal induction of GSase and that it requires specific associations between Müller cells and neurons.

These are presently largely hypothetical considerations, but they suggest directions for further studies on the role of Müller glia-neuron interactions in GSase induction and in still other aspects of differentiation in this and other neural systems.

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