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SELECTIVE REINNERVATION OF TWO CELL POPULATIONS IN THE ADULT PIGEON CILIARY GANGLION

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SUMMARY

1. Presynaptic fibres innervating the adult pigeon ciliary ganglion were cut 2 mm proximal to the ganglion. The modification of transmission in the ciliary and choroid cell populations was studied after periods of 6 hr–100 days.

2. Transmission failed after 2 days and for the next 10 days there was no transmission through the ganglion. Long latency responses in both ciliary and choroid nerves were first observed at 13–15 days, and the latency decreased toward control values in 40 days. Electrical transmission reappeared in the ciliary population in about 26 days.

3. Presynaptic fibres innervating the ciliary population have higher conduction velocities and lower electrical thresholds than those innervating the choroid group. This relation was maintained throughout the reinnervation process. Fibres innervating the extraocular muscles also regenerated and achieved conduction velocities similar to their control values.

4. In two experiments out of seventeen, a few fast conducting, low threshold fibres, presumably stray ciliary fibres, innervated choroid cells and induced electrical transmission.

5. It is concluded that each group of cells, ciliary and choroid, was reinnervated in a highly selective manner by its original class of presynaptic fibres, and that the presynaptic ciliary elements cause the specializations necessary for electrical transmission.

INTRODUCTION

The orderly arrangement of cells in the nervous system seems to result from cytospecificity of the individual elements involved. Experimental evidence supporting this hypothesis arises from studies of the ontogenesis

of the nervous system and on functional restoration after nerve section (Sperry, 1965). In higher vertebrates, due to negligible regeneration in the central nervous system (Sugar & Gerard, 1940; Windle, 1956), most studies have been confined to the somatic peripheral segment, especially the skeletal neuromuscular junction. The results indicate a lack of selectivity during nerve regeneration (Weiss & Hoag, 1946; Bernstein & Guth, 1961; Miledi & Stefani, 1969; but see also Feng, Wu & Yang, 1965).

One case of selective reinnervation in higher vertebrates was first described by Langley (1897*a*,*b*) and later confirmed by Guth & Bernstein (1961) in autonomic ganglia. From the restoration of proper physiological function after preganglionic nerve section, they concluded that the cells of the cat superior cervical ganglion had been reinnervated by their original class of presynaptic fibres. The structural and functional complexity of the superior cervical ganglion (De Castro, 1932; Perri, Sacchi & Casella, 1970) hampers further analysis of the reinnervation. The factors responsible for selective reinnervation can be better evaluated in the avian ciliary ganglion with its simple neuronal organization and accessibility during all stages of development. This study of reinnervation was therefore carried out on the ciliary ganglion of pigeons after preganglionic oculomotor nerve section.

The pigeon ciliary ganglion, like that of the chicken, consists of two distinct cell populations, choroid and ciliary, that can be differentiated anatomically as well as physiologically (Marwitt, Pilar & Weakly, 1970). The axons of the choroid group terminate in the smooth muscle coat of the choroid where transmission is muscarinic (L. Landmesser & G. Pilar, unpublished observations.) The ciliary group is distinguished by the presence of myelin lamellae around the soma and synaptic apparatus and innervates the iris and ciliary body. In the bird the iris muscle is striated and transmission is nicotinic (Pilar & Vaughan, 1969*a*, *b*). The ciliary group is also distinguished by a dual, electrical and chemical, mode of ganglionic transmission (Martin & Pilar, 1963; Hess, Pilar & Weakly, 1969). In both groups chemical transmission is cholinergic and a single presynaptic fibre synapses with each ganglion cell.

The electrical coupling serves as a useful independent label of synaptic events, important in determining the occurrence of selective reinnervation. Furthermore, a study of reinnervation in these fibres may provide a better understanding of the factors necessary for electrical transmission if physiological changes during regeneration can be correlated with definite anatomical structures (Martin & Pilar, 1964; Hess *et al.* 1969; Brightman & Reese, 1969).

The results of the present study show that each group of cell populations was reinnervated by its original class of presynaptic fibres, and that the

specializations necessary for electrical transmission can be brought about by the presynaptic element.

METHODS

The left oculomotor nerves of thirty-two adult pigeons (*Columba livia*) were severed intracranially under methoxyfluorane anaesthesia (Marwitt *et al.* 1970) approximately 2 mm (range 1·7–2·2 mm) proximal to the ciliary ganglion. A brief description of the surgical procedure follows. Skin and dermal muscles were sectioned (parallel to the orbit posterior edge) by an electrosurgical unit (Bovie, Ritter Co.). Part of the parietal and squamosal bones was removed to expose the optic lobe. A wedge-shaped cut was made through the dura mater which was then reflected toward the orbit. Slight pressure was exerted to deflect the optic lobe and thereby maximize the space between it and the internal squamosal bone. The optic lobe was followed to the emergence of the optic nerve, marked by the ophthalmic branch of the internal carotid artery. The oculomotor nerve is then visible as it enters the fissure dorsal to the optic foramen. At this point the nerve is in close contact with the internal carotid artery and temporal rete. The nerve was sectioned with iridectomy scissors and the proximal and distal stumps were observed to retract leaving a gap of approximately 1 mm. This allowed a maximal chance of mixing of the nerve fibres during regrowth (Young, 1942). Completeness of section was also evident from the dilation of the pupil on the operated side.

Special care was taken to avoid bleeding. In the few cases where the blood supply to the oculomotor nerve was disturbed, the birds were sacrificed immediately and not used. The wound was closed by sewing the dermal muscles to the insertion of the temporalis muscle in the temporal fossae.

Healing occurred without complications in a few days and in some cases penicillin (10–20,000 u./lb.) was given I.M. as a prophylactic measure. Infections occur rarely in operated pigeons. They seem to possess a high level of natural immunity to the bacteria existing in the normal laboratory environment (i.e. pneumococos; Bateman & Rowley, 1969).

The ciliary ganglia together with pre- and post-synaptic nerves were isolated from the operated birds under brief ether anaesthesia from 6 hr to 100 days after sectioning the oculomotor nerve. The extracellular stimulating and recording techniques have been described previously (Martin & Pilar 1963; Hess *et al.* 1969). The same plastic chamber was employed in all experiments and recordings were made from the post-synaptic ciliary and choroid nerves with a constant distance between the stimulating electrodes on the oculomotor nerve and the ganglion, and between the ganglion and the post-synaptic recording electrodes. Therefore latencies from different experiments were directly comparable. The measurements were made between the initiation of the stimulus artifact and the foot of the post-synaptic response, elicited at threshold strength of stimulation. Conduction velocities were determined from the lower threshold presynaptic nerve fibres.

RESULTS

Responses from the ciliary and choroid nerves

The post-synaptic control responses elicited by maximal electrical stimulation of the oculomotor nerve are shown in the uppermost row of Fig. 1*A*, *B*. This preparation was isolated from an unoperated bird. The

response from the ciliary nerves (*A*) was characteristically bimodal as was previously shown by Hess *et al.* (1969). The second, longer latency peak of the response could be blocked by cholinergic blocking agents, high magnesium-low calcium (0·5 mM-Ca, 3–5 mM-Mg in contrast to normal Tyrode containing 3 mM-Ca and 1 mM-Mg) and was due to chemical transmission. The initial shorter latency peak of the ciliary response was resistant to the above treatments and has been shown with intracellular recording to be due to direct electrical coupling between pre- and post-synaptic elements (Martin & Pilar, 1963).

In the cells contributing to the first peak, current flow from the presynaptic spike is sufficient to reach threshold and discharge them. The remainder of the cells in which coupling is subthreshold, are discharged by the release of acetylcholine (Marwitt *et al.* 1970). However, the entire ciliary population is capable of being discharged by the electrical coupling potential (Marwitt *et al.* 1970).

The response recorded from the choroid nerves (Fig. 1*B*) had only a single peak due to cholinergic chemical transmission. The latency of the ciliary chemical response was always significantly shorter ($1{\cdot}8 \pm 0{\cdot}5$ msec, mean $\pm$ S.D.) than the choroid latency (Marwitt *et al.* 1970). This occurs, in part, because the ciliary population is innervated by larger diameter preganglionic fibres that conduct at $6{\cdot}9 \pm 3{\cdot}0$ m/sec (mean $\pm$ S.D.). The fibres that innervate the choroid cells conduct at $3{\cdot}4 \pm 0{\cdot}7$ m/sec (mean $\pm$ S.D.). In addition the post-ganglionic fibres from the ciliary cells are also larger than those from the choroid cells.

Subsequent responses in Fig. 1 were obtained at various times after oculomotor nerve section indicated in each row. These responses were selected because they were typical of changes observed during the re-innervation process.

Complete transmission failure occurred in both groups of cells 46 hr after nerve section and for approximately 10 days thereafter no transmission occurred. Thirteen days after nerve section, a progressive constriction of the pupil on the operated side indicated restoration of transmission through the ganglion.

The electrophysiological signs of this restoration are presented in the lower three rows of Fig. 1. A unimodal response of 5 msec latency first appeared in the ciliary nerve at 13 days. It was entirely the result of chemical transmission since it was blocked by superfusing the preparation with D-tubocurarine (2×10^{-6} g/ml.). There was no response from the choroid nerves. At 15 days, responses were observed in both ciliary and choroid nerves, with a latency of 6 msec in the ciliary and 13 msec in the choroid. Both responses were blocked with hexamethonium (10^{-4} g/ml.) Responses obtained from the ciliary nerves 36 days after nerve section (bottom row), were bimodal and of similar form and latency to the control

response. The choroid nerves showed a unimodal response with a 6 msec latency, also similar to the choroid control. The second peak of the ciliary response and the entire choroid response was blocked by cholinergic blockers. The first short latency peak of the ciliary response resisted the synaptic blocking agents, indicating the reappearance of electrical coupling in the ciliary neurones.

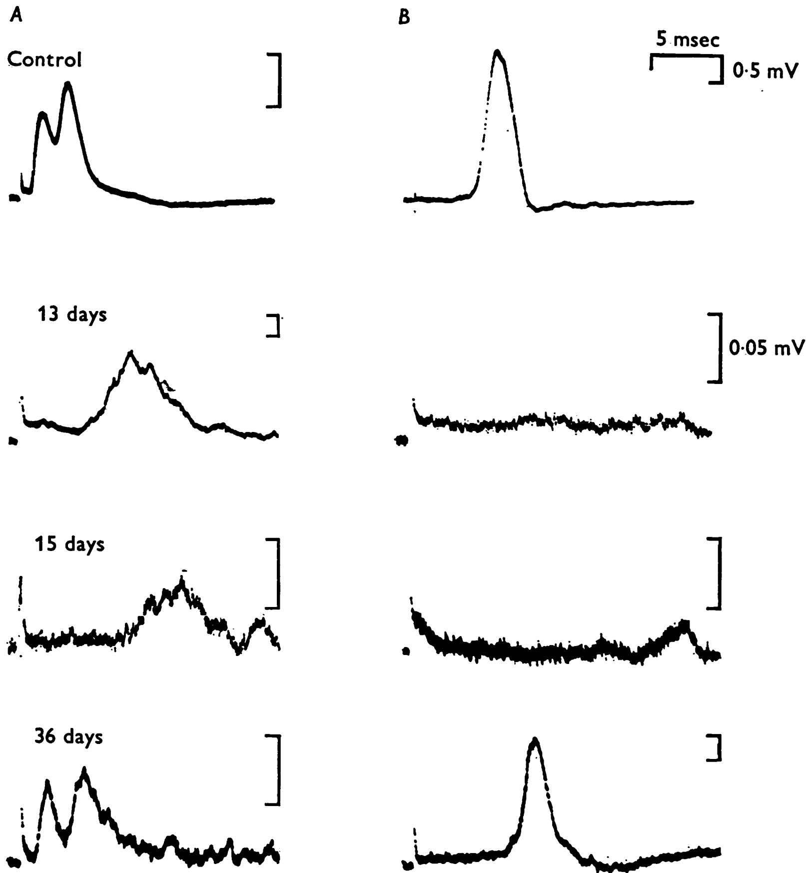

Fig. 1. Extracellular responses obtained from adult pigeon ciliary and choroid nerves elicited by electrical stimulation of the oculomotor nerve: ciliary *A*; choroid *B*. Top row shows control response in ciliary; longer latency, unimodal response in choroid. The next three rows show responses obtained at various indicated times after section of the oculomotor nerve. The pair of responses in each row were obtained from the same experiment. The responses in the bottom row from a 36-day reinnervated bird do not differ significantly from the control responses except for a slightly longer latency in the choroid.

Latencies of ciliary and choroid responses during regeneration

The time courses of latency changes are illustrated in Fig. 2 which shows the results obtained from ciliary (upper graph) and choroid (lower graph) nerves from 6 hr to 100 days after nerve section. In both graphs, the dashed line, fitted by eye, indicates changes in latency of transmission after denervation and during reinnervation. The line is discontinuous between day 2 and day 13 reflecting transmission failure. The series of denervation experiments, concerned with early changes in synaptic transmission up to 2 days after nerve section, will be the subject of separate papers (G. Pilar & J. N. Weakly, unpublished observations; G. Pilar & L. Landmesser, unpublished observations) and will not be further discussed here.

In order to minimize the latency fluctuations that are not directly related to the process of reinnervation, birds of comparable size and age were used in all experiments. The latencies shown in Fig. 2 were compared with previously published control values (Marwitt *et al.* 1970) obtained from unoperated birds. The latencies of both responses, initially quite long, gradually decreased during reinnervation to approach control values. They therefore provide a reliable indication of the time course of the reinnervation process. In contrast, response amplitudes, as seen in Fig. 1, were generally smaller than control values, fluctuated considerably, and were not correlated with time after nerve section.

In the upper graph of Fig. 2, the filled circles represent the latencies of chemically mediated ciliary responses; the open circles, the latencies of electrically mediated responses. Restoration of transmission, first entirely chemical, occurred 13 days after nerve section. The latency of the chemical response, initially three times longer than the control value, gradually decreased during the next 2 weeks. At 26 days after nerve section, electrical coupling reappeared with a slightly longer latency than normal. It is noteworthy that the latency of the electrical response did not change with time after the operation, once it initially reappeared. In the lower graph of the same Figure, the latencies of the choroid responses are plotted. A response first appeared at 2 weeks, and was of considerably longer latency than the control. The latency gradually decreased to approach a steady value at about 4 weeks. When latencies of ciliary and choroid responses from the same time after nerve section were compared, the ciliary response was always the shorter of the two, maintaining the original distinction between them. After 40 days both latencies reached steady values that were slightly longer than those of the controls. Only one experiment did not conform to this time sequence. In this case transmission was not restored at 20 days.

In two of seventeen reinnervated ganglia studied short latency

responses, such as the ones seen in Fig. 3*A* and *B* (arrows), were recorded from the choroid nerves. These responses were resistant to ganglionic blocking agents, and from previously mentioned criteria were judged to represent cells in which transmission was electrically mediated. In both cases the response amplitudes were very small, apparently representing

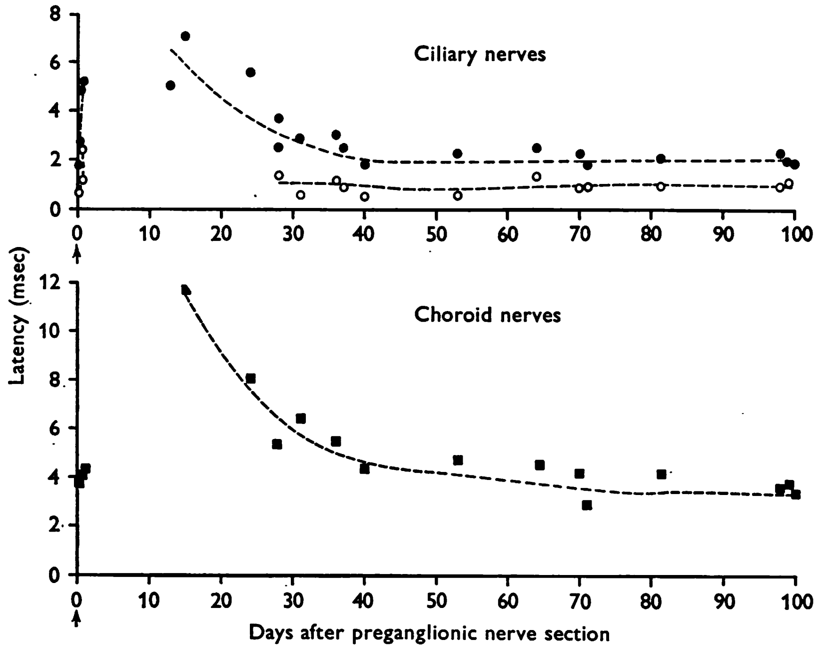

Fig. 2. Latency of extracellularly recorded post-synaptic responses as a function of time after oculomotor nerve section indicated by arrow. Upper graph: ciliary response showing electrical (filled circles) and chemical (circles) components; electrical components do not reappear until 26 days. Lower graph: latency of choroid response (squares). Curves fitted by eye. The initial points represent changes at short times after denervation. The discontinuity of the dashed line indicates transmission failure 46 hr after nerve section. The latencies of both ciliary and choroid responses are longer during the initial stages of reinnervation. From 40 days, the latency of the responses are within the range of the control values: $1{\cdot}8 \pm 0{\cdot}5$ msec (mean $\pm$ S.D.) for the ciliary chemically mediated response, $0{\cdot}6 \pm 0{\cdot}3$ msec (mean $\pm$ S.D.) for the ciliary electrically mediated response and $3{\cdot}6 \pm 0{\cdot}5$ msec (mean $\pm$ S.D.) for the choroid response.

only a few cells. In these and previous studies (more than 100 experiments) such short latency choroid responses were never observed in normal, unoperated birds (Marwitt *et al.* 1970). The presynaptic fibres mediating this response in Fig. 3*A* (arrow) had a conduction velocity of 5 m/sec and were lower threshold than the fibres mediating the remainder of the

choroid response. These latter fibres had a conduction velocity of 2 m/sec. The conduction velocity of the fast conducting fibres was similar to that of fibres mediating the ciliary response (not shown) in the same preparation. The results in these two cases indicate that presynaptic fibres similar to those normally innervating ciliary cells, innervated choroid cells and induced electrical coupling.

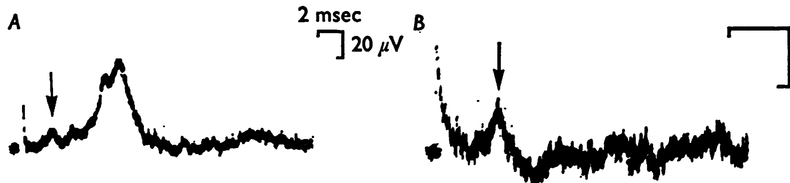

Fig. 3. Responses obtained from choroid nerves of two operated birds after oculomotor section: (*A*) 40 days, (*B*) 100 days. Both responses show initial small electrical components (arrows) with latencies of 1·3 msec (*A*) and 1·2 msec (*B*). In *A* the second, longest latency peak represents chemical transmission (see text). No chemical transmission is present in *B*.

Conduction velocity of the regnerating presynaptic fibres

The longer latencies observed in both the ciliary and choroid responses can be explained in part by slower conduction velocities of the presynaptic fibres. In Fig. 4, the conduction velocities of the fibres innervating the ciliary cells (circles) and choroid cells (squares), are plotted as a function of time after preganglionic nerve section. The control values (mean ± S.D.) determined in a previous study (Marwitt *et al.* 1970) are shown as vertical bars on the ordinate. The conduction velocities of the regenerating fibres are initially slow, but in both groups gradually increase and by 35–60 days fall within the range of the control values; similar changes were observed in regenerating mammalian fibres by Berry, Grundfest & Hinsey (1944). However, at each interval after nerve section, the fibres innervating the ciliary population conducted more rapidly and were of lower threshold than those innervating the choroid.

The increase in conduction velocity with time presumably reflects the maturation and increase in diameter of the regenerating axons. The abruptness of this change, especially in the ciliary presynaptic fibres between 30 and 40 days, may result from remyelination of the regenerating axons. Under conditions very similar to the present experiments, Cajal (1928) observed myelination to take place 25–30 days after nerve section, occurring first in larger diameter axons.

The graph showing the increase in conduction velocity of the ciliary fibres with time has been combined in Fig. 5 with the latencies of the electrical response. The electrical response first reappears when the

conduction velocity increases abruptly at approximately 30 days. Since the latency of the electrically coupled transmission is within the range of control values as soon as it reappears, this electrical response seems to be mediated by myelinated presynaptic fibres. At the very least, re-establishment of electrical coupling is temporally correlated with remyelination. While such a correlation could be fortuitous, it gives added support to the postulated participation of myelin in electrical coupling (Hess *et al.* 1969).

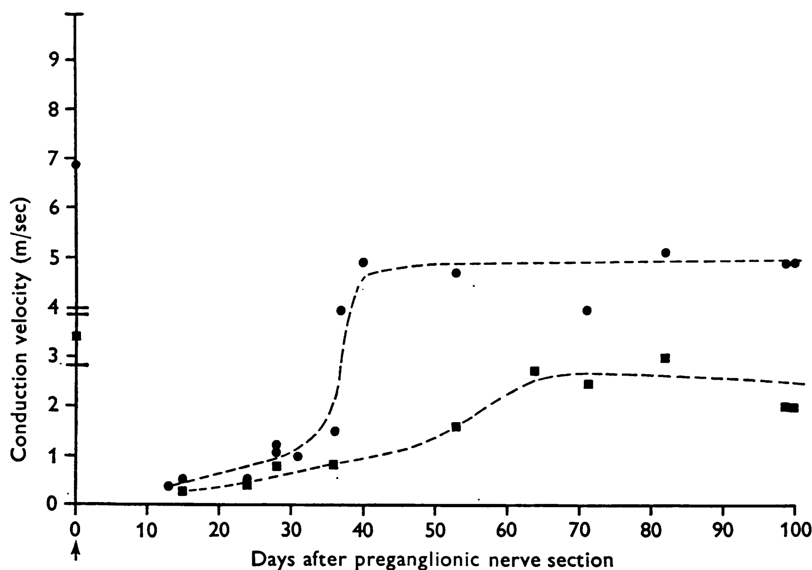

Fig. 4. Conduction velocity of preganglionic fibres innervating ciliary (circles) and choroid cells (squares) as a function of time since oculomotor nerve section (arrow). By 35–60 days the velocities of both have approached control values; $6{\cdot}9 \pm 3{\cdot}0$ m/sec (mean ± S.D.) for the ciliary fibres and $3{\cdot}4 \pm 0{\cdot}7$ m/sec (mean ± S.D.) for the choroid fibres.

The delay in the reappearance of electrical transmission, some 15 days after the restoration of chemical transmission, as seen in Fig. 2, is similar to the sequence of events during embryonic and post-hatching development. In chicks, chemical transmission has been observed as early as Hamburger & Hamilton's stage 35 (1951), electrical transmission not appearing until stage 41 (G. Pilar & L. Landmesser, unpublished observations). In pigeon squabs only chemical transmission is present at hatching time, electrical transmission appearing 10 days later (Hess *et al.* 1969).

Nerve fibres innervating extraocular muscles

Within the common oculomotor nerve the parasympathetic fibres presynaptic to the ciliary ganglion run in conjunction with somatic motor fibres innervating the extraocular muscles. The possibility of ganglionic innervation by these somatic motor fibres after section of the common

extraocular nerve was explored in several experiments. Conduction velocity measurements were used to distinguish the fibres innervating the extraocular muscles as had been done previously to distinguish between the two groups of preganglionic fibres. The control conduction velocity of the fibres innervating the internal rectus, inferior oblique, and superior and inferior rectus muscles was determined from unoperated birds and found

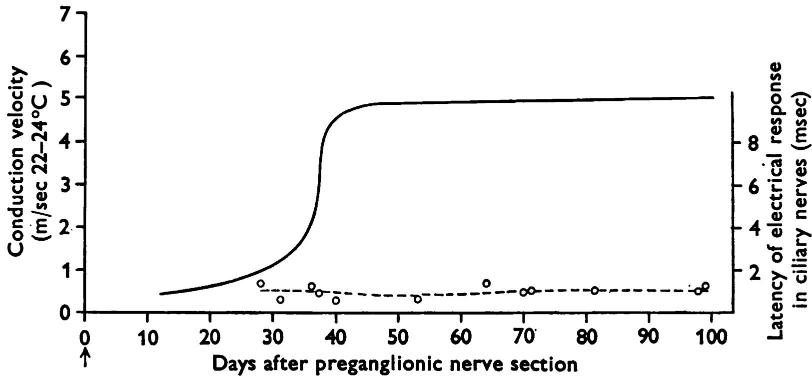

Fig. 5. Graph relating conduction velocity and appearance and persistence of electrical transmission. The continuous curve shows changes in the conduction velocity (from Fig. 4) of regenerating fibres presynaptic to the ciliary population as a function of time after preganglionic nerve section. The circles indicate latencies of the electrical ciliary response (from Fig. 2) which was observed only after 26 days.

TABLE 1. Conduction velocity of nerves innervating extraocular muscles (22–24° C)

	Control (m/sec)	Reinnervated (m/sec)
	16	13
	15	15
	13·5	11·3
	17	14·5
	18	16
	17·5	11·4
Mean ± S.D.	16·1 ± 1·7	13·5 ± 1·9

to be 16·1 ± 1·7 m/sec (mean ± S.D.) (Table 1). Measurements were then made on three preparations 80–100 days after nerve section, when conduction velocities from preganglionic regenerated fibres had reached stable values within the control range. The mean conduction velocity of the fibres innervating the extraocular muscles was 13·5 ± 1·9 m/sec (mean ± S.D.). The conduction velocity of preganglionic ciliary fibres from one of these preparations was 5 m/sec and that of the preganglionic choroid fibres

2 m/sec. Thus it seems that after regeneration, the somatic efferent fibres to the extraocular muscles retain their original peripheral distribution as do the autonomic fibres preganglionic to the ciliary ganglion.

DISCUSSION

This study indicates that the restoration of synaptic connexions in the adult ciliary ganglion after preganglionic nerve section is of a highly selective nature and that presynaptic ciliary fibres are capable of bringing about the anatomical specializations required for electrical transmission. If this did not occur, a proportion of short latency choroid responses and long latency ciliary responses should be observed. Yet responses obtained from operated birds after 30 days were always similar to those shown in the bottom of Fig. 1 and did not differ significantly from the controls. Similarly, latency and conduction velocity of responses from regenerated nerves to the extraocular muscles were indistinguishable from control responses. The degree of selectivity observed during the re-innervation was remarkable considering that both cell populations are part of the autonomic system, cholinergic, and contained within the same ganglion. Furthermore, from our results it seems that the larger ciliary presynaptic fibres reached the ganglion cells before the choroid fibres, yet having the option to connect with both groups of cells, they reinnervated the ciliary population almost exclusively.

An alternative explanation for these results is that the ganglion and muscle cells are capable of influencing the fibre diameter and hence the conduction velocity of the presynaptic fibres synapsing with them. However, observations from other systems do not support this idea (Cajal, 1928; Young, 1942; Vera, Vial & Luco, 1957; Close & Hoh, 1968; Landmesser, 1969). Regenerated myelinated fibres tend to be smaller than normal (Cajal, 1928; Young, 1942) but the occurrence of a complete reversal in the relative conduction velocities of the fibres innervating the two ganglion groups and the extraocular muscles seems highly unlikely.

The restoration of the electrical mode of transmission, almost wholly restricted to the ciliary population, is added evidence that selective reinnervation occurred and that the ganglion cells did not substantially alter the conduction velocity of the fibres that synapsed with them. Fast conducting, presumably ciliary, fibres were able to bring about electrical transmission in a few cases in choroid cells. The short latencies of these electrical responses obtained from the choroid nerves indicate that the choroid cells did not alter the conduction velocity of the fast conducting fibres that synapsed with them.

An attempt was made to sample all stages of reinnervation, rather than

an arbitrary or terminal stage. Therefore the sequence of events was carefully defined in time, and should allow meaningful anatomical correlations at selected times. It also helps to exclude the possibility of initial random regrowth followed by retraction or degeneration of inappropriate synaptic connections.

The 13–15 day period before ganglionic transmission was resumed can be entirely explained by the time taken by the regenerating axons to reach the ganglion cells. This time can be approximately computed from previous data obtained from somatic nerves. Autonomic fibres do not differ in their regeneration rate (De Castro, 1930). Two to 3 days are needed for the cells in the central stump to initiate the metabolic changes necessary for growth (Cajal, 1928; Grafstein & Murray, 1969). Cajal concluded that an additional ten days are required for the regenerating proximal sprouts to span the 1 mm gap caused by nerve transection. One or 2 days more are taken by growth through the 2 mm peripheral stump (Cajal, 1928; Young, 1942). Since transmission resumed 13 days after preganglionic section, it seems there is no appreciable interval between the time the nerve fibres come into contact with the ganglion cells and when they are capable of transmitter release. At this time the regenerated preganglionic fibres were immature, as judged from their conduction velocity. This observation differs from that of Gutmann (1942) on regenerating muscle nerves where there is a delay between the arrival of nerve fibres at the muscle and restoration of transmission. The 10–12 day period before nerve sprouts invade the distal stump is sufficient for complete degeneration of axons in the peripheral stump (Cajal, 1928) and disorganization of the synaptic structures (De Castro, 1930). Therefore, these junctional structures must be completely reformed by the regenerating presynaptic fibres.

The highly specific manner in which the ciliary ganglion is reinnervated warrants an investigation of the causes for only partial restoration of response amplitude in the postganglionic nerves. It is possible that only a portion of the regenerating preganglionic fibres reach the ganglion due to lethal damage of their cell bodies or physical obstacles to nerve regeneration. However, it is also possible that fibres which regenerate to inappropriate ganglion cells are unable to form functional synapses and subsequently degenerate. Experiments currently in progress should distinguish which of these alternatives is correct.

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