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ENHANCED CHEMOSENSITIVITY OF CHICK PARASYMPATHETIC NEURONES IN CO-CULTURE WITH MYOTUBES

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SUMMARY

1. The influence of target interaction upon the electrophysiological properties of dissociated ciliary ganglion cells was investigated by testing the sensitivity of the neuronal somal membrane to ionophoretically applied acetylcholine (ACh). Variations in the percentage of cells responsive to the transmitter were measured with time in culture.

2. Twenty-four hours after plating, all cells respond to an ionophoretic pulse of ACh with a depolarization. However, 1 week after plating (between 7 and 14 days) most of the neurones are unresponsive, and highly responsive cells (> 100 mV peak depolarization/nC) are extremely rare. At even later times in culture, neurones sensitive to the transmitter are again more frequent.

3. When neurones are plated onto pre-formed pectoral myotubes, however, ACh sensitivity is maintained throughout a 3 week culture period. Neuromuscular junctions are formed by the neurones, and when sufficient neurones are present, all the muscle fibres tested show evidence of functional synaptic transmission. Chemosensitivity to ACh is not maintained by neurones in muscle-free microcultures even when responsive neurones in muscle-containing microcultures are present on the same cover-slip.

4. Interneuronal synaptic contacts, defined by ultrastructural criteria, are formed in cultures of neurones alone, but evidence of widespread functional synaptic interaction between cells was not found at 7–14 days in culture.

5. It is concluded that the maintenance of ACh sensitivity of cultured ciliary ganglion cells is enhanced by the presence of muscle in co-culture. The interneuronal synaptic contacts observed are apparently not as potent a stimulus as co-culture with muscle for the full expression of the cholinergic phenotype under these culture conditions.

INTRODUCTION

Our previous study (Vaca, Tuttle & Pilar, 1982) examined the dependence of a neurone upon interaction with its target during development by detailing the

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inductive influence of muscle on the cholinergic synthetic and secretory capacity of cultured ciliary ganglion cells. The present report attempts to extend our knowledge of this relationship between target interactions and neuronal physiology through investigation of the influence muscle in co-culture and the opportunity to form neuromuscular junctions has upon ACh chemosensitivity in the parent neurone. Moreover, cultures observed ultrastructurally in the course of the study were found to contain numerous morphological synaptic contacts among the neurones. These interneuronal contacts are a deviation from the normal behaviour of these cells *in vivo*. In fact, such contacts were not observed even when the developing ciliary ganglion cells were deprived of their targets by early eye deprivation (Landmesser & Pilar, 1974*a*). This observation prompted us to further investigate physiologically these abnormal synapses. In brief we found that functional synaptic interaction between ganglion cells during the initial 3 weeks in culture was rare, if present, perhaps due to the loss of chemosensitivity on the ganglion cell somas. This hyposensitivity to ACh on the part of the ganglion cells cultured alone is not detected in neurones in co-culture with a target tissue. Furthermore, we have substantiated the evidence, discussed in the previous paper (Vaca, Tuttle & Pilar, 1982), for the existence of interneuronal synapses in cell culture alone, as well as for the formation of competent neuromuscular contacts in co-culture with muscle. A preliminary report of these findings has been published in abstract form (Pilar & Tuttle, 1978; Vaca, Tuttle & Pilar, 1979).

METHODS

Cell culture

Most of the culture methods used have been reported previously (Tuttle, Suszkiw & Ard, 1980). In brief, ciliary ganglia were removed from white Leghorn chick embryos at embryonic stages 30–36, dissociated by gentle trypsinization and mechanical disruption, and plated as a cell suspension onto collagen-coated plastic cover-slips. For some experiments, the collagen substrate was restricted to $\frac{1}{2}$ mm diameter spots spaced on the cover-slip. As ciliary ganglion cells do not readily adhere to untreated plastic surfaces, restriction of the substrate to small spots leads to the formation of individual 'microcultures' (See Furshpan, McLeish, O'Lague & Potter, 1976). Culture medium was 80% (v/v) Eagle's Minimal Essential Medium (MEM), supplemented as before, and 10% each of heat-inactivated horse serum and chick embryo extract (8 days).

Co-cultures of skeletal muscle and ganglion cells were prepared essentially as in Nishi & Berg (1979). Pectoral muscle from 11-day chick embryos was minced, mechanically dissociated to myoblasts, pre-plated to remove fibroblasts (Yaffe, 1968), and plated on collagen coverslips in either normal growth medium or 85% (v/v) MEM with 15% bovine fetal serum. After 48 hr, this initial medium was replaced with one containing 10^{-5} M-cytosine arabinoside (Sigma Chemical). Following antimetabolite treatment for 24–48 hr, fresh growth medium was reintroduced, and the ganglion cells were plated onto the monolayer of fused myotubes. One half of the medium of all cultures was replaced every 2–3 days with fresh growth medium. Incubation was at 34 °C in 96% air 4 %CO_2.

Electrophysiology

Techniques for intracellular recording from the cultured ciliary ganglion neurones were as described (Tuttle *et al.* 1980). Intracellular recording from myotubes in the co-cultures was in a 50% saline, 50% culture medium mixture with 10 mM-HEPES and 3 mM added $CaCl_2$. Acetylcholine (ACh) was applied onto the neurones ionophoretically from high-resistance (80–150 MΩ) micropipettes filled with 3M-ACh, using a constant current source (Dreyer & Peper, 1974).

Fig. 1 illustrates the techniques used to study the ACh sensitivity of the ganglion cells in culture. After a neurone was impaled a depolarizing current was injected and action potentials were elicited;

data were taken only from successful penetrations of cells with normal electrical excitability (Tuttle *et al.* 1980). Passage of a long depolarizing current pulse resulted in repetitive firing, a response characteristic of ganglion cells *in vivo* (Martin & Pilar, 1963). The ionophoretic pipette was then moved so its tip lay near the cell surface, and a negative pulse delivered (bottom trace); the range of pulse durations applied varied from 1 to 40 msec. In the first trial illustrated, this did not elicit a response from the neurone (upper trace). The ionophoretic pipette was then moved closer to the cell, and a depolarization obtained in response to another pulse of ACh. After moving the pipette even closer, the ACh response reached its maximum. A pulse of reverse polarity applied through

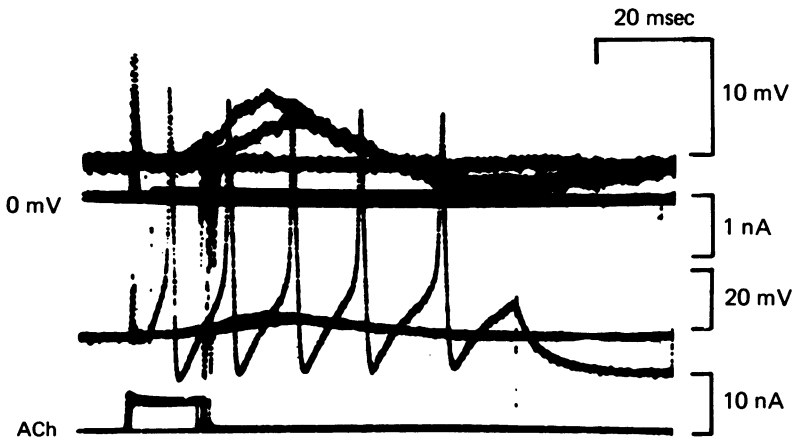

Fig. 1. Intracellular recordings from a ciliary ganglion cell in culture, illustrating the method used to study ACh sensitivity. The records are superimposed from the traces of different trials and are photographed from a storage osciloscope screen. Top trace is a.c. recording at higher gain of depolarization responses, the same ones which appear in the third trace from the top, recording d.c. at a lower gain. The second trace was used as a zero reference and also displayed the current injected through the recording pipette to stimulate the neurone directly. The response to this stimulation is shown in the third trace and consists of repetitive action potential discharge. Lower traces show the ACh ionophoretic pulses. For explanation of the recording technique see text.

the ionophoretic pipette at the same position failed to elicit a response, demonstrating that direct electrical coupling of the ionophoretic pipette to the neuronal membrane was not present (not shown). ACh sensitivity was determined as mV peak depolarization/nC of ionophoretic charge applied.

The existence of neuromuscular contacts was studied during intracellular recording from myotubes, and oscilloscope traces were photographed on moving film or the same signals recorded on chart paper. D-Tubocurarine (DTC) and Tetrodotoxin (TTX) were added to the recording chambers at the concentrations indicated in the text.

Electron microscopy

Cultures were prepared for electron microscopy by first rinsing the coverslips in 0·1 M-sodium cacodylate buffer pH 7·4 for 10–15 min and then fixing in 2% glutaraldehyde for 1–1·5 hr. The cultures were rinsed again in buffer and then post-fixed in 2% osmium tetroxide for one hour. Both glutaraldehyde and osmium tetroxide were made up in 0·1 M-sodium cacodylate buffer. It was more convenient to use collagen-coated glass cover-slips or tissue culture dishes for ultrastructural work, as the plastic cover-slips tended to yield poor results upon embedding. The cover-slips were rinsed once again and dehydrated in an alcohol series (60, 70, 80, 95, 100%), and then embedded in Epon 812. The Epon was dropped onto the cover-slip in an amount small enough to prevent overflow from the edges of the cover-slip. The cover-slips were placed in a 60 °C oven for 48 hr and allowed

to polymerize. After curing the cover-slips were viewed under phase contrast and areas of interest for sectioning were scored with a carbide marker. The glass cover-slips were carefully lifted away from the epoxy leaving a shiny surface corresponding to the underside of the culture. The marked areas were then carefully sawed out with a jewellers saw and mounted, shiny surface up, onto a blank Epon block. Thin sections were cut on a Porter Blum MT-2 Ultramicrotome with a diamond knife and picked up on 200 mesh copper grids, stained with uranyl acetate and lead citrate and viewed in a Philips 300 electron microscope. Approximately 150–200 successive sections were cut for each block and all sections were viewed. Areas that showed contacts between cells were photographed.

RESULTS

Morphological contacts between dissociated ganglion cells

Dissociated ganglion cells plated alone readily formed what appear to be synaptic contacts by ultrastructural criteria. Details of some of these morphological synapses are shown in Pl. 1, taken from cultures fixed at 7 days *in vitro* (Pl. 1 *A*, *B* and *C*) and after 11 days (Pl. 2 *D*). The ganglion cells grow as flattened spheroids on the collagen surface, with the nucleus usually bulging upward. We have noticed that the contacts are more frequent on the lower part of the cell, nearer the substrate and thus often away from the nucleus. The images presented are quite typical. In all cells observed by serial sectioning, several contacts per cell were confirmed. Also, essentially random sections taken from routine cultures invariably display some evidence of specialized contacts. It is not possible to determine from these images if all the apparent synaptic profiles were formed between different neurones, or if some or all were 'autapses' (Van der Loos & Glaser, 1972). However, in certain cases interneuronal contacts definitely were present. This was deduced from higher magnification images in which the same longitudinally sectioned neuronal process was seen to make contact with first one neurone and then another.

Finding these apparent synaptic profiles between the neurones in culture was somewhat unexpected, although certainly not without precedent in other systems (See Discussion). *In vivo*, if the optic vesicle is removed during development, thus depriving the neurones of target tissues, no such connexions are formed (Landmesser & Pilar, 1974*a*). Thus, dissociation and culture of the neurones *in vitro* either releases them from an *in vivo* constraint against interneuronal contact formation or aspects of the culture regimen and/or environment induce the formation of aberrant contacts.

Perhaps more unexpected was an inability to detect synaptic interaction between ganglion cells by intracellular recording. Such interactions are quite common in cultures of other structures (Burton & Bunge, 1981; Nelson, Neale & MacDonald, 1981). Stable intracellular recordings (longer than 5 min) were obtained from over 100 neurones in fourteen different culture sets specifically to detect synaptic interactions, and no definitive evidence of either spontaneous synaptic potentials or elicited synaptic activity was detected. Recordings were taken at room temperature; at 32–34 °C; in growth medium; in Tyrode saline; and in saline with twice the usual Ca^{2+} concentration (4mM). Various recording and stimulation schemes used are illustrated in Fig 2. Simultaneous recordings were made from cell pairs that under Nomarski interference and Hoffman modulation contrast appeared to be connected.

Direct intracellular stimulation of each in turn failed to reveal any evidence of synaptic activity. Also, single cell recordings during extracellular stimulation with a low-resistance saline-filled pipette of apparently connected nearby neurones and bundles of neuronal processes failed to elicit synaptic interaction. All of the neurones examined had stable resting potentials and were capable of discharging normal action

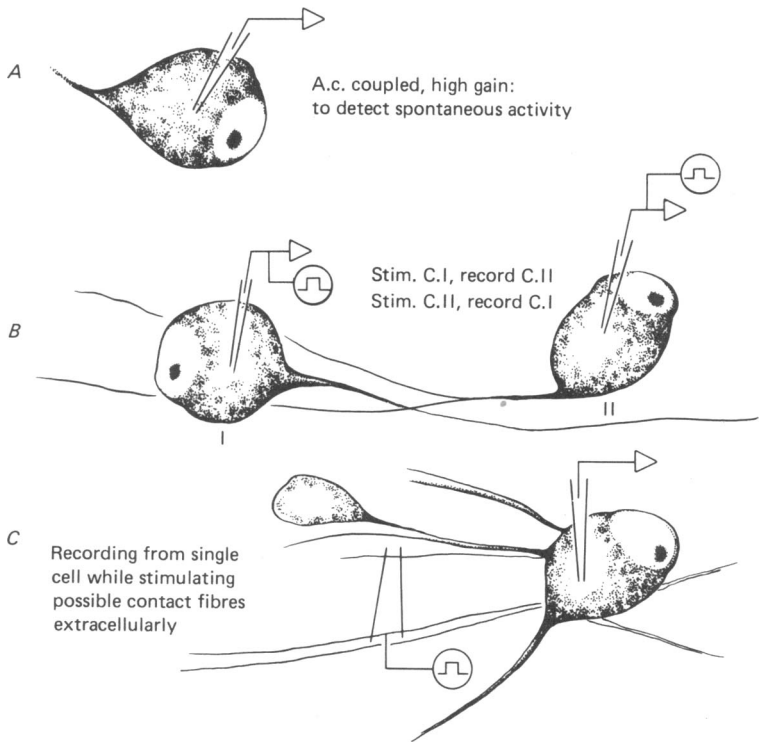

Fig. 2. Diagrammatic representation of three different techniques utilized to study synaptic interaction between ciliary neurones in culture. In *A*, intracellular recordings of over 100 cells at 1 mV/cm were monitored for several minutes to detect spontaneous activity. In *B*, pairs of neurones were studied which appeared to make synaptic contact under phase contrast observation. Twenty-five cell pairs were impaled and following stimulation of cell I, recordings were obtained from cell II. In a subsequent trial the recording and stimulation were reversed. In *C*, bundles of axons and cell processes converging upon a cell were stimulated extracellularly with a saline-filled micro-electrode during intracellular recording from the object neurone. No responses were detected with any of the stimulation paradigms.

potentials. A description of some of the active and passive membrane electrical properties of these cultured neurones has been published (Tuttle *et al.* 1980).

Most of these recordings were obtained from neurones dissociated at embryonic stage 31–32, and kept in culture for 7–14 days. This time frame circumscribes that in which the ultrastructural evidence suggests contacts are prevalent and mature. Thus, the cultures lacked any evidence of functional synapses, despite the widespread occurrence of morphologically normal synaptic profiles. While negative evidence

cannot rule out the possibility of functional synapses between ciliary ganglion neurones, they are certainly less prevalent than the ultrastructural evidence suggests.

ACh sensitivity of cultured ganglion cells

We have explored the basis for this failure of transmission at apparent interneuronal synapses by examining the pre- and post-synaptic processes that serve chemical transmission. Defects in transmitter synthesis, storage or release were eliminated as

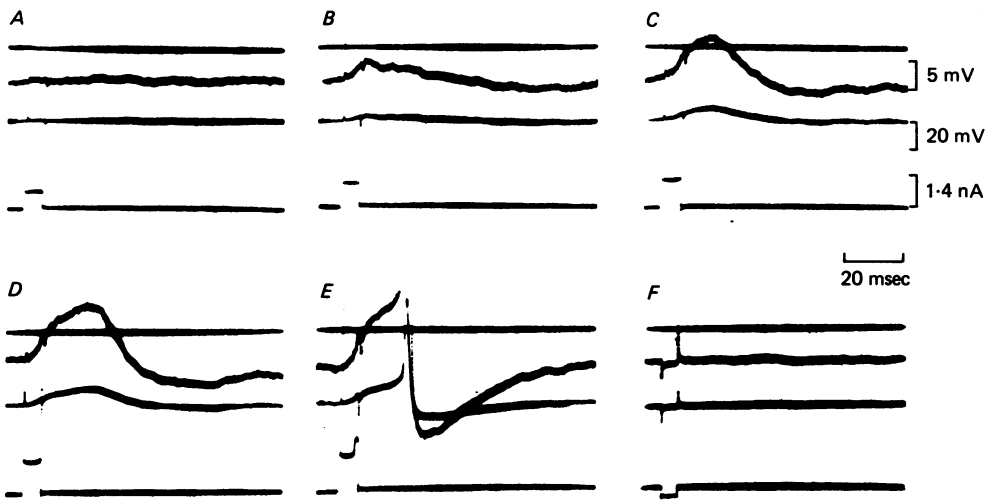

Fig. 3. ACh sensitivity of a ciliary neurone 24 hr after plating. The ionophoretic pulse was increased in amplitude from *A* to *E*, until the depolarization elicited in the cell (second and third trace) reached threshold and an action potential was elicited. Note in *E* that the ACh pulse has a notch at the end, due to current-dependent changes in electrode resistance (see Dreyer & Peper, 1974). In *F*, a test for direct electrical coupling between the ionophoretic micropipette and the neurone is shown. At the end of the ACh series, pulses of reverse polarity were applied. No response was seen in this cell.

possibilities because the neurones were found to be competent with respect to transmitter metabolism and release (Vaca *et al.* 1982). Furthermore, ciliary ganglion neurones in culture readily and rapidly form functioning neuromuscular junctions with skeletal myotubes (see p. 96 of this paper; Betz, 1976; Obata, 1977; Nishi & Berg, 1977; Slaaf, Hooisma, Meeter & Stevens, 1979). A remaining possibility was a defect in post-synaptic chemosensitivity at the contact sites.

Because transmission through the ciliary ganglion is established quite early in development (Landsmesser & Pilar, 1972) prior to the time of dissection for culture; and because transmission is purely chemical at this stage and mediated by ACh, it was not surprising to detect sensitivity to ACh soon after the neurones were plated. Fig. 3 shows responses obtained by the ionophoretic application of ACh onto freshly dissociated neurones from a high-resistance micro-electrode.

Once a stable penetration with the recording pipette was established, the ionophoretic pipette was moved under visual guidance to a spot adjacent to the cell soma.

The response to 8 msec pulses through the ACh pipette was used to position it as close to the membrane as possible without direct coupling. The neuronal response to a graded series of pulses of ACh was then determined. In Fig. 3 increasing depolarizing pulses were applied (*A–E*) and a larger response (second trace from upper) was elicited with each step increase of the ACh ionophoretic current, until threshold was reached and an action potential was initiated (*E*).

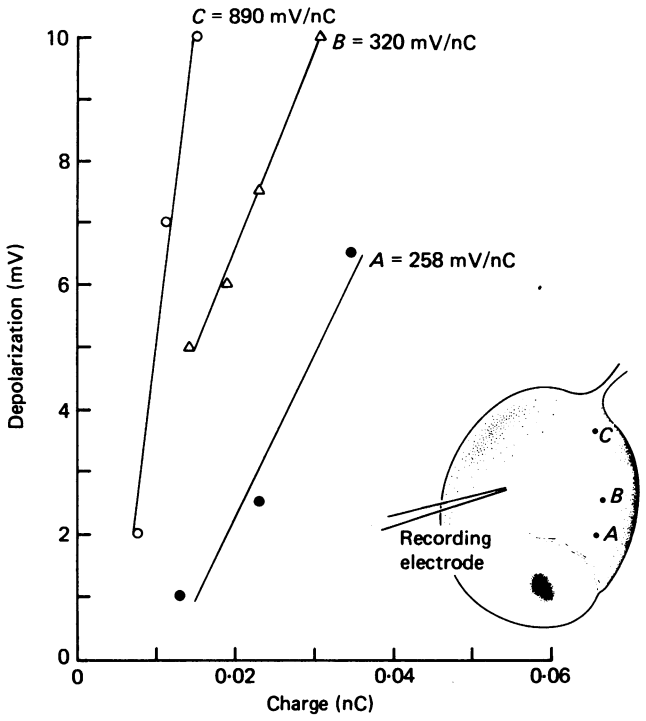

Fig. 4. Localization of ACh sensitivity in a ciliary ganglion cell from a 2-day old culture. Letters in the cell diagram show the position of the micropipette during three ACh ionophoretic sequences. The high sensitivity in *C* closely matched the usual location of synapses in the intact ganglion. It is possible that *A* and *B* actually tested the same area of sensitivity, as the spatial resolution of this method has not been determined. The relatively high level of sensitivity found for this example was not unique, though in most cases areas of this level of sensitivity were not found.

In this ganglion cell sensitivity seemed highest in restricted spots, as is the case of other parasympathetic neurones *in vivo* (Harris, Kuffler & Dennis, 1971). Fig. 4 illustrates this more clearly, as well as the method used for quantitating sensitivity to ACh. Total estimated ionophoretic charge was plotted on the abscissa and peak depolarization (in mV) on the ordinate. The slope of a regression line through several responses was taken as the estimate of ACh sensitivity in mV depolarization per nC of charge. As can be seen in Fig. 4, three different spots of the soma of this cell were tested, and the position of highest sensitivity was near the axon hillock, where synapses are normally found *in vivo* (Hamory & Dyachkova, 1964).

Recordings from ganglion cells from older embryos (stage 34–36) could be obtained immediately after dissociation (and at all subsequent times in culture), but it was difficult to record from stage 31–32 ganglion cells before 7 days in culture, due to their smaller size and apparent fragility. Nonetheless, there is no evidence to suggest that the situation with the younger neurones would be different. Furthermore, their subsequent developmental course in culture is similar.

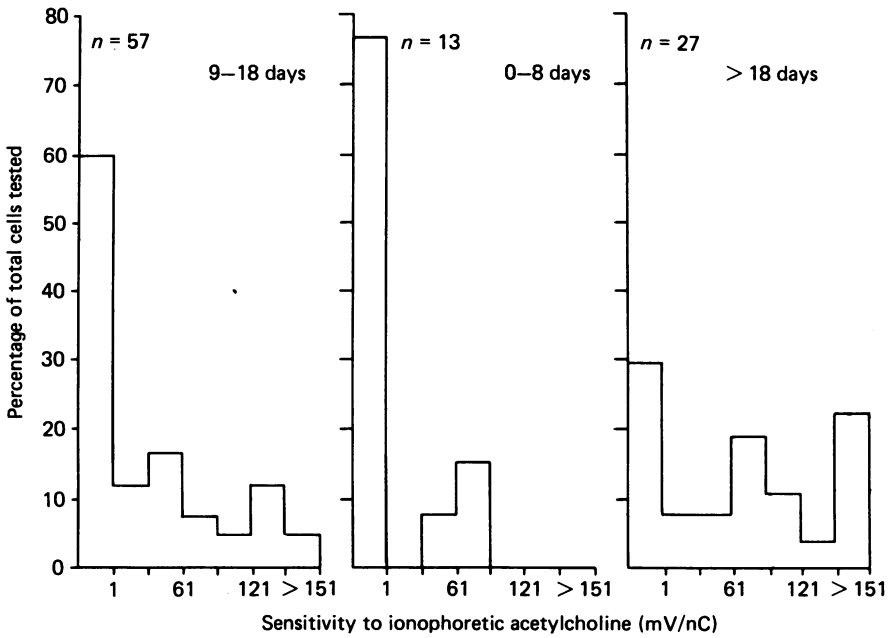

Fig. 5. Changes of ACh sensitivity with time in culture. In the first week a great range of sensitivity was found, although the higher values tended to occur in neurones tested within 2 days. Between days 9 and 18 a very small percentage of responsive cells were found, even with ACh pulses of longer duration and greater current strength. After 18 days in culture, responses to ACh again became more frequent, and fewer totally unresponsive neurones were in evidence.

The sensitivity to ACh found initially became less apparent after some time in culture. By the second week after plating, large numbers of unresponsive cells could be found. This is shown in Fig. 5, where the distribution of ACh sensitivities (in mV/nC) found at various times after plating is presented. During the second week in culture, almost 80% of the neurones plated alone lacked demonstrable sensitivity to the transmitter and no 'highly sensitive' neurones were found. This scarcity of active receptors to ACh, and perhaps a lack of aggregation at limited areas of membrane, is probably sufficient to explain the failure to observe transmission between the cultured neurones. These tests included recordings from over 100 neurones in more than twenty different cultures from several different culture sets.

To ascertain if ACh was indeed released from the micropipette, in some cases when no response to ionophoretic pulses in neurones were obtained, the same pipette was

used to stimulate cultured muscle fibres in the same culture dish. This was possible in microcultures, where in some of the islands only myoblasts were grown, and large ACh responses were routinely observed.

At longer times in culture, up to 30 days, a larger percentage of the ganglion cells had demonstrable sensitivity to the ionophoretically applied transmitter (Fig. 5). However, we have not yet thoroughly studied transmission in older cultures. In

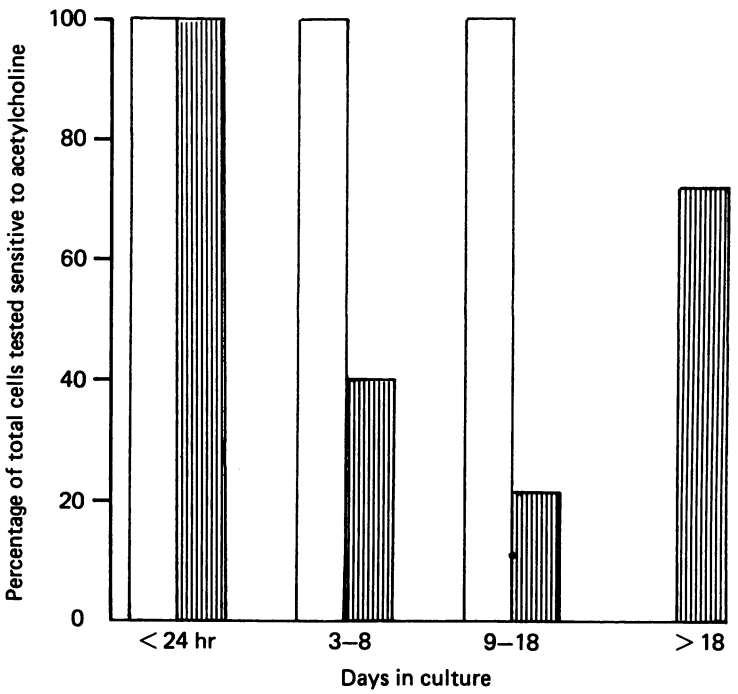

Fig. 6. Influence of co-culture with myotubes on the ACh sensitivity of dissociated ciliary ganglion cells. The open bars indicate neurones in co-culture; hatched bars are for neurones cultured alone. At 24 hr cells alone and in co-culture displayed the same ACh sensitivity. By 3 days only 40 % of the neurones alone tested retain their sensitivity. This proportion decreases to 22 % between days 9 and 18, while the neurones in co-culture with muscle retained their ACh sensitivity throughout the testing period.

general, these neurones appeared to be evenly sensitive to ACh, yielding essentially the same responsiveness over the whole exposed somal membrane. As was reported previously (Tuttle *et al.* 1980), myotubes occasionally appear in cultures of neurones plated alone, perhaps due to the unintentional inclusion of a few extraocular myoblasts in the cell suspension. When this occurs, fusion of the myotubes invariably begins at 10–14 days in culture, and after another week or more, cross-striated profiles can sometimes be seen under phase contrast.

Because ciliary ganglion neurones *in vivo* (Landmesser & Pilar, 1974*b*) and under certain culture conditions (Nishi & Berg, 1977) depend upon myotubes for their survival, it was of interest to determine what effect the presence of this target tissue would have on the chemosensitivity of the cultured neurones. The influence was

dramatic. Fig. 6 presents a comparison of the percentage of sensitive cells found in cultures of neurones plated alone and those plated onto previously formed monolayers of myotubes. To date, no ciliary ganglion neurone in co-culture with myotubes has failed to respond to ACh (97·6 + 15·5 mV/nC mean ± S.E. of the mean), regardless of the time in culture (up to 18 days). Sensitivity in this case was also relatively even over the somal surface at 10–14 days in culture, though the possibility of areas of relatively higher sensitivity especially at earlier times in culture cannot be definitely eliminated at this time. A preliminary examination of interneuronal transmission in these cultures suggests it may be active, as spontaneous events similar in appearance to e.p.s.p.s are recorded from the neurones (J. Tuttle, unpublished observations).

In some of the cultures from which the above data were obtained, the myotubes and neurones were cultured in 'microcultures' (see Methods). In this way, the chemosensitivity of neurones in microcultures with myotubes present was compared to that of neurones in microcultures lacking the muscle target, while both were cultured in the same dish. The great difference in responsiveness to transmitter remained. The presence of mytubes in accessible co-culture enables the neurones to retain active receptors to ACh.

These experiments may at first seem to rule out a role for a diffusible substance released from muscle in maintaining neuronal chemosensitivity. However, the small amount of muscle in the microcultures may be insufficient to supply an adequate dose for such a hypothetical trophic substance.

Ciliary neurone–myotube junctions

In addition to examining the ACh sensitivity of ganglion cells, the presence of functional neuromuscular junctions formed in neurone–myoblast co-cultures was also ascertained. Recordings were taken from myotubes at 4–12 days after the neurones were plated into the culture, and at all times it was relatively easy to observe neuromuscular activity indicative of synapse presence.

In Pl. 2*A* light micrographs of co-cultures of neurones and muscle cells are shown. The picture was taken with Hoffman modulation contrast illumination and illustrates the typical organization and density of cells used in this and in the previous study (Vaca *et al.* 1982). After plating, some neurones settled between muscle cells on the collagen substrate, while many of neurones settled onto muscle cells, where they grew in close apposition to the muscle cell membrane, apparently without the formation of specialized contacts.

In Pl. 3 such neurones are seen at the ultrastructural level to be separated by a 30 nm gap from the muscle cell. Similar separation is observed between the adjacent neurone. Although no serial reconstructions of the entire relationship between neuronal soma and myotube were made to completely rule out the formation of specialized contacts, it appears that axonal processes usually grow along the muscle for some distance before making junctional contacts. This can be seen in Pl. 2*B* where the ganglion cells (*) send axonal processes various distances along the myotube before terminating in bouton-like structures (arrows) similar to previous observations (Fischbach, Berg, Cohen & Frank, 1976). We have not yet completed the study of the fate of interneuronal synapses in co-culture conditions, but they are still present when neuromuscular junctions are formed (G. Crean, unpublished observations).

Intracellular recordings were obtained from myotubes in these co-cultures and the presence of spontaneous junctional potentials was used as indicative of the presence of neuromuscular contacts. The records of Fig. 7 show muscle spontaneous activity selected from an uninterrupted moving film. The presence of several different rise times strongly suggests that the muscle cell is innervated by at least two different active loci. Similar recordings were obtained from myotubes from other cultures.

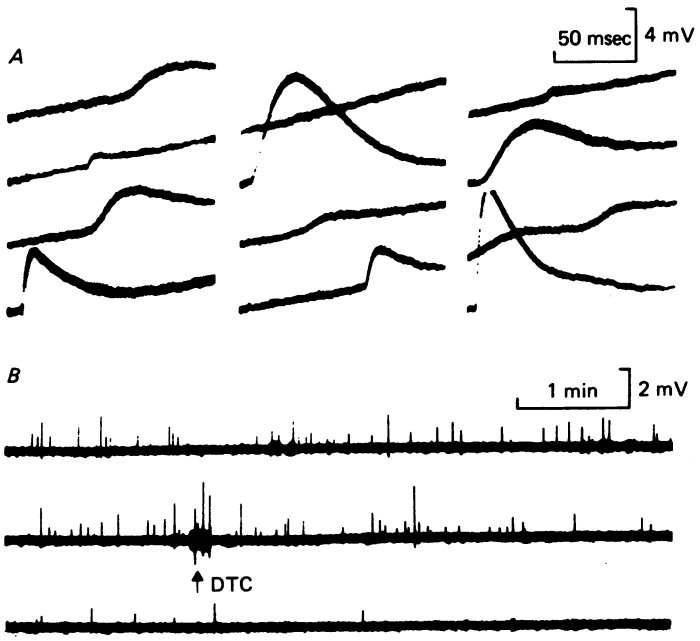

Fig. 7. Functional neuromuscular junctions formed between ciliary neurones and pectoral myotubes. Spontaneous potentials shown in *A* were selected from moving film taken during intracellular recording from the myotube 7 days after neurones were added to the culture. (Resting potential 74 mV). The chart recording in *B* is of spontaneous activity from another cell, showing the effect of 2·6 μM/l. of DTC added to the recording chamber. Shortly after the addition of DTC, the spontaneous activity disappeared, without changes in the muscle resting potential (70 mV).

Resting transmembrane potentials of the myotubes were from −68 to −74 mV and spontaneous potential amplitudes varied from 0·4 to 8 mV. Frequency of occurrence of the spontaneous potentials varied from 0·13 to 1·1 Hz. Similar potentials were recorded after the addition of TTX to the culture, at a final concentration of 10 μg/ml. Although in some cases the frequency of occurrence of spontaneous events decreased from 0·23 to 0·02 Hz, in most cases TTX had no discernible effect on spontaneous potential occurrence. This seems to indicate that most of the nerve cells are quiescent, without spontaneous action potentials, a characteristic that was already seen when neurone electrical activity was studied directly (Tuttle *et al.* 1980). Finally, the addition of DTC to the bath reduced the response amplitudes until the spontaneous potentials finally disappeared. Fig. 7*B* is a continuous recording from an innervated

myotube and the arrow indicates the addition of 2·6 μM/l. of DTC. Shortly after this application, all electrical activity ceased.

These recordings are a clear indication that the ciliary cells form active junctions with muscle; together with the evidence previously obtained on transmitter metabolism and release (Vaca *et al.* 1982), it is clear that ACh is mediating neuromuscular transmission. In a few instances when recording intracellularly from a muscle cell, by careful placement of a second stimulating electrode near axon bundles, it was possible to elicit a muscle action potential, and sometimes a burst of subthreshold junction potentials. A consistent effort was not made to record this evoked activity. The relative ease with which muscle fibres are innervated suggests that ciliary neurones form functional contacts with muscle frequently. But we could not with certainty say if all neurones in the culture formed junctions.

DISCUSSION

The developmental importance of cellular interactions has been underscored by the results of the study of neural influences on muscle physiology (Gutmann, 1976). The influence of nerve on the differentiation of muscle is well documented (see for review Fischbach, Frank, Jessell, Rubin & Schuetze, 1979). However, the target that a neurone synapses with, in many cases muscle, also has important influences on the full phenotypic differentiation of nerve cells (Patterson, 1978). Synapse formation by the ciliary ganglion cells upon an appropriate target tissue has been correlated *in vivo* with a spectrum of developmentally significant events, including the induction of the enzymes related to neurotransmission (Chiappinelli, Giacobini, Pilar & Uchimura, 1976), ultrastructural development (Pilar & Landmesser, 1976), increases in toxin-binding sites (Chiappinelli & Giacobini, 1978), and a period of neuronal cell death related to interneuronal competition (Landmesser & Pilar, 1974*b*, 1976; Pilar, Landmesser & Burstein, 1980).

In this report we present evidence that maintenance of the ACh sensitivity of cultured ciliary ganglion cells is contingent on their interaction with muscle cells, and that the observed formation of synapses between ciliary cells does not equally support their chemosensitivity. This mechanism may reflect developmentally significant events which help to establish correct connectivity. In general, such a phenomenon would allow only specific targets to influence the transmitter sensitivity in the parent cell.

These two aspects of the present work will be discussed separately.

(*a*) *Ineffective synapses*

The data presented in this paper do not absolutely establish the presence of chemically ineffective synapses between ciliary ganglion neurones in cell culture. We did not examine the same ending both electrophysiologically and morphologically. However, the data strongly suggest that the interneuronal contacts invariably seen with the electron microscope are not as easily detected as transmitting junctions. Because the ganglion cells in culture readily take up Ch and synthesize and release the neurotransmitter ACh (Vaca *et al.* 1982), presynaptic metabolic deficiencies are not present. Therefore, the high frequency with which cells unresponsive to ACh are

encountered, perhaps in addition to a lack of matching between contact areas and areas of sensitivity, provide the probable explanation for the failure to detect functional interneuronal transmission.

Ciliary ganglion neurones in culture alone have been reported to display an increase in the number of α-BTX binding sites with time of culture (Messing & Kim, 1981) reaching a plateau at seven days. Results similar in time course but quantitatively different (greater α-BTX binding) have been reported during *in vivo* development (Chiappinelli & Giacobini, 1978). If α-BTX labelled functional ACh receptors in the ganglion this result would contradict the present observations. This apparent discrepancy could be explained if α-BTX bound to a different membrane component than the ACh receptor in chick autonomic ganglia (Carbonetto, Fambrough & Muller, 1978; Ravdin & Berg, 1979). The actual situation is at present unclear.

Further, morphological synapses ineffective due to post-synaptic insensitivity have been reported previously in cell cultures of other peripheral neurones: sympathetic and dorsal root ganglia (unpublished observations in Furshpan *et al.* 1976; Scott, Engelbert & Fisher, 1969; Miller, Varon, Kruger, Coates & Orkand, 1970). In these instances, though, the transmitter released at the interneuronal or autosynaptic contacts was 'foreign' to the post-synaptic neurone. The experiments dealing with cultured sympathetic neurones (Furshpan *et al.* 1976) deserve a further comment. The silent synapses formed by sympathetic neurones are different from those formed by the cholinergic neurones described in the present study. Sympathetic neurones are not ordinarily sensitive to their own transmitter whereas ciliary ganglion neurones are. The ciliary neurones both receive and form cholinergic synapses, and are functionally innervated at the time of dissection. This fact allowed the fluctuation in transmitter responsiveness that followed dissection and dissociation to be detected and examined.

The basis for the failure of ciliary ganglion cells, when plated alone in culture, to maintain active receptors to their physiological transmitter is unknown. Neuronal cell culture necessarily involves both denervation and axotomy of the embryonic neurones, and each of these insults has been shown to alter transmitter responsiveness (Kuffler, Dennis & Harris, 1971; Brenner & Martin, 1976) and in some cases reduce survival (Pilar *et al.* 1980). The axon reaction and its profound effects on neuronal morphology and metabolism are also well known. It is of interest that reestablishment of connexions with the periphery is necessary to restore synaptic transmission in the axotomized superior cervical ganglion (Purves, 1975).

Although it should be noticed that several investigators have studied ACh sensitivity of cultured neurones (O'Lague, Potter & Furshpan, 1978; Wakshull, Johnson & Burton, 1979), no attempt has been made to study the temporal changes of the neurotransmitter sensitivity with time in culture. In one study avian sympathetic neurones in cell culture are able to synthesize and insert new α-bungarotoxin receptors into the membrane (Carbonetto & Fambrough, 1979) and the number of toxin binding sites does decrease after 5 days *in vitro*.

The appearance of synaptic contacts may have some relevance for the survival of neurones *in vitro*. Until our success in maintaining long term cultures of dissociated ciliary neurones (Tuttle, 1977; Tuttle *et al.* 1980) it was reported that ciliary ganglion cells do not remain viable longer than a week unless co-cultured with potential target tissues. (Helfand, Smith & Wessels, 1976; Nishi & Berg, 1977). Later these

observations were confirmed and extended in several other laboratories (Nishi & Berg, 1979), especially with the isolation of a ciliary ganglion 'neuronotrophic' factors (Varon, Manthorpe & Adler, 1979; Bonyhady, Hendry, Hill & McLennan, 1980; Manthorpe, Skaper, Adler, Landa & Varon, 1980). Thus a growth protein is thought to be essential for cell survival and it can be supplied by the target tissues, similar to the NGF for sympathetic neurones (Thoenen & Barde, 1980). It is an intriguing possibility that the survival of peripheral neurones involves growth factors most readily obtained through specialized contacts (Landmeser & Pilar, 1974*a*, *b*, 1976).

(*b*) *Trophic effect of muscle*

The effect of co-culture of myotubes with ciliary ganglion neurones upon subsequent neuronal development in culture is dramatic. The present data demonstrate the ability of target tissue to enhance neuronal chemosensitivity. Myotubes also stimulate ciliary ganglion cells to higher levels of choline acetyltransferse activity, acetylcholine synthesis, and transmitter release (Vaca *et al.* 1982). These data thus demonstrate a broad trophic effect of muscle in co-culture upon the ganglionic neurones. These target tissue interactions were more readily examined in cell culture because *in vitro* the neuronal dependence upon their target for survival was circumvented.

The influence of innervation and activity upon the distribution of ACh receptors in muscle has been well studied (Diamond & Miledi, 1962; see Dennis, 1981 for review), and the spread of chemosensitivity following the denervation of parasympathetic neurones has been examined (Kuffler *et al.* 1971). We have added to this growing list of intercellular influences on chemosensitivity a retrograde effect, a target-cell influence on neuronal transmitter responsiveness. However, the exact mechanism of this effect remains to be demonstrated; whether a diffusible factor released by muscle if present in high concentration will suffice, or neuromuscular junction formation alone is the necessary interaction. Bidirectional (trophic) synaptic interaction has been demonstrated for the regulation of transmitter-related enzymes (Black, 1978), thus a similar regulatory scheme for transmitter receptors is not without some precedent. The present work further suggests that synapse formation with an inappropriate target, other neurones in the same culture, may not provide an equally effective trophic stimulus. However, we have not excluded the possibility that large numbers of functionally very active interneuronal synapses might support the maintenance of chemosensitivity on a level equal to that of neuromuscular interaction. Activity through newly formed and mature synapses has been hypothesized to serve as an important signal for the regulation of neurodevelopment and transmitter metabolism (Harris, 1981). Separating the influences of synaptic activity from trophic action would be most instructive.

It is tempting to try to place the present results in a developmental context. At the time of dissection for culture (stage 31–34) ciliary ganglion neurones have been committed to a cholinergic path of development. Choline acetyltransferase is present in sufficient levels for ACh synthesis and pre-ganglionic cholinergic transmission has been established (Chiappinelli *et al.* 1976). Furthermore, this stage of development can be reached in the absence of target tissues (Landmesser & Pilar, 1974*a*). Past this stage *in vivo*, a dependence upon the target tissue for neuronal survival ensues, and the competition between neurones normally leads to a period of cell death for

the unsuccessful members of the population (Landmesser & Pilar, 1974*a*, *b*, 1976; Pilar *et al.* 1980). Removal of the neurones from the embryo at this time seems to interrupt the sequence and, with proper culture conditions, release the neurones from the absolute requirement for target interaction. However, the neurones alone in culture seem less capable of further development toward the mature state. Without the additional influence of target tissues (and perhaps activity), the already activated cholinergic genetic programme seems to remain in a 'holding pattern' and the cultured neurones exist in a somewhat immature state, without the full expression of all normal characteristics.

A recent abstract (Margiotta & Berg; *Soc. Neurosci. Abstr.* **7**, p. 596, 1981) presents evidence of synaptic interaction between ciliary neurones in co-culture with muscle and, much less often, between ciliary neurones in cultures lacking muscle but in medium with 3% (v/v) embryonic eye extract. This preliminary data, if further substantiated, would seem to confirm the beneficial effect of co-culture with muscle. The apparent rare occurrence of functional synaptic interaction between neurones cultured alone may suggest that such is possible, depending perhaps on culture conditions, but this conclusion must await a more detailed presentation of the evidence.

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EXPLANATION OF PLATES

Calibration bar for electron microscope pictures 1 μm; for light micrographs 10 μm.

PLATE 1

b = boutons, arrow indicates pre- and post-synaptic densities. *A*, *B*, *C*, are synaptic contacts observed with the electron microscope from ciliary neurones cultured alone for 7 days. *D* is from a neurone culture at 11 days *in vitro*. Synaptic vesicle profiles can be recognized in all endings with an increase in number apparent from *A* to *D*. Some of the vesicles have a large dense core; mitochrondria and some large vesicular membranes (*B*, *C*, *D*) are also seen. Pre- and post-synaptic specializations in the area of synaptic contact are clearly recognized.

PLATE 2

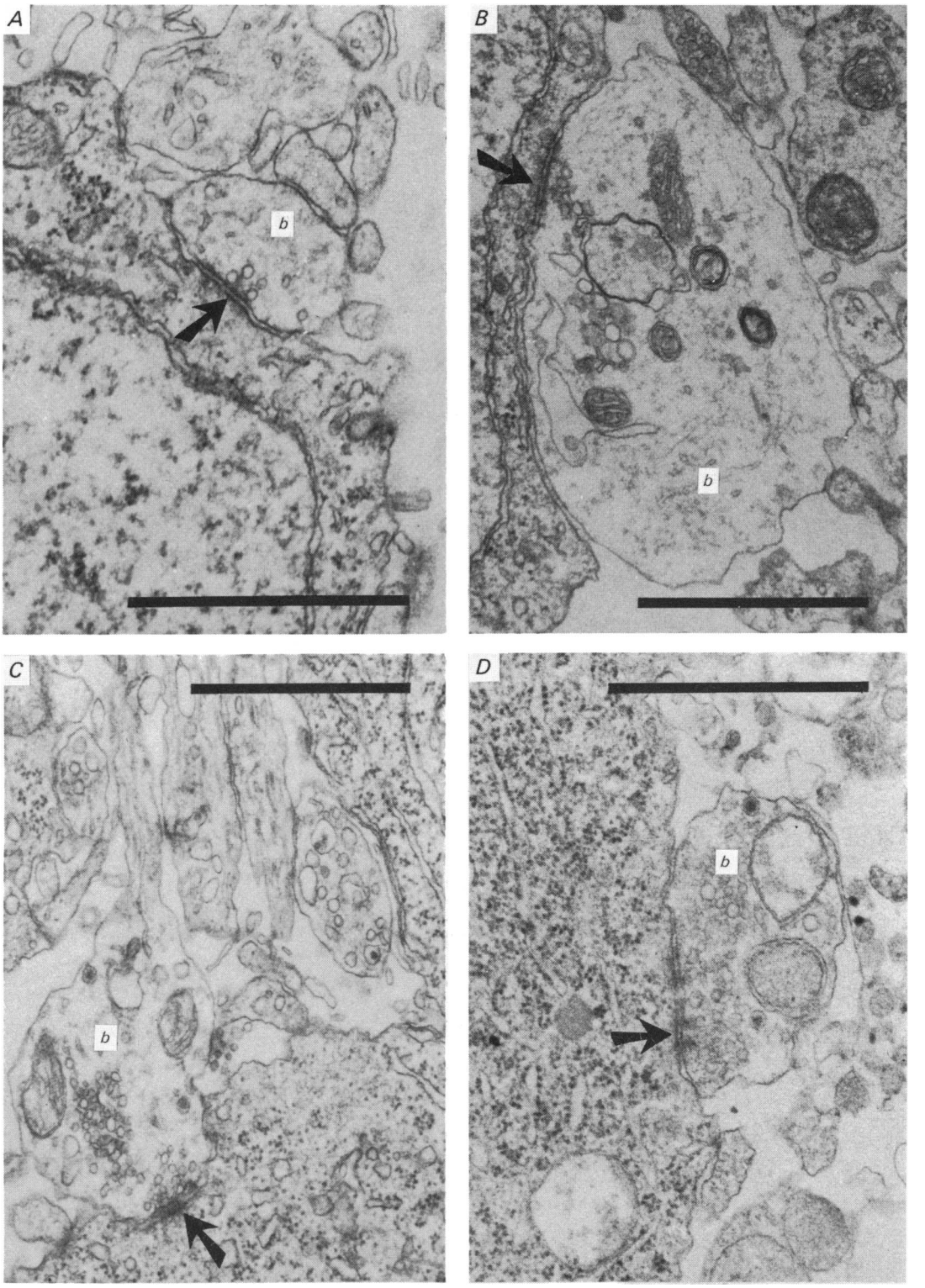
Light micrographs of ciliary ganglion cells and muscle in culture 7 days after the addition of the neurones. Prints were taken with Hoffman modulation contrast optics.

A is a large field to illustrate the density and distribution of the cellular elements in the co-culture. Some neurones (*) attached to the muscle, while the rest grew between muscle fibres, which appeared longitudinally arranged. Neural processes are seen growing between the neurones and myotubes.

In *B* a restrictive view of the co-culture showing three neurones (*) attached to a myotube. Neuronal processes have been extended from the cell at the right (short) and from the cell in the left (longer). The processes trailing along the muscle seem to terminate in a round expansion (bouton?) as indicated by the arrows.

PLATE 3

Low power electron micrograph of two neurones (*n*) together and myotube (*m*) separated by a 30 nm narrow gap. No ultrastructural specializations are distinguished between the neurones and the myotube. Notice the apparent absence of organized glial investment.


A
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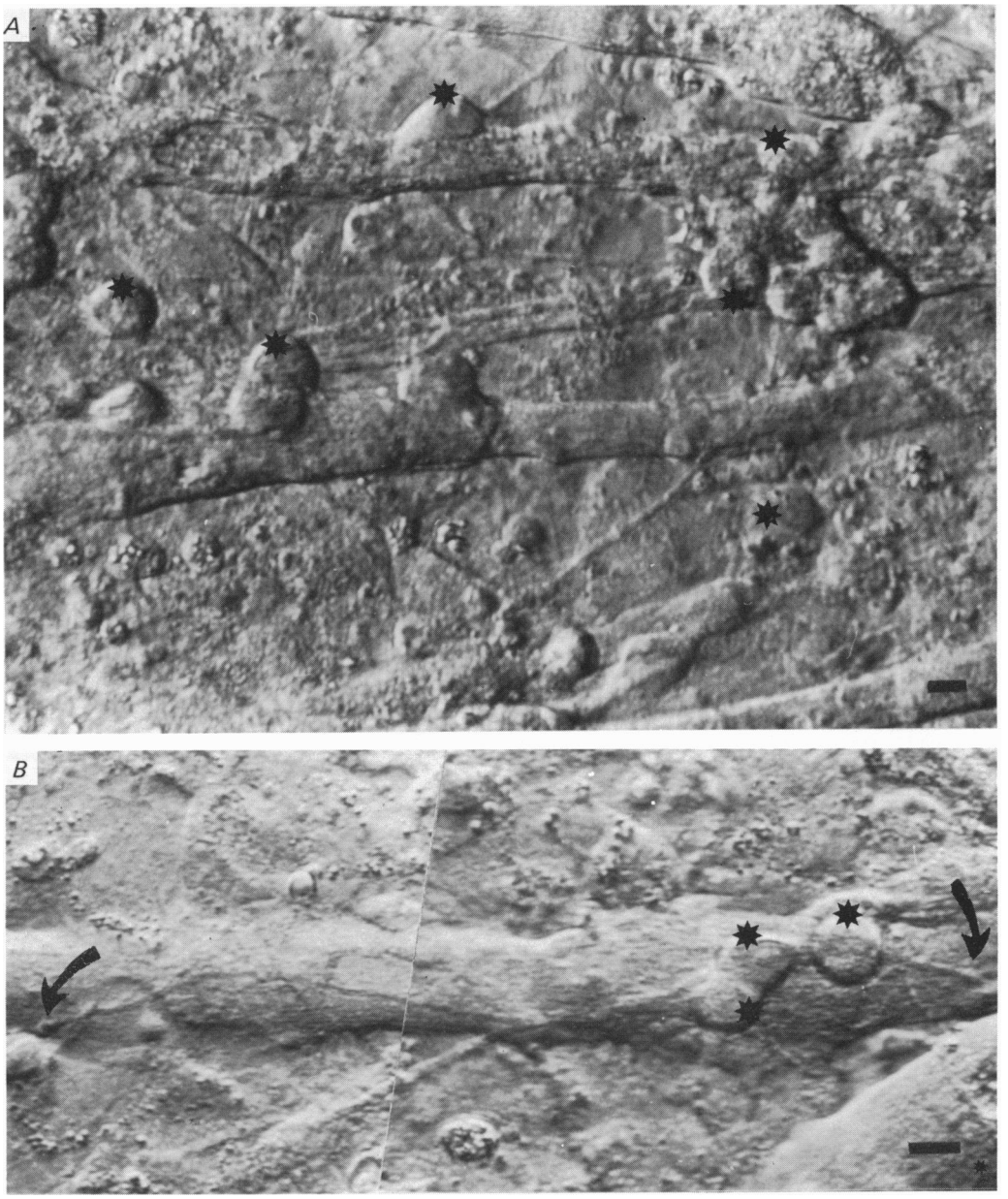
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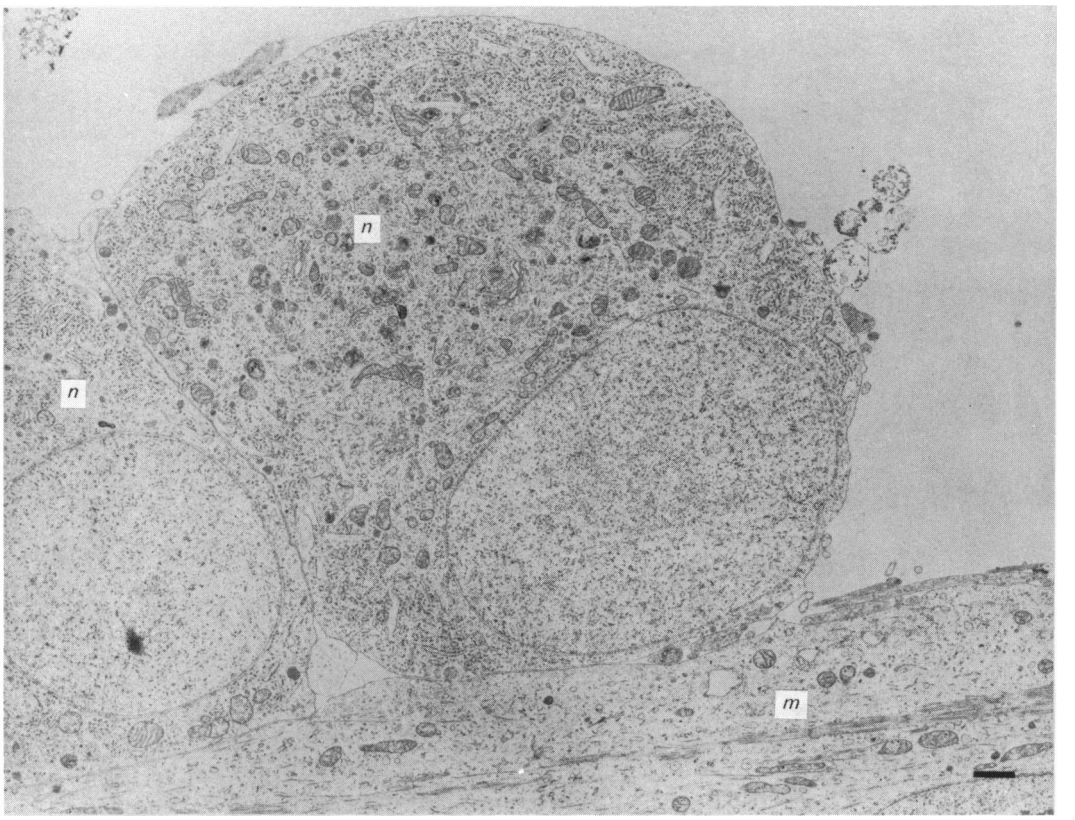
n
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