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INDUCTION OF CHOLINERGIC ENZYMES IN CHICK CILIARY GANGLION AND IRIS MUSCLE CELLS DURING SYNAPSE FORMATION

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SUMMARY

1. In chick ciliary ganglia and irises, cholineacetyltransferase (ChAc) and acetylcholinesterase (AChE) activities were measured from the fifth day of incubation until 1 week after hatching. The changes in enzyme activity were correlated in time with previous electrophysiological and morphological findings of synapse formation in these tissues.

2. At Stage 26 (Hamburger & Hamilton, 1951; before synapse formation in the ganglia) low activities of ChAc (12 ± 4 [mean $\pm$ S.E.] p-mole ACh synthesized/hr) were measured in the iris nerve terminals, indicating that ganglion cells are biochemically differentiated, immediately after cell migration is completed. The specific activities of ChAc and AChE rose during development and these increases were closely related to the onset and maturation of ganglionic and iris synaptic transmission. These increases in enzyme activities can be used in cholinergic synapses as an index of synapse formation.

3. The 200-fold specific increase of ChAc in iris nerve terminals which occurs at Stage 34 probably reflects an increase in synthesis of the enzyme in ganglion cells and suggests that the formation of the iris neuromuscular junction triggers the enzyme induction. It is implied that the cell responds to a signal ascending the axon from the terminal.

4. The initial increase of AChE specific activity in the ganglion occurs after transmission is established in all cells between Stage 30 and 34 and is mainly due to enzyme synthesis by the ganglion cells. In the iris there is a twofold increase in specific activity after the formation of neuromuscular junctions which probably reflects enzyme induction in the muscle

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subneural region. It is concluded that the specific induction of AChE in post-junctional cells is due to an influence of the prejunctional element.

5. During synaptic formation in the ciliary ganglion, reciprocal interactions between the neurones and their targets result in the induction of ChAc in the prejunctional elements and AChE in the post-junctional cells.

INTRODUCTION

In previous reports the onset and development of cholinergic transmission in chick embryo ciliary ganglia and iris neuromuscular junctions were described (Landmesser & Pilar, 1972, 1974*a*, *b*). Subsequently, it was established that coinciding with the formation of iris junctions at Stage 36–40 (Hamburger & Hamilton, 1951), the ciliary ganglion cells showed a characteristic organization of ribosomes into polysome clusters and a dramatic increase in rough endoplasmic reticulum, R.E.R. (Pilar & Landmesser, 1976). This was followed by a normally occurring period of cell death, and it was concluded that ganglion cells probably die because they fail to form, or at least maintain, a sufficient number of synapses with limited peripheral targets (Landmesser & Pilar, 1974*a*, *b*, 1976). The above developmental events appeared to be due to interactions between ganglion cells and the peripheral target organ, the iris.

To further characterize these cellular interactions biochemically, the activities of cholineacetyltransferase (ChAc) and acetylcholinesterase (AChE) were measured in ganglia and irises from the fifth day of incubation until 1 week post-hatching. The changes in enzyme activity were correlated in time with the previous electrophysiological and morphological findings (Landmesser & Pilar, 1972, 1974*a*, *b*, 1976; Pilar & Landmesser, 1976).

A similar study on the chick ciliary ganglia was recently published by Sorimachi & Kataoka (1974), but most of their measurements were done on only the later stages of development, and the enzyme distribution in pre- and post-ganglionic elements was not ascertained.

In the present study a special effort was made to assay enzyme activity in embryonic stages before and at the onset of synaptic transmission. In order to overcome the difficulty inherent in this parasympathetic ganglion (that cholinergic enzymes are localized in both pre- and post-synaptic elements), enzyme activities in the iris muscle, which contains the nerve terminals of the ciliary cells, were also determined. Since both enzymes are transported from the soma to the terminals, the presence of ChAc and AChE in these nerve endings was used as an indicator of the enzyme activity in the ganglion cell soma. Further unpublished observations on this system have suggested that the activity of ChAc in the nerve

terminals is proportional to its activity in the cell soma (H. Uchimura, G. Pilar, E. Giacobini and J. Suszkiw, unpublished). Therefore, a change in this enzyme activity in the iris is probably an expression of similar responses in the ganglion cell somas.

Reciprocal modulatory influences between pre- and post-synaptic elements resulting in an alteration of enzymes related to neurotransmission have been described in developing sympathetic ganglia (Black, Hendry & Iversen, 1971, 1972; Thoenen, Saner, Kettler & Angeletti, 1972; Dolezalova, Giacobini, Giacobini, Rossi & Toschi, 1974; Hendry, 1975), in combined cultures of spinal cord and muscle cells (Giller, Schier, Shainberg, Fisk & Nelson, 1973), and between tectal and retinal cells also in culture (Adler & Teitelman, 1974).

In the present paper similar interactions are described between parasympathetic elements and further evidence for the influence of the peripheral target organ on the maturation of nerve cells was obtained.

A brief report of these findings has appeared elsewhere (Pilar, Chiappinelli, Uchimura & Giacobini, 1974).

METHODS

Biochemical assays were done in ciliary ganglia and irises dissected out from chick embryos from Stage 26 until hatching and in 1-day and 1-week-old chicks in 4° C cold Tyrode solution, buffered with 5% CO_2 in O_2. These conditions were similar to the ones used for the physiological experiments (Landmesser & Pilar, 1972). Fertilized white Leghorn eggs were incubated and at selected times the embryos removed from the shell and staged in the dissecting dish according to Hamburger & Hamilton (1951). Embryos were not sexed, but the chicks were males. Birds were killed by decapitation and pre- and post-ganglionic trunks were cut close to the isolated ganglia, and ciliary nerves were sectioned at the external edge of the iris ring. At early stages iris rings were removed with small portions of choroidal tissue. Two ganglia were pooled at Stages 26–32 and two irises at Stages 24–38. Tissue isolation from the youngest embryos was completed in 30 min and in later stages in 2–5 min. Controls were done in specimens maintained in oxygenated solution for 1 hr, and these determinations were similar to the ones obtained at the shortest times. Tissues were transferred directly into small ground glass homogenizers kept in liquid N_2. The samples were homogenized, ½ hr later, in 0·5% Triton X-100 and 0·05% bovine serum albumin solution at 2° C. Maximal AChE and ChAc activity was obtained with these concentrations. Homogenates were stored at −65° C until the assays were performed, always within 2 days.

Biochemical assays. ChAc activity was assayed using the radiochemical method of Fonnum (1975): 2 µl. homogenate were added to 20 µl. buffer substrate solution. The final concentrations (in mM) were: [1-^{14}C]acetyl-coenzyme A, New England Nuclear, Boston, Mass., 0·1 (specific activity 50–55 cm/mM); choline bromide, 10; eserine sulphate, 0·2; NaCl, 300; and 0·05% (v/v) thioglycolic acid in 50 mM sodium phosphate buffer, pH 7·40. The concentration of substrate used (0·1 mM) gave maximum enzyme activity and no further increase was obtained when 0·2 mM acetyl-coenzyme A was added (see Fonnum, 1975).

After 30 min incubation at 38° C, labelled acetylcholine, ACh, was extracted with

kalignost in acetonitrile into toluene scintillation fluor and radioactivity was counted by liquid scintillation spectroscopy at 92% efficiency.

The method of Koslow & Giacobini (1969) was used to determine AChE enzyme activity with the following modification: a kalignost (sodium tetraphenylboron) solution for ACh extraction was used instead of reinneckate (Fonnum, 1969). The final concentrations of the incubation mixture were: 1·0 mM acetylcholine iodide [acetyl-1-^{14}C], New England Nuclear (specific activity 3–5 mc/mM) and 90 mM sodium phosphate buffer, pH 7·2. Buffer substrate solution, 20 μl., was added to 2 μl. homogenate in a microtube, shaken thoroughly and incubated for 15 min at 30° C. Iso-ompa (tetraisopropylpyrophosphoramide) was added to the buffered substrate to inhibit non-specific cholinesterase (butyrylcholinesterase, BuChE) activity. Maximum inhibition of butyrylcholinesterase was obtained with 1×10^{-5} M iso-ompa, and the range of concentration explored varied from 5×10^{-4} M to 1×10^{-5} M. The cholinesterase activity in the presence of the inhibitor in the incubation medium was 95–100% of the total activity obtained without iso-ompa, which suggested that practically all enzyme activity measured in the tissues was due to AChE.

After extraction of ACh with kalignost, the radioactivity of [^{14}C]acetate produced was counted in a solution of 4 ml. ethanol and 10 ml. toluene fluor scintillation cocktail with 89% efficiency. In different samples homogenized in distilled water total protein was assayed by the method of Lowry, Rosenbrough, Farr & Randall (1951) using bovine serum albumin as a standard. All chemicals were reagent grade.

The enzyme activities (mean $\pm$ S.E. of mean) are expressed as p-mole ACh synthesized/hr for ChAc, or n-mole ACh hydrolysed/hr for AChE either: per ganglion; per microgram protein; or per cholinergic unit (enzyme activity per ganglion cell with attached synapses, not shown). Because the cellular elements of the tissues grow at different rates, and because of changes in enzyme localization during development as well as the presence of cell death in some stages of embryogenesis, it was difficult to decide which of the three ways of expressing enzyme activity gave the most meaningful estimation for relation to the developmental events described in this paper. However, since similar qualitative changes were shown with all three units of measure used, this problem did not hinder the interpretation of results. It should be noted that the ciliary ganglion contains two distinctly grouped cell populations, ciliary and choroid. The choroid cells innervate the vascular smooth muscle of the eye. Although the time at which these cells form neuromuscular junctions has not yet been determined, preganglionic fibres establish ganglionic synapses with both choroid and ciliary cells simultaneously (Landmesser & Pilar, 1972) and cell death had a similar time course for both populations (Landmesser & Pilar, 1974*b*). Further, when the portion of the ganglion containing predominantly choroid cells was assayed separately (A. Burt and C. Narayanan, unpublished) the enzyme activities were found to be similar to the ciliary portion. It is therefore likely that the changes in enzyme activity described in this paper occurs in both cell populations.

Time course of physiological and morphological changes in ganglia and irises during embryogenesis. Since most of this paper's conclusions will be based on the correlation of the enzyme activities with the previous findings on the development of these tissues, an illustration of the sequence of these events is shown in Fig. 1. The graphs were re-plotted on the same time scale of embryonic development from previously published data (Landmesser & Pilar, 1974*a*, Fig. 2, and *b* Figs. 2 and 7). Fig. 1*A* shows the total number of cells in the ganglia; *B*, percentage of ganglionic transmission; and *C*, the formation of neuromuscular junctions between the ciliary cells and the iris muscle. The critical period of ganglionic synapse formation occurs from Stage 26 to 31 when all ganglion cells can be synaptically activated. Ganglion cell death occurred between Stages 34 and 39 coinciding with the genesis of iris junctions.

RESULTS

As necessary background for the present study, the distribution of enzymes in the cellular elements of ganglia and irises was determined. This was required since both pre- and post-synaptic cells are cholinergic and therefore have a similar complement of cholinergic enzymes involved in the synthesis and break-down of ACh. A short description follows, these

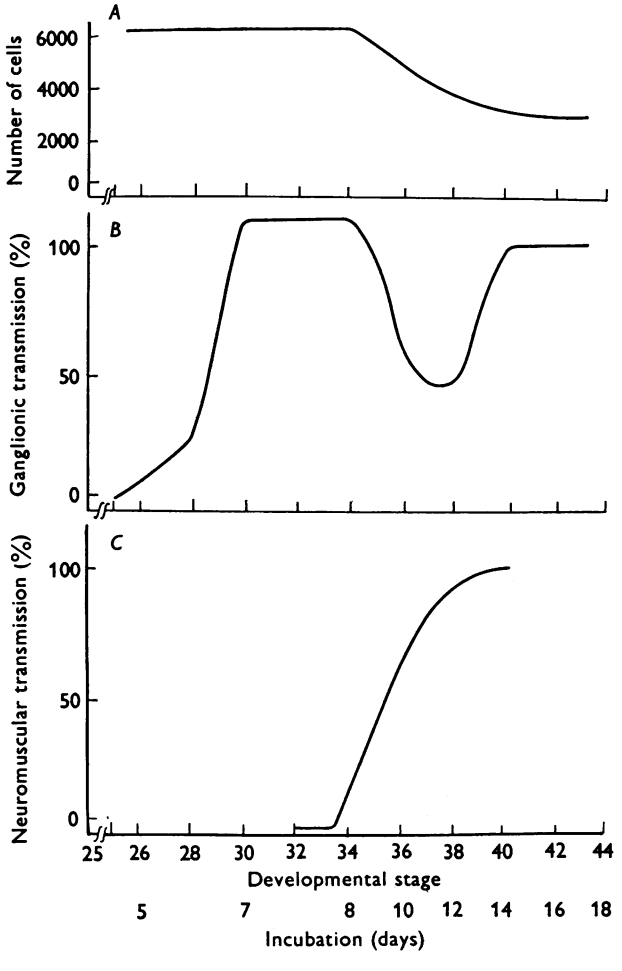

Fig. 1. Morphological and physiological correlates of synaptic transmission during embryonic development. *A*, number of cells in ciliary ganglion; *B*, percentage transmission through ciliary ganglion synapses; *C*, percentage transmission through iris neuromuscular junctions; from Landmesser & Pilar (1974*a*, *b*).

results being published in brief form elsewhere (Suszkiw, Uchimura, Giacobini & Pilar, 1973).

In order to differentiate presynaptic and post-synaptic enzymes in the ciliary ganglion, the fibres entering the ganglion from the central nervous system were severed close to the ganglion. Enzyme activities were

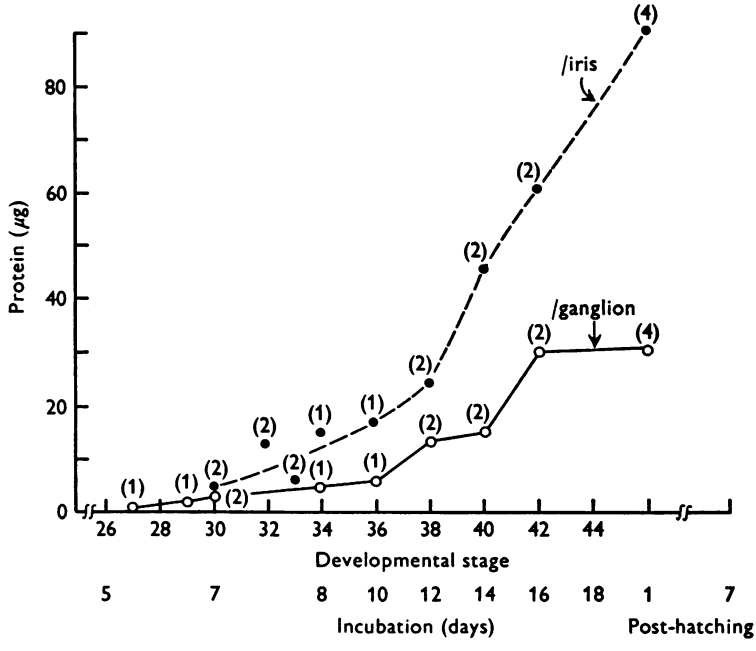

Fig. 2. Developmental pattern of total protein increase per ganglion (continuous line) and per iris (dashed line). Numbers in parentheses indicate number of samples.

determined in the decentralized ganglion 3 days after the operation, at which time all enzyme activity measured was assumed to be post-synaptic. Similarly, to separate the contributions of the ciliary nerve terminals and the iris muscle cells to enzyme activities in the iris, the ciliary nerves were severed and the denervated iris assayed for enzyme activities 3 days later.

In the ciliary ganglion of adult white King pigeons 60% of the total ChAc measured is localized in the preganglionic elements; the rest, 40%, is post-ganglionic. Thirty % of the AChE is presynaptic, while most of the enzyme (70%) is concentrated in the ganglion cells. Control experiments were done in ganglia isolated from 1-week-old chicks and a similar distribution of enzyme activities was found.

In the pigeon iris practically all ChAc and 15% of the total AChE activity measured is in the ciliary nerve endings. The remaining 85%

AChE is localized in the iris muscle. In the chick iris identical localization for ChAc was obtained. However, when AChE was assayed in specimens taken from both albino and white Leghorn chicks, as much as 40% of the enzyme disappeared after degeneration of the ciliary nerve terminals. No explanation other than species differences could be given to interpret this discrepancy. While the remaining 60% was clearly post-synaptic, its subcellular localization in the muscle was not determined. In striated muscle 50% of the AChE biochemically measured is localized in the end-plate regions, the remainder is associated with the sarcoplasm (Giacobini & Holmstedt, 1960; Kovács, Kojér & Balogh, 1961).

The total protein measured in ganglia (continuous line) and irises (dashed line) is shown as a function of embryonic age in Fig. 2. As expected there is a steady increase from early stages with a more rapid growth after 10 days.

ChAc activity in nerve cells during development

In Fig. 3*A* are plotted the changes in ChAc activity in ganglia during the entire developmental period studied. Log. scales were used in the ordinate of this and similar graphs (see also Fig. 5), because of the large (in this case 1400-fold) apparently exponential increase in enzyme activity (from 53 ± 3 p-mole/hr at Stage 26 to $70{,}100 \pm 4700$ p-mole/hr at 7 days post-hatching). It is worth while to note that this graphic representation visually enhances the deviation from the mean especially for the smaller values (see Fig. 3*B*). A similar rise is also observed when the activity is expressed per microgram protein, but now the increase is approximately eightyfold. The early increment between Stages 28 and 32 was not seen by Sorimachi & Kataoka (1974) who found instead a rise in ChAc only after day 14. Our enzyme values are 30% lower than the ones obtained by these investigators at early stages and 10–20% higher at later stages. This might consequently explain their failure to observe the initial increase of specific activity.

One possible explanation for the discrepancies can be advanced. If during the dissection of the minute ganglia small portions of pre- and post-synaptic nerves are left attached, the total enzyme activity measured will be increased to a greater extent at earlier stages. At early times the activity of ChAc in ganglia is so small that the attached nerves contribute significantly to the total values measured.

In Fig. 3*A* it can be seen that during the period of cell death, the specific activity remains initially unchanged followed by a sudden gain at Stage 38.

In order to localize the enzyme changes shown in Fig. 3*A* which occur in both preganglionic and post-ganglionic cells, the activity of the ciliary nerve terminals (Fig. 3*B*) was measured. The total activity is initially low and steady until Stage 32 when a sudden 200-fold increase takes place. If the time course of the initial levels of activity in Fig. 3*A* and *B* are

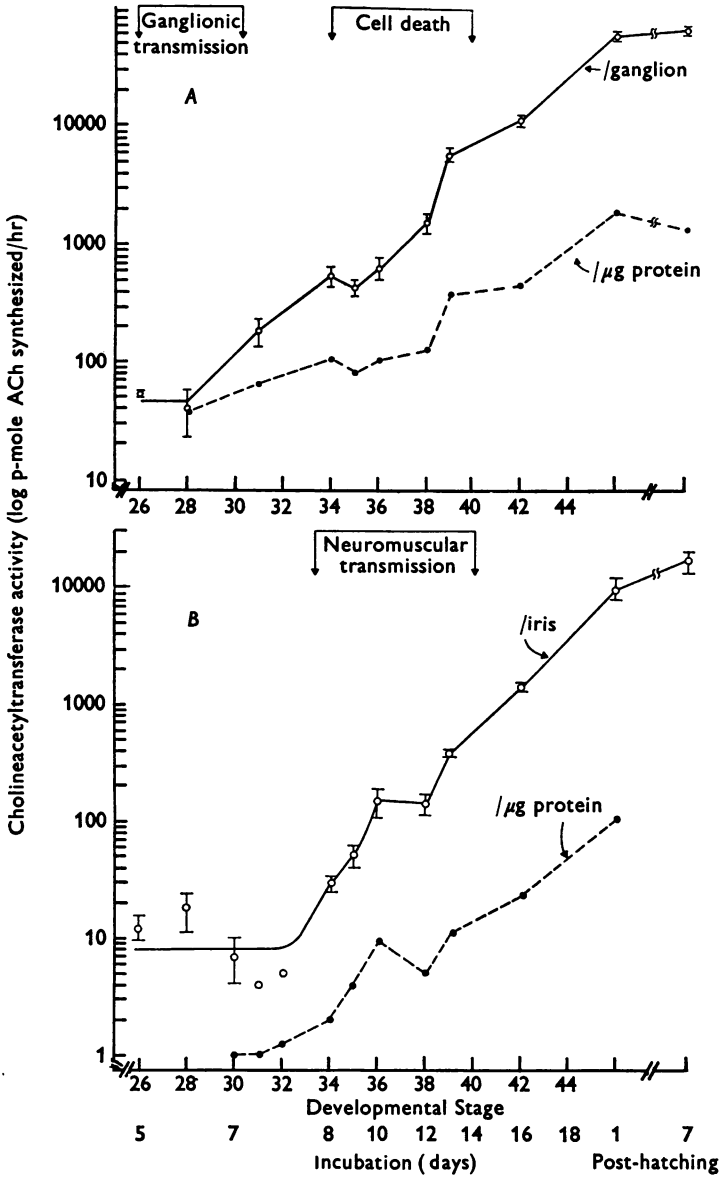

Fig. 3. Variations of ChAc activity during development. *A*, ChAc in ciliary ganglion, expressed per ganglion (continuous line) and per microgram protein (dashed line). *B*, ChAc in iris, expressed per iris (continuous line) and per microgram protein (dashed line). Vertical bars represent mean ± S.E. of mean in this and following Figures.

compared, it is possible to conclude that the observed increase in the ganglion preparation (Fig. 3*A*) up to Stage 32 must be occurring in the preganglionic elements. Thereafter there is a contribution of the post-synaptic cells, since ChAc activity increases in the iris nerve terminals (Fig. 3*B*). Since no synthesis of this enzyme occurs in the nerve terminals, the increased ChAc activity which takes place in the developing terminals of both the preganglionic and the ganglion cells is due to enzyme transported by axonal flow. The augmented activity in the neurone somas, however, probably reflects an increment in enzyme synthesis.

In Fig. 4 the relationship between ChAc in terminals and cell somas is further illustrated at the early critical periods and correlated with the time course of synapse formation electrophysiologically studied.

In the iris terminals (Fig. 4*A*) before the initiation of neuromuscular transmission (Stages 26–34), when axons (Landmesser & Pilar, 1974*b*) are in close proximity to epithelial cells (the precursors of the striated muscle, Lucchi, Bortolami & Callegari, 1974), weak but detectable ChAc activity is measured (12 ± 4 p-mole ACh synthesized/hr per iris). Its presence is an indication that ganglion cells are synthesizing the enzyme.

Coinciding with the initiation of neuromuscular transmission, there is first at Stage 34 a small but steady increase of ChAc followed by a marked increment at Stage 38. These temporal relations between enzyme development and junction formation suggest that iris synaptogenesis triggers an increased synthesis of ChAc in cell somas and a consequent axonal transport to the terminals. This implies that the cell responds to a signal ascending the axon from the terminal.

The possibility that the increase is triggered by anterograde influence of the preganglionic central neurones (located in the accessory nucleus) on the ganglion cells can be eliminated by analysing Fig. 4*A* and *B*. By Stage 30 all ganglion cells have functioning synapses on them (Fig. 4*B*, dashed line) but there is no increase of ChAc (continuous line) in their nerve terminals until Stage 36. In other words, there is not an immediate synthesis of the enzyme in ganglion cells elicited by ganglion synaptogenesis.

The early ChAc increase in preganglionic elements occurring from Stage 26 to 34 could also be triggered by synapse formation, similar to the feed-back mechanism operating in the iris neuromuscular junction.

Changes of AChE activity with embryonic age

The AChE activity expressed per ganglion increases sevenfold in the short period between 6 and 8 days (from 41 ± 13 n-mole/hr at Stage 26 to 269 ± 18 n-mole/hr at Stage 34) and thereafter reaches mature values (587 ± 57 n-mole/hr at 7 days post-hatching) (Fig. 5*A*, continuous line).

The AChE specific activity increase is in time course similar to the total activity but of smaller magnitude (fourfold) except that after Stage 34 there is a substantial decrease. This indicates that during cell growth the control of AChE synthesis is independent of the synthesis of proteins in general. Two times higher values of AChE activity and a different time

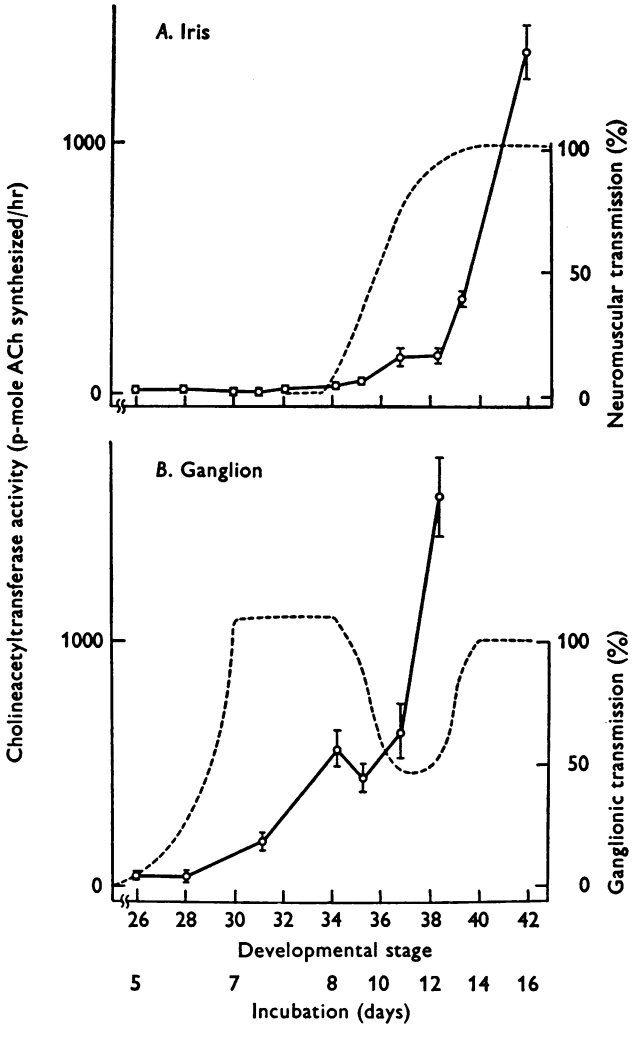

Fig. 4. Relationship between ChAc activity and percent transmission. *A*, ChAc activity per iris (continuous line) compared with percent neuromuscular transmission through iris (dashed line). *B*, ChAc activity per ganglion (continuous line) compared with percent ganglionic transmission (dashed line).

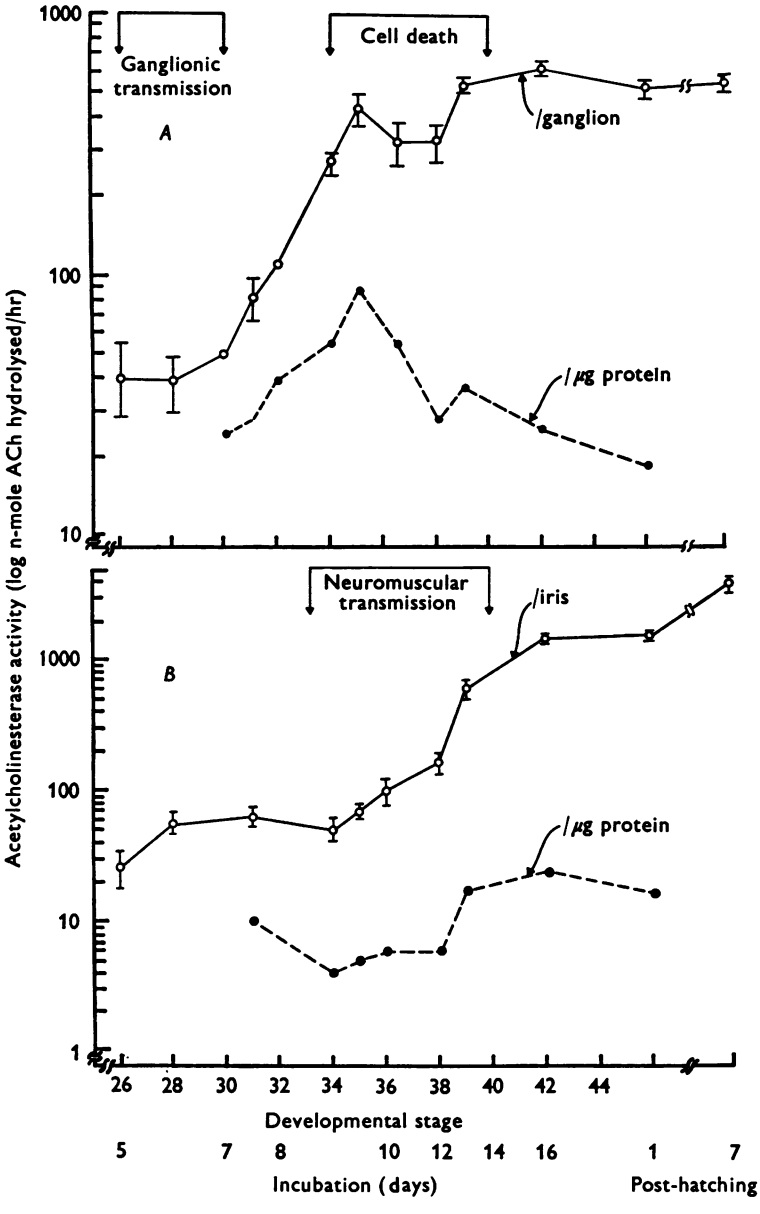

Fig. 5. Variations of AChE activity during development. *A*, AChE in ciliary ganglion, expressed per ganglion (continuous line) and per microgram protein (dashed line). *B*, AChE in iris, expressed per iris (continuous line) and per microgram protein (dashed line).

course were reported by Sorimachi & Kataoka (1974) in their investigation. They observed a specific enzyme activity decrease only after hatching, and had no measurements for the early stages.

In the iris (Fig. 5*B*) steady values were found up to Stage 34, after which there was an increment in total AChE on the order of fifteenfold. When, on the other hand, specific activity was computed, there was only

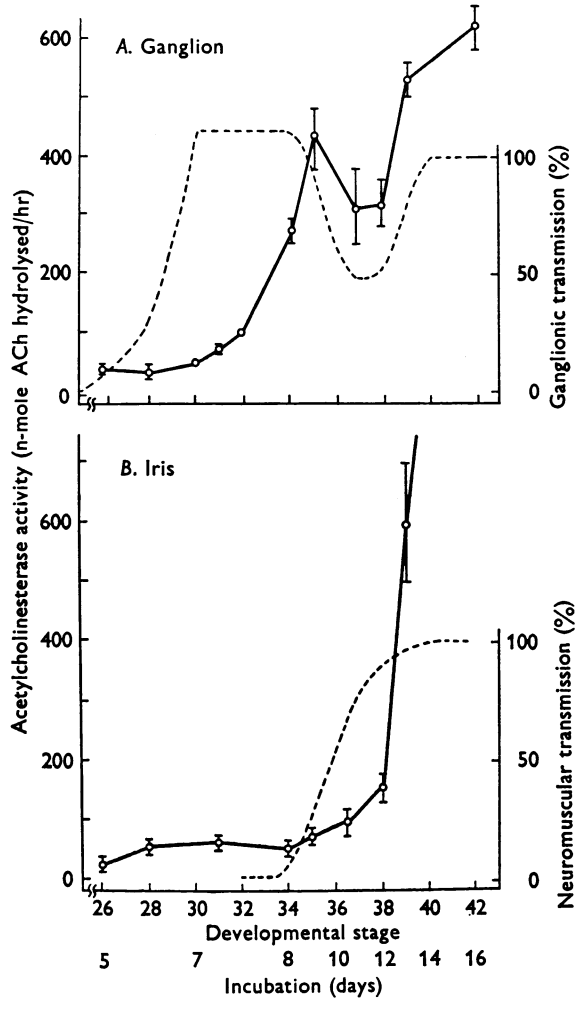

Fig. 6. Relationship between AChE activity and percentage transmission. *A*, AChE activity per ganglion (continuous line) compared with percentage ganglionic transmission (dashed line). *B*, AChE activity per iris (continuous line) compared with percentage neuromuscular transmission through iris (dashed line).

a twofold rise at Stage 38, the values before and after the increase remaining steady. It seems likely that during the steady periods, the rate of AChE synthesis is similar to the rate of total protein synthesis.

In Fig. 6 total AChE activity is plotted together with the time sequence of synapse formation in ganglia (*A*) and irises (*B*). It can be seen that the ganglionic enzyme activity increase occurs after transmission has been established in all ganglion cells at Stage 30 (dashed line), and that during the period of cell death (transmission decrease in Fig. 6*A*, see also Fig. 1*A* and *B*) there is a decrease of activity of similar time course.

Although the localization of the induced enzymatic activity is uncertain (either pre- or post-synaptic, see first paragraph Results), it can be tentatively concluded that the increments occur in ganglion cells because: 70% of the measured ganglion enzyme activity is post-synaptic in the chick; and the period of AChE decrease closely follows the disappearance of ganglion cells.

If this conclusion is correct, it is possible to interpret the initial small activity increase in the iris between Stage 34 and 38 (Fig. 6*B*) as AChE transported to the nerve terminals from the cell somas. This increase is not due to enzyme induction because there is no specific activity increase during this period (dashed line Fig. 5*B*). However, the increment in specific activity which is seen at Stage 38 and due to induction (Fig. 5*B*) seems to be mainly localized in iris muscle, possibly in the junctional region. The histochemical localization of AChE reaction products in electron microscopic photographs of ciliary axons by day 9 (Stage 35) and in the subneural apparatus of the iris muscle by day 13 (Stage 39) support these interpretations (Lucchi *et al.* 1974).

In conclusion, it is suggested that the specific induction of AChE in ganglia and irises is related to synapse formation, probably takes place in the post-junctional structures, and is due to an influence of the prejunctional elements. Specific induction is defined as a discriminative increase of AChE specific activity.

DISCUSSION

This study demonstrates that the specific increase of ChAc and AChE can be used in cholinergic synapses as an indicator of synapse formation and suggests that the genesis of these junctions triggers the rise of ChAc activity in the prejunctional element, and the induction of AChE in the post-junctional cell. This hypothesis is based on the close temporal correlation between these biochemical measurements and the electrophysiological and anatomical events (Landmesser & Pilar, 1972, 1974*a*, *b*, 1976) occurring in these tissues during development.

Only very low levels of ChAc are necessary to maintain functional ganglionic transmission during the initial stages of synaptogenesis, and at this time the synaptic ultrastructural specializations are minimal (Landmesser & Pilar, 1972, 1976). Later there is a progressive increase of enzyme activity, although the synapses are still probably of low quantal content with few synaptic vesicles present in the terminals (Landmesser & Pilar, 1972, 1974*a*). At Stage 41 there is a large increase of vesicles and synaptic ultrastructural specializations are distinctly observed (Landmesser & Pilar, 1972, 1974*a*, 1976). It seems that maturation of the synapse in the ganglion takes place following the period of cell death (Landmesser & Pilar, 1974*b*) and occurs after the final pattern of neural connexions (Landmesser & Pilar, 1976) has been established. If the observed increase of ChAc activity actually reflects an increase in ACh synthesis, it is possible to suggest that the large number of vesicles which appear at Stage 41 are used to store transmitter which is not needed for immediate release.

The increase in synthesis of ChAc which coincided with the formation of contacts between central neurones and ganglion cells, and between ganglion cells and muscle supports the role of the target organ in triggering the cell into its secretory state. This was also suggested by the coincident organization of ribosomes into polysome clusters and an increase in R.E.R. described in these ganglion cells (Pilar & Landmesser, 1976). The synthesis of ChAc is considered to be a phenotypic expression of a differentiated cholinergic neurone, and indeed, ganglion cells are in fact synthesizing ChAc before they are synaptically connected. It can be concluded that immediately after ganglion cell migration is completed at Stage 25 (Hammond & Yntena, 1958), the nerve cells are electrically (Landmesser & Pilar, 1972) and biochemically differentiated. Therefore, the influence of the neuronal interaction on enzyme control is quantitative rather than qualitative. Furthermore, it has been shown that autonomic nervous system neuroblasts before and during migration are potentially able to differentiate into adrenergic or cholinergic cells (Le Douarin & Teillet, 1974). This and the above suggest that differentiation occurs immediately after cell migration is completed.

The increase of AChE specific activity is chronologically correlated to synapse formation in ganglia. Its localization in synaptic membranes, however, requires further investigation since in light microscope histochemical studies AChE was not found in ganglion cell cytoplasm until Stage 36 (Taxi, 1961), and there is no report of its presence in surface membranes at later stages (Koenig, 1965). It has been suggested that the induction of AChE specific activity is correlated with neuronal growth (Burt, 1968). This hypothesis is not supported by these results since, in

spite of the continued growth and differentiation of the ganglion cells after Stage 34 (long after synapse formation, Hess, 1965; Landmesser & Pilar, 1974*a*), the specific enzyme activity was observed to decrease.

Since the time courses of specific induction for AChE and ChAc in ganglia are different (compare Figs. 3*A* and 5*A*), it is clear that the synthesis of these enzymes is regulated by separate mechanisms. Furthermore, electrical coupling between cells can be eliminated as a vector of the described cellular influences, since in the ciliary ganglion electrical coupling appears only at Stage 38, when the activities have already been enhanced.

During the presentation and discussion of these results it has been assumed that the increased enzyme levels are due to enhanced protein synthesis, but the levels can also be a function of the rate of protein degradation (Pitot & Peraino, 1964, see Schimke, 1974, for review). The precise mechanism of these developmental enzymatic changes remains to be investigated. However, because of the exponential rise in activity, it is likely that the augmented ganglionic enzyme activity is due to a net increase in the number of enzyme molecules resulting from increased synthesis (Schimke, Sweeney & Berlin, 1964). Likewise, the trans-synaptic induction of tyrosine hydroxylase in developing sympathetic ganglia is due to increased synthesis of the enzyme (Black, Joh & Reis, 1974).

Enzymatic regulation also occurs in adult tissues. In the pigeon ciliary ganglion it was shown that the integrity of the presynaptic element is needed in order to maintain the synthesis of ACh (Pilar, Jenden & Campbell, 1973), and ChAc by ganglion cells (H. Uchimura, G. Pilar, E. Giacobini and J. Suszkiw, unpublished).

It is possible that the signal which triggers enzyme induction at the initiation of neuronal contacts is similar to the signal responsible for maintenance of this effect during later stages of development and in adult neurones. In the last two instances synaptic function may play a role. In adult sympathetic ganglia ChAc activity in preganglionic terminals was induced by an increase of action potential frequency in post-synaptic fibres (Oesch & Thoenen, 1973), and trans-synaptic regulation of post-synaptic enzymes has been postulated to be dependent on transmitter, or membrane depolarization, or both (see review by Thoenen, 1974, and Costa, Guidotti & Hanbauer, 1974). Similarly, during development, it has been shown that ACh or its depolarizing effect regulates an increase of tyrosine hydroxylase in superior cervical ganglion cells (Black & Geen, 1974). In the ciliary ganglion system it is possible that ACh may be involved in the induction of AChE.

Another chemical substance which has been shown to be important for

growth and differentiation of the sympathetic neurone is nerve growth factor (Thoenen *et al.* 1972). However, in parasympathetic ganglia nerve growth factor has no effect (Levi-Montalcini & Angeletti, 1968; Coughlin, 1975) and it is therefore unlikely to mediate these enzyme inductions.

The hypotheses described above may explain the effects of the pre-synaptic element on the post-synaptic cell but cannot be applied to the increase in preganglionic ChAc. In developing sympathetic ganglia it has been suggested that initial contacts between neurones are necessary for this effect, although continued differentiation of the post-ganglionic neurone is not required (Hendry, 1975). In our experiments functional synapse formation did in fact coincide with the enzymatic changes. However, it is possible that these two events are not causally related and that both are brought about by a different type of cell interaction.

One possibility would have a similar mechanism to the one suggested for selective adhesion between cells. This would hypothesize that when the pre- and post-ganglionic elements come in contact with each other, complementary membrane molecules, such as enzymes and their substrates, form complexes at the reacting cell surfaces (Roseman, 1974). The reacting neurone membrane would then undergo changes that not only maintain the adhesion between the elements but also trigger enzymatic induction. A specific messenger could be released or a specific site in the membrane activated. These hypothetical reactions can take place simultaneously in both pre- and post-synaptic elements during ganglionic development and induce enzyme formation.

It is conceivable then that both adhesion and synaptic formation can be mediated by the same process and that part of this molecular reaction would be the initial step involved in enzyme induction. This hypothesis requiring experimental confirmation would obviate the need for specific inductor substances and offers a unified mechanism to explain complex interactions occurring between developing neurones.

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