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SYNAPSE FORMATION AMONG DEVELOPING SENSORY NEURONES FROM RAT NODOSE GANGLIA GROWN IN TISSUE CULTURE

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SUMMARY

1. Sensory neurones from new-born rat nodose ganglia were grown in tissue culture, either with or without the ganglionic satellite cells, in order to investigate influences of satellite cells on sensory neurone development.

2. To learn more about the post-natal development of nodose ganglia in rats neuronal counts of the ganglion were made at three different developmental stages. There were no significant differences of neuronal number in nodose ganglia in new-born rats, rats 3 weeks of age, and adult rats.

3. Up to 60% of the neurones formed synapses with each other when they developed in culture without ganglion satellite cells. Pharmacological experiments indicated that the transmitter at these synapses was ACh and the post-synaptic receptors were nicotinic.

4. Neurones co-cultured with satellite cells rarely formed functional synapses and most (85%) were not sensitive to ACh: 75% of neurones cultured without satellite cells were ACh sensitive.

5. These results provide evidence that mammalian sensory neurones form synapses among each other in culture. The results also suggest that ganglionic satellite cells prevent functional synapses among these neurones from occurring, in part because the neurones do not express ACh sensitivity when co-cultured with satellite cells.

INTRODUCTION

The nodose ganglion is a cranial sensory ganglion located in the periphery, whose axons run in the vagus nerve to provide sensory innervation to much of the viscera including the heart, the lungs, the trachea and the gut (Paintal, 1973). The soma of neurones in mammalian sensory ganglia are totally engulfed by satellite cells and are completely devoid of synapses (Lieberman, 1976; Pannese, 1981). One objective of this present study was to determine if ganglionic satellite cells influence the development of sensory neurones, especially regarding the neurone's ability to receive synapses. A previous study showed that some neurones from nodose ganglia of new-born rats were capable of both forming and receiving synapses when the neurones developed in tissue culture, but functional synapses among neurones did not occur when they were co-cultured with muscle (Baccaglini & Cooper, 1982*a*, *b*); as the proportion of neurones surviving in those cultures was low, it was uncertain

if those neurones forming synapses would have developed into authentic sensory neurones had they been left *in vivo*.

The present study shows that the majority of neurones taken from rat nodose ganglia at birth receive functional synapses from other nodose neurones when grown in dissociated cell cultures. These synapses are cholinergic and the post-synaptic receptors are nicotonic. The results also show that the incidence of functional synapses is dramatically reduced when the neurones are co-cultured with satellite cells from the ganglion.

METHODS

Cell preparation

Nodose ganglia, which lie along the vagus nerve at the level of the bifurcation of the carotid arteries, were dissected under sterile conditions from new-born rats (C.D. strain, Charles River, Canada) which were killed by cervical dislocation. Previously the ganglia were dissociated mechanically (Baccaglini & Cooper, 1982*a*); however, the yield was low, only representing approximately 2–5 % of the neurones in the intact ganglia. In this study, in order to improve the yield, the ganglia were dissociated enzymatically. The enzyme solution consisted of: Hanks Balanced Salt Solution without Ca^{2+} and Mg^{2+} (Gibco), supplemented with penicillin (100 u./ml) and streptomycin (0·25 mg/ml) (Gibco), glutamine (200 mM-Stock solution, Microbiological Associates, 0·2 mg/ml), and glucose (0·01 g/ml) to which collagenase (1 mg/ml, Worthington Type I) and protease neutral (Dispase, Grade I, 1 mg/ml, Boehringer Mannheim) were added. The ganglia were first placed in this enzyme solution for 15–20 min at 37°, and then transferred to a second enzyme solution, similar to the above solution except that collagenase was omitted. The ganglia remained in this second solution for 2–2½ h at 37°, and at 15 min intervals the solution was triturated with a fire-polished glass pipette. The cell suspension was then washed twice with serum-containing medium to inactivate the enzymes, and in some cases, pre-plated in growth medium in a 60 mm plastic culture dish (Falcon) for 30–45 min at 37 °C and 5 % CO_2 in an attempt to reduce the number of fibroblasts. The cells were collected and cultured.

Culturing method

The culturing methods were similar to those described previously (Baccaglini & Cooper, 1982*a*; see also Hawrot & Patterson, 1979). Briefly, the culture media consisted of L-15 media supplemented with vitamins and co-factors, rat serum (5 %), and 7S nerve growth factor (NGF; 1 μg/ml). Cytosine arabinoside (5–10 μM, Sigma) was added to the growth media during the first week in order to kill dividing cells. The cell suspension from twenty dissociated ganglia was plated on collagen-coated Aclar (Allied Chemical Co.) cover-slips into approximately twenty-five modified culture dishes (see Hawrot & Patterson, 1979).

The cultures were maintained in a humid atmosphere of 95 % air–5 % CO_2 at 37° and fed every 3–4 days with growth media, usually 2 ml per dish.

Histology and neurone counts

Nodose ganglia were dissected out from new-born, 3 week old, and adult rats, and fixed in 3 % glutaraldehyde, post-fixed in osmium tetroxide, stained in uranyl acetate, and embedded in Epon. Neurone counts of intact ganglia were estimated by cutting serial 1 μm sections through the ganglia and counting the number of neurones that contained a nucleolus. In individual sections the number of nucleoli per nucleus was often not greater than one whereas examination of serial sections indicated an average of two nucleoli per nucleus. Based on this consideration the number of neurones that contained a nucleolus was divided by two. To avoid errors due to counting the same nucleolus twice or missing nucleoli completely, estimates of the average size of nucleoli in these neurones were obtained. Nucleolus size was estimated by examining approximately twenty-five nucleoli in serial 1 μm sections; these nucleoli were never seen to extend further than four consecutive 1 μm sections or less than three 1 μm sections. Consequently, only every fourth 1 μm serial section was used to estimate neurone numbers. In some cases only every twelfth or sixteenth serial 1 μm section was used to count neurones with nucleoli, but to correct for the missing sections

the total numbers were multiplied by 3 or 4 respectively; these approximations did not differ significantly from the estimates obtained when every section was counted but it did make the cell counting procedures much more rapid.

The size of the neurones was estimated from the diameter of neurones that were sectioned through the nucleolus. The number of neurones in the cultures was obtained directly by counting them in living culture using phase optics.

Electrophysiology

The electrophysiological recording techniques were similar to those described previously (O'Lague, Potter & Furshpan, 1978; Baccaglini & Cooper, 1982*a*). The recording micro-electrodes were filled with 3 M-KCl (resistances in perfusion fluid 60–120 MΩ) and were used to record voltage and pass current simultaneously.

Perfusion fluid

The perfusion media contained: 10% (v/v) basal L-15 medium, NaCl (140 mM), KCl (5·4 mM), $CaCl_2$ (2·8 mM), $MgCl_2$ (0·18 mM), HEPES (10 mM, B.D.H.), glucose (5·6 mM), choline chloride (0·07 mM), Phenol Red (0·03 M), glutamine (2 mM), and penicillin–streptomycin (as in growth medium). The pH was usually 7·3–7·4. All drugs used were dissolved in the perfusion media.

ACh sensitivity. This was tested by means of a pressure ejection system described previously (Baccaglini & Cooper, 1982*b*). Briefly, ACh was applied from a micropipette that was filled with ACh solution (10^{-3}–10^{-4} M) and connected to a compressed air supply; the pipettes, whose tips were 10–20 μm, could be positioned under visual control, and the pressure (5–20 kPa) was gated by a solenoid valve. The drug solution quickly flooded a region several tens of micrometres in diameter: some dilution of drug occurred before it reached the cell body; the exact concentration is unknown but it was previously shown that 10^{-3} M-ACh in the pipette yielded a maximal depolarizing response (Baccaglini & Cooper, 1982*b*).

Agents added. These were: acetylcholine chloride (ACh; Sigma); hexamethonium chloride (Sigma); D-tubocurarine chloride (Sigma); atrophine sulphate (Sigma); 5-hydroxtryptamine (5-HT; Sigma).

RESULTS

Intact nodose ganglia

The nodose ganglion lies along the vagus nerve and the individual neurones intermingle with the myelinated and unmyelinated axons which run through the ganglion. The proximal and distal ends of the ganglion merge with the vagus nerve so that the exact limits of the ganglion are difficult to detect with the dissecting microscope. Since there is little information on the number of neurones contained in the nodose ganglia of rats, and since such information was pertinent to the present study, the vagus nerve was sectioned serially at the level of the ganglia and neurones counted using the light microscope (see Methods). Fig. 1 shows the distribution of neurones along the vagus for one adult nodose ganglion and indicates the proximal–distal extent of the ganglia. The proximal–distal extent was determined for each nodose ganglia that was counted to be certain that all neurones in the ganglia were included. The results from five ganglia processed in this way indicate that the adult nodose ganglion in rats contains 6012 ± 589 (mean ± S.E. of mean) neurones. The neurone cell bodies are $35{\cdot}7 \pm 1{\cdot}27$ μm (mean ± S.E. of mean) ($n = 25$) in diameter.

To determine if the number of neurones in the adult ganglia are already established at birth, neuronal counts of nodose ganglia were determined for animals at three different ages: new-born, 3 weeks, and adult. The results of these counts, shown in Fig. 2, indicate that the number of neurones remains constant from birth onwards. At birth the neurone cell bodies are $21{\cdot}8 \pm 0{\cdot}8$ μm (mean ± S.E. of mean) ($n = 25$) and

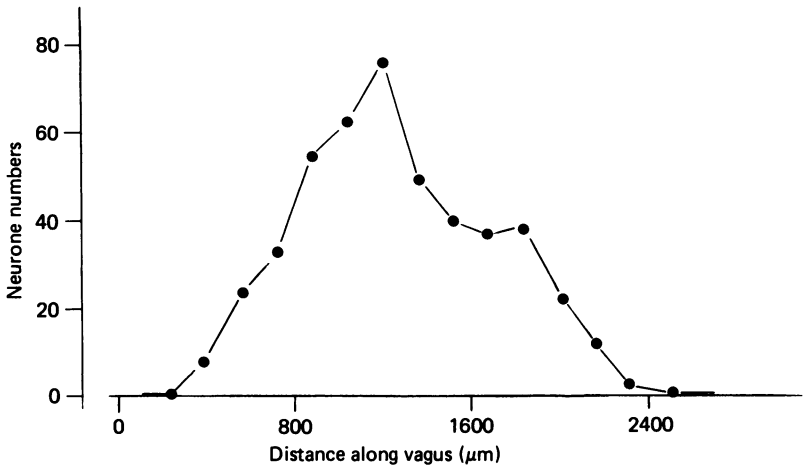

Fig. 1. Distribution of neurones in the nodose ganglion. This graph shows the distribution of nodose neurones along the vagus nerve at the level of the ganglion in the adult rat. The results were obtained from serial sections of the vagus: the number of neurones are shown at intervals of 160 μm. There are approximately 6000 neurones in the ganglion.

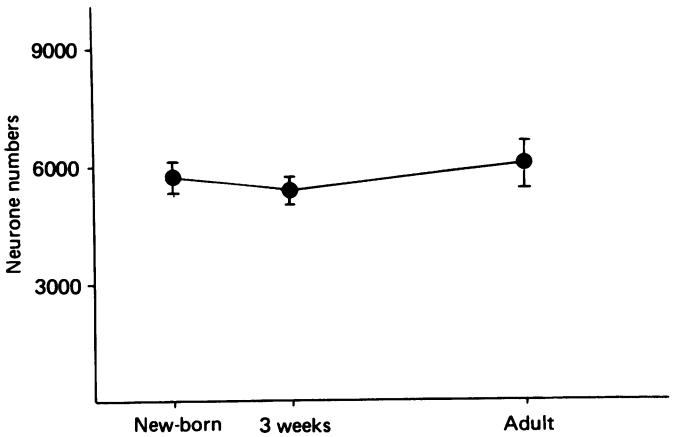

Fig. 2. Neurone numbers in the nodose ganglion *vs.* age. The number of neurones (means $\pm$ S.E. of means) in the nodose ganglion of rats is shown for three different ages: new-born ($n = 5$), 3 weeks ($n = 4$), and adult ($n = 5$). These numbers were determined from serial sections of the nodose ganglion and show no significant difference in the number of neurones in the nodose from birth onwards.

by 3 weeks they are $27{\cdot}5 \pm 0{\cdot}8$ μm (mean $\pm$ S.E. of mean) ($n = 25$). Plate 1 shows representative sections for each age.

Nodose neurones in cell culture

Nodose ganglia from new-born rats were dissociated with enzymes and cultured on collagen-coated cover-slips in growth media (see Methods). The prominent cell types from the ganglion, neurones, as well as satellite cells, including fibroblasts and

Schwann cells, grow in these cultures initially. The neurones, whose cell bodies remain almost spherical, 10–20 μm in diameter, are easily distinguishable with phase optics from the smaller satellite cells, which are 'spindle'-shaped, 10–12 μm long and 2–3 μm at their widest portion. It is more difficult, on the other hand, to distinguish with the light microscope the neuronal processes from the many thin cytoplasmic extensions emanating from the satellite cells in 1–2 day old cultures. An example of a 2 day old culture is shown in Pl. 2*A*.

The number of neurones in these cultures, determined by counting them directly in living cultures 1–2 days after plating, was often as much as 65–70 % of the neurones in the intact ganglia.

The satellite cells in some 2 day cultures were eliminated with cytosine arabinoside, a purine base analogue that kills dividing cells. By 5–7 days almost all satellite cells had disappeared and the neurones were allowed to develop for a further 3–5 weeks by themselves. During this time, the soma can grow to 30–45 μm in diameter and the neurites, now visible, form a dense mesh on the bottom of the culture dish (see Pl. 2*C*).

In cultures not treated with cytosine arabinoside the neurones developed together with the satellite cells. During the first week in culture the satellite cells both enlarged in size and increased in number to form a monolayer. The neurones grew to about 30–45 μm in diameter and many neurones appeared flatter than those in cultures without satellite cells; the neuronal processes were, for the most part, obscured by the monolayer (see Pl. 2*B*). In some cultures it was necessary to prevent overgrowth by the fibroblasts by either exposing the cultures to low doses of radiation (1250 rads from ^{60}Co source), sufficient to prevent cells from dividing but not great enough to kill them, or by exposing the culture for a brief period to cytosine arabinoside which reduced the number of dividing cells. Neither treatment had any effect on neurone number. The loss of neurones was usually less than 15 % during the first week in culture, regardless of treatment with cytosine arabinoside. During the subsequent 3–5 weeks neuronal loss was approximately 10 %, whether the satellite cells were present or not.

Synapse formation among neurones in culture in the absence of satellite cells

Intracellular recordings from neurones that had developed without satellite cells for 2–3 weeks indicated that abundant synapse formation had taken place. Many neurones, over 60 % in some cultures, had e.p.s.p.s; some of these e.p.s.p.s were large enough to evoke action potentials (see Fig. 3). Usually these e.p.s.p.s occurred spontaneously, at frequencies approximately 0·5–2/min; in some neurones, however, the e.p.s.p.s only became evident after an action potential had been triggered: these e.p.s.p.s usually occurred several milliseconds after the action potential and were often intermittent, following only 1/3–1/5 of the stimuli (see Fig. 3).

Recordings were made from synaptically interacting pairs of neurones to demonstrate more clearly that these spontaneously depolarizing potentials were in fact synaptic potentials, and also to estimate the number of neurones forming synapses in these cultures. When pairs of neurones within a microscopic field (400 μm in diameter) were chosen at random for recording, approximately 15–20 % were synaptically connected. The degree of synaptic interaction varied from pair to pair

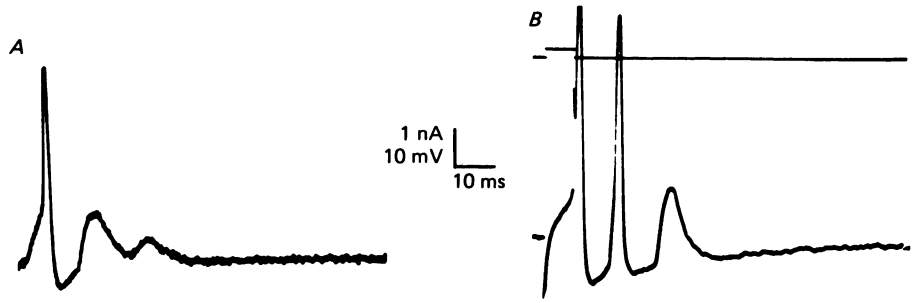

Fig. 3. Spontaneous synaptic potentials. These voltage traces are intracellular recordings from two different neurones showing examples of synaptic potentials. In *A*, the potentials occurred spontaneously and some were large enough to trigger action potentials. In *B* a single action potential, triggered by stimulating the neurones with a brief current pulse (upper trace), evoked two synaptic potentials on itself. These synaptic potentials presumably originated from other neurones in the culture: the first synaptic potential was large enough to reach threshold.

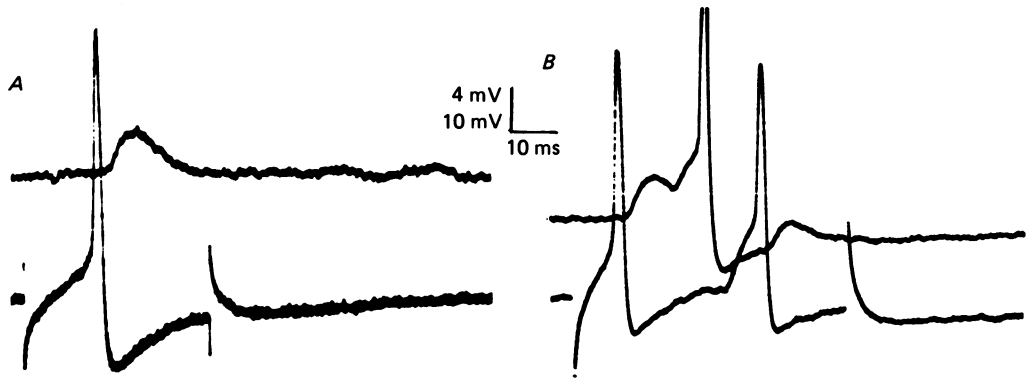

Fig. 4. Synaptic interaction. These two examples show the variation in degree of synaptic interaction between neurones. In *A*, stimulation of an action potential in one neurone (bottom trace) evoked a single e.p.s.p. in the follower neurone (upper trace). In *B*, stimulation of a single action potential (lower trace) resulted in several e.p.s.p.s in both the follower neurone (upper trace) as well as the stimulated neurones; some of these e.p.s.p.s were large enough to trigger action potentials. In this and other Figures the capacitative artifacts mark the beginning and end of the current pulse used for stimulation. The vertical scale is 4 mV for the upper trace in *A*, and 10 mV for the other traces.

and from culture to culture. In some cases an action potential in one neurone evoked a single e.p.s.p. on the other, whereas in the other extreme an action potential in one initiated a complex series of e.p.s.p.s, some large enough to trigger action potentials on both neurones of the pair. Two such examples are shown in Fig. 4. Rarely (less than 10% of the pairs examined) were synaptically linked pairs of neurones reciprocally connected in that stimulation of either neurone evoked an e.p.s.p. on the other; usually only one neurone of a pair evoked e.p.s.p.s.

Identification of transmitters and post-synaptic receptors

The synaptic interactions were reversibly reduced upon changing the external solution to one containing raised Mg^{2+} concentration. In six out of six interactions Mg^{2+} raised from 0·56 mM (control) to 10·8 mM reduced the synaptic potential by more than 90%.

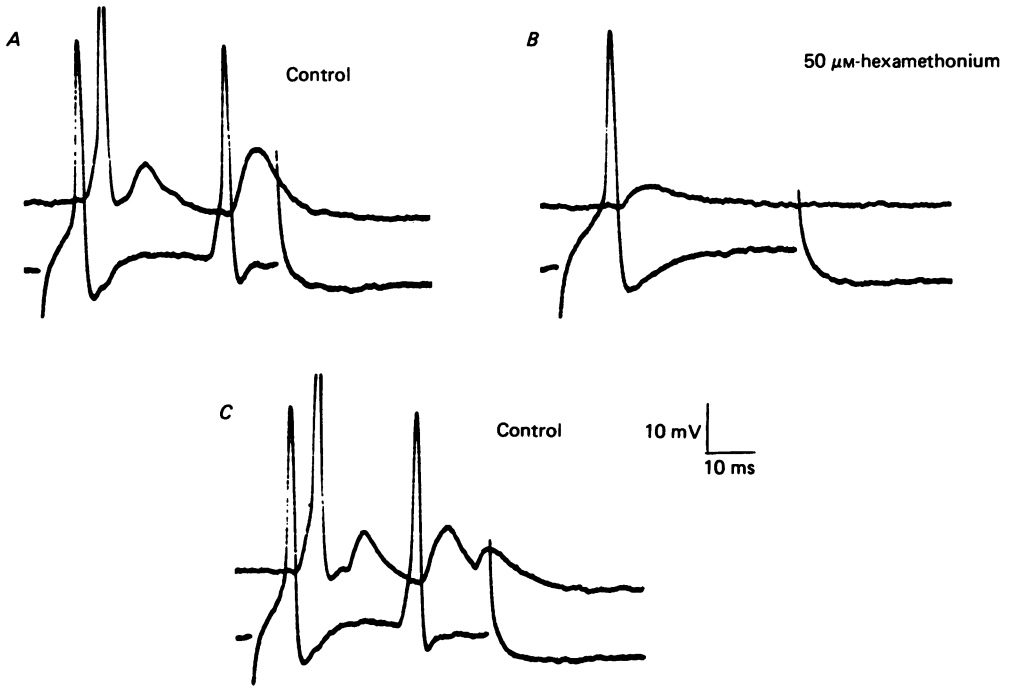

Fig. 5. Reduction of synaptic potentials by hexamethonium. These three pairs of traces are intracellular voltage recordings from two neurones. *A* is in control solution and shows that stimulation of a single action potential in one neurone (lower trace) evoked e.p.s.p.s in both the follower neurone (upper trace) and itself; some e.p.s.p.s were large enough to trigger action potentials. *B* shows recording from the same two neurones within minutes after perfusing the culture with 5×10^{-5} M-hexamethonium; the stimulated action potential now evoked a single e.p.s.p. much reduced in amplitude on the follower neurone. The e.p.s.p. continued to decrease in amplitude and within 10 min it was difficult to resolve from the base line. The e.p.s.p.s on the driver neurone were also abolished. *C* is 20 min after returning to control solution and shows that the synaptic interaction was restored.

The identity of the transmitter was investigated by pharmacological means. Twelve out of twelve interactions were reversibly reduced by more than 90% in 50–100 μM-hexamethonium; six out of six interactions were also reversibly reduced by more than 90% in 50–100 μM-curare, whereas in five out of five interactions 1 μM-atropine had no effect. These concentrations are typical for nicotinic receptors on autonomic neurones. Fig. 5 shows an example of the reversible blockade by 50 μM-hexamethonium.

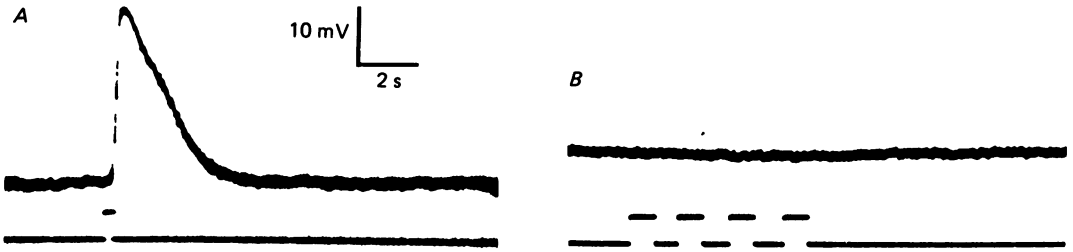

Fig. 6. Response of neurones to applied ACh. This Figure demonstrates the response of two neurones to ACh (10^{-3} M in the pipette) pressure ejected onto their cell bodies. For both *A* and *B* the upper trace represents the membrane potential and the lower trace the duration the drug is applied. For the neurone in *A*, brief application of ACh caused a rapid depolarization; the neurone repolarized as the drug diffused in the bath. In *B*, repeated application of the drug had no detectable effect on the membrane potential of an insensitive neurone.

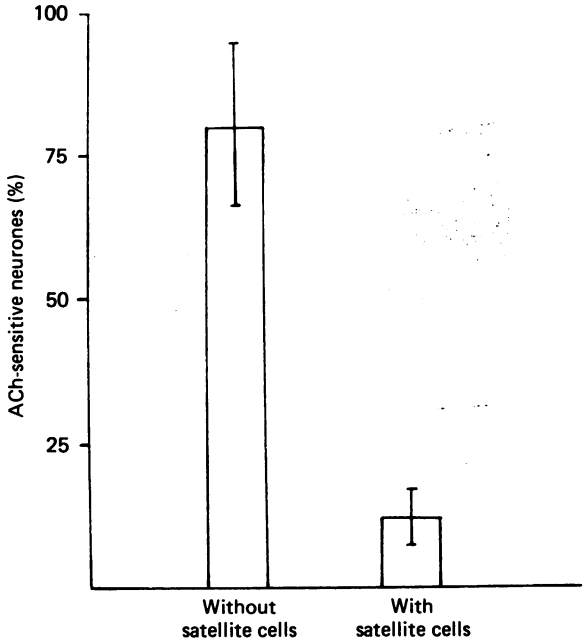

Fig. 7. Influence of satellite cells on expression of ACh sensitivity. The neuronal cultures were divided into two groups, those co-cultured with ganglionic satellite cells (right) and those cultured without satellite cells (left). Neurones in each group were chosen at random and tested for their sensitivity to ACh: the left column represents 183 neurones from eleven cultures; the right column totals 203 neurones from twelve cultures. There is a clear reduction in the proportion of ACh-sensitive neurones in cultures containing satellite cells.

Lack of functional synapses among neurones co-cultured with satellite cells

The synaptic interactions, described above, existed among neurones developing in culture without satellite cells. The incidence of synaptic interaction among neurones was rare for neurones co-cultured with satellite cells. Less than 5% (9 out of 212) of the neurones showed spontaneous synaptic potentials, and less than 2% (one out of

fifty-three) of randomly chosen pairs of neurones were synaptically linked in these cultures.

Influence of satellite cells on ACh sensitivity

In contrast to neurones developing without non-neuronal cells most neurones co-cultured with satellite cells were insensitive to ACh. Neurones were tested for sensitivity to ACh by pressure ejecting ACh (10^{-3}–10^{-4} M in the pipette) onto the soma while monitoring the neurone's membrane potential (see Methods). Most neurones fell clearly into two categories: sensitive neurones, which usually depolarized by 20–40 mV upon the application of ACh (a small number (10%) however, depolarized by only 5–10 mV) and insensitive neurones, which did not depolarize due to ACh application. An example of a sensitive and insensitive neurone is shown in Fig. 6.

The proportion of neurones sensitive to ACh was determined for the two types of cultures, those containing satellite cells and those without. Over 75% of the 183 neurones from eleven cultures that had developed for 1–3 weeks without satellite cells were sensitive to ACh, whereas only 15% of the 203 neurones from twelve cultures containing satellite cells were ACh-sensitive (see Fig. 7).

To test if the lack of effect by ACh on neurones co-cultured with satellite cells was because the drug could not have access to the neurone, 20 mM-K^+ was applied to the neurones; twelve out of twelve neurones showed rapid depolarization to high K^+. Furthermore, three out of six neurones not sensitive to ACh did respond when 5-HT (10^{-4} M) was pressure ejected onto their somas.

DISCUSSION

This study extends previous work in two important ways: it shows that sensory neurones from nodose ganglia of new-born rats will form cholinergic synapses among each other when developing in culture, and it demonstrates that satellite cells from the ganglion prevent functional synapses from occurring, at least in part by inhibiting the expression of ACh sensitivity by these neurones. While there is much evidence indicating that virtually all neurones in nodose ganglia of adult mammals are sensory (Paintal, 1973), there is little information on the development of this ganglion. One possibility which needed to be examined was that many neurones in the nodose ganglion of new-born rats are not necessarily those which develop into sensory neurones found in the ganglion in adult rats. For example, perhaps some neurones in the new-born ganglion represent a transient population migrating down the vagus nerve *en route* to some other location, possibly to some parasympathetic ganglion, and by chance happen to be located in the nodose ganglion at birth. Evidence in favour of this possibility would necessarily complicate the interpretation of the experiments presented here, since the neurones in this study were derived from the nodose ganglion of new-born rats. To examine the above possibility, neurone counts were made of the nodose ganglion from rats at different post-natal ages: new-born, 3 weeks, and adult. As shown in Fig. 2 there is no significant difference in the number of neurones in the ganglion at the three different ages. In addition, results from [^{3}H]thymidine experiments show that nodose neurones in rats undergo their last cell division between embryonic day 11 and embryonic day 15 (Altman & Bayer,

1982), indicating that there is not a continual turnover of neurones in the ganglion post-natally. The results of neuronal counts, considered together with the results from the [^{3}H]thymidine experiments, strongly suggest that neurones in the nodose ganglion of new-born rats continue to develop into sensory neurones found in the nodose ganglion of adult rats. Since the neurones grown in culture in the present study can represent up to 70% of the neurones in the intact nodose ganglia of new-born rats, it seems reasonable to conclude that most, if not all, of the neurones in these cultures would have continued to develop into sensory neurones had they been left in the animal.

Synapses are not present in peripheral sensory ganglia in mammals (Lieberman, 1976; Panesse, 1981), yet over 50% (more than 200 tested) of the cultured neurones received functional synapses when the neurones developed without ganglionic satellite cells. In addition, as many as 15–20% of randomly selected pairs of neurones could be shown to be synaptically coupled, suggesting that many neurones in these cultures are forming synapses. The exact number of synapsing neurones is difficult to quantify with the methods used because the neurites ramify extensively throughout the culture and some neurones could be synapsing with neurones that were not being monitored at that time. As a result the interactions go undetected.

The pharmacological experiments on these synapses indicate that acetylcholine is the transmitter, a similar result to a previous study on dissociated nodose neurones in culture (Baccaglini & Cooper, 1982*a*). The transmitter released by nodose neurones *in vivo* is unknown; however, there are experiments that suggest ACh could be a transmitter for some nodose neurones (Matsumura & Kolle, 1961; Vera & Luco, 1967; Fujiwara, Kurahashi, Mizuno & Nakamura, 1978; Falempin & Rousseau, 1983). Other substances implicated as transmitters for nodose neurones are substance P (Lundberg, Hokfelt, Nilsson, Terenius, Renfeld, Elde & Said, 1978; Katz & Karten, 1980), glutamate (Talman, Perrone & Reis, 1980) and 5-HT (Gaudin-Chazal, Portalier, Puizillout & Vigier, 1983). In addition, the pharmacological experiments indicate that the post-synaptic receptors are nicotinic, similar to those on autonomic neurones.

There is some evidence that in the chick the satellite cells from developing nodose ganglia can differentiate into autonomic neurones (Ayer-Le Lievre & Le Douarin, 1982). It seems unlikely that this phenomenon was occurring in these cultures largely because it is only when satellite cells were eliminated from the cultures that synapse formation became apparent; when neurones were co-cultured with satellite cells functional synapses were rare.

This difference in the incidence of synapse formation between neurones developing with or without satellite cells cannot be explained by postulating two different populations of neurones whose survival depends upon the culture conditions because the initial yield is high and the subsequent neurone death is low; the difference is more probably a consequence of some satellite cell influence(s) that affects synapse formation. One satellite cell influence, which may be related to synapse formation, prevented the expression of ACh sensitivity by these neurones. Most neurones (75%) developing without satellite cells were ACh sensitive; these neurones also received functional synapses. Few neurones (15%) co-cultured with satellite cells were sensitive to ACh, and functional synapses were rare. The relationship between ACh sensitivity and synapse formation in these neurones in culture needs to be explored

further. It is tempting to speculate that the lack of synapses in peripheral sensory ganglia *in vivo* might be the result of some interaction between ganglionic satellite cells and sensory neurones, especially in view of the close association that develops between these two cell types.

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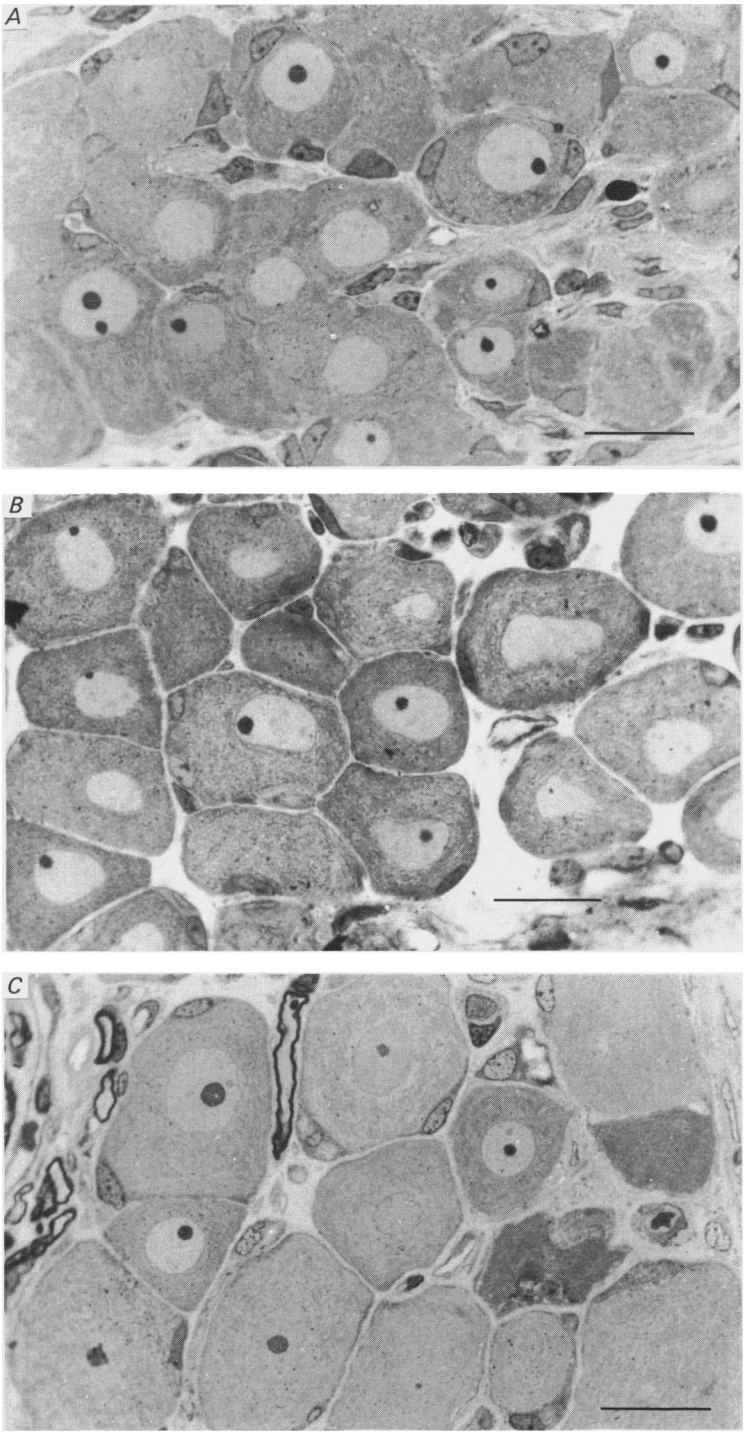
EXPLANATION OF PLATES

PLATE 1

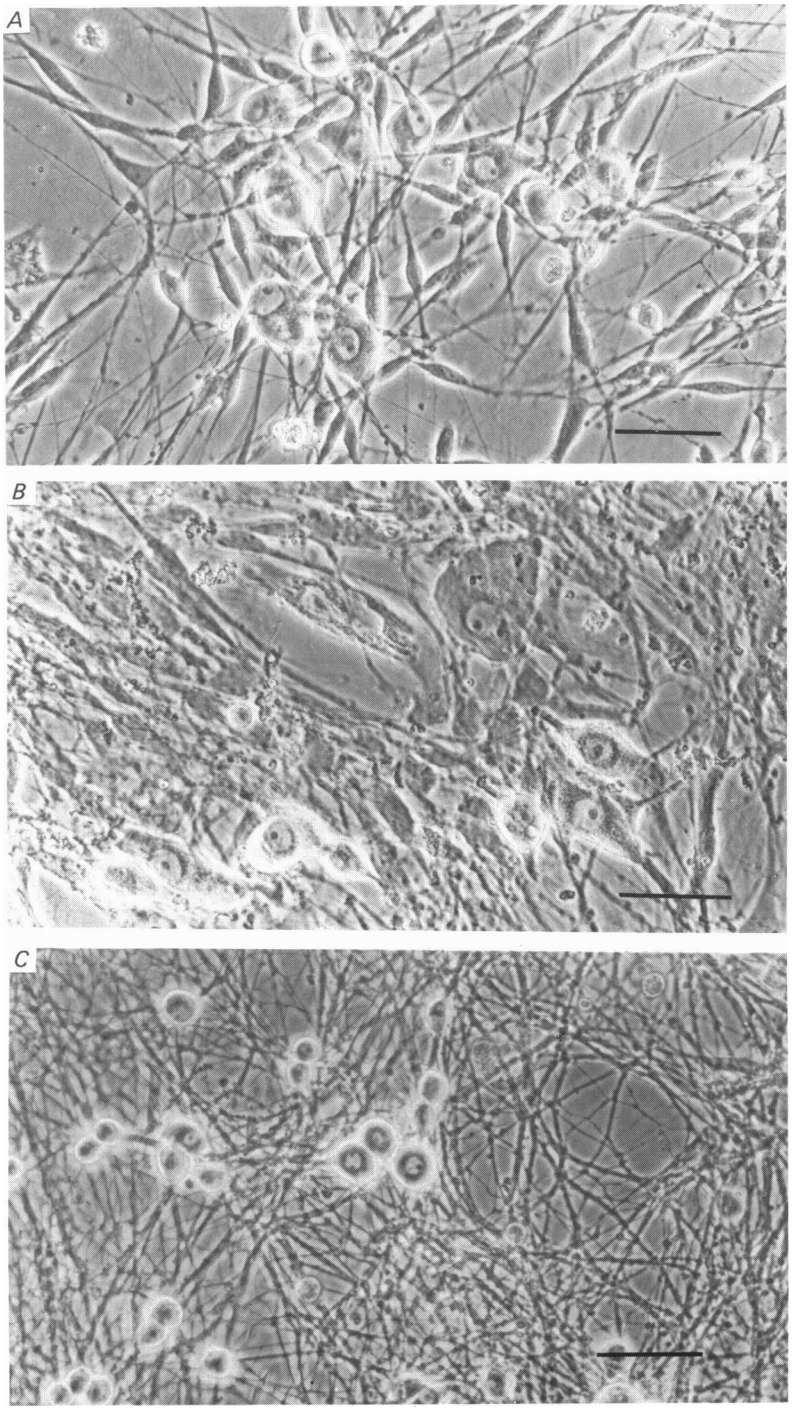
This shows representative 1 μm sections, stained with Toluidine Blue, from intact nodose ganglia at three different ages: new-born (*A*), 3 weeks after birth (*B*), and adult (*C*). Each section shows neurones and satellite cells; the myelinated axons are also apparent. The bar is 25 μm.

PLATE 2

This shows three phase micrographs of nodose neurones in culture. *A* was taken 2 days after plating and shows both the neurones (large spherical cells) together with satellite cells. *B* shows neurones co-cultured with satellite cells 23 days after plating: the satellite cells have grown and almost cover the bottom of the culture dish obscuring the neuronal processes. *C* shows the neurones developing in the virtual absence of satellite cells and was taken 27 days after plating. The cell bodies and neuronal processes are now visible. The bar is 40 μm in *A*, 60 μm in *B* and 80 μm in *C*.



(*Facing p.* 274)


A
B
C