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HYDROCORTISONE-MEDIATED REGULATION OF GENE EXPRESSION IN EMBRYONIC NEURAL RETINA: INDUCTION OF GLUTAMINE SYNTHETASE

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SUMMARY

Functional differentiation in the embryonic chick neural retina is marked by a sharp increase in the accumulation of glutamine synthetase (GS). Hydrocortisone and related 11-β-hydroxy corticosteroids induce prematurely GS in the embryonic retina. The induction involves gene expression which results in increased rate of GS synthesis and accumulation. Properties of hydrocortisone receptors in retina cytosol are described and compared with those from other embryonic tissues. Binding of hydrocortisone–receptor complexes to retina nuclei was studied to estimate the number of nuclear binding sites related to GS induction. GS is not inducible in dispersed retina cells; inducibility for this enzyme depends on retinotypic organization of the cells. It is suggested that histotypic cell contacts in the embryonic retina are involved in the mechanisms which control induction by the steroid of this gene-controlled expression characteristic of the differentiation of these cells.

INTRODUCTION

Embryonic differentiation involves two kinds of interdependent informational processes: differential gene expression, *i.e.* "output" of genomic information which results in changing patterns of macromolecular synthesis; and communication between cells by signals that specify which genes are to be expressed in a given cell at a particular stage of development. Hormones represent one class of such intercellular signals in that they can trigger in target cells specific changes in macromolecular synthesis. Although hormones have long been implicated in various normal and pathological aspects of embryonic development, their exact effects in differentiation have not been clearly understood. Recent advances in molecular endocrinology have promoted embryologists to explore in greater detail experimental systems suitable for studying interactions of hormones with embryonic cells.

The system which will be described here, in outline, is the embryonic neural retina in which we are studying how a corticosteroid regulates the induction of an enzyme associated with the differentiation of this tissue. The enzyme is glutamine synthetase (GS), an important biochemical marker of functional differentiation and maturation in the embryonic neural retina. GS catalyzes the conversion of glutamate to glutamine which plays an important role in a variety of biosynthetic pathways. In nerve cells, GS is of special interest because it is thought to be associated with membranes and synaptic structures, and because of the implication of glutamine in neurotransmission. The work reviewed here was done on chick embryo retinas which have several important advantages for biochemical and biological studies on this problem. For detailed description of this system and review of previously published work see [1].

GS IN THE EMBRYONIC RETINA

The neural retina develops from a relatively simple epithelial tissue in the early embryo, into a complex and highly specialized neural structure. In the chick embryo, the period of development starting on day 16 of incubation is particularly important, because during that time the retina undergoes functional differentiation and maturation as a visual system. One of the biochemical events that mark the onset of this critical period is a very sharp increase in the level of GS starting on day 16 of development (Fig. 1). During early stages of embryonic development the activity of GS in the retina is very low; its sharp rise on day 16 is triggered by cortical steroids which increase in the embryonic circulation shortly before that time.

PRECOCIOUS INDUCTION OF RETINA GS BY HYDROCORTISONE

This situation raised a self-evident, but important, question: does the retina become responsive to this hormonal effect only after it had reached day 16 of development, or can GS be hormonally induced prematurely long before the retina had attained this stage of differentiation. In other words, is it possible to alter the timetable of a developmental process in this neural tissue by hormonal intervention.

To examine this question [1], retina tissue from chick embryos of different ages was explanted into organ cultures and to these cultures various corticosteroids were added; we will discuss here only the

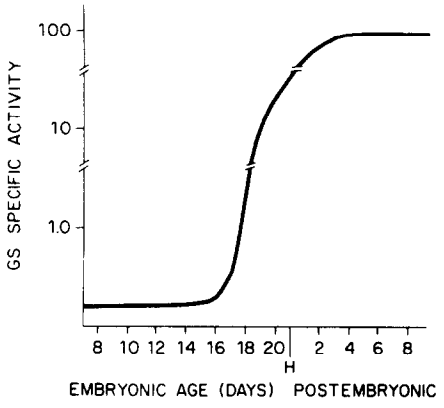

Fig. 1. The developmental pattern of glutamine synthetase (GS) specific activity in the neural retina of the chick embryo and after hatching (H). From [1].

effect of hydrocortisone. After 24 h in culture, GS activities were examined and it was found that the steroid elicited significant increases in GS levels in premature retinas; even in retinas from 8-day embryos the steroid elicited induction of the enzyme. This precocious induction of GS by hydrocortisone was specific only for the neural retina and was not obtained in other embryonic tissues. Therefore, we conclude that in the retina, an enzymic differentiation which normally occurs late in development can be induced considerably ahead of the normal time, *i.e.* in highly premature cells, by supplying them with the steroid inducer. This implies that the cytoplasmic receptors for this corticosteroid are present in embryonic retina cells long before these receptors are normally scheduled to function in GS induction (and, in fact, before a functional adrenal cortex is presented in the embryo); and furthermore, that the genes involved in the induction of GS in the retina can respond to the steroid signal long before the cells had reached the appropriate stage of development.

The dose–response relationships between different corticosteroids and GS induction *in vitro* in retinas from 12-day chick embryos have been studied [1]. The induction of retina GS is essentially limited to 11-β-hydrocorticoids (Fig. 2), hydrocortisone being the most effective inducer. Other hormones do not induce GS in the retina, nor is GS inducible by cyclic AMP or its derivatives [2].

HYDROCORTISONE RECEPTORS IN RETINA CELLS

It has been shown in several systems that interactions of steroids with target cells involve binding of the hormone to cytoplasmic receptors [3, 4]. It was, therefore, obviously important to demonstrate whether hydrocortisone receptors are present in embryonic neural retina and to determine how their level in these cells changes with embryonic development. Using hydrocortisone as the binding steroid, cytosol preparations from retinas, and the charcoal assay or DEAE-Sephadex column chromatography for determinations of amounts of hydrocortisone receptors, we found [5] to some surprise, that receptor level in the retina is very high in young embryos and declines gradually with age (Fig. 3); it then shows a transient increase around day 16–17, *i.e.* at the time of the normal rise of GS in the embryonic retina. Quite different age profiles for hydrocortisone receptors were found in the brain and liver of the chick embryo in which GS is not inducible, and in serum (Fig. 3). Therefore, the developmental profile of hydrocortisone receptors in the embryonic neural retina is quite unusual. We cannot explain, at present, the significance of the high level

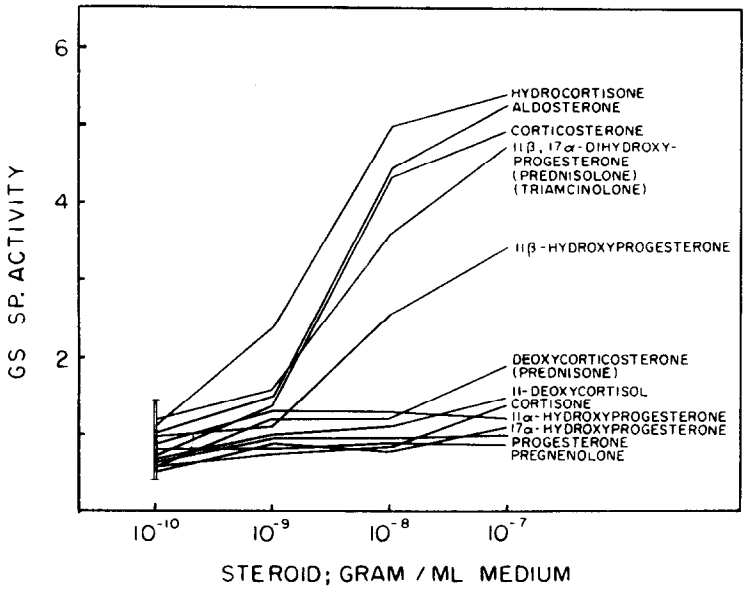

Fig. 2. Precocious hormonal induction of GS in cultures of neural retina tissue from 12-day chick embryos; dose-response effects of various steroids during 24 h in culture. From [1].

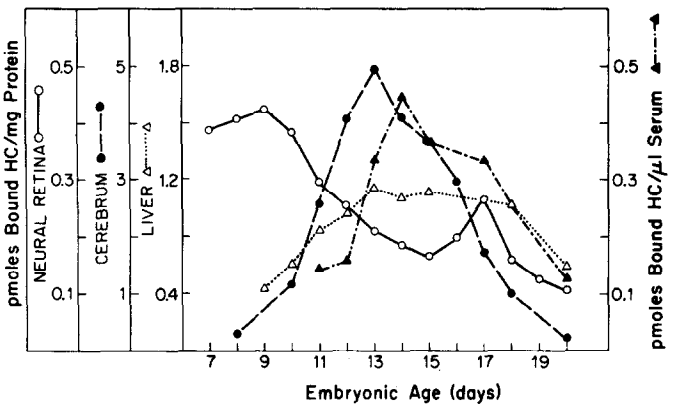

Fig. 3. Developmental profiles of receptor activity for hydrocortisone in different tissues and in serum of chick embryos. From [5].

of hydrocortisone receptors in the very young retina cells, long before the existence of a functional adrenal in the embryo. While their presence in the premature retina is consistent with the precocious inducibility of GS, their high level at early stages of development and their subsequent decline suggest that these proteins may have additional, as yet unknown functions in the early differentiation of neural retina cells.

In addition to their unique developmental profile, hydrocortisone receptors in the retina differ in still other ways from hydrocortisone receptors in other tissues of the chick embryo. First, our evidence shows [5] that embryonic retina contains two major classes of hydrocortisone-binding-sites, a low affinity and a high affinity site which lack co-operativity and differ significantly in their equilibrium dissociation constants for hydrocortisone binding. In the embryonic brain a single site predominates. This conclusion was derived from column-chromatography analysis of hormone–receptor complexes, and from Scatchard analysis of receptor saturation data [5]. Second, in contrast to hydrocortisone receptors from other tissues of the chick embryo, the retina receptors are highly sensitive to unoptimal temperatures and pH. They are readily inactivated above 38°C; they lose activity below pH 7·0 (Fig. 4).

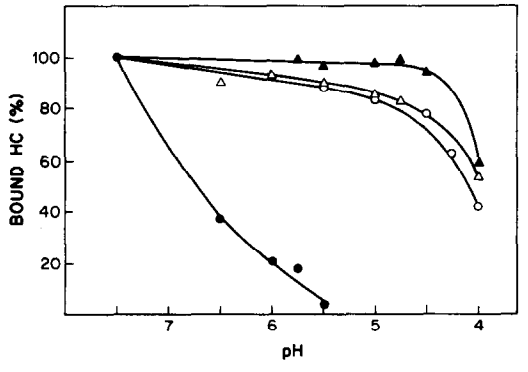

Fig. 4. Sensitivity to low pH of the activity of hydrocortisone receptors from embryonic neural retina and other tissues. Portions of cytosol preparations were adjusted to the indicated pH values, centrifuged, and the supernatant was readjusted to pH 7·5. The total hydrocortisone binding activity remaining after this treatment was expressed as percentage of the untreated control. Receptor preparations were from retina (●——●), liver (▲——▲), cerebrum (○——○), and optic tectum (△——△). From [5].

Such tissue-type differences in the properties of hydrocortisone receptors, may be related to the specific effects of the hormone, *i.e.* to the fact that hydrocortisone induces GS only in the retina, and induces other enzymes in other tissues. One could imagine that differences in receptor properties may result in different affinities of the receptor–hormone complex for specific parts of the genome and this could lead to different gene expressions. Suggestive in this direction are findings that isolated retina nuclei bind hydrocortisone–receptor complexes from retina cytosol, but do not bind hydrocortisone receptors from the brain [6].

GENE EXPRESSION IN GS INDUCTION

Turning now to the question of gene control in this system, it is known that the steroidal induction of GS in the retina requires transcription and results in an increase in the amount of enzyme-specific RNA templates; the consequent rise of GS activity is due to *de novo* synthesis and accumulation of the enzyme [7, 8, 9, 1]. Radioimmunoprecipitation measurements with anti-GS antiserum have shown that, shortly after the addition of the steroid newly synthesized GS begins to accumulate and its level increases 10-fold in 24 h. Measurements of the rates of GS synthesis (Fig. 5) showed that the hormone elicited a rapid increase in the rate of GS synthesis, so that after 8 h of induction the rate was 15 times greater than in controls. Finally, immunoprecipitation of nascent enzyme from pulse-labeled polysomal profiles of the retina (Fig. 6) showed that after induction there was significantly greater precipitation in the region of 12–15 polyribosomes [10]. Since this polysomal region corresponds to the estimated size of mRNA for GS, this evidence further indicates that GS induction involves an increase in the amount

of templates active in the synthesis of this enzyme. Consistent with this conclusion is the fact that GS induction is prevented by blocking RNA synthesis at the time of hormone addition [7, 11]. Taken as a whole these and other results [6, 9, 10, 12] have led to the working assumption that GS induction is elicited by the hydrocortisone–receptor complexes entering the nucleus, binding to specific sites on the chromatin and thereby effecting transcriptional events which lead to accumulation of active templates for GS synthesis [1].

BINDING OF HYDROCORTISONE RECEPTOR COMPLEXES TO NUCLEI

To test this assumption, we have begun to study the association of the steroid–receptor complexes with the retina genome. We determined that when retina tissue is supplied with hydrocortisone, receptor–hydrocortisone complexes become rapidly translocated from the cytoplasm into the nuclei [12]; with increasing doses of hydrocortisone, free receptors for this steroid are depleted from the cytosol and, at the same time, the amount of receptor–steroid complexes in the nuclei increases (Fig. 7). This increase in the nuclear content of steroid–receptor complexes corresponds to increases in the level of GS.

Having entered the nucleus, some of the steroid–receptor complexes are sequestered, or bound, to nuclear acceptor sites. To estimate the number of these nuclear binding sites in retina cells, we used an optimalized cell-free system which contained isolated retina nuclei, retina cytosol and tritiated hydrocortisone [6, 12]. Figure 8 shows an experiment in which different amounts of nuclei were used. The results indicate that binding of receptor–complexes

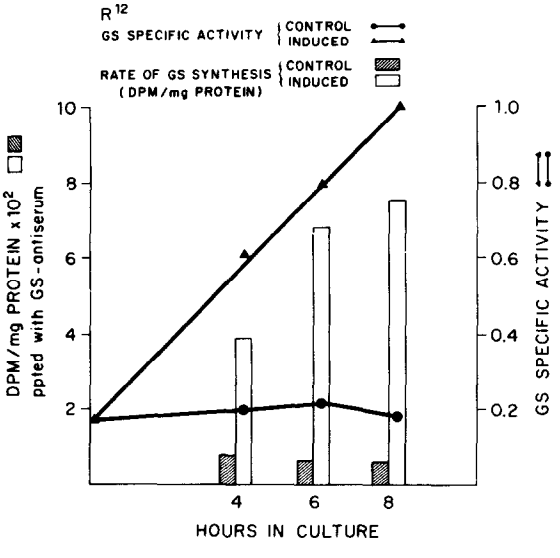

Fig. 5. Induction of GS in cultures of 12-day embryonic chick retina tissue: changes in the rate of enzyme synthesis measured by immunotitration of radioactively labeled (15 min) enzyme; for experimental details see Ref. [9]. Comparison of GS specific activities and rates of GS synthesis in induced and control retinas. From [9].

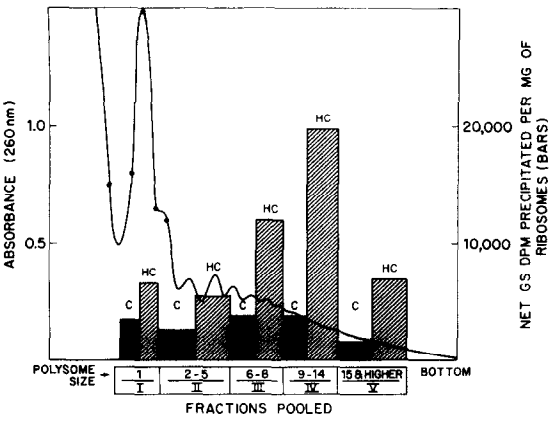

Fig. 6. Immunoprecipitation by anti-GS gammaglobulin of fractionated polysomes from induced and uninduced retinas. For details see Ref. [10]. C—control (uninduced); HC—induced with hydrocortisone. From [10].

to nuclear sites is a function of nuclear concentration; *i.e.* at low concentrations of nuclei the binding sites became saturated and binding plateaued; at higher concentrations of nuclei, the amount of receptors in the cytosol became limiting with respect to the available nuclear sites.

The nuclear binding sites became saturated at approximately 0·5 pmoles of steroid per mg DNA. Assuming that one receptor hormone complex binds to one nuclear site, we calculate from the available data that the total number of nuclear binding sites for this receptor–complex is in the range of 1500 per retina nucleus. This number is considerably smaller than that reported for dexamethasone–receptor binding-sites in hepatoma nuclei [13], or in rabbit fetal lung cells [14]. The question remains how many of these 1,500 nuclear binding sites in the retina genome which are occupied under conditions of saturation with the steroid–receptor complexes are actually involved in the induction of GS.

THE EFFECT OF PROFLAVINE

Of the several possible approaches to this problem, we will mention one which offers some promise. It was discovered that the diaminoacridine proflavine, which intercalates into DNA, blocks preferentially GS induction in the retina at a pre-translational level [15]. A concentration of proflavine which prevents almost completely GS induction, inhibits less than 10% of total RNA and protein synthesis. While the inhibitory effect of proflavine in the retina is not limited exclusively to GS, it is definitely not widespread or lethal to the cells. We assumed that proflavine blocked GS induction by competing with hydrocortisone–receptor complexes for nuclear binding sites [15]. If this were the case, treatment of retina with proflavine should reduce nuclear binding of the steroid–receptor complexes. This proposition was examined in the cell-free system and we found

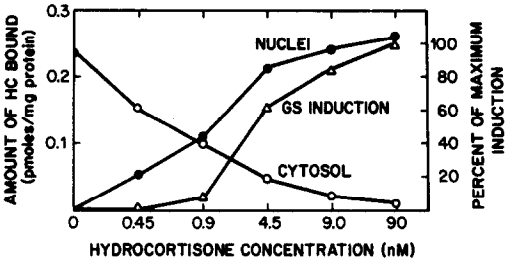

Fig. 7. Distribution of bound radioactive hydrocortisone (HC) in cytoplasm and in nuclei of embryonic retina in relation to GS induction, following a 60 min incubation of the retina in the presence of [^{3}H]-HC. GS activity was assayed in parallel cultures. For details see Ref. [12].

that, in fact, proflavine reduced to 20% nuclear binding of the receptor complexes [12].

It would thus appear that of the 1500 nuclear binding sites for the steroid–receptor complexes only 300 represent the fraction which includes those sites that are specifically related to GS induction. We expect that refinements of this experimental approach, based on the use of selective inhibitors of gene expression may further narrow down this number closer to those sites that are involved specifically in GS induction.

CELLULAR ORGANIZATION AND GS INDUCTION

I would like to mention briefly yet another regulatory mechanism relevant to hormonal control of GS in the retina. Having seen that the enzyme can be induced *in vitro* in intact retina tissue, we now ask if it can be induced also in separated, individual retina cells; in other words, are specific cell contacts and tissue organization involved in the hormonal regulation of this gene expression, or does each cell respond to the steroid inducer independently of the others.

To answer this question, cell suspensions were prepared from retinas of 10-day chick embryos, the cells were placed in Petri dishes to make monolayer cell cultures, and hydrocortisone was added to these dispersed cell cultures. The results (Fig. 9) showed that in the dispersed cells GS induction failed to take place [16, 17]. This failure of GS induction was not

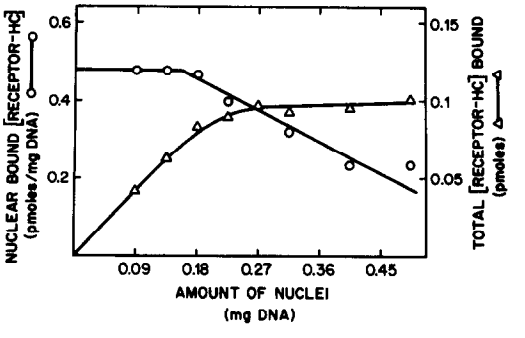

Fig. 8. Binding of hydrocortisone-receptor complex (constant amount) by isolated retina nuclei (increasing amounts). For details see Refs. [10, 12].

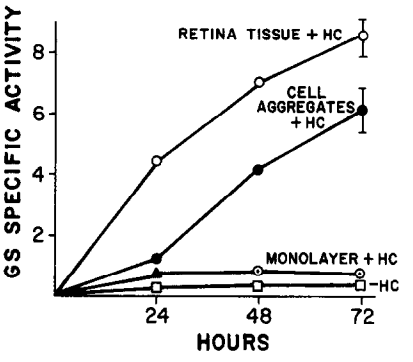

Fig. 9. Induction of GS by hydrocortisone (HC) in cultures of embryonic (10-day) neural retina tissue, primary cell monolayers and cell aggregates. For details see Refs. [16–18].

due to trivial causes; the steroid hormone was taken up by the cells, and the enzyme did not leak out. Induction did not take place because in the dispersed cells the rate of GS synthesis did not increase above the non-induced level. However, if the dispersed cells were reaggregated into multicellular clusters within which they re-establish histotypic cell contacts and reconstruct retina tissue, they became again responsive to GS induction. Figure 9 shows the correlation between levels of GS induction in the three states of cellular organization: bell monolayers, tissue, and cell aggregates [18].

Further analysis of this situation led us to conclude that in the neural retina, tissue-specific cell contacts and retinotypic cell organization are a critical factor in cell responsiveness to the hormonal induction of GS. If the characteristic multicellular organization of the retina is disrupted, the individual cells are not responsive to GS induction. This implies that conditions at the cell surface may affect the competence of these cells to react to steroidal signal intended to evoke a specific gene expression [18]. The dependence of GS induction on histotypic cell organization may reflect the close link between this induction and retina differentiation: in apparent contrast, enzymes involved primarily in homeostatic adaptation can be induced in monolayer cell cultures [4].

The mechanisms which control this critical relationship between cell contact, cell surface properties and induction by a steroid hormone of a differential gene expression in embryonic retina cells are unknown. The significance of this problem obviously extends beyond this particular system. The involvement of the cell surface in regulating various cell functions is now generally recognized; its presently suggested role also in the regulation of cell responsiveness to corticosteroid action raises important questions which merit further study in this and other systems.

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