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THE ONSET AND DEVELOPMENT OF TRANSMISSION IN THE CHICK CILIARY GANGLION

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SUMMARY

1. The onset and development of transmission has been studied electrophysiologically in the isolated chick ciliary ganglion from Stage 25 (Hamburger & Hamilton, 1951) until 28 days after hatching. Ultrastructure of the synapses was concomitantly investigated.

2. Synaptic transmission began at Stage 26½ and was 100 % in both cell groups, ciliary and choroid, by Stage 33. It was initially chemical until Stage 41 when effective electrical coupling first appeared in the ciliary population. The proportion of electrically transmitting synapses increased to 80 % by 1–2 days post-hatching.

3. Few morphological synapses were present at Stage 33½ when all ganglion cells were transmitting. A scarcity of synaptic vesicles persisted until late in embryonic development when all ciliary cells possessed calyces. At hatching the calyces were filled with synaptic vesicles.

4. Initial synaptic contacts were by fine terminal branches often on the intricate processes of early ganglion cells. Calyces formed from Stage 36½ and there was a concomitant retraction of ganglion cell processes, so that by Stage 40 all ciliary cells had simple calyces. The calyx was a transitory structure, which from the first week post-hatching began to break up into a cluster of boutons.

5. Chemical post-synaptic potentials (PSPs) were at Stage 40 long (30× the membrane time constant) and further prolonged by eserine. By Stage 43, PSPs had become markedly shortened and were unaffected by eserine. No simple explanation can be offered for the changes in PSP time course and sensitivity to anticholinesterases during development.

6. Intracellular records from Stage 40 ciliary cells, which all possess

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calyces, showed 1–2 mV amplitude, diphasic, fast decaying electrical coupling potentials (CPs). Later in development the CPs became 20–40 mV amplitude, more slowly decaying and monophasic. This seemed to be correlated with faster presynaptic conduction velocities and myelination of the cell soma. Such changes in CPs may reflect a shift from capacitative to more resistive coupling and point to several factors contributing in varying degrees to the electrical transmission.

7. Presynaptic fibres innervating ciliary cells were from the start of lower threshold and faster conduction velocity than those innervating ciliary cells, as occurred in the adult. It is concluded that these preganglionic fibres were probably specified by the time transmission starts and that they selectively innervated the proper post-synaptic cells.

INTRODUCTION

Ultrastructural studies have described the development of synaptic contacts between neurones (Glees & Sheppard, 1964; Bunge, Bunge & Peterson, 1967; Wechsler & Schmekel, 1967; Crain & Peterson, 1967). Some observations have been made at the time of onset of function in the mammalian spinal cord (Bodian, 1966, 1968) and cortex (Molliver & Van der Loos, 1970), yet function has been estimated only qualitatively from behavioural reflexes or evoked surface potentials. Correlations between synaptic function and structure are difficult to determine without knowledge of the physiological state at the anatomically observed developmental stages.

The adult avian ciliary ganglion has been shown to contain two homogeneous cell populations, ciliary and choroid, that can be anatomically and functionally distinguished (Marwitt, Pilar & Weakly, 1971). The ciliary population possesses an electrical as well as chemical mode of transmission. Electrophysiologically determining the degree and nature of transmission through this ganglion during development should allow correlations with ultrastructure that are less subject to sampling errors than similar studies based on behavioural or gross electrical recordings alone.

Furthermore, determination of the time at which synaptic transmission begins is essential in interpreting the various developmental studies done using this ganglion. It has been shown that removal of the periphery (Amprino, 1943; Cowan & Wenger, 1968) or the afferent input (Levi-Montalcini & Amprino, 1947) affect the ganglion cells at certain developmental stages. The state of synaptic function at these critical times, as well as the sequence of functional maturation of the pre- and post-synaptic elements, has heretofore been unknown.

Therefore, the onset and maturation of synaptic transmission has been studied electrophysiologically in the chick ciliary ganglion from Stage 25 (Hamburger & Hamilton, 1951) until 28 days after hatching. Synaptic fine structure has been described for four physiologically distinct developmental stages, and correlation between the physiological and histological findings are discussed. The formation of calyces is also described. A brief report of this work has appeared elsewhere (Pilar & Landmesser, 1971).

METHODS

Physiology

The presynaptic oculomotor nerve together with ciliary ganglion and post-synaptic ciliary and choroid nerves were isolated from chick embryos from Stage 25 of Hamburger & Hamilton (4½ days) until 28 days post-hatching. Fertile Leghorn eggs were incubated in the laboratory (Humidaire Co., New Madison, Ohio, Incubator no. 50). Stimulating and recording techniques, previously described (Martin & Pilar, 1963*a*), were modified by the use of suction electrodes with interchangeable polyethylene tips of various diameters. Electrical responses were differentially amplified (MPA-6, Transidyne General Co., Ann Arbor, Michigan). Degree of transmission through the ganglion was estimated by comparing the area under the post-synaptically recorded responses to both suprathreshold pre- and post-ganglionic stimulation (see text-fig. 1*A*, *B*). Conduction velocity was measured by applying supramaximal stimuli at decreasing increments of distance along the presynaptic nerve, and measuring the changes in latency of the peak of the compound post-synaptic response obtained. Such latencies when plotted as a function of distance, measured with an ocular micrometer, were usually well fit by a straight line, whose slope gave the conduction velocity (Text-fig. 1*C*). When determinations failed to produce straight lines, values were discarded.

Intracellular recording techniques also have been described (Martin & Pilar, 1963*a*). In some cases irises with intact ciliary nerves were used to assess the degree of functional innervation of the periphery. The degree of closure of the iris as a result of maximal post-ganglionic electrical stimulation was compared with that induced by superfusion with KCl (100 mM) or acetylcholine choride (ACh) (10^{-6} M) (see Pilar & Vaughan, 1971, for details). All experiments were done at room temperature (22–24° C).

Morphology

After the post-synaptic response to maximal preganglionic stimulation was obtained, the ganglion was removed and fixed in 4% phosphate-buffered glutaraldehyde at 4° C. Subsequent recording of the post-synaptic response to maximal post-ganglionic stimulation allowed comparison of the degree of transmission and ultrastructure for the same ganglion. Ganglia were fixed overnight, post-fixed in 1% osmium tetroxide, embedded in Epon, stained with lead citrate or uranyl acetate, sectioned, and viewed on an RCA electron microscope. In order to identify the two cell populations, thick sections of the whole ganglion were stained with toluidine blue and observed with the light microscope. Subsequently thin sections were made of selected areas for electron microscopy. In some cases irises and ciliary nerves were also studied. Nerve counts were obtained from photographic montages of electron microscopic plates at a magnification of 5000×.

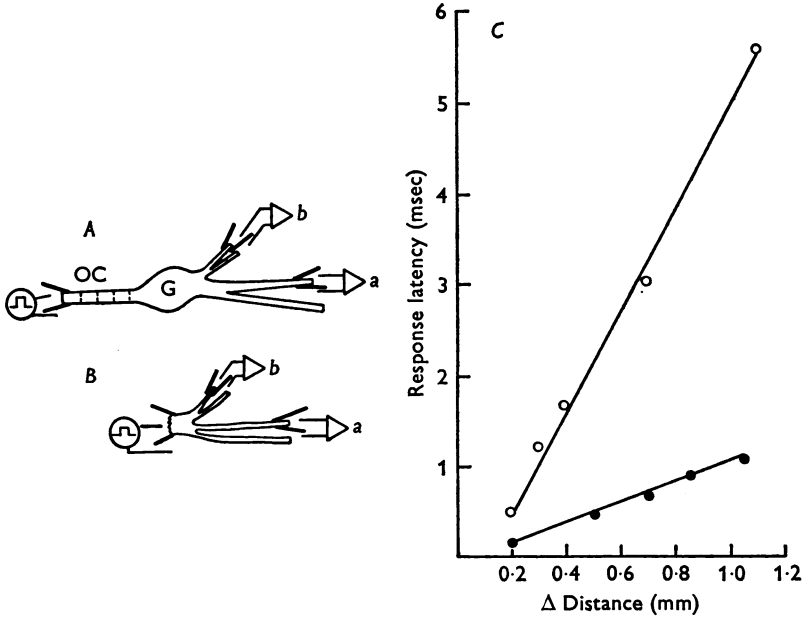
RESULTS

Physiology

Extracellular recordings from ciliary and choroid nerves

Degree of transmission

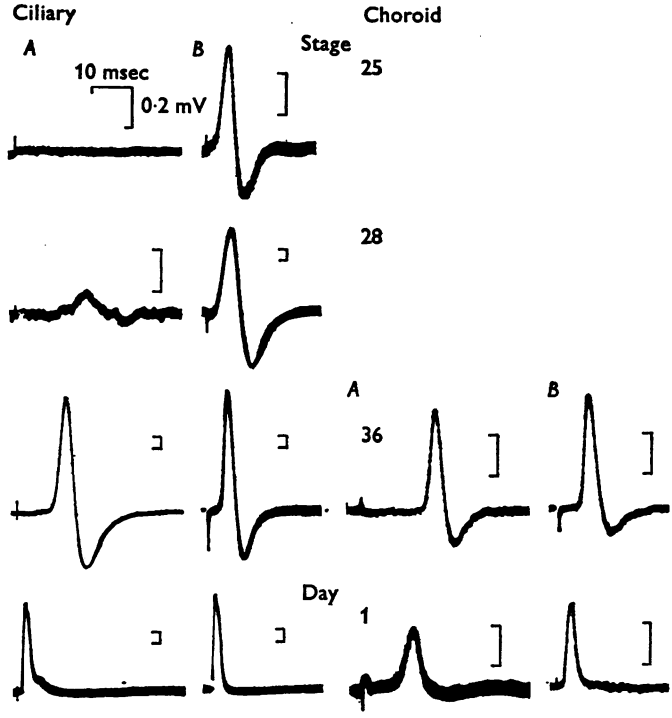
At Stage 25 ($4\frac{1}{2}$ days), stimulation of the oculomotor nerve failed to produce a response in the post-synaptic ciliary nerves (Text-fig. 2, upper row *A*). However, stimulation just distal to the ganglion produced the post-synaptic response shown in the upper row, Text-fig. 2*B*. Therefore, at a time when there was no evidence of transmission through the ganglion, differentiated ganglion cells with axons capable of conducting action potentials were present. That such diphasic responses were actually pro-



Text-fig. 1. Determination of conduction velocity and degree of transmission (*A*). Stimulating suction electrode is advanced along the oculomotor nerve at distances indicated by the dashed lines. Latencies and amplitude of ciliary (*a*) and choroid (*b*) responses are recorded from the post-synaptic nerves. *B*, ganglion was removed and stimulating suction electrodes applied directly to the proximal post-synaptic nerves, responses recorded as in *A*. From the ratio of the area under the pre- and post-synaptic responses, the degree of transmission was estimated (*C*). The slopes of the lines obtained by plotting latencies of ciliary (filled circles) and choroid (open circles) responses as a function of distance gave conduction velocities of 0·9 m/sec for the presynaptic ciliary fibres and 0·17 m/sec for the choroid.

pagated and not electrotonically recorded was apparent from the fact that response latency measured at several increasing distances from the stimulating electrode increased consistent with a conduction velocity of 0·19 m/sec.

Transmission was first observed in the ciliary population at Stage 26½ (5 days). The presynaptically evoked responses obtained during the next



Text-fig. 2. Post-synaptically recorded ciliary and choroid responses elicited by pre (*A*) and post (*B*)-synaptic stimulation at the developmental stages indicated in the centre of Figure. At Stage 25 there is no transmission through the ganglion; at Stage 28 the presynaptically evoked response is only 77 % of the post-synaptically evoked response. By Stage 36 the ciliary and choroid presynaptically evoked responses are similar in magnitude to the respective post-synaptically evoked responses, indicating 100 % transmission. The ciliary presynaptic response at day 1 consists of a large electrically mediated peak, followed by a smaller chemically mediated peak; the choroid presynaptic response consists of only a single chemically mediated peak.

several stages (26½ to 28) were small, somewhat dispersed in latency (Text-fig. 2*A*, second row) and completely blocked by repetitive stimulation at 5/sec. They were only 7–30 % of the post-synaptically evoked responses

which were more sharply peaked and not blocked even at 20/sec stimulation. This presumably represented an early stage in the development of transmission when only a small proportion of the ganglion cells were activated. Choroid nerves were too fine to dissect and record from at these stages.

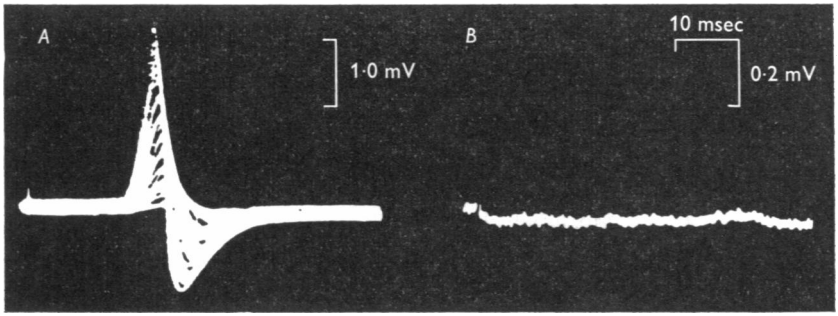
Responses could be obtained from both ciliary and choroid nerves from Stage 31 (7 days) and the proportion of transmitting cells in all preparations had reached 100% by Stage 33. Responses from this stage were similar to those obtained from Stage 36 ciliary and choroid nerves where the pre- (*A*) and post-synaptic (*B*) responses were of similar amplitude (Text-fig. 2, third row).

However, many early chick embryo cells are electrically coupled (Sheridan, 1968). If extensive coupling occurred between ganglion cells, a few chemically active synapses could activate a large number of cells, invalidating the procedure for estimating degree of transmission. While it is difficult to rule out this possibility, there is evidence against it. The responses to presynaptic stimulation from a Stage 33 ganglion with 100% transmission were very finely graded, indicating a large number of individually activated ganglion cells (Text-fig. 3*A*). Furthermore, antidromic stimulation of various ciliary branches (see Fig. 1 in Marwitt *et al.* 1971) did not evoke responses from any other branch, as might have been expected if a large number of cells were coupled, provided that there was invasion of the cell soma. Extensive anatomical sampling of the Stage 33½ ganglion did not reveal any specialized contacts between ganglion cells (see anatomical description below). Post-synaptically evoked responses at this stage were also smoothly gradable.

It also might be proposed that only a small number of ganglion cells had differentiated axons in the nerves at Stage 33½. However, the number of axons (0·7 μ mean diameter, unmyelinated) in the ciliary nerves 1·5 mm distal to the ganglion was determined from a photographic montage of electron microscope plates and found to be approximately 8000. In adult birds, the ciliary nerves contain approximately 3000 ± 600 axons (Marwitt *et al.* 1971). It would therefore appear that most of the ciliary ganglion cells have differentiated axons in the ciliary nerves at this time. The larger number of axons in the embryonic nerve count may merely result from terminal branching since the count was made nearer to the iris than in the adult counts.

Transmission in both cell populations was from the time of its onset until Stage 41 entirely chemical. Responses could be completely blocked by 10^{-6} M D-tubocurarine that did not modify nerve conduction. In the adult and young chick, the ciliary population shows a dual mode of electrical and chemical transmission (Martin & Pilar, 1963*a*, *b*). Electrical trans-

mission appeared in the ciliary population at Stage 41, and the proportion of electrically transmitting cells increased with time (see below). A bimodal response from a newly hatched chick is seen in the bottom row *A* of Text-fig. 2. The larger, shorter latency peak represents cells that are discharged by electrical coupling potentials, the latter peak by those that are discharged by chemical post-synaptic potentials (PSPs) (Martin & Pilar, 1963*b*; Marwitt *et al.* 1971). Choroid cells possessed only the chemical mode throughout development.



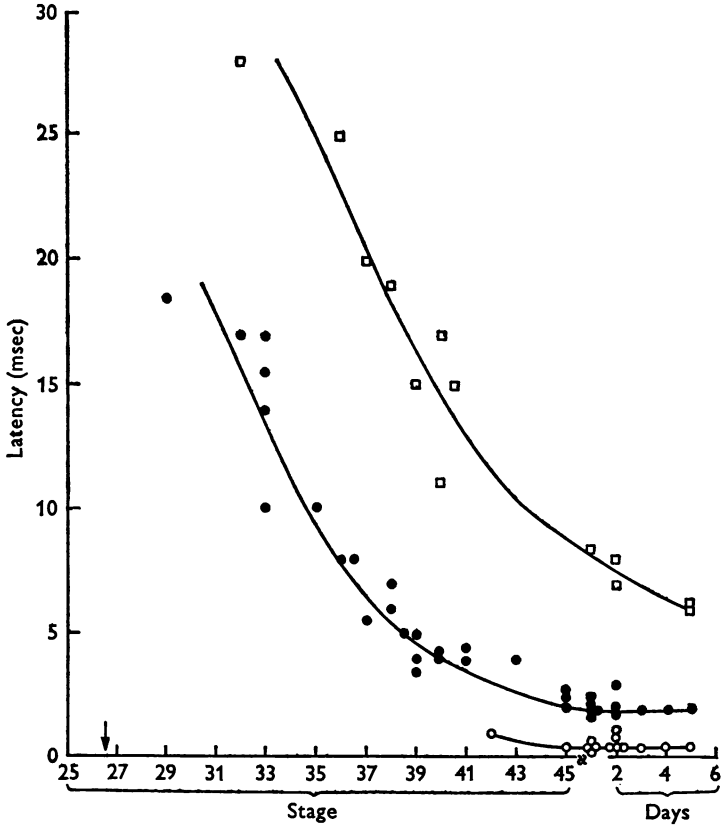
Text-fig. 3. *A*, presynaptically evoked response recorded from one ciliary branch at Stage 33. The stimulus intensity has been slowly increased to show that the response was finely gradable at this stage. *B*, lack of response upon antidromically stimulating the ciliary branch recorded from in *A*, and recording from another branch. Similar records were obtained no matter which branches were stimulated or recorded from.

Latencies of ciliary and choroid responses

Latencies of both ciliary and choroid responses, initially quite long, decreased during development. Choroid responses were throughout development of longer latency than ciliary responses. In Text-fig. 4, latencies of ciliary (filled circles) and choroid (squares) responses, normalized for a conduction distance of 1 mm both pre- and post-synaptically, are plotted as a function of developmental stage. Latencies of electrical ciliary responses (open circles) from the time they appear at Stage 41, were similar to those obtained from newly hatched chicks. Such alterations in latency and a time lag of similar length between the onset of chemical and the appearance of electrical transmission were also seen during post-hatching development of pigeon squabs (Hess, Pilar & Weakly, 1969), and during nerve regeneration in the adult pigeon (Landmesser & Pilar, 1970).

Conduction velocities

In the newly hatched chick, the difference in latency between the ciliary and choroid responses results because ciliary cells are innervated by faster conducting preganglionic fibres, and have larger diameter axons as well (L. Landmesser & G. Pilar, unpublished observations). Likewise, throughout embryonic development preganglionic fibres innervating ciliary cells



Text-fig. 4. Latency of presynaptically evoked response recorded from the ciliary and choroid nerves from different embryonic stages to 5 days post-hatching. Chemical ciliary responses (filled circles) are always of shorter latency than choroid responses (squares). Onset of transmission (Stage 26½) indicated by arrow. Until Stage 29 it was not possible to record from and determine the conduction velocities of both pre- and post-synaptic nerves, necessary to normalize for differences in conduction distance. Electrical ciliary responses (open circles) are not observed extracellulary until Stage 42, when their latencies are not significantly different from later stages. Curves fitted by eye.

always had lower thresholds and conducted more rapidly than presynaptic choroid fibres. Mean values for ciliary (filled circles) and choroid (squares) responses obtained from values plotted in upper graph, Text-fig. 5, up to Stage 40 were $0{\cdot}57 \pm 0{\cdot}04$ m/sec (mean $\pm$ S.D.) and $0{\cdot}2 \pm 0{\cdot}08$ (mean $\pm$ S.D.) m/sec respectively. These slow presynaptic conduction velocities were due to the small size and unmyelinated nature of the embryonic fibres (see anatomical description below) and for the most part explained the large differences in latency shown in Text-fig. 4. Similar slight differences in conduction velocity have allowed Carpenter & Bergland (1957) to distinguish components with large latency differences in unmyelinated embryonic chick sciatic nerve. As observed in the upper graph of Text-fig. 5, presynaptic conduction velocity increased with time in both populations, more abruptly from Stage 40 (14 days) especially in the ciliary population, presumably as the result of myelinization. Such large increase in conduction velocity was also correlated with myelination in developing chick sciatic nerve (Carpenter & Bergland, 1957).

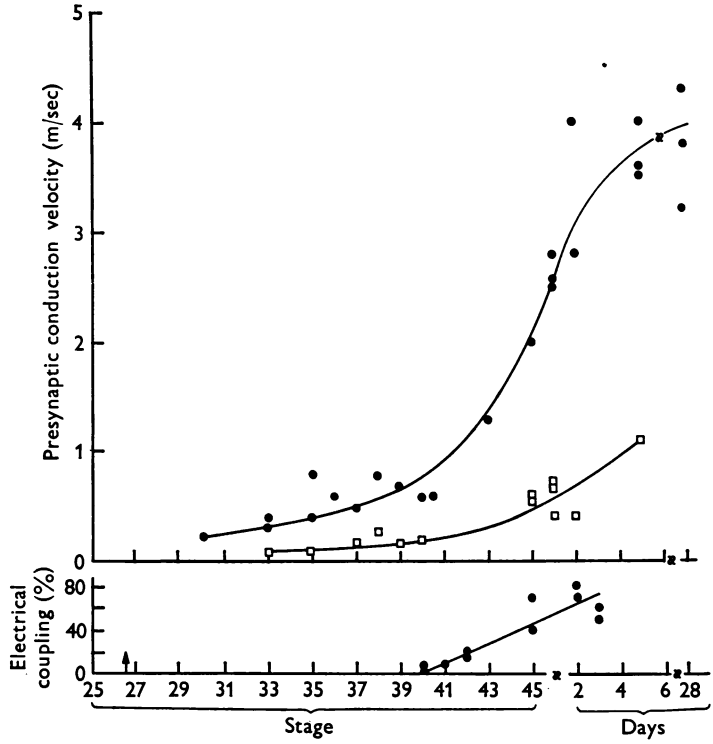
Transmission block with repetitive stimulation always occurred at lower frequencies in the choroid population. For example, the Stage 33 choroid response was completely blocked at 5/sec, the ciliary response only at 20/sec. This difference persists in the chick where ciliary responses are blocked at 40/sec and choroid at 20/sec.

Thus, from the time synapses become functional, preganglionic fibres mediating the ciliary responses can be distinguished from those mediating the choroid responses. Differences between post-synaptic conduction velocities from ciliary and choroid nerves were also evident from Stage 30 when measurements from choroid nerves first became tenable. Mean values for ciliary and choroid conduction velocities from Stage 30 until Stage 40 were $0{\cdot}26 \pm 0{\cdot}09$ m/sec (mean $\pm$ S.D.) and $0{\cdot}16 \pm 0{\cdot}03$ m/sec (mean $\pm$ S.D.) respectively. In the adult pigeon and chick post-synaptic ciliary fibres also conduct about twice as fast as choroid fibres (L. Landmesser & G. Pilar, unpublished observations).

Similar changes in conduction velocity have been observed during reinnervation in the adult pigeon (Landmesser & Pilar, 1970). There, the increase in presynaptic conduction velocity was accompanied by an increase in the proportion of electrically transmitting cells. This also occurred during embryonic development as can be seen by comparing the upper graph of Text-fig. 5, where conduction velocity is plotted as a function of developmental stage, with the lower graph of the same Figure, showing percent of electrically transmitting ciliary cells.

In the present experiments, employing suction electrodes, the proportion of electrically transmitting cells in newly hatched chicks was approximately 70 %. In previous experiments using a fixed bath in which pre- and post-synaptic nerves lay

in a channel filled with Vaseline, the degree of suprathreshold coupled cells was usually only 20–40 % at hatching, increasing to 100 % during the next few weeks (see also Martin & Pilar, 1963*b*). The discrepancy between these and the previous experiments seems to result from the different experimental conditions. However, in both cases, intracellular records reveal that all ciliary cells possess electrical coupling potentials at hatching. The reasons in the present experiments favouring a greater proportion of suprathreshold coupling potentials at hatching are not known.



Text-fig. 5. Conduction velocity (upper graph) and degree of electrical coupling (lower graph) from embryonic Stage 25 until 28 days post-hatching. Upper graph shows that conduction velocity of presynaptic ciliary (filled circles) and choroid (squares) nerve fibres increases during development, especially rapidly after Stage 40. Conduction velocity of presynaptic ciliary fibres is always faster than that of choroid fibres. Percent of electrical coupling, determined from ratio of area under electrical peak to area under whole ciliary response (see Hess *et al.* 1969), increases from zero at Stage 39 to 75 % at 2 days post-hatching.

Peripheral connexions

It was of interest to know if ciliary and choroid cells had made contact with their periphery at the time they were first innervated. This was initially tested for the ciliary population, by observing the degree of iris closure to maximal post-synaptic stimulation. Contractile responses could

only be elicited from Stage 36, when ganglionic transmission was well established. However, lack of responses at earlier times was not evidence of lack of innervation. For irises that did not contract with nerve stimulation also failed to contract when superfused with either KCl or ACh. Subsequent observations with the electron microscope showed such irises to consist of myoblasts lacking both organized myofibrils and sarcoplasmic reticulum. However, as early as Stage 25, small unmyelinated nerve fibres were seen running in the iris tissue. It seems that at least the ciliary cells were in contact (not necessarily synaptic) with the periphery before they were functionally innervated by preganglionic fibres.

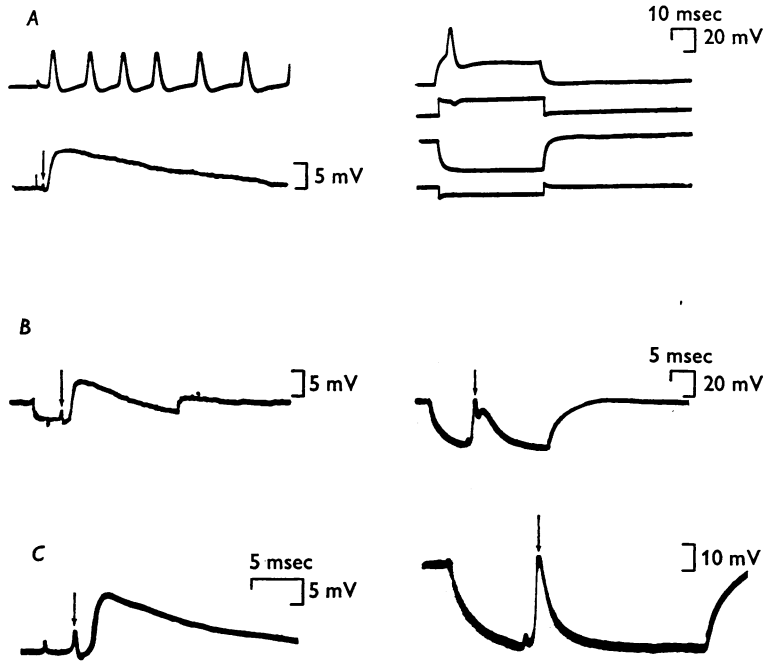
Intracellular recordings

Synaptic potentials

Intracellular recordings could be successfully obtained from Stage 38½ ganglia, six days after the onset of transmission through the ganglion. Resting potentials (35–50 mV) were lower than values obtained from young chicks (50–70 mV) (see also Martin & Pilar, 1963*a*), but impalements were often stable for at least 30 min.

Orthodromic stimulation usually gave rise to a burst of ganglion cell action potentials (up to twenty) in contrast to single spikes elicited in chicks. The first six spikes of such a burst can be seen in the left of Text-fig. 6*A*. Antidromic stimulation of the ciliary nerves produced a single spike (not shown), as did depolarizing pulses (Text-fig. 6*A*, right). When spikes were blocked with hyperpolarizing pulses (Text-fig. 6*B*, left) or when the excitatory post-synaptic potential (PSP) did not reach spike threshold (Text-fig. 6*A*, lower trace left) long time course PSPs were revealed. The PSP shown in Text-fig. 6*A*, left, is preceded by a subthreshold electrical coupling potential (arrow). Most of the ciliary cells are not electrically coupled at this stage. The time constant for decay of the PSP was 80 msec; the membrane time constant only 2·5 msec. Since the PSP did not follow the membrane time constant, prolonged transmitter action, including possible prolonged release, seemed probable. PSPs from newly hatched chicks also had decay times in excess of the membrane time constant, but usually only by a factor of 3–4 (twenty cells) (also Martin & Pilar, 1964). The membrane time constants of embryonic cells ranged from 1·5 to 4·3 msec (twelve cells), similar to the values for newly hatched chicks. However, embryonic PSPs had time constants 20–40 times the membrane time constant. Such prolonged decay times persisted until Stage 43 (2 days before hatching). The rather abrupt shortening of the falling phase of the PSP can be seen by comparing in Text-fig. 6*B*, the Stage 40 PSP, on the left, with the PSP recorded from a 1-day chick, on the right.

Changes in coupling potentials were also observed. Initially the amplitude was 1–2 mV, at Stage 40 (Text-fig. 6*B*, right). The subthreshold coupling potentials observed in every ciliary cell at Stage 40 had almost all become suprathreshold by hatching time (20–40 mV amplitude). Time course changes were also noted. The embryonic coupling potential seen in Text-fig. 6*C* left, (arrow) has a fast decay time and is diphasic. Similar such small diphasic potentials were previously observed in pigeon squabs

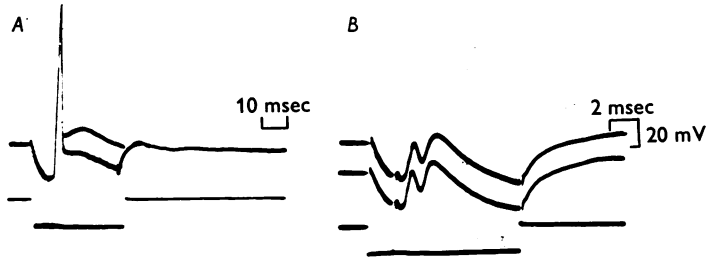


Text-fig. 6. Intracellular responses. *A*, left shows burst of spikes elicited by a single orthodromic stimulus in embryonic cell (Stage 38½). Lower left trace shows prolonged PSP. Right shows results of passing current pulses directly through cell. Current record is bottom trace of each pair. *B*, left, showing PSP from Stage 40 embryo with small electrical coupling potential (CP) and prolonged PSP seen during hyperpolarizing pulse to block spike initiation. Right, from 1 day chick showing much shortened PSP, and larger, CP. *C*, left, showing small, diphasic CP from Stage 40 embryo; right, larger monophasic CP from same cell as *B*, right. Chemical post-synaptic potential was blocked by 10^{-6} M-dTC. Arrows indicate electrical coupling potentials.

at 2 weeks post-hatching (see Fig. 2 in Hess *et al.* 1969) which approximately corresponds in developmental sequence to a Stage 40 chick embryo. Later in development the coupling potential became monophasic and decayed more slowly, approximating the membrane time constant, as seen in Text-fig. 6*C* right.

The effects of anticholinesterases

The prolonged PSPs seen during embryonic development seem to result from prolonged transmitter action. While time constants of PSPs from young chicks were in excess of membrane time constants, they were not significantly increased by the anticholinesterases, eserine or neostigmine (Text-fig. 7*B*). Similar observations have been made by Dennis, Harris & Kuffler (1971) on many of the parasympathetic ganglion cells in the frog heart. The possibility that anticholinesterases were without effect because they failed to reach the synaptic areas or because cholinesterase was absent was excluded. Anticholinesterases potentiated the excitatory effect



Text-fig. 7. Effect of eserine on PSPs. *A*, hyperpolarizing pulse was insufficient to block action potential from Stage 43 cell, but falling phase of underlying PSP is clearly seen (bottom superimposed trace). Addition of eserine (upper trace) greatly prolonged falling phase of PSP. *B*, PSPs from a 1-day chick showing no effect (top trace) of eserine (10^{-6} g/ml., superfused for 1 hr) on the time course of control (bottom trace).

of externally applied ACh by a factor of 500–1000. After recovery from depolarization, the persisting block of the PSP was also enhanced by anticholinesterases (G. Pilar & L. Landmesser, unpublished observations).

These effects of anticholinesterases on externally applied ACh occurred in the early embryo as well. Yet anticholinesterases greatly prolonged the slowly decaying PSPs in the early embryo (Text-fig. 7*A*). Cholinesterase has been histochemically demonstrated throughout this period of development (Koenig, 1965).

Opposite changes have been observed in developing chick neuromuscular junctions in tissue culture where anticholinesterases are initially without effect (Kano & Shimada, 1971).

Ultrastructure

In the newly hatched chick the ciliary and choroid cells can be distinguished. Ciliary cells have larger, myelinated somas, with presynaptic calyces occupying approximately 25 % of the cell surface at the pole where the axon emerges (Marwitt *et al.* 1971; see also Martin & Pilar, 1964).

Choroid cells are smaller, unmyelinated, and have scattered bouton synapses indented in the ganglion cell soma. These features have been previously described by Hess (1965). Both groups are spatially separated within the ganglion. Very small spine-like 'pseudodendrites' project from the ciliary cells into the cytoplasm of the calyx (Hámory & Dyachova, 1964; Hess, 1965; Takahashi & Hama, 1965). Choroid synapses always abut directly on the soma.

Stage 25–26½ (4½–5 *days*). *Onset of transmission*

At Stage 25, before transmission through the ganglion was detected, many ganglion cells could be recognized, although ciliary and choroid populations were not distinguishable. Some undifferentiated cells could not be classified as either neuroblasts or glioblasts. Bundles of unmyelinated axons were seen between ganglion cells. Since at that time, the ganglion cells possess well developed axons, the pre- or post-synaptic nature of these axon bundles could not be ascertained.

At the time of onset of transmission (Stage 26½) there was a large increase of unmyelinated fibres in the ganglion but since transmission became 100% only between Stages 30–33, no attempt was made to systematically sample the ganglia from these intermediate stages. A Stage 33½ ganglion with 100% transmission was completely sampled by observing sections every 10 μ.

Stage 33½ (8 *days*). 100% *chemical transmission*

At this stage the ganglion was composed of small (15–20 μ diameter) roughly spherical cells with eccentric nuclei. The cells contained many free ribosomes, sparse rough endoplasmic reticulum, scattered mitochondria, and prominent Golgi apparatuses. Approximately one third of the cells were opposed to other ganglion cells for as much as 6 μ without glial separation, as has been described by Pannese (1968), but lacking both the 'adhesion plaques' and 'zona occludens' that he observed in developing chick spinal ganglia. The question of such cells being electrically coupled arises, especially considering the method of estimating degree of transmission. However, there seems to be no evidence that these regions are sites of effective electrical coupling. Probably such sites of apposition may only exist because the cells have not yet been completely enveloped by satellite cell processes.

A large proportion of the ganglion volume was occupied by bundles of unmyelinated nerve fibres (0·3–1·5 μ diameter) containing neurotubules and for the most part not separated by glial elements (Pls. 1*A*, *B*, 2*A*). Such axon bundles occupied large portions of the ganglion cell surface (Pl. 2*A*), the remainder being covered with glial processes (Pl. 1*A*) except

in the areas of ganglion cell apposition described above. No structures morphologically similar to growth cones (Tennyson, 1970) were observed. Light microscopy at this stage revealed a distinct choroid population of smaller cells on one side of the ganglion. However, unlike the newly hatched chick where the types of synapses on ciliary and choroid cells are distinct, all Stage 33½ ganglion cells were similar, possessing many long and intrincate processes that were interposed with the axon bundles forming a dense neuropile (Pl. 1*A* and *B*). Some of these processes clearly emerged from the soma and contained mostly free ribosomes. Others had only neurotubules and occasional ribosomes. It is not clear if these latter processes were extensions of the axon hillock or represented the initial segment of the post-synaptic axon.

Specialized synaptic contacts with pre- and post-synaptic membrane thickenings were extremely infrequent and contained only few pleiomorphic, agranular synaptic vesicles, which showed greater size variation than in mature synapses. Many of the observed synaptic contacts occurred on the cell processes.

Stage 36½ (10 *days*). *Calyx formation*

From this stage on, most of the anatomical descriptions will deal with the ciliary population, because this cell group undergoes dramatic changes during development.

By Stage 36½ the cells were larger, approximately 25 μ in diameter, and most axonal profiles were ensheathed by glia. Calyces were first observed on some ciliary cells at this stage (Pl. 2*C*), and by Stage 39, were seen on all ciliary cells (Pl. 3*A*). Before Stage 36½ only bundles of unmyelinated nerve endings were in contact with cells. Pl. 2*B* shows a portion of ganglion cell in contact with many fine nerve endings containing scattered synaptic vesicles. These overlapping fingers of cytoplasm seem to be continuous with the larger terminal branch (Pl. 2*B* left) which in turn is continuous with the calyx type ending seen in Pl. 3*C*. One can also observe in the same calyx, left, parallel double membranes running well within the cytoplasm (asterisk). The area between the membranes is electronlucent, distinguishing it from the abundant smooth endoplasmic reticulum which contains a denser material. It would seem that calyx formation, as seen at this stage, involves either a coalescence of the many fine initial terminals, or a filling in of the interstitial areas of terminal branches, so that the many endings expand over the cell surface resulting in a calyx. In this context, the membranes seen within the cytoplasm would represent areas of plasma membrane that had been internalized.

The other possibility is that one bouton expands with simultaneous retraction of the others. However, the initial synaptic boutons already

possess morphologically defined synaptic structures. If retraction occurred, one should observe synaptic endings in various stages of regression. This was not seen at any period studied. If calyx formation results from coalescence or filling in of branches, all terminal branches must originate from the same neurone, since light microscopical studies have revealed only one afferent axon impinging on these cells (see Marwitt *et al.* 1971, for references).

Abundant smooth endoplasmic reticulum (Pl. 2*C*) also has been observed by Mugnaini (1971) in developing cerebellum, and by Lentz (1967) in regenerating nerve, and may in the present case be related to rapid growth or expansion of the terminal. Calyx formation must also be accompanied by a retraction of the many ganglion cell processes seen at earlier stages. Multivesicular bodies and dense membrane bound granules as described by Mugnaini (1971) in developing cerebellar cortex were also seen, and may be related to lytic activity, perhaps of unnecessary membrane material.

Stage 39 (14 *days*). *Subthreshold electrical coupling*

The calyx, seen in Pl. 3*A*, is typical of the afferent terminations on ciliary cells at this time, having extensive areas of apposition with the post-synaptic cell. Smooth endoplasmic reticulum and mitochondria are abundant in the calyx, but synaptic vesicles are still sparse, and seen only near the synaptic membrane thickenings. A few large dense core vesicles were occasionally observed. The rarity of synaptic vesicles is similar to previous stages. There is a clear zonation of ganglion cell rough endoplasmic reticulum, most occurring in the peripheral cytoplasm. The remainder of the cell contains mostly free ribosomes. Calyces were present on the axon hillock pole of the cell, as in the newly hatched chick. The somas were as yet devoid of myelin although satellite cells ensheathed the soma and calyx.

Two-day chick. Suprathreshold electrical coupling

The most obvious differences between the calyx at this stage and Stage 39 is a large increase in the number of synaptic vesicles, which now occupy the entire calyx, instead of being restricted to a few active zones (Pl. 3*B*). During the first week post-hatching some of the calyces began to be broken up into a mass of boutons by a process of invagination of calyx fingers into the soma, and intrusion of somal processes into the calyx, previously observed by Hámory & Dyachova (1964).

Ciliary cells were now surrounded by myelin lamellae, which tended to be less compact at the axon hillock pole where the preganglionic fibre terminated.

Gap junctions, the other ultrastructural feature presumably correlated with electrical coupling (Brightman & Reese, 1969) have been observed by Takahashi & Hama (1965) in chick ciliary cells but not in the embryonic ganglion. While both Brightman & Reese (1969) and G. Pilar and L. Landmesser (unpublished observations) have observed gap junctions in adult pigeons and young chicks using lanthanum as an extracellular marker, as yet the method has proved too variable for us to obtain a quantitative estimate of gap junctions during embryonic development.

DISCUSSION

The various findings described in the results will be discussed under three separate headings.

Specification and differentiation of synaptic connexions

This study of the normal development of the chick ciliary ganglion has produced several observations related to specification and the manner in which cells become initially connected. Furthermore, the ciliary and choroid populations differ sufficiently, morphologically and functionally, to warrant investigation into what controls or can modify their differentiation. These results do not rule out that some preganglionic axons are in anatomical contact with ciliary ganglion cells before differentiation, as observed by Tello (1923), but in any case their presence is not needed for initial ganglion cell differentiation (Levi-Montalcini & Amprino, 1947). Since the present results show that ciliary ganglion cells have differentiated and are in contact with their periphery before they are functionally innervated, it is possible that the periphery influences the type of preganglionic fibres forming synapses or the type of synapses so formed. This possibility can be investigated using this preparation. It is known that removal of the optic vesicle at Stages 11–13 (Amprino, 1943; Cowan & Wenger, 1968) results in ganglion cell degeneration only from Stage 35 on. Since the present experiments have shown that synapses are already formed at Stage 26½–33, it will be possible to determine if cells never in contact with their periphery (as a result of optic vesicle removal at Stage 11–13) are ever innervated. If synapses are formed, the effect of the periphery on ganglion cell specification, i.e. the type of preganglionic fibres making contact, can be resolved. If synapses are not formed, this functional deafferentation may contribute to the observed ganglion cell degeneration.

Since presynaptic ciliary fibres always conduct faster than presynaptic choroid fibres, it is probable that the presynaptic fibres have already been specified by the time that transmission starts, and that they selectively innervate the proper post-synaptic cells from the beginning. This does not rule out the possibility that many random anatomical (not necessarily functional) connexions are formed. In fact, the existence of many cell

processes during the period of synapse formation may increase the chance that each cell type comes into contact with the proper type of presynaptic fibre. For after connexions are formed, there is retraction of most of these processes.

Ultrastructure and transmission

The substantial alterations in ultrastructure that occur at rather precise times during development can be correlated with the electrophysiological findings. The onset and development of synaptic transmission has been traced and changes observed at definite stages.

The initiation of transmission (Stage 26½) coincided with the appearance of morphologically defined synaptic contacts, although their occurrence was rare, even at Stage 33½ when all cells were transmitting. Such paucity of synapses was also observed by Bodian in the developing mammalian spinal cord (Bodian, 1966, 1968).

Few synaptic vesicles were observed in terminals from early ganglia, a condition that persisted until Stage 41. If the observed synaptic vesicles represent quanta of transmitter (Katz, 1966) quantal content would be thought to be low at these synapses, as was observed in developing nerve–muscle junctions in tissue culture (Robbins & Yonezawa, 1971). However, neither failures nor large fluctuations in PSP amplitude were observed in the present study. Therefore, if the number of releasable quanta is indeed low, release probability may be high and consequently the Poisson law does not apply. But it would be premature to discuss other possible alternatives without additional experimental evidence.

Another developmental feature observed is the formation of calyces on the ciliary cells. In some respects these observations are similar to Morest's (1968) light microscopy description of many fine nerve branches as precursors to calyces on the cells of the mammalian trapezoid body. However, unlike the calyces of the trapezoid body, those of the ciliary ganglion are transitory, since they break up into a cluster of boutons after 2–3 weeks. What actually causes the formation of a calyx from the many fine initial contacts and its later breakup is unknown.

The physiological significance of the calyx is also elusive, although it has been implicated in electrical transmission (Martin & Pilar, 1964). It does seem to foreshadow the appearance of electrical coupling and occurred on all ciliary cells by stage 40 when fast and diphasic electrical coupling potentials were uniformly obtainable.

In the model proposed to explain the electrical coupling (Martin & Pilar, 1964), such relatively fast decaying and slightly diphasic coupling potentials would result from a greater ratio of capacitative/resistive coupling in a system with homogeneous membrane properties; i.e. the time constant of the

synaptic membranes do not differ from the time constant of the non-synaptic membranes. The large area of apposition of pre- and post-synaptic membranes would be the only required morphological specialization. From Stage 42 through the first week post-hatching, the electrical coupling potentials become larger, monophasic and more slowly decaying. Such potentials require a greater proportion of resistive coupling and synaptic membranes with time constants less than the remainder of the cell. The only ultrastructural change observed at this time is myelination of the cell soma, the calyces remaining unaltered.

The myelinization would increase the resistance of the somal membrane, insuring that more of the coupling current would leave through the axon hillock area. Such increased resistance could also compensate for a decrease in synaptic membrane resistance, as the result of gap junctions (Brightman & Reese, 1969; Takahashi & Hama, 1965) and still maintain a total input resistance in the observed range of 25 MΩ. The breaking up of the calyx during the first 2 weeks post-hatching does not impair electrical transmission. This was also observed in developing pigeon squabs (Hess *et al.* 1969). In summary several factors seem to contribute to effective electrical coupling, although the relative importance of each may vary during development.

Time course of post-synaptic potential

Initially the chemical post-synaptic potentials are prolonged and further lengthened by anticholinesterases. However, during the latter part of development PSPs become markedly shortened and are unaffected by anticholinesterases. Similar changes in time course of excitatory junctional potentials were observed in developing mouse vas deferens (Furness, McLean & Burnstock, 1970) although the transmitter and mechanism of transmitter inactivation are different in these adrenergic synapses.

The shortening of the PSP rather abruptly is possibly due to a shortening of transmitter secretion for which we have no evidence. The early long PSPs cannot be explained by temporal summation because each ciliary cell has a single calyx. Since spontaneous miniature synaptic potentials were not observed, a comparison could not be made between their time course and that from elicited PSPs.

Another possibility is that later in development diffusion of transmitter from the synaptic cleft is fast enough to terminate transmitter action. If diffusion were sufficiently slower at early embryonic stages hydrolysis of ACh might then become rate limiting. This would explain the lengthening of the early long PSPs by eserine, and the lack of effect of anticholinesterases on the more mature, shorter PSPs. However, there are no obvious structural alterations that can be correlated with such postulated changes in diffusion.

In light of recent evidence relating to the action of anticholinesterase another possibility should be considered. Eserine might combine with ACh receptors making their complex with ACh more stable (Kuba & Tomita, 1971). Receptor properties would then have to alter during development. Finally, the shortening of the PSP time course could result from receptor desensitization if more acetylcholine is released at later stages when synaptic vesicles are abundant, since receptor desensitization is readily produced in these synapses (G. Pilar & L. Landmesser, unpublished observations).

From the above discussion it seems that there is no simple explanation for the function of cholinesterase in synaptic transmission of this ganglion.

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EXPLANATION OF PLATES

The following abbreviations are used in all plates: a, axonal process; C, calyx; GC, ganglion cell; G, Golgi apparatus; g, glial process; M, myelin; n, nucleus; p, ganglion cell process; SC, satellite cell. Calibration bars indicate 1 μ.

PLATE 1

A, region of neuropile from a Stage 33½ ganglion. Many ganglion cell processes are interposed with bundles of neurotubule containing unmyelinated axons. Early stages of synapse formation (arrows) include thickening of pre- and post-synaptic membranes and a few vesicles. Glial processes cover parts of the ganglion cell surface but do not yet ensheath the individual unmyelinated axons.

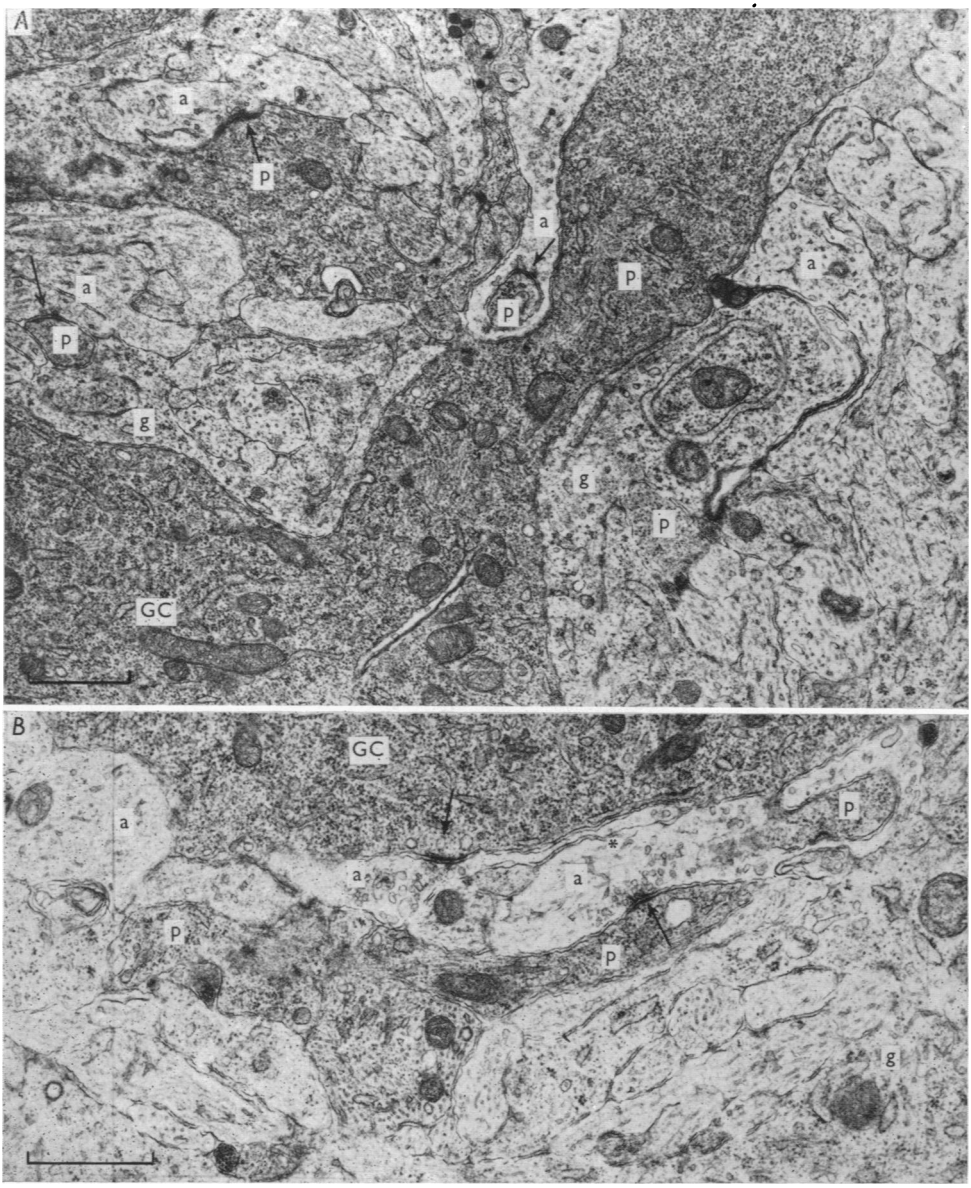
B, early synapses from a Stage 33½ ganglion, with pre- and post-synaptic membrane thickenings and clusters of vesicles, occur both directly on the cell soma and on a ganglion cell process. Such synaptic contacts were quite rare in occurrence at a stage when transmission was 100%. Nerve endings contain neurotubules, mitochondria and vesicles. Asterisk marks area where two fingerlike nerve endings overlap, and may indicate the first stage of calyx formation from initial fine, separate endings (see text, p. 705, and Pl. 2*B*, *C*).

PLATE 2

A, region of ganglion cell soma from Stage 33½ embryo. Bundles of unmyelinated axons, seen in close apposition to the ganglion cell soma, were very common at this stage. Scattered ribosomes, and Golgi apparatus, are seen in the soma.

B, stage 36½ ciliary cell showing the unmyelinated axon branches terminating in many fine fingers of overlapping cytoplasm that contain scattered synaptic vesicles. Specialized synaptic contacts are indicated by arrows. Some of these fingers are continuous with one end of a presumptive calyx, the remainder of which appears in Pl. 2*C* rotated 90°.

C, the calyx covering the large portion of the ganglion cell surface contains neurotubules, scattered mitochondria and smooth endoplasmic reticulum. Two specialized synaptic contacts are indicated by arrows. The asterisk lies between two stretches of double membranes within the calyx cytoplasm. Such internalized membranes, a common occurrence at this stage, may result either from coalescence of the fine finger-like endings seen in Pl. 2*B*, or by a filling in of the interstitial areas of the fine branches of the nerve ending.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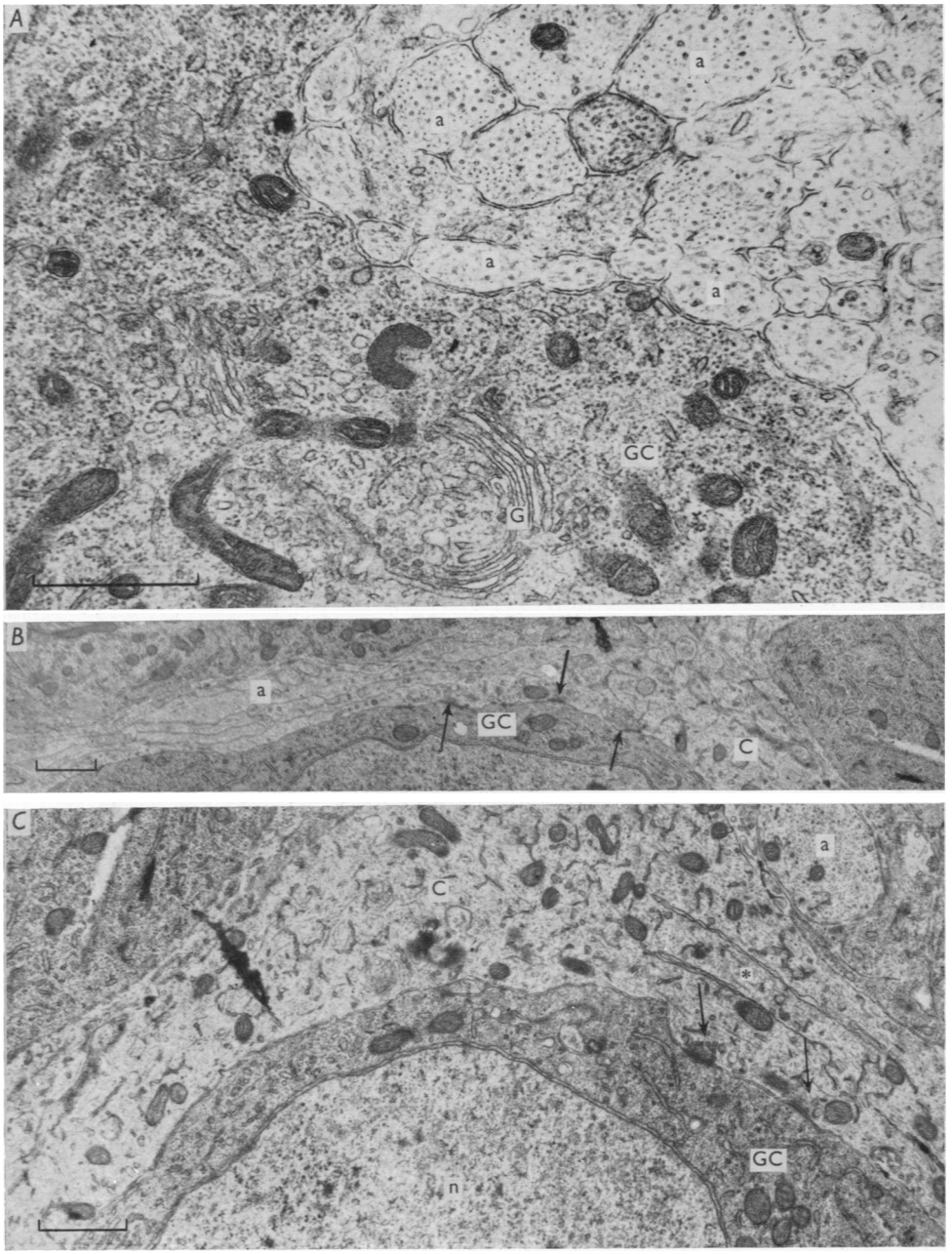
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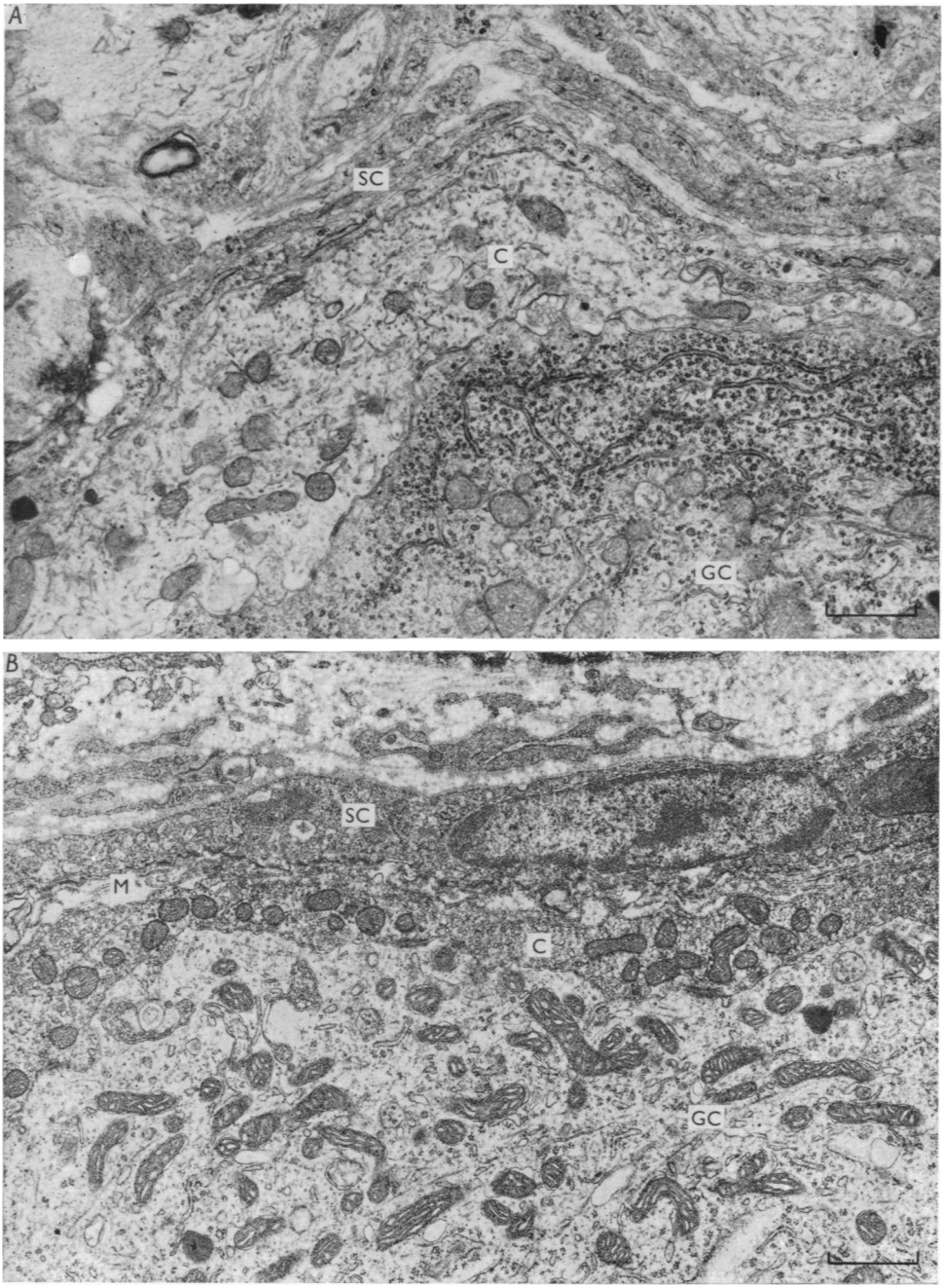
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PLATE 3

A, part of a Stage 39 calyx. Although calyces are now apposed to ganglion cells for large areas, synaptic vesicles are still very sparse and, as indicated by this picture, were often absent. Mitochondria and smooth endoplasmic reticulum are abundant in the calyx, and the ganglion cell shows a clear band of rough endoplasmic reticulum in the peripheral cytoplasm. Overlapping layers of satellite cell cytoplasm ensheath the cell and calyx, and most of the axons at this stage.

B, region of calyx from a 2-day chick, showing striking increase in number of synaptic vesicles. Ganglion cell cytoplasm no longer contains a region of rough endoplasmic reticulum in the periphery. Myelin lamellae now surround cell and calyx.