Ultrastructural and histochemical observations in the developing iris musculature in the chick

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SUMMARY

Irises from chick embryos and from 2- and 3-week-old chicks were studied using ultrastructural and histochemical methods in order to clarify the relationship between cell loss in the ciliary ganglion and the establishment of permanent peripheral connections between the ciliary neurons and the iris muscle. The iris muscle undergoes morphological and biochemical differentiation between 11 and 13 days of incubation. This period coincides with the critical period in the development of the ciliary ganglion when massive cell degeneration occurs. During this period, the iris develops typical sarcomeric structure, with AChE activity in the nuclear envelope, Golgi, and the “T” system. At 15 days of incubation AChE activity is found localized in discrete areas on the muscle fiber, forming specific neuromuscular junctions. Between 13 and 15 days of incubation, there is a shift in the localization of AChE activity in the iris muscle, from the sarcoplasmic structures to the junctional membranes.

Few synaptic terminals are observed in the iris musculature prior to 11 days of incubation. There is a marked increase in the number of synaptic terminals between 11 and 13 days of incubation which also coincides with the period of cell loss in the ciliary ganglion. The establishment of neuromuscular junctions at 15 days of incubation corresponds with the period when the number of neurons in the ciliary ganglion has attained the adult level. The time table of the events described above, leads us to conclude that during development only those neurons in the ciliary ganglion which make peripheral contacts survive, and only such contacts differentiate into mature neuromuscular junctions on the iris muscle. This will imply that neurons which are doomed to die, although they may send out fibers to the periphery, do not make peripheral contacts before death.

INTRODUCTION

The important question as to how the temporal and morphological changes of the cells in a neuronal center might influence the sequential development of a peripheral structure they innervate or vice versa, has been a subject of continuing debate. The avian iris is unique in that it comprises striated muscle with parasympathetic innervation, and has proven to be especially favourable
for the study of nerve-muscle relationship during development, since the ciliary ganglion which innervates the iris has been studied thoroughly under normal and experimental conditions (Landmesser & Pilar, 1970, 1974a, 1974b, 1976; Narayanan & Narayanan, 1976, 1978).

Although Lucchi, Bortolami & Callegari (1974) have reported a study of the fine structure of the iris musculature in chick embryos, to our knowledge, there has been no study implicating the development changes in the iris muscle with the early morphogenetic events occurring in the developing ciliary ganglion. The following study using ultrastructural and histochemical methods was undertaken to examine the relationship between the ciliary ganglion and the iris at various stages of development of the chick embryo, and to determine the mechanism by which a lasting relationship comes to be established between the nerve fibers and the muscle. The results of our study indicate a possible relationship between the pattern of localization of acetylcholinesterase (AChE) activity in the developing iris musculature and its innervation and cell death in the ciliary ganglion.

**MATERIALS AND METHODS**

Fertile eggs were obtained from Babcock strain of fowls maintained in the animal care facility of this Medical Center. Eggs were incubated in a forced-draft incubator at 37-5 °C and relative humidities between 65 and 70 %. Iris from chick embryos from seven days of incubation until hatching and also from 2- and 3-week-old chicks were used in the present study. Iris from embryos were staged according to Hamburger and Hamilton (1951) stage series and fixed by immersion in early stages. For later stages, 13 days onwards, they were perfused through the heart using a fixative containing 2 % paraformaldehyde and 2.5 % glutaraldehyde in a 0.1 M cacodylate buffer. Iris were then carefully dissected out with an iris knife and Watchmaker's forceps while being viewed under a stereomicroscope. They were radially cut into small slices, and fixed overnight in some fresh fixative. The tissues were then postfixed in 1 % osmium tetroxide, dehydrated in alcohol and propylene oxide, and embedded in Epon. Pieces of iris representative of each developmental stage were incubated to demonstrate localization of AChE following the technique of Lewis and Schute (1966), postfixed in Dalton's fixative, dehydrated and embedded in epon. Control studies consisted of similarly prepared tissues incubated in the absence of substrate, and tissues subjected to AChE inhibitor diisopropylfluorophosphate (DFP) $1 \times 10^{-6}$ M to $1 \times 10^{-4}$ M. Sections, 1 μm thick, were first stained with toluidine blue and examined under a microscope to select the area to be thin sectioned. Thin sections were cut using a Porter and Blum MT 2 ultramicrotome, double stained with uranyl acetate and lead citrate (Reynolds, 1963).
RESULTS

The iris at seven days of incubation (stage 31)

The iris of a chick embryo at seven days of development comprises almost entirely myoblasts and fibroblasts. The myoblasts are spindle-shaped cells with large nuclei which are granular in appearance and more electron dense than that of later stages. Many free ribosomes and small polysomal clusters are found evenly distributed in the cytoplasm. A well developed Golgi apparatus associated with smooth endoplasmic reticulum is also observed. When treated for AChE localization very faint staining was observed in the nuclear envelope and RER. Some of the myoblasts are found to be in apposition with other myoblasts presumably preparatory to fusion. A few of the myoblasts are elongated at this stage, and sheets or bundles of thin filaments are found beneath the plasma membrane, often extending almost into the fingerlike extensions at each end of the myoblast.

The iris at nine days of incubation (stage 35)

At nine days of incubation, the iris shows several multinucleated cells resulting from a fusion of myoblasts to form a syncitium. The spindle-shaped myoblasts are found to fuse laterally as well as end to end forming a myotube. The cells increase in size with growth of cytoplasm which contains a greater amount of granular endoplasmic reticulum and polyribosomes. Just like the iris of seven days the perinuclear cisternae and the rough endoplasmic reticulum show moderate AChE activity. Thick and thin myofilaments are found in higher number in the cytoplasm of these myotubes. Frequently, a large golgi apparatus is found from which vesicles appear to be budded off. Some of these vesicles are of the coated type. Unmyelinated nerve fibers with varicosities containing synaptic vesicles are often observed in the field. AChE is found to be localized at the axonal membrane of these nerve fibers at this stage.

The iris at 11 days of incubation (stage 37)

At 11 days of incubation, in the iris, myotube formation is very important. Typical sarcomeric structure is not yet established at this stage. Most of the cells show aggregation of thick and thin filaments among which are found darkly stained bodies that are supposedly precursors of the Z bands (Fishchman, 1972). Occasionally, cells are found showing beginnings of sarcomeric organization. AChE reaction product is generally found in the nuclear envelope and the cisternae of the granular endoplasmic reticulum of these myotubes at this stage. However, those cells which show some sort of sarcomeric organization also show AChE localization in the sarcotubular system. Numerous axons with varicosities and synaptic vesicles are found to traverse the muscle territory.
Fig. 1. Electron micrograph of a nerve terminal on a muscle fibre having myofilaments and Z bodies from the iris of a 11-day chick embryo. 51 300 ×. Bar, 0·5 μm.

Fig. 2. Electron micrograph of an iris muscle fiber from a 13-day-old chick embryo showing synaptic terminals filled with clear vesicles. (arrows). Note the absence of synaptic densities at this stage. 27 700 ×. Bar, 0·5 μm.
AChE is also found localized in the axolemma of these nerves. At 11 days, occasional synaptic terminals with clear round vesicles have been observed on myotubes containing myofilaments, (Fig. 1).

The iris at 13 days of incubation (stage 39)

In the iris of 13 days of incubation, the muscle cells exhibit characteristics of well differentiated muscles with nuclei arranged in a row and organized sarcomeric structure. Between 11 and 13 days of development, the myofilaments are organized into A and I bands, and a well developed tubular system is established (Figs 3, 5). Figure 3 demonstrates the localization of AChE in the nuclear envelope, in the Golgi, and in the ‘T’ system, suggesting active synthesis of the enzyme by these cells. At this stage, numerous nerve terminals with synaptic vesicles are found on the muscles (Fig. 2). However, they do not show characteristics of established neuromuscular junctions such as intimate association between the nerve and the muscle, or the localization of AChE in the membranes of the nerve, or the muscle, or the synaptic clefts. Figure 4 shows a muscle fiber with AChE activity in the sarcotubular system and an associated nerve terminal with no AChE localization in the junctional membranes. Occasionally, terminals can be observed showing partial localization of the enzyme indicative of early stages in the formation of neuromuscular junctions (Fig. 5).

The iris at 15 days of incubation and later stages

From 15 days onwards there is not much difference in the structure of the iris muscle cells from that of the iris in the hatched chick. Organized myofibrils are found in the majority of the cells, with well-developed sarcotubular system showing good localization of AChE. Well-developed synaptic terminals with clear round vesicles are found on most muscle cells. One significant feature of the iris musculature of 15 days which is not seen in earlier stages, is the specific localization of AChE at the neuromuscular junctions (Figs 6, 7). Figure 6 shows a fiber innervated by a multiple nerve ending while Fig. 7 shows one innervated by a single large terminal extending over a considerable length of the muscle.

Iris of hatched chick and later ages

The iris muscle of hatched chicks is not very different from that of later embryonic ages. The nerve terminals on the iris muscle are large, well developed, and filled with clear round vesicles. The axons are myelinated at this stage. After hatching there is a noticeable change in the pattern of AChE localization in the muscles. The reaction product localized in the sarcotubular system is very faint, while that localized at the neuromuscular junctions is very prominent. A definite shift in the localization of AChE from the sarcoplasmic structures to the junctional membranes is evident. From then on the enzyme activity is
confined mostly to the junctional membranes, which constitutes the adult pattern (Figs 8, 9).

**DISCUSSION**

A definite pattern in the development of AChE activity in the iris musculature is demonstrated in this study. At nine days of incubation AChE is found localized in the nuclear envelope and in the granular endoplasmic reticulum, and nowhere else. Between 11 and 13 days of incubation, there is an increase in the activity of AChE in the myotubes suggesting active synthesis of AChE which is now localized in the nuclear envelope, RER, Golgi apparatus, and in the sarcotubular system. At 15 days of incubation, in addition to its presence in the above sarcoplasmic structures there is a significant accumulation at the neuromuscular structures with a simultaneous increase in the junctional membranes.

A consistent pattern is also observed in the development of synaptic terminals on the muscle cells. Ciliary nerves are found in the territory of the iris musculature at nine days of incubation, and synaptic terminals are found in proximity to the myoblasts and myotubes only at 11 days of incubation. Between 11 days and 13 days of incubation, there is an increase of nerve terminals associated with the muscle cells. The concurrent increase of AChE activity in the muscle cells at the same period, suggests an inductive influence of the nerves over the muscle. AChE activity is found localized at the junctional region only at 15 days of incubation, when we see a more intimate association of the nerve terminals and the muscle. From the above results it appears that the induction of AChE activity in the iris muscles occurs in two steps:

1. An initial induction of active AChE synthesis by developing myotubes triggered by the formation of initial neuromuscular contacts between the ciliary nerves and the iris muscle fibers.

2. A later induction of AChE localization in developing end plates highly suggestive of mature neuromuscular junctions. The last step could be considered as a maturational process, since this precedes the appearance of neurally-mediated pupillary reflex which occurs between days 16 and 17 in the chick embryo (Heaton, 1970).

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Fig. 3. Electron micrograph of an iris muscle fiber from a 13-day-old chick embryo with AChE localization shown by the dark reaction product, in the nuclear membrane (a), in the Golgi (b), and in the sarcotubular membrane (c), 27 700 x. Bar, 0.5 µm.

Fig. 4. Electron micrograph of the iris of a 13-day-old chick embryo treated for AChE activity. Arrow shows a nerve terminal on the muscle. Note the distribution of AChE reaction product in the 'T' system and its absence in the junctional membranes. 28 500 x. Bar, 0.5 µm.

Fig. 5. Electron micrograph of the iris from a 13-day-old chick embryo treated for AChE activity showing partial localization of AChE reaction product in the myoneural junction. (arrows) 20 500 x. Bar, 0.5 µm.
Fig. 6. Electron micrograph of an iris muscle of a 15-day-old chick embryo showing AChE localization in the neuromuscular junction, (arrows) involving a multiple nerve ending. 24 500 ×. Bar, 0.5 μm.

Fig. 7. AChE localization in the neuromuscular junction in the iris of a 15-day-old chick embryo, involving a single large nerve terminal, extending over a considerable length of the muscle. 24 500 ×. Bar, 5 μm.
Fig. 8. Electron micrograph of the iris muscle from 1-day-old chick showing AChE localization in the neuromuscular junction involving several nerve terminals. Note the reduction of AChE in the sarcotubular system of the muscle cell. 14 800×. Bar, 1 μm.

Fig. 9. Electron micrograph of the iris muscle from a 1-day-old chick showing AChE localization in the neuromuscular junction involving a single nerve terminal. Note the absence of AChE in the muscle cell on the right. 14 800×. Bar, 1 μm.
In our studies bouton terminals are observed between 11 and 13 days of incubation and AChE localization in myoneural junctions between 13 and 15 days of incubation. This seems to indicate that the terminals induce enzyme localization in the junctional membranes by continued contact with the muscle for a certain period of time. This is in agreement with the findings of Atsumi (1971), that localized activity, the initial sign of endplate formation occurred one to two stages after the formation of the bouton terminals. He proposed that the nerve endings might induce enzyme localization by contact with the myotube for a certain period of time.

Our results agree with those of Chiappinelli, Giacobini, Pilar & Uchimura (1975) who observed an increase of choline acetylase (ChAc) in the ciliary ganglia and an increase of AChE in the iris muscle at about nine days of incubation. They suggest a reciprocal interaction between the neurons and their targets, resulting in the induction of ChAc in the prejunctional elements and AChE in the postjunctional elements, both triggered by the formation of neuromuscular junctions. However, our results show that the induction of enzymes in the prejunctional and postjunctional elements must have been mediated by the initial neuromuscular contacts since no mature neuromuscular junctions are observed during this period.

It is clear from the present study that between 11 and 13 days of incubation, the period of maximal cell loss in the ciliary ganglion, we observe an increase in the number of synaptic terminals loosely associated with the iris muscle. Mature neuromuscular junctions are not observed during this period. It is only at 15 days of incubation when the cell number in the ciliary ganglion has attained close to adult levels that mature neuromuscular junctions are observed. Although no counts were made to determine the actual number of synaptic terminals at each stage, our observations show that a decrease in cell number in the ciliary ganglion corresponds with an increase in synaptic terminals on the iris muscle. This would support our contention that cells which die do not make peripheral contacts while only those cells which make successful contacts with the periphery survive.

This investigation was supported in part by the National Institutes of Health-National Institutes of child Health and Human Development No. RO1-HD12064. The authors wish to express their grateful thanks to Mr Tom Lee for competent technical assistance and Miss Toni Santee for secretarial help.

REFERENCES


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(Received 19 March 1980, revised 10 October 1980)