J. Physiol. (1982), **324**, *pp.* 429–439
With 1 plate and 5 text-figures
Printed in Great Britain

ELECTROPHYSIOLOGICAL STUDIES OF NEW-BORN RAT NODOSE NEURONES IN CELL CULTURE

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(*Received 5 June 1981*)

SUMMARY

1. Neurones of the nodose ganglion of the vagus nerve were dissociated from new-born rats and grown in the virtual absence of non-neuronal cells and in the presence of nerve growth factor.

2. The resting potentials of the neurones ranged from −40 to −80 mV. Action potentials were of short duration, with no inflexion on the falling phase; others were of longer duration with a hump on the falling phase.

3. The inward current of the action potential was carried either predominantly by Na^+ or by Na^+ and Ca^{2+}.

4. Tetrodotoxin (1 μM) blocked the Na^+ channels of some neurones but in other neurones the Na^+ channels were partially or completely resistant to tetrodotoxin (1–10 μM).

5. Many neurones formed excitatory synapses on neighbouring neurones which were blocked or greatly reduced by conventional ganglionic nicotinic antagonists. This indicates that these neurones secreted ACh and expressed ACh receptors at these synapses.

6. The accompanying paper (Baccaglini & Cooper, 1981) reports the effect of co-culturing nodose neurones with non-neuronal cells on the expression of functional nicotinic receptors.

INTRODUCTION

The mammalian nodose ganglion is an autonomic sensory ganglion whose axons run in the vagus nerve to provide sensory innervation to most of the viscera, including heart, lungs, trachea and gut (Paintal, 1973). Little is known about the post-natal development of nodose neurones. To learn more we have grown dissociated nodose neurones from new-born rats in tissue culture. Our initial interests were in the electrophysiological properties of the surface membrane, the development of sensitivity to transmitter substances, and the identity of neurotransmitters secreted by these neurones.

In this paper we describe some electrophysiological properties of the neurones developing in the virtual absence of non-neuronal cells. We also report that many

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neurones form cholinergic synapses with each other in these culture conditions. In the following paper (Baccaglini & Cooper, 1981) we report that the incidence of cholinergic interaction is significantly reduced when the neurones are co-cultured with either heart myocytes or skeletal myotubes. In part the lower incidence of cholinergic interaction is due to a reduction in the number of neurones sensitive to ACh. Preliminary accounts of this work have previously been published (Cooper & Baccaglini, 1979; Baccaglini & Cooper, 1979).

METHODS

The methods used to culture the neurones are similar to those described by Mains & Patterson (1973) and are described in detail by Hawrot & Patterson (1979). Briefly, nodose ganglia were obtained from new-born rats (CD) strain, Charles River Breeding Lab. The nodose ganglion lies along the course of the vagus nerve, close to the bifurcation of the carotid artery (it can be easily distinguished from the superior cervical ganglion). The animals were killed by cervical dislocation and the ganglia dissected under sterile conditions. In some experiments ganglia were obtained from 20-day-old rat embryos; the mother was asphyxiated with CO_2 and the embryos were promptly removed to a physiological saline solution. The ganglia were initially placed in plating media (described below) and mechanically dissociated with watchmaker forceps. A suspension of single cells and small clumps from sixty ganglia from three litters were plated into the centre well of six modified culture dishes (described below) on plastic cover-slips coated with a dried film of rat-tail collagen. The cultures were maintained in a humid atmosphere of 95% air–5% CO_2 at 36 °C and fed every 3–4 days with 'growth' medium (described below), usually 2 ml. per dish.

The culture dishes were described previously (see Hawrot & Patterson, 1979). Briefly, 10 mm holes were cut in the centre of plastic Petri dishes (Falcon, 35 mm) and then covered with a cover-slip (polystyrene) fixed to the outside with paraffin wax. The inner surface, which formed the bottom of the shallow well, was coated with rat-tail collagen; the dishes were sterilized with ultraviolet light.

Medium. The medium was similar to that described by Mains & Patterson (1973). L-15 medium (North American Biological Inc.) was supplemented with imidazole, aspartic acid, glutamic acid, proline, cystine, β-alanine, vitamin B_{12}, inositol, choline chloride, lipoic acid, biotin, *p*-aminobenzoic acid, fumaric acid and co-enzyme A. The medium was sterilized with washed nuclepore filters (pore size, 0·2 μm) and stored at 4 °C.

Plating medium. To 100 ml. of basal L-15 medium was added: glucose (0·6 g in 2 ml. of H_2O), glutamine (1 ml. 200 mM stock solution, Microbiological Associates), penicillin–streptomycin (1 ml. of 10,000 u. penicillin/ml. and 10 mg streptomycin/ml., Grand Island Biological Co.).

Growth medium. Sterile 150 mM-$NaHCO_3$ (17 ml.) was added to 83 ml. basal L-15 medium. To this were added: methocel (0·6 g; Dow Chemical Corp.), glucose, glutamine and penicillin–streptomycin as in the plating medium; in addition adult rat serum (5 ml.) and nerve growth factor (100 μg in 0·1 ml. H_2O). 7S NGF was prepared by gel filtration through Sephadex G-100 followed by DEAE-cellulose fractionation (Varon, Nomura & Shooter, 1967; Bocchini & Angeletti, 1969).

To prevent multiplication of non-neuronal cells the cultures were either irradiated (5000 rad at 200 rad/sec from ^{60}Co source), or incubated with growth medium containing 10 μM-cytosine arabinoside (Sigma) for two periods of 48 hr each.

Recording techniques. The recording techniques were similar to those described by O'Lague, Potter & Furshpan (1978). Briefly, the culture dishes were placed in a holder on the stage of an inverted phase microscope. The temperature of the cultured neurones was maintained at 34–37 °C. A perfusion fluid (see below) was supplied to the dish at a constant rate (usually 1 ml./min) by an adjustable pump (Extracorporeal) and withdrawn by a suction tube. Reservoirs containing different solutions (see below) were selected by a multichannel valve without interrupting the flow. An Ag:AgCl electrode at ground potential was connected to the central well by a salt bridge containing 3 M-KCl in 2·5% agar. The recording micro-electrodes were filled with 3 M-KCl or occasionally with 4 M-K acetate (resistance in perfusion fluid 60–120 MΩ) and were used to record voltage and pass current simultaneously. It was possible to record continuously from a neurone for several hours.

Perfusion media. The 'normal' medium contained: 10% (w/v) basal L-15 medium, NaCl (140 mM), KCl (5·4 mM), $CaCl_2$ (2·8 mM), $MgCl_2$ (0·18 mM), $NaHCO_3$ (12·5 mM), NaH_2PO_4 (0·56 mM), glucose (5·6 mM), choline chloride (0·07 mM), imidazole (0·8 mM), phenol red (0·03 mM), glutamine (2 mM), and penicillin–streptomycin (as for plating medium). The pH was usually about 7·4 and kept at this value by bubbling a mixture of air: CO_2 (95:5), when necessary. In the 'Na^+-free' medium glucose (280 mM) replaced NaCl, and $NaHCO_3$ and NaH_2PO_4 were replaced by HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; pH 7·4; 10 mM). All other components were the same as in 'normal' perfusion medium.

When a channel-blocking agent (e.g. tetrodotoxin) altered the excitability of a neurone so that the initially suprathreshold current pulse no longer gave rise to an action potential, the strength of the current pulse was increased by a factor of 1·5 to test further the ability of the cell to produce an action potential in the presence of the agent. The result of such a test is reported here only if the action potential returned to at least 80% of its initial value after washing with normal saline for 30–40 min.

The effects of cholinergic antagonists. On successive stimulation of the presynaptic ('driver') neurone, ten to twenty trials in normal solution were recorded; the average of those trials was then compared with the average of a similar number of trials in the presence of various concentrations of a blocking agent, unless the block was complete. If the post-synaptic ('follower') neurone fired in response to synaptic activation the size of the e.p.s.p. was taken as threshold membrane potential of the follower neurone, a lower limit.

Agents added. Tetrodotoxin (TTX; Sigma Chemical Co.); $CoCl_2$ (Fisher Scientific Co.); hexamethonium chloride (Pfalz & Bauer); atropine sulphate (Merck); D-tubocurarine (Mann Research Lab.); chlorisondamine and pempidine tartrate (gifts from Dr E. Marder).

RESULTS

Growth of nodose neurones in culture

During the first 24 hr in culture the neurones became attached to the collagen film and sent out processes. The processes tended to adhere and progressively formed a network of increasing density. A phase-contrast micrograph of a typical 3-week-old living culture is shown in Pl. 1. While most neurones have round cell bodies, some are more flattened; the nucleus, containing one to two nucleoli, usually lies near the centre of the cells. Neurones occur either singly or in small clumps. During the first 3–4 weeks the neurone cell bodies grew from approximately 10–15 to 35–40 μm in diameter. We typically plated 500–600 neurones per dish. Cell death was less than 10% over the 4–6 week period when nerve growth factor (NGF) was added to the growth medium.

NGF requirement. The dependence of the neurones on NGF was investigated in five platings. For each plating dissociated neurones were randomly divided into two lots; one was plated in regular growth medium (see Methods), the other in an identical medium except that NGF was omitted. Four days after plating no major difference was seen between the cultures in presence and absence of NGF, either in the number of neurones or in the appearance of the neurites. However by 10 days the NGF-free cultures had fewer and smaller neurones and the neurite network was less extensive than those containing NGF. By 20–25 days fewer than 10% of the neurones remained in the NGF-free cultures, and these neurones were small (approximately 10–20 μm in diameter) and had thin, virtually unbranched processes. In NGF-free cultures we did see a few healthy-looking neurones; these cell bodies were usually located over non-neuronal cells. Perhaps non-neuronal cells released NGF or some other factor that promotes neuronal survival.

Electrophysiological properties

Resting potential. When neurones were impaled with a micro-electrode, there was often an abrupt change in potential (−60 to −70 mV) but more frequently there was a jump to −30 and −40 mV followed by a slower change to an average steady level of −50 to −60 mV. This resting potential was often maintained for hours. In cultures 10 days old the average resting potential was less and remained steady for shorter times.

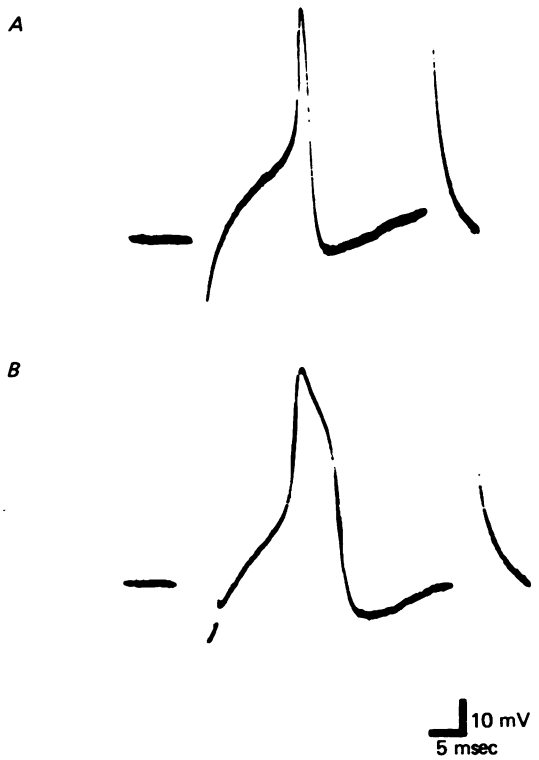

Fig. 1. Action potentials elicited by injection of depolarizing current pulses. *A*, the action potential has no apparent inflexion on the falling phase (resting potential −48 mV). *B*, the action potential is of longer duration than in *A* and has a pronounced inflexion on the falling phase (resting potential −50 mV). The beginning and end of the current pulse are marked by artifacts.

Action potential. Action potentials varied in duration and in the extent to which a hump was present on the falling phase. In some neurones the action potential had a distinct hump on the falling phase (Fig. 1*B*), while in other neurones this hump was completely lacking (Fig. 1*A*) and in some the inflexion was present to an intermediate extent. The after-hyperpolarization was generally 5–10 mV in amplitude and lasted 10–30 msec. Many neurones fired repetitively when stimulated with a current pulse which lasted for 150 msec.

The ionic composition of the inward current of the action potential was investigated by the application of selective blocking agents. Fig. 2 shows the variation of these

neurones in their sensitivity to TTX; TTX (1–10 μM) either abolished the action potential (three of fifteen neurones), reduced it in rise time and amplitude (ten of fifteen; range of reduction of amplitude 7–48 %), or had no effect (two of fifteen). Four neurones whose action potential was either unaffected or only partially blocked by TTX were stimulated in Na^+-free solution (see Methods). In each case the action potential was reversibly abolished, suggesting that some of the nodose neurones have Na^+ channels that are TTX-resistant (Fig. 2*B*).

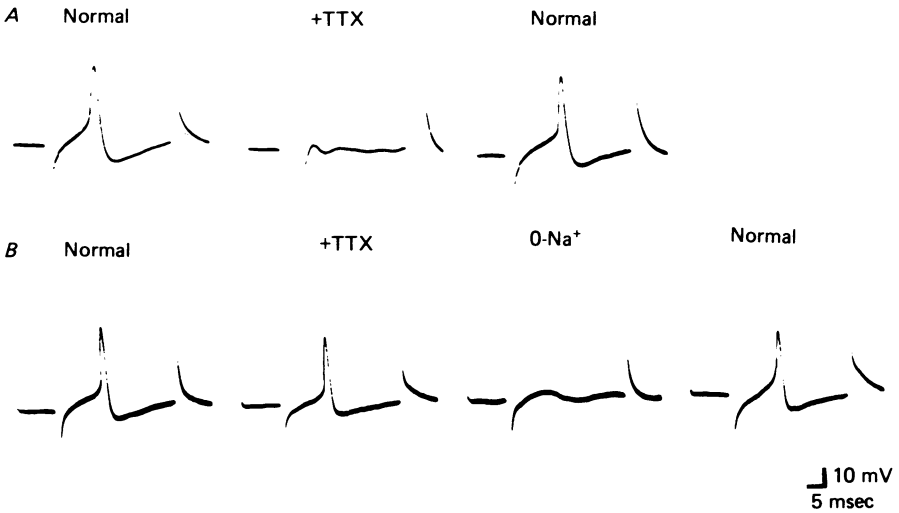

Fig. 2. Na^+ action potentials. *A*, addition of 1 μM-TTX to the perfusion solution (middle trace) reversibly blocked the action potential (resting potential −55 mV). *B*, the action potential was unaffected by addition of 3 μM-TTX but blocked completely and reversibly in Na^+-free solution (resting potential −40 mV). The beginning and end of the current pulse are marked by artifacts. The action potentials were retouched for clarity.

In early experiments we substituted Na^+ with choline; however we found that the neurones depolarized by 10–15 mV in this medium (see also Gallego & Eyzaguirre, 1978). In most subsequent experiments we substituted Na^+ with glucose and found no change in resting potential.

The presence of an inward Ca^{2+} current, suggested by the inflexion on the falling phase of the action potential, was tested by the addition of $CoCl_2$ (2·5 mM) to the perfusion solution. The action potential was reduced in amplitude in nine of eighteen neurones tested (range 6–56 %); no effect on it was observed for the other nine neurones tested. This suggests that in some of the nodose neurones an inward Ca^{2+} current contributed to the action potential. An action potential in which both Na^+ and Ca^{2+} contribute to the inward current is shown in Fig. 3.

Synaptic interactions

When nodose neurones were grown in the virtual absence of non-neuronal cells many of the neurones, particularly in cultures more than 4 weeks old (up to 50 % in some cultures), had fast-rising spontaneous e.p.s.p.s which often reached threshold. To obtain an estimate of the number of neurones that could evoke such e.p.s.p.s

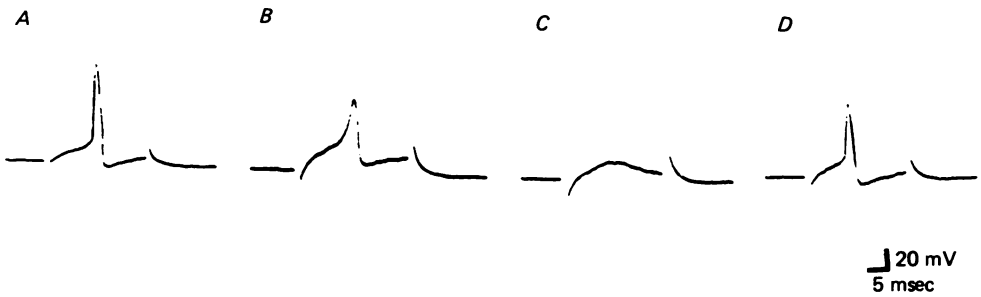

Fig. 3. Contribution of both Na^+ and Ca^{2+} to the inward current. *A*, action potential evoked in normal perfusion medium. *B*, the addition of 1 mM-TTX reduced the amplitude and prolonged the action potential. *C*, the action potential in *B* was blocked competely by the addition of 2·5 mM-$CoCl_2$. *D*, these effects were reversed by returning to normal perfusion solutions (resting potential −50 mV). The beginning and end of the current pulse are marked by artifacts. The total time between *A* and *D* was 110 min. The action potentials were retouched for clarity.

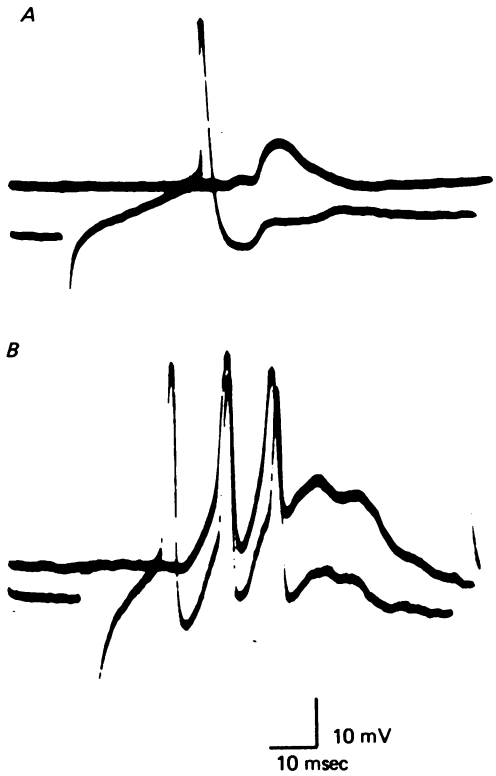

Fig. 4. Synaptic interactions. In each case one neurone (driver; bottom trace) was stimulated by passing depolarizing current pulses. *A*, the evoked action potential caused an e.p.s.p. in the other cell (follower; top trace) at a latency of approximately 5 msec. The e.p.s.p. was followed by another, in both the follower and driver neurones. *B*, the evoked action potential caused a barrage of suprathreshold activity in both the follower and driver neurones.

('driver neurones') pairs of neurones were chosen at random in a microscopic field (approximately 300 μm in diameter), and each neurone was stimulated in turn while recording from both. An example of such a synaptically linked pair is shown in Fig. 4. Routinely between twenty and forty such pairs per culture were sampled and the number of driver neurones determined. In this culture condition (few non-neuronal cells), as many as 20–30 % of the neurones were found to be drivers by this test. This

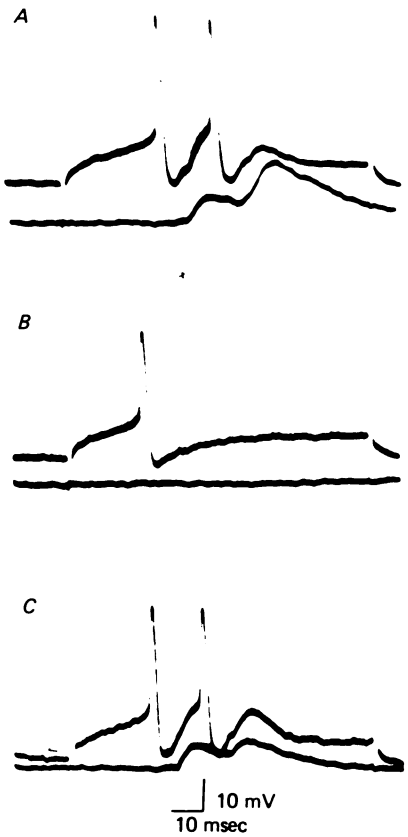

Fig. 5. Reversible block by a nicotinic-cholinergic antagonist of evoked e.p.s.p.s. *A*, an action potential evoked in the driver (top trace) caused e.p.s.p.s in the follower (bottom trace) and in itself. *B*, same two neurones as *A* 10 min after 5×10^{-4} M-hexamethonium was added to perfusion media. *C*, 10 min after returning to control solution *A*.

is probably an underestimate of the true value because the processes of each neurone spread widely throughout the culture; a neurone which failed to elicit an e.p.s.p. in the tested neurone might have driven other neurones not tested.

Evidence that ACh was the major transmitter of these synapses. The spontaneous e.p.s.p.s disappeared in minutes after adding hexamethonium (5×10^{-5}–5×10^{-4} M) to the perfusion solution. In addition, in all seventeen cases tested, hexamethonium caused a significant reduction of the evoked e.p.s.p.s; in eight of these the reduction was complete (100 %), and in the remaining nine the reductions ranged from 62 to

96%. On the other hand, 10^{-6} M-atropine either had no effect on the e.p.s.p.s (four of six) or it slightly reduced the amplitude (6% in one case, 23% in the other). At concentrations sufficient to affect nicotinic receptors atropine did reduce the e.p.s.p.s; 10^{-4} M reduced e.p.s.p.s by 63–99% in four of four and 5×10^{-4} M caused 100% reduction in both of two e.p.s.p.s tested.

The evoked e.p.s.p. was also reduced in amplitude by the addition of other nicotinic-cholinergic antagonists: D-tubocuranine (5×10^{-5} M; 60–100% reduction), chorisondamine (5×10^{-5} M; 85–90% reduction), pempidine (10^{-5} M; 70–85% reduction). These results indicate that ACh is released at these synapses in culture and that most or all of the relevant post-synaptic receptors are similar to the conventional nicotinic cholinergic receptors of autonomic ganglia.

DISCUSSION

The objective of this paper was to study the growth and electrophysiological properties of nodose neurones from the new-born rat in cell culture.

To find cholinergic synapses between the neurones when cultured in the absence of neuronal cells was unexpected. No synapses have been reported in mammalian primary sensory nerves *in vivo* or *in vitro*, with the notable exception of the mesencephalic nucleus neurones (reviewed by Lieberman, 1976). The transmitters of adult mammalian nodose neurones are unknown. There is indirect evidence that some nodose neurones in the cat may release ACh (Matsumura & Kolle, 1961; Vera & Luco, 1967; Fujiwara, Kurahashi, Mizuno & Nakamura, 1978). In addition, immunohistochemical techniques have demonstrated substance P to be located in cell bodies of rat nodose neurones (Lundberg, Hökfelt, Nilsson, Terenius, Rehfeld, Elde & Said, 1978), and L-glutamate has also been raised as a possible transmitter for rat nodose neurones innervating baroreceptors (Talman, Perrone & Reis, 1980). In our experiments we have determined pharmacologically that ACh is released at synapses made between nodose neurones in culture. We could not judge using these electrophysiological techniques how many neurones released ACh, nor did we determine what other transmitters were synthesized and released by these neurones.

The post-synaptic receptors at these synapses had similar pharmacology to conventional ganglionic nicotinic receptors. It had previously been shown that some vagal afferent terminals had nicotinic receptors (Gray & Diamond, 1957; reviewed by Paintal, 1973). In addition, Armett & Ritchie (1961) showed that many unmyelinated fibres in the vagus nerve were sensitive to ACh; some of these axons presumably had their cell bodies in the nodose ganglion (Agostoni, Chinnock, Daly & Murray, 1957). Recently Higashi (1980) reported that many neurone cell bodies in the nodose ganglion of the rabbit were sensitive to ACh. In the accompanying paper (Baccaglini & Cooper, 1981) we report further experiments on the sensitivity of these cultured neurones to ACh and other transmitter substances.

To achieve long-term growth of these developing nodose neurones (4–6 weeks) in cell culture in the absence of non-neuronal cells, it was necessary to supplement the growth media with NGF; without it most neurones did not survive longer than 5–7 days. This finding is in agreement with Hedlund & Ebendal (1980) who showed that explant cultures of 10-day-old chick nodose ganglia require NGF for maintenance and

fibre outgrowth. However Lindsay (1979) reported that dissociated embryonic rat nodose neurones can survive in culture with astocytes for 6 days in the absence of NGF and presence of anti-sera to NGF. In addition Johnson, Gorin, Brandeis & Pearson (1980) reported that new-born guinea-pigs, exposed to antibodies directed against NGF from early fetal development, showed no change in nodose ganglion cell numbers, even though the superior cervical ganglion was dramatically reduced. We routinely used a 7S NGF preparation which contains many other components including NGF (Thoenen & Barde, 1980). It is conceivable that an unidentified component of the 7S NGF was necessary for the suvival of the neurones in our cultures.

As yet no information is available on the electrophysiological properties of developing nodose neurones *in vivo*. We routinely recorded resting potentials of 40–60 mV (occasionally some were as high as 80 mV). Similar values have been reported for adult neurones in nodose ganglia of the rabbit (Jaffe & Sampson, 1976) and the cat (Gallego & Eyzaguirre, 1978). Higher values were usually recorded in neurones more than 18 days in culture compared to neurones 10–14 days in culture; this may reflect the larger cell body size of the former, although a developmental change in ionic channels cannot be excluded.

The action potential amplitudes and shapes of cultured neurones were similar to those reported for adult nodose neurones *in vivo* (Jaffe & Sampson, 1976; Gallego & Eyzaguirre, 1978): an inflexion in the falling phase was pronounced in some neurones, whereas in others it was barely detectable. The inward current of the action potential of cultured neurones was carried by either Na^+ alone, or by significant amounts of both Na^+ and Ca^{2+}. Similar results were found for adult cat nodose neurones using a tissue slice preparation (Gallego & Eyzaguirre, 1978). In addition the action potentials of many cultured neurones were not abolished in the presence of TTX. TTX-resistant Na^+ channels have been described in some developing neurones (reviewed by Spitzer, 1979) as well as in adult mice dorsal root ganglion neurones (Yoshida, Matsuda & Samejima, 1978). Information is not yet available on the effects of TTX on adult nodose neurones.

Even though the neurones in this study originate from the nodose ganglion, we have no direct evidence that these neurones would have developed into sensory neurones had they been left *in vivo*. Conceivably they could either be displaced sympathetic or parasympathetic neurones present in the nodose ganglion at the time of culture. However, conventional parasympathetic neurones do not depend on NGF for their maintenance *in vivo* or *in vitro* (Levi-Montalcini & Angeletti, 1968; Helfand, Riopelle & Wessels, 1978). In addition, authentic sympathetic neurones cultured with muscle form abundant cholinergic synapses with each other (O'Lague, Macleish, Nurse, Claude, Furshpan & Potter, 1975; Nurse, 1977); nodose neurones in co-culture with muscle form few cholinergic synapses (Baccaglini & Cooper, 1981). Therefore, tentatively we conclude that the cholinergic and cholinoceptive nodose neurones in culture would have developed into sensory neurones if they had been left *in vivo*. Possibly these neurones altered a normal pattern of differentiation in response to environmental ones *in vitro*.

Expert assistance was provided in various aspects of this work by Delores Cox, Joseph Gagliardi, Sandra James and Doreen McDowell. We thank P. Walicke and A. Doupe for providing NGF.

Valuable advice was received from D. D. Potter and E. J. Furshpan. This work was supported by an American Heart Association Grant no. 78364 and in part by the Dysautonomia Foundation. P.I.B. was a post-doctoral Fellow supported by the Whitney Foundation. E.C. was a post-doctoral Fellow supported by the Muscular Dystrophy Association.

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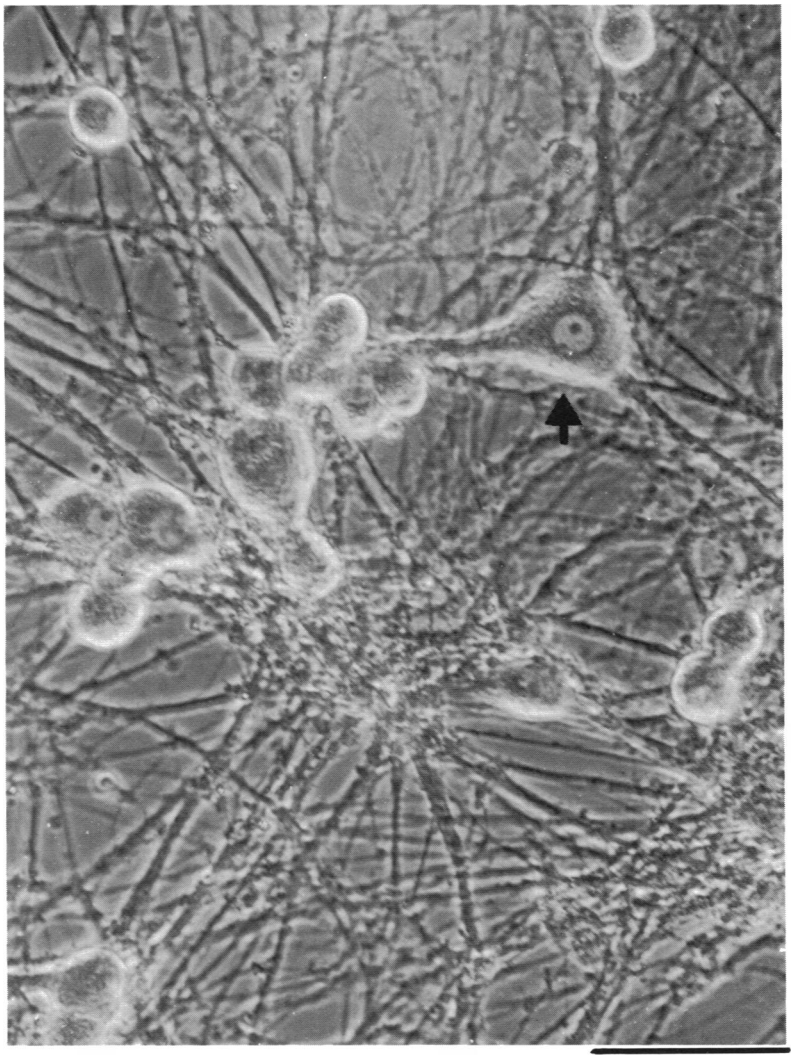

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EXPLANATION OF PLATE

Phase micrograph of neurones dissociated from the nodose ganglion of new-born rats; 3 weeks in culture. Note the virtual absence of non-neuronal cells. Most neurones have round cell bodies, but a few have a more flattened, triangular shape (arrow). The processes form a dense meshwork. Calibration bar is 50 μm.