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SYNAPTIC TRANSMISSION AND CELL DEATH DURING NORMAL GANGLIONIC DEVELOPMENT

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SUMMARY

1. During normal embryonic development of the chick ciliary ganglion, cell death over a 4-day period (Stages 35–39) reduces the number of ganglion cells by half, from 6500 to 3200. Both ciliary and choroid populations are affected by approximately the same amount.

2. Previous to cell death, preganglionic fibres form functional synapses on all ganglion cells, indicating that synapses form on cells which are destined to die.

3. Shortly before the period of cell death, there is a failure of transmission in approximately half the cells. Some evidence suggests that transmission failure in at least some of the cells is of preganglionic origin.

4. Cell death is nearly synchronous with the establishment of peripheral connexions by ganglion cells, at least with respect to the ciliary population which forms functional synapses with iris muscle. This implies that those cells which die do so because they have failed to form adequate peripheral connexions.

5. It is suggested that many of the cells in which transmission has failed die, bringing transmission through the ganglion back to 100%. However, transmission failure appears to be a transitory phenomenon in other cells which survive and probably results from death of their preganglionic elements. Restoration of transmission would then be brought about by the formation of new or more effective synapses by surviving preganglionic fibres.

INTRODUCTION

In the preceding paper, it was shown that early removal of the target organs of the neurones in the chick ciliary ganglion did not prevent the establishment of functional ganglionic synapses. However, between Stages 35–36 of Hamburger and Hamilton ganglionic transmission failed

abruptly and was closely followed by extensive ganglion cell death (Landmesser & Pilar, 1974).

A substantial cell death was observed in the contralateral control ganglion at the same time. It thus appeared that in the ciliary ganglion, as in many neuronal structures, extensive cell death is a part of normal development (Levi-Montalcini & Levi, 1943; Hamburger, 1958; Hughes, 1961). Since peripheral ablation serves to accentuate this death, it is generally assumed that during normal development, nerve cells die because they have failed to form proper peripheral connexions (Hughes, 1961; Prestige, 1967*a*, *b*).

Most evidence for this comes from spinal cord, where motoneurones die shortly before the limbs become motile (Hamburger, 1958; Hughes, 1961; Hughes & Prestige, 1967). A more quantitative study by Hughes & Egar (1972) showed that the large drop in the total number of nerve cells, both sensory and motor, contributing to the innervation of the anuran hind limb, was accompanied by a similar loss of axons in the sciatic nerve. They interpreted this to mean that most cells which died had probably already sent axons into the nerve.

Since the ciliary ganglion is relatively homogeneous and both ganglionic transmission and formation of peripheral connexions can be assessed, we decided to look at transmission through the normal ganglion at the critical Stages (35–40), especially in order to see if this period coincides with the formation of peripheral synapses. The process of synaptic maturation had been studied in the chick ciliary ganglion (Landmesser & Pilar, 1972), but transmission in the normal ganglion had not been quantitatively assessed during this period.

METHODS

Most methods including electrophysiological, histological and electron microscopical were described in the preceding paper. The measurement of conduction velocity of nerve fibres has been described (Landmesser & Pilar, 1972).

In this paper, additional experiments were done to determine the time at which ganglion cells form synapses with the iris. A circular cut is made in the eyeball opposite from the pupil, and the retina and lens are removed. The half eyeball is mounted in a chamber, containing oxygenated Tyrode, so that light may be shined through the pupil. The aperture of the pupil can then be measured with an ocular micrometer. After measurement of the resting diameter, the ciliary nerves were stimulated supramaximally at 10–30/sec until maximal closure of the iris was achieved (1–2 min). The maximal closure to nerve stimulation was compared with that induced by 150 mM-KCl solution. For further discussion of the contractile properties of the iris in an adult bird see Pilar & Vaughan (1971).

RESULTS

Failure of transmission during development

It was demonstrated that following early removal of the periphery, transmission through the ciliary ganglion was normal until Stages 35–36 when it failed in most cells (Landmesser & Pilar, 1974). At approximately the same time most of the ganglion cells degenerated. A rather striking cell death was observed in control ganglia at the same stages, but the proportion of transmitting cells at these stages had not been previously determined. An earlier report (Landmesser & Pilar, 1972) showed that all ganglion cells with axons in the post-ganglionic nerves were innervated by Stage 33. While transmission was 100 % in both cell populations between Stage 40 to hatching, it had never been quantitatively investigated from Stages 34 to 39. In Fig. 1 left, responses elicited by preganglionic stimulation and typical of Stages 30–34 can be seen, this record being obtained from a Stage 33 animal. In both ciliary and choroid populations, the area under the response curve brought about by pre- (Fig. 1*A*) and post-ganglionic (Fig. 1*B*) stimulation was equal, which indicates that all ganglion cells with axons in the post-ganglionic nerves were transmitting.

In order to assess whether most cells had actually sent axons into the post-ganglionic nerves by this stage, an electron micrographic montage of the ciliary and choroid nerves was made of a Stage 30 animal and the number of axons counted. There were altogether 14,291 ciliary and choroid fibres which is in excess of the approximately 6500 ganglion cells counted at the same stage. Therefore, most ganglion cells have functioning synapses on them by Stage 30. As reported in the preceding paper the excess of axons over ganglion cells probably results from branching of axons as they leave the ganglion, this being extensive at early stages.

However, between Stages 35 and 38 there was an abrupt failure in transmission in both ciliary and choroid populations. This can be seen in Fig. 1 right where the preganglionically evoked responses (*A'*) at Stage 37 are only 70 % (ciliary) and 36 % (choroid) of the post-ganglionically evoked responses (*B'*).

Fig. 2 summarizes the percentage of transmission for ciliary (filled circles) and choroid (squares) populations at various developmental stages. It can be seen that following a period of 100 % transmission between Stages 30 and 34, there is a drop in transmission to approximately 50 % between Stages 36 and 38. Then by Stage 40 transmission has returned to 100 % where it remains until hatching. It is noteworthy that this failure occurs in both populations to approximately the same extent.

As reported in the previous paper, the number of neurones in control ganglia is nearly halved between Stages 36 and 38, decreasing from 6500

at Stage 34 to approximately 4000 by Stage 38. The dotted line in Fig. 3 traces the number of cells at various stages as a percentage of the maximal cell number (6500) and is taken from the curve of Text-fig. 3 in the previous paper. It can be seen that transmission failure precedes cell death, this being especially true for Stages 36–38.

As the cell number reaches its final value between Stages 38–40, transmission returns to 100 % in both populations where it remains until hatching.

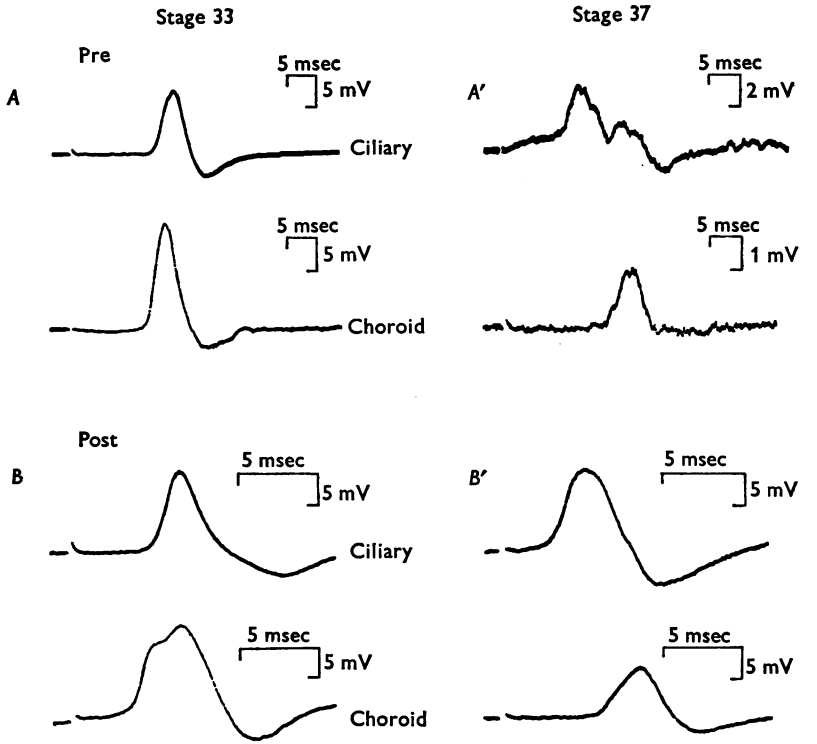

Fig. 1. Comparison of % transmission at Stages 33 and 37 for ciliary and choroid populations. Left: the area under the response curves recorded from the Stage 33 post-synaptic ciliary and choroid nerves is the same whether brought about by pre- (*A*) or post-ganglionic (*B*) stimulation, indicating 100 % transmission. Right: at Stage 37 the area for preganglionically evoked responses (*A'*) is less than that for post-ganglionic evoked responses (*B'*), indicating partial failure of transmission.

As in the case where the periphery is removed (Landmesser & Pilar, 1974) we explained the return to full transmission by death of the non-transmitting cells. The remaining cells where transmission is unimpaired would then represent the entire ganglion. It is improbable, although not

entirely ruled out, that any cells would be added to the ganglion at this stage by either cell division or delayed migration.

While cell death certainly plays a role, it does not seem that it can account entirely for the low value to which transmission falls and its subsequent return to 100 %. This can be seen by considering the number

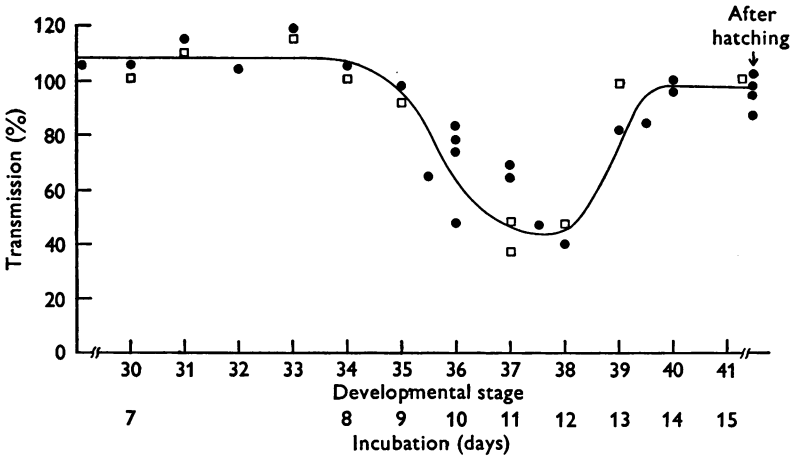

Fig. 2. Per cent transmission in ciliary and choroid populations at different developmental stages. Transmission in both ciliary (filled circles) and choroid (squares) groups is 100 % until Stages 35–39 when it drops transiently to approximately 50 %. Curve fitted by eye.

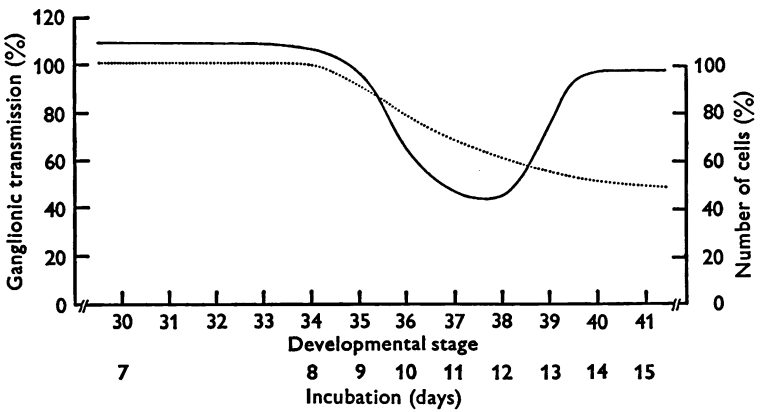

Fig. 3. Comparison of % transmission and the number of ganglion cells at different stages. While both cell number (dotted line) and transmission (continuous line) drop at approximately the same time, transmission failure precedes cell loss, especially at the later stages. This graph is a combination of the transmission failure taken from Fig. 2 and the percentage of cells from Fig. 3 (Landmesser & Pilar, 1974).

of transmitting ganglion cells at any stage (obtained by multiplying the total cell number by the percentage of transmission). In Fig. 4 this is plotted as a dashed line which can be compared with the total number of cells anatomically present (continuous line).

The number of transmitting cells drops precipitously between Stages 34 and 37 from 6500 to 2000. If all non-transmitting cells died, one would expect the final number of ganglion cells to be 2000. However, the average number of transmitting cells returns to somewhat more than 3000 between Stages 38 and 40.

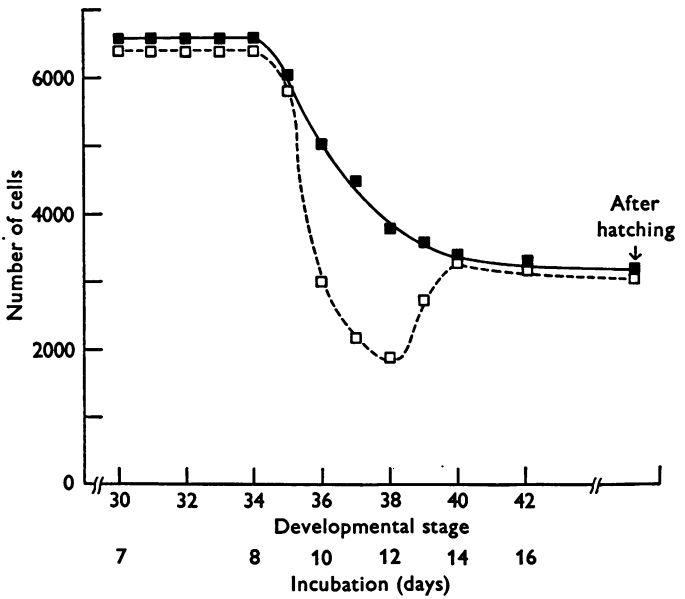

Fig. 4. Comparison of the number of transmitting ganglion cells with total cell number. Total number of cells/ganglion (filled squares) declines monotonically to approximately 50 % of the original number between Stages 34 and 40. During the same stages, the number of transmitting cells (open squares: total number of cells × % transmission) drops to a minimum of 2000 and returns to approximately 3000.

This result could be artifactual and would be obtained if axons of degenerating cells persisted as viable for several days after degeneration of the cell body had proceeded to such a degree that it was no longer counted as a viable neurone. This would produce low estimates of transmission. If many cells not counted because they were pycnotic were actually viable, this would also contribute to the error, for in this case the total cell number counted would be low and thus the number of transmitting cells would be underestimated.

However, another possibility is that transmission failure does occur transiently in a number of cells between Stages 36 and 38 only to resume by Stage 40. Some electron microscopic evidence of degenerated presynaptic terminals on normal appearing ganglion cells may correlate with this physiological observation (G. Pilar and L. Landmesser, unpublished observations). Support for this is also derived from the experiments where the periphery was removed. In these cases transmission was sometimes zero between Stages 36 and 38, yet always at least 500 cells persisted until Stage 42.

In summary, between Stages 35 and 40 cell number declines to half the original value. The drop in transmission during this critical time is also approximately 50 %. Since transmission fails in both cell populations it is of interest to know if cell death is similar for both ciliary and choroid groups.

In most cases only the total number of cells per ganglion was determined. This was done because, while the ciliary and choroid populations are, as a whole, distinct, there are always some cells that cannot be unambiguously assigned to either group, especially at early stages. However, in one Stage 34 ganglion an attempt was made to assign cells to either ciliary or choroid populations based on previously described anatomical characteristics (Marwitt, Pilar & Weakly, 1971; Landmesser & Pilar, 1972, 1974). In this case there was a total of 6512 cells with 3087 cells in the ciliary and 3446 cells in the choroid population. In one Stage 38½ ganglion, the total number of cells was 3741. In this case there were 1793 ciliary and 1823 choroid cells. In both cases, the number of cells not assigned to either group was only about 100. It can be seen therefore that in general both cell populations are reduced to the same extent. While not quantitatively assessed, both cell types died at approximately the same time.

An understanding of why transmission fails will require intracellular recording between Stages 36 and 38, which may prove difficult. However, some pertinent observations can be made from extracellular recordings. Throughout development until Stage 36, the responses recorded from the post-synaptic ciliary nerves to a single preganglionic shock are unimodal. An example from a Stage 34 ciliary nerve is shown in Fig. 5*A*.

Between Stages 36 and 39 a second peak, as shown in Fig. 5*A'*, was consistently observed. Both peaks were blocked by synaptic blocking agents such as DTC, and thus represent chemical transmission. Furthermore, the conduction velocity of the preganglionic fibres contributing to each peak was found to be the same in several experiments. For example, in the Stage 37 response of Fig. 5*A'* the first and second peaks represented preganglionic fibres that conducted at 0·33 and 0·35 m/sec respectively.

In another case at Stage 38, the conduction velocities were 0·34 and 0·30 m/sec. Therefore the difference in latency between the two peaks cannot be explained by differences in conduction velocity of preganglionic fibres.

Nor can it be explained by differences in conduction velocity of the post-ganglionic nerves, for a second peak was not seen when the post-ganglionic nerves were stimulated directly (Fig. 5*B'*) as is usually the case in controls (Fig. 5*B*).

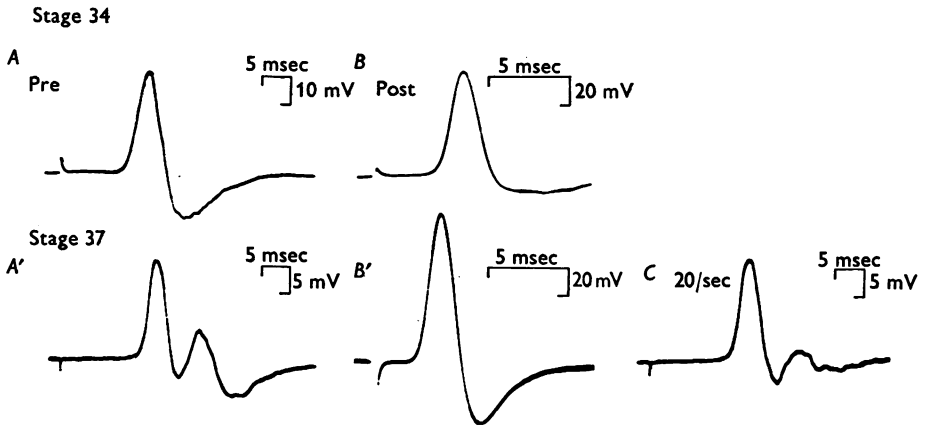

Fig. 5. Ganglionic transmission during the period of transmission failure. In a Stage 34 ganglion the compound action potential recorded from the ciliary nerves is unimodal whether stimulation is pre- (*A*) or post-ganglionic (*B*). During period of transmission failure at Stage 37, the response in the ciliary nerves to preganglionic stimulation (*A'*) consists of two peaks, while direct stimulation of the ciliary nerves (*B'*) results in a unimodal response. In *C*, the second peak of the Stage 37 preganglionically evoked response is blocked by 20/sec repetitive stimulation for 5 sec, which leaves the first peak unaltered.

We assumed that the extra delay must occur in the ganglion and might represent delay at failing synapses. In fact, the synapses contributing to the second peak, do fatigue more easily than those contributing to the first. In Fig. 5*C* one can observe that preganglionic stimulation of 20/sec almost completely blocked the second peak without affecting the first peak. It should be pointed out that action potentials from directly stimulated ciliary nerves at this stage do not fail until 40/sec.

When transmission is blocked with either hexamethonium (10^{-4} M) or DTC (5×10^{-6} M) both peaks are reduced by a similar amount (Fig. 6*A* and *B*). This might indicate that the amount of transmitter released to a single stimulus as well as post-synaptic properties are initially normal. Transmission failure, first detected as an inability to maintain transmitter

output, would then be largely presynaptic. This must be tested more directly with intracellular recordings.

Labelled α-bungarotoxin could be used to tell if any change in post-synaptic acetylcholine receptors had occurred. However, preliminary results indicate that α-bungarotoxin does not bind to the ganglionic acetylcholine receptors (Z. Hall and G. Pilar, unpublished observations).

Is cell death correlated with peripheral connexions?

Since the cell death observed normally is similar in time of occurrence to that induced by peripheral ablation, one is tempted to speculate that approximately half of the cells fail to form peripheral connexions and therefore die. In order to see if cell death correlated with the development of peripheral connexions (at least for the ciliary population) the time of functional innervation of the iris was determined.

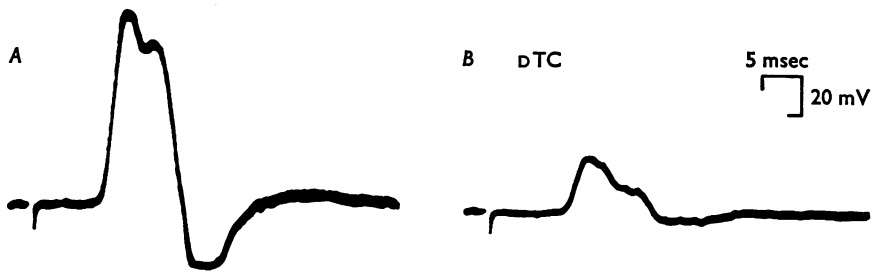

Fig. 6. Blockage of transmission with D-tubocurarine in a Stage 38 ganglion. *A*, control response showing two peaks. *B*, response after 15 min in 5×10^{-6} M DTC, showing partial blockage of both peaks.

The time at which the iris could first be activated by ciliary nerve stimulation was compared with the mechanical response elicited by direct stimulation of the contractile elements by KCl. There was the possibility that synapses were made with iris fibres before they became contractile. In this case, it would not be possible to determine the time of innervation by observation of iris closure brought about by stimulation of the ciliary nerves.

However, it can be seen from the graph (Fig. 7) that a mechanical response can be induced by KCl before it can be elicited by nerve stimulation. Although it is still possible that synapses form on non-contractile fibres before Stage 33, the present method provides evidence that synapses become functional on many of the muscle fibres between Stages 35 and 36. At this time, synapses can also be observed with the electron microscope on some iris muscle fibres. However, many partially differentiated

myoblasts and myotubes are still present (G. Pilar and L. Landmesser, unpublished observations).

While it was observed that nerve fibres from ganglion cells grow out into the eyeball to the region of the iris as early as Stages 25–30, cell death does not occur until Stages 35–38. Since it is at precisely this time that functional synapses on iris muscle can be detected, it can be inferred that cell death is related to the formation of synapses in the periphery.

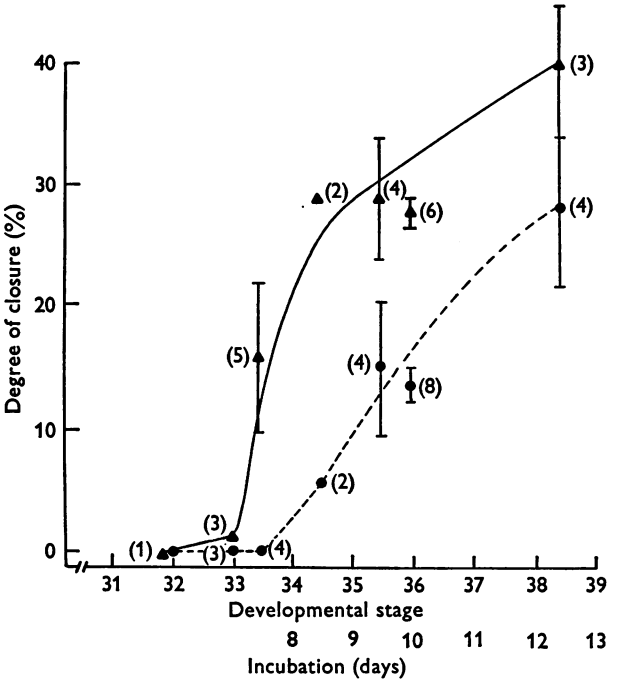

Fig. 7. Degree of closure of iris at various developmental stages. The maximal degree of iris closure, expressed as % of the resting pupil diameter, $\frac{\text{(resting pupil diameter – pupil diameter during stimulation)}}{\text{resting pupil diameter}}$ brought about by repetitive electrical stimulation of the ciliary nerves (filled circles) can be compared to that brought about by superfusion with 150 mM-KCl (triangles). The values represent the mean ± S.E., number of experiments for each point in parentheses, curve fitted by eye.

DISCUSSION

During the normal course of embryonic development, every second nerve cell in the ciliary ganglion dies. This substantial cell loss is less than that occurring in the amphibian ventral horn (Hughes, 1961; Prestige, 1967*b*) where nine out of ten neurones die, but it is similar to that in the

trochlear nucleus (Cowan & Wenger, 1967) where the peripheral field is a simple homogeneous target much as in the ciliary ganglion.

It is generally postulated that this cell death is due to failure of the neurones to form peripheral connexions. This is consistent with the finding that cell death in the ciliary ganglion is nearly synchronous with the formation of functional synapses with the iris muscle. Most of the cells that die have already sent axons into the post-ganglionic nerves. Of course, in this as in all the previous studies on histogenetic neurone death, it has not been possible to quantitatively assess synapse formation in the periphery. It is not clear whether those cells that survive have formed synapses while those that die have not. While ganglion cells send axons into contact with the periphery fairly early (Stages 25–30) cell death does not occur until synapse formation some time later.

Looking at the present experiments from a different aspect, the cells in the ciliary ganglion are themselves the peripheral target of the neurones in the accessory oculomotor nucleus in the brain (Yoshida, 1953; Cowan & Wenger, 1968) which give rise to the preganglionic fibres. Cowan & Wenger did not mention cell death in normal accessory nuclei but they did, however, find obvious death in the accessory nucleus following early removal of the optic vesicle, but only after Stage 35. Our results show that most ganglion cells have been synapsed on by that time. Thus the preganglionic fibres have had a chance to compete for available ganglion cells before death ensues and it seems reasonable to propose that those preganglionic neurones which die have failed to form sufficient ganglion cell synapses. This would result if some preanglionic fibres had never formed any connexions, and would be further accentuated when many ganglion cells themselves begin to die.

The question arises as to whether proper peripheral connexions are required to prevent cell death, or if any connexion would suffice. In this respect, it is important to note, that conduction velocity measurements (Landmesser & Pilar, 1972) show that few if any ganglion cells grow down the wrong nerves; the axons from the ciliary cells down the choroid nerve and vice versa. Thus most of the ganglion cells do not seem apt to synapse with the wrong target tissue, an occurrence that might be expected to be greater in the limb where any given motoneurone is confronted with a larger number of possible synaptic targets, making synapse formation with the wrong target more likely. This might contribute to the larger amount of cell death in the ventral horn, although further speculation is unjustified until more is known about the manner in which the limb is innervated.

Yet even in the ciliary ganglion, with a simple target, fully half the neurones die. This overproduction of neurones may be required to saturate

the periphery with synapses, yet it is difficult not to wonder at the apparent waste. Cells devoid of peripheral connexions, and therefore destined to die are also synapsed upon by preganglionic fibres.

In the ciliary ganglion it was observed that transmission failure precedes cell death. Since, we know of no other functional studies, comparisons with other systems are not possible. As pointed out in the results, the observed transmission failure may be artifactual, yet it is probable that at least some preganglionic fibres die and appear in stages of fibrous degeneration, before the ganglion cells upon which they are synapsing die. Such death of preganglionic fibres may be non-selective in the sense that it results simply because the fibre has not formed enough ganglion synapses. This would be accentuated when ganglion cells themselves begin to die.

The transient drop in transmission would then be explained, for degeneration of some preganglionic fibres would leave some ganglion cells without synapses, or at least with a subthreshold number of synapses. As surviving preganglionic fibres formed new synapses or more extensive synapses on these cells, transmission would return to normal. The drop in transmission that we have observed occurs just after the ultrastructural change from multiple bouton synapses to a single calyciform ending per ciliary cell (Landmesser & Pilar, 1972, 1974). Thus, at least some of the preganglionic fibres might have been out-competed for available ganglion cells.

It is not possible to say at present, whether these results have any direct bearing on specificity or on the proper matching up of preganglionic fibres with ciliary cells. Since both ganglion cell types are innervated at approximately the same time (Landmesser & Pilar, 1972), and die at nearly the same time, whatever matching up and competition that occurs seems synchronous for both cell populations. Thus one cell type does not mature, receive synapses, and die, before the second type receives synapses. This would tend to downplay the importance of a temporal sequence in the formation of selective connexions in this system, which has, however, been postulated to be important elsewhere (Gottlieb & Cowan, 1972).

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