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Synaptic Functions in Rat Sympathetic Neurons in Microcultures. I. Secretion of Norepinephrine and Acetylcholine

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This is the first of a series of four papers that describes the use of a sensitive "microculture" procedure for examining the neurotransmitter profile of a neuron by assaying the transmitter(s) it releases. Sympathetic principal neurons isolated from the superior cervical ganglia of neonatal or adult rats were grown for 10 d to several months on small islands of cardiac myocytes (island diameter, ca. 0.5 mm). To assay transmitter status a neuron and a myocyte in the same microculture were impaled with microelectrodes, the neuron was stimulated and the pharmacology of the effect(s) on the group of electrically coupled myocytes, and on the neuron itself, was investigated. Because the growing axonal processes were confined to the island, the innervation of the myocytes became dense; transmission from neuron to myocytes occurred reliably and was often intense. Most experiments were done on islands containing only a single neuron so that the observed effect(s) on the myocytes could be confidently assigned to that neuron. After the physiological assay, the fine structure or cytochemistry of the neuron was often examined. With single-neuron microcultures the physiology and anatomy of the neuron, including the fine structure of its synaptic endings and varicosities, could be correlated unambiguously.

During the course of this work, we have observed five pharmacologically distinct effects exerted on the myocytes by either neonate- or adult-derived neurons. Three of these effects, one exerted at least in large part by adenosine and the others by agents still under study (one appears to be 5-HT), are described by Furshpan et al. (1986), Matsumoto et al. (in press), and D. Sah and S. G. Matsumoto (unpublished observations). This paper is concerned with evidence for secretion by these neurons of norepinephrine (NE) and acetylcholine (ACh). The physiological effects of the secretion of these two substances onto the myocytes (excitation and inhibition, respectively) were generally similar to those reported *in vivo*. The minimal latencies of the responses were short, probably due to the high density of innervation. ACh secreted by a neuron onto itself, at autapses, evoked fast nicotinic EPSPs. We have not detected autaptic effects attributable to the secretion of NE. A minority of the neurons were detectably only adrenergic or only cholinergic. The incidence of these transmitter states was strongly dependent on culture age and culture conditions; in a heterogeneous group of about 300 reasonably well-characterized neurons about 17% (12% of neonate-derived) were apparently purely adrenergic and about 10% (13% of neonate-derived) were apparently purely cholinergic. The remainder secreted various combinations of transmitters as described in the other papers of this series (Furshpan et al., 1986; Matsumoto et al., in press; Potter et al., 1986).

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During the last decade, there has been considerable study of the development, in culture, of synaptic functions of sympathetic neurons dissociated from ganglia of the neonatal rat. One of the principal findings has been that the developing neurons are plastic with respect to expression of adrenergic and cholinergic properties. During the first few days after being placed in "mass cultures" that contain several thousand neurons, adrenergic properties are expressed under all growth conditions. In certain culture conditions the neurons continue to express adrenergic properties, but in other culture conditions (e.g., coculture with cardiac cells or growth in medium conditioned by such cells) cholinergic properties detected by biochemical, fine structural, and electrophysiological techniques develop over a period of several weeks; strong evidence was obtained that the development of cholinergic properties is not due to selection between two populations of neurons, each capable of expressing only a single transmitter status, but to a change in the status of neurons that would otherwise have remained adrenergic (for discussion and references, see Bunge et al., 1978; Landis, 1980; O'Lague et al., 1978c; Patterson, 1978). This induction of cholinergic properties is produced, at least in part, by release of a "conditioned-medium factor" (CM factor) from the cardiac or other nonneuronal cells (Fukada, 1980; Patterson and Chun, 1977a, b; Swerts et al., 1983; Weber, 1981); a similar activity is present in serum (Wolinsky and Patterson, 1985). The induction by CM factor can be largely blocked by elevation of K^+ in the medium from 5 to 20 mM, by the addition of veratridine, or by chronic electrical stimulation (Walicke et al., 1977). There is evidence for a similar transition during the development of the cholinergic sympathetic innervation of the plantar sweat glands of the rat *in vivo* (Landis, 1983; Landis and Keefe, 1983; Yodlowski et al., 1984) and for adrenergic/cholinergic plasticity during development of neural crest derivatives in the avian embryo (for discussion see Le Douarin et al., 1981).

The experiments described in this series of papers arose out of an interest in the transmitter status of individual sympathetic, neonate-derived neurons during the transition from adrenergic to cholinergic status. Methods were devised for growing the neurons singly, or in small numbers, in "microcultures" 0.3–0.5 mm in diameter (Furshpan et al., 1976; Landis, 1976; Reichardt and Patterson, 1977). To assay transmitter status phys-

iologically, the neurons are cocultured with cardiac myocytes and sometimes with another neuron. The synaptic effect(s) of neuronal impulses on the myocytes or on the neuron(s) are then determined. After the physiological assay, the microculture can be fixed for cytochemical or electron microscopical study; when only one neuron is present, a direct correlation of the function of the neuron with the fine structure of its synaptic endings and varicosities is readily made, a correlation difficult to achieve in intact mammalian tissues (but see McGuire et al., 1984). A method for dissociating viable principal neurons from sympathetic ganglia of adult rats was devised by Johnson (1978; see also Wakshull et al., 1979a, b); it has proved possible to study such adult-derived neurons in microcultures and to compare their transmitter functions with neonate-derived neurons.

During the course of examining a large number of microcultures, we became increasingly aware of the surprising complexity of transmitter status in these neurons, whether derived from neonatal or adult rats. In this first paper of the series, we describe (1) our current procedures for making and recording from microcultures; (2) the characteristics of the synaptic interactions mediated by the classical transmitters of sympathetic neurons, norepinephrine (NE) and acetycholine (ACh); and (3) observations on the fine structure of microcultures and correlations between structure and function. In the second paper (Potter et al., 1986), we describe the unconventional corelease by many of these neurons of NE and ACh, and we report direct evidence for the change in transmitter status by individual neurons from adrenergic to cholinergic. In the third paper (Furshpan et al., 1986), we present evidence for a neuronally evoked inhibition of the myocytes apparently caused primarily by adenosine (purinergic transmission). In a fourth paper (Matsumoto et al., in press) we describe nonadrenergic excitatory effects on the myocytes, one type of which is apparently due to secretion of 5-HT (see also D. Sah and S. G. Matsumoto, unpublished observations) and we summarize the remarkable diversity in transmitter status that arises from the expression of these several transmitters to varying extents and in varying combinations. Preliminary reports of some aspects of this work have appeared (Furshpan et al., 1976, 1982; Landis, 1976; Potter et al., 1980, 1981, 1983).

Materials and Methods

The experiments reported in this and the other three papers of this series were done with principal neurons, dissociated from the superior cervical ganglia of newborn or adult rats; the neurons were grown in microculture on islands of cells, dissociated from neonatal rat hearts. Many of the techniques for cell isolation, cell culture, and electrophysiological recording were identical to those previously reported for experiments on mass cultures (Bray, 1970, 1973; Mains and Patterson, 1973; O'Lague et al., 1978a); modifications of these procedures for the present work are emphasized here.

Culture techniques

To make microcultures small droplets of collagen solution were applied to a "nonwetting" coverslip. After the droplets had dried, dissociated heart cells were plated onto the coverslip. The heart cells adhered preferentially to the collagen dots to form separate circular islands (350–500 μm in diameter). Dissociated neurons were later plated at a low density so that some of the islands received only a single neuron. The details of the procedure are as follows.

Culture media

The composition of the dissecting medium (a bicarbonate-free medium based on Leibovitz's L-15), in which the hearts were minced and the ganglia mechanically dissociated, is given in Mains and Patterson (1973), where it is referred to as plating medium. However, the more recent source of the dry L-15 has been Flow Laboratories, and NGF was usually omitted.

The heart-plating medium, used for washing and plating the dissociated heart cells, was made by adding 10% (vol/vol) fetal bovine serum (Microbiological Associates) to the dissecting medium.

The L-15 CO_2 medium, in which the microcultures were routinely grown, was as given in Mains and Patterson (1973), except that we omitted methocel and BSA (and the source of the dry L-15 was Flow Laboratories). This medium differed from heart-plating medium in that it contained bicarbonate (26 mM), NGF (7S, 1 μg/ml), and "fresh vitamin mix" [per 100 ml: 10 μg 2-amino-4-hydroxy 6,7-dimethyltetrahydropterine ($DMPH_4$); 1 mg ascorbic acid; 100 μg glutathione). Rat serum (5% vol/vol) replaced the fetal bovine serum. The NGF concentration in the growth medium was approximately that found by Chun and Patterson (1977) to maximize survival of the neonate-derived neurons and the synthesis of protein, NE, and ACh.

"High-K^+" medium (see Walicke et al., 1977) was identical to L-15 CO_2 except that it contained 20 mM K^+ (the excess K^+ replaced Na^+).

Culture dishes

Plastic petri dishes (Falcon, 35 mm) were modified as follows. A round hole (10–12 mm in diameter) was cut in the center of each dish bottom and then covered by a plastic coverslip fixed to the outside of the dish with an inert silicone polymer (Sylgard, Dow Corning). The coverslip, thinner than the original dish bottom, improved microscopic visualization of the cells; it also formed the bottom of a shallow well that retained fluid over the cells during feeding. The coverslip had to be sufficiently hydrophobic to discourage direct adhesion of cells, yet hydrophilic enough to allow prolonged adherence of the collagen dots; it also had to be optically clear and nontoxic. During much of this work we used specially ordered polystyrene coverslips (Lux), referred to as "nonwetting" because they had not been treated to enhance wetting and adhesiveness for cultured cells. When these coverslips became unavailable, we tested a variety of other plastics and found several types from which usable coverslips could be cut: (1) polystyrene "planing slides" (Flow Laboratories; suggested to us by Dr. P. H. O'Lague); (2) Aclar (Allied Chemical Co.; see Masurovsky and Bunge, 1968); and (3) Petriperm (Hereus; supplied by Tekmar).

Success with "planing slides" was increased when the plastic was first washed with detergent (Haemo-Sol). Aclar appears to be more hydrophilic than "nonwetting" polystyrene but was usable when precautions were taken to reduce or reverse adherence of cells to areas of plastic not covered by collagen (see below). Petriperm is so thin and flexible that it often formed nonflat coverslips. It was usually supported by a stiffer coverslip cut from a planing slide. One surface of the Petriperm is too hydrophobic to allow proper adherence of the collagen dots; the other, like Aclar, is more hydrophilic than "nonwetting" polystyrene but was also usable.

Collagen dots

To guide placement of the collagen droplets and provide an address for each microculture, a grid of 25 squares (each 1.2 × 1.2 mm) was scratched on the outside surface of the coverslip (except when Petriperm was used) with a flat array of six needles soldered together. The collagen solution, prepared by a modification of the method of Bornstein (1958), was applied with a microelectrode (tip broken to 20–250 μm diameter) attached to a hypodermic syringe. One droplet (ca. 10–30 nl) was placed near the center of each grid square on the inner surface of the coverslip. The droplets were roughly hemispherical and usually 400–450 μm in diameter; the acetic acid solution evaporated in a few minutes, leaving insoluble dots of reconstituted collagen. The dishes were sterilized with UV light.

Cell preparations

Heart cells

Hearts taken from newborn rats were minced with fine scissors (into ca. 1 mm^3 bits) and enzymically dissociated at 37°C with collagenase [Worthington, Type CLS; 1 mg/ml in buffered Hank's balanced salt solution (pH ca. 7.4; $NaHCO_3$, 7.5 mM) without Ca^{2+} or Mg^{2+}]. Unless otherwise noted, both the atria and ventricles were dissociated. Often the proportion of atrial cells was increased by taking the atria from 10 hearts and the ventricles from 3; usually only the posterior one-half to two-thirds of each ventricle was taken to reduce nonmuscular tissue and blood vessels. Cells dissociated during an initial digestion (15 min) were discarded; cells harvested with the aid of trituration during and after a second digestion (20–30 min) were washed 2–3× in heart plating

medium to remove collagenase, irradiated with 4000–5000 rad at a dose rate of about 100 rad/sec from a ^{60}Co source, and washed once more. In some early platings only cells from a third digestion period (also 20–30 min) were harvested. When Aclar or Petriperm coverslips were to be used, the heart cells were preplated in Falcon tissue culture dishes (for 60 min) to remove the more rapidly adhering cells (mostly fibroblasts). Aliquots (0.3 ml) of the cell suspension (in heart plating medium) were plated into the dry central wells of culture dishes prepared as described above. The dishes were incubated, usually at 35°C (in an air atmosphere) and observed every 20–30 min. When adherent cells—those not dislodged by a gentle stream of medium from a pipette—had nearly covered the collagen dots but were still nearly absent from the bare plastic (20 min–2 hr), nonadhering cells were washed away and removed. The dishes were rinsed once with growth medium (L-15 CO_2 with 5% rat serum, without NGF or fresh vitamin mix) and then incubated in the same medium at 37°C until neurons were added (usually 1 d, but up to 14 d, later).

In cases in which more than a few cells adhered to the bare plastic, a common occurrence with Aclar and Petriperm coverslips, the off-island cells could usually be removed by a jet of culture medium too weak to dislodge many cells from the islands. We also found that cells failed to stay attached to the bare plastic for prolonged periods; in some cases, well-isolated islands were obtained by keeping heart-cell cultures for 1–2 weeks before neurons were added.

Within a day after they were plated, the heart cells flattened and, on most islands, formed a continuous monolayer covering the collagen dot. Myocytes could be recognized by their contractions; many of the non-contracting cells had the appearance of fibroblasts. During the first few days it was common for the myocytes to beat spontaneously and, within an island, synchronously. During the ensuing one to several weeks, many islands stopped beating spontaneously; however, the myocytes retained their ability to contract when stimulated electrically or chemically, and usually continued to react synchronously throughout the life of the culture. Both spontaneously beating and silent myocyte layers were useful for assaying neuronal transmitter release. However, when the myocytes beat irregularly, it was often difficult to distinguish incidental changes in frequency from weak synaptic effects. Such microcultures were usually abandoned; in a few cases, the beating was suppressed by a small steady hyperpolarizing current passed through the myocyte-recording electrode.

The useful lifetime of a microculture was usually limited by the stability of the myocyte layer. When the layer was initially sparse, it usually became progressively more sparse over a period of a few weeks; because innervated islands were often less stable than uninnervated ones, the neurons may have contributed to this loss of myocytes. When the myocyte layer was initially thick, it often balled-up; as neurons were not usually visible in the ball, such cultures were generally discarded. Microcultures usually suffered one of these fates in less than 2 months, although some microcultures remained stable for 4–5 months.

Neurons

Neurons were dissociated from the superior cervical ganglia of albino rats (Charles River CD), either newborn or adult (the adults were at least 8 weeks old and weighed 200–450 gm). Neurons obtained from neonatal ganglia will be called neonate-derived and those from adult ganglia, adult-derived. Ganglia taken from newborn rats were mechanically dissociated as described by Bray (1970). In addition, to break up clusters of neurons, the cell suspension was forcibly ejected 3× through a hypodermic needle (23 gauge, 2.5 cm long) onto the bottom of a plastic petri dish (Hawrot and Patterson, 1979). Ganglia from adult rats were dissociated with proteolytic enzymes by a modification of the method of Wakshull et al. (1979a). The ganglia were desheathed by dissection and then incubated (at 37°C) in collagenase (Worthington Type CLS, 1 μ/ml) in Hank's balanced salt solution (Ca^{2+} and Mg^{2+} free) for 1 hr without trituration. At the end of 1 hr, this enzyme solution was replaced with one containing the same concentration of collagenase with the addition of Dispase (Boehringer-Mannheim, 4 mg/ml). This suspension was triturated every 15 min using a 5 ml pipette until a microscopic examination of the cell suspension revealed that most of the ganglia had dissociated into single cells (usually 1–2 hr). The cells were then washed twice in growth medium and irradiated (5000 rad) as described above. The cell suspension obtained from six neonatal or eight adult ganglia was usually plated into the central wells (containing heart-cell islands) of 18–24 dishes. At this density some of the islands received no neurons, while some received one or several neurons. There were usually two to four single-neuron islands per culture dish. Before the neurons were plated, the heart-cell cultures were fed L-15 CO_2 (with NGF), and thereafter 2–3× per week.

Electrophysiological techniques

Perfusion system

Intracellular recordings from heart cells and neurons were made with the culture dish held on the stage of an inverted microscope. Sterile perfusion solution (control or drug-containing) flowed continuously through the central well of the culture dish; the temperature of the incurrent solution was kept near 36°C. The volume of fluid in the central well was about 0.1 ml; the rate of perfusion was usually 0.3–0.4 ml/min. Perfusion fluid containing drugs could be selected from several reservoirs with a multichannel valve without interruption of flow. Because of the dead space in the connecting tubing and the well, it took 1.5–2 min before the first effects of a drug solution appeared; maximal effects were usually observed within 5 min. Washout times, particularly for high concentrations of drugs, were often longer (5–30 min). The dish holder, perfusion system, and temperature-control arrangements were as previously described (O'Lague et al., 1978a) with the following alterations:

1. Perfusion fluid was delivered to the culture by a peristaltic pump (Holter; Extracorporeal) rather than by gravity.

2. During the course of these experiments, several variants of perfusion fluid were used. The one most frequently used was made by adding (per 100 ml): 10 ml basal L-15 CO_2 [83% basal L-15 (see Mains and Patterson, 1973) and 17% 150 mM $NaHCO_3$]; 9 ml 10× concentrate of Hank's balanced salt solution, without Ca^{2+} or Mg^{2+} (Gibco); choline chloride, 1 mg (72 μM); glucose, 600 mg (33 mM); glutamine, 29.2 mg (2 mM); penicillin, 20,000 U; streptomycin, 20 mg; calcium chloride, 311 mg (2.8 mM). In some cases, $NaHCO_3$ (1.6 ml of a 150 mM solution) was added. The final concentrations of major ions were (mM): Na^+, 140; K^+, 5.4; Ca^{2+}, 2.93; Mg^{2+}, 0.18; HCO_3, 2.6 or 5.0; H_2PO_4, 0.56).

3. Gases were not bubbled through the perfusion reservoirs.

Electrodes

Microelectrodes were made on a commercial puller (Sutter Instruments) from glass tubing (Haer; #30–31) and were filled with 3 M KCl. With suitable electrodes (resistance, 60–100 MΩ), antivibration mounting of the microscope and micromanipulators (e.g., Micro-G), and precautions to make the perfusion stream smooth and steady, intracellular recordings from neurons and myocytes were maintained routinely for several hours and, in favorable cases, for 8–10 hr. A grounded Ag:AgCl electrode was connected to the central well of the culture dish through a pool of 3 M KCl and an agar–saline salt bridge.

Drug application

Transmitter agonists were usually applied to the microculture locally in brief "puffs" ejected from a pipette (tip diameter, 5–10 μm) by pressure pulses controlled by a solenoid valve (Choi and Fischbach, 1981). Antagonists were usually applied in the perfusion stream.

Drug sources

ACh, atropine sulfate, norepinephrine bitartrate, adenosine, and reserpine were obtained from Sigma Chemical Co.; atenolol from Stuart/ICI; hexamethonium chloride from Pfaltz and Bauer; phentolamine (Regitine) and reserpine phosphate from Ciba-Geigy; DL-propranolol from Ayerst and Sigma; sotalol from Mead Johnson; 8-phenyltheophylline from Calbiochem; and alprenolol from Astra. It should be noted that atropine sulfate contains two atropine moieties.

Morphological techniques

At the end of a recording session, the addresses of the assayed microcultures in the 5 × 5 array were noted and a sketch or photograph made in order to identify the recorded cells for subsequent observation by electron microscopy.

Some cultures were fixed with 3% glutaraldehyde in 0.12 M phosphate buffer, pH 7.3, for 20 min at room temperature and either processed immediately or left overnight in fixative at 4°C. Cultures were rinsed with phosphate buffer, postfixed with 1.3% OsO_4 in phosphate buffer for 30 min, stained *en bloc* overnight with 1% uranyl acetate in 0.1 M acetate buffer, pH 5.1, dehydrated with ethanol, and embedded under vacuum in a thin wafer of Epon 812 directly on the coverslip. Other

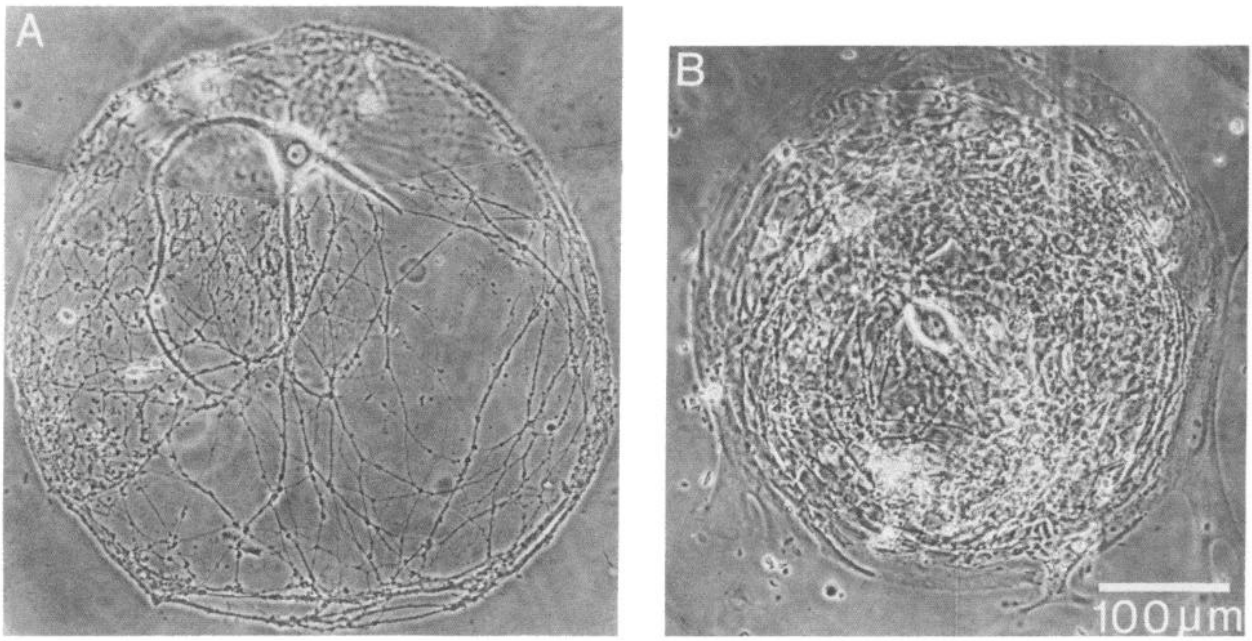


Figure 1. Phase-contrast micrographs of two microcultures: *A*, 40 d old; *B*, 25 d old. In the atypical microculture in *A*, few cardiac cells were plated initially and few, if any, of these survived; the neuritic tree can be well seen. Note the network of axon-like processes confined to the collagen island and the three thicker dendrite-like processes that arose from the cell body (at 4, 6, and 10 o'clock). The microculture in *B* was more typical, although the layer of heart cells was thicker than average; the neuronal cell body is visible in the center, but the neurites are largely obscured by the cardiac cells.

cultures were fixed with cold 3.5% potassium permanganate for 20 min (Richardson, 1966). During the course of these experiments, permanganate was used both with phosphate buffer (0.1 M, pH 7.3) and without (dissolved in distilled water). Our impression is that the unbuffered permanganate yielded better morphology than the buffered. After permanganate fixation, the cultures were thoroughly rinsed with cold 0.1 M acetate buffer, pH 5.1, stained overnight with 1% uranyl acetate in the same acetate buffer, rinsed again with the acetate buffer, dehydrated with ethanol, and vacuum-embedded in Epon 812.

A square of Epon containing the appropriate microculture was cut from the wafer and in most cases mounted with Araldite glue, culture side up, on a blank Epon block. The coverslip was removed either before or after mounting. Thin sections were cut parallel to the plane of the coverslip and mounted on either 1 × 2 mm Formvar-coated slot grids or on Gilder hexagon grids. In some cases, the coverslip was removed after embedding the culture and the culture surface covered with fresh resin; after polymerization, sections were cut perpendicular to the plane of the coverslip. In most cases, semiserial thin sections were then cut. In several cases, however, serial thin sections were cut and mounted in order on the slot grids. Three of these cells that had been sectioned perpendicular to their major processes were serially reconstructed. Every third section was examined for synapses, whose positions were recorded, and a montage of the cell was made from sections taken at 3 μm intervals. Two-dimensional drawings were made from the montage. Thin sections from aldehyde–osmium-fixed material were stained with lead citrate, while those from potassium permanganate-fixed material were examined without further staining.

In some cases, the cultures were preincubated with an exogenous catecholamine: 10 μM 5-hydroxydopamine (5-OHDA; Regis Chemical Co., Sigma) was dissolved in perfusion medium, and the culture was incubated for 30 min at 37°C before permanganate fixation.

The average proportion of small granular vesicles (SGV) was determined for each of a number of physiologically characterized neurons. Synapses and varicosities were photographed at 15,000×. The numbers of small clear synaptic vesicles (SCV) and SGV present in each profile were counted on prints at 40,000×, and the percentage of vesicles that contained granular precipitate was determined.

Results

Natural history of the microcultures

The neurons that initially adhered to heart-cell islands, whether derived from neonatal or adult ganglia, soon extended axonal processes (see below) that branched repeatedly and eventually formed an extensive arborization, so that no part of the island was far from an axonal process. Dendrite-like processes (see below) were apparent after 1 week to 10 d in culture. The microculture of Figure 1*A* was in a dish into which few viable heart cells had been plated, and most of these were eventually lost; in the absence of heart cells, the solitary, neonate-derived neuron and its processes can be seen clearly. Note that the neurites were confined to the collagen island, which is outlined by circumferential bundles of axons, and that the neuron's axonal processes appear to make contact with the thick dendrite-like processes that extend from the soma. In the more typical microculture of Figure 1*B*, the thick heart-cell layer obscures the neurites, but the neuronal cell body can be clearly seen. Observations made with the EM indicate that many strands in the axonal network (e.g., Fig. 1*A*) consisted of many (10–30) individual branches of the axon, each branch exhibiting numerous varicosities along its length (see Fig. 3).

The heart cells, in addition to being a source of the factor that induces cholinergic function in the neurons (Landis, 1980; O'Lague et al., 1978b; Patterson and Chun, 1974, 1977a, b), served as target cells to detect neurotransmitter released by the neurons. The assay was made by recording the membrane potential of a myocyte with an intracellular microelectrode. The release of neurotransmitter was evoked by electrical stimulation of the neuron through a microelectrode inserted into its cell body; the microelectrode also monitored the electrical activity of the neuron.

During the first week or so in culture, the cell bodies of neonate-derived neurons were small (10–15 μm diameter) and easily damaged by impalement. In addition, the synaptic effects on the myocytes produced by either neonate- or adult-derived neurons were generally weak, presumably because the axonal outgrowth onto the myocytes was still sparse; the chance that release of a transmitter would go undetected was correspondingly high. Because the growing axonal branches of the neurons were confined to the microculture, the innervation of the myocytes became progressively denser. By about 2 weeks, synaptic interaction between neurons and myocytes occurred reliably and was

often strong. Almost all the observations reported in this series of papers were made on microcultures older than 10 d. In several cases where synaptic interaction between the neuron and the myocytes was weak, ultrastructural examination of the island disclosed a sparse axonal arbor and relatively few axonal varicosities. We occasionally encountered neurons that produced no detectable synaptic effect on the myocytes. These neurons were not studied further, and it is not known whether they released inadequate amounts of neurotransmitter or whether they released transmitter molecules to which the myocytes were insensitive.

There was considerable variability in the number of cardiac cells per microculture among platings and even within the same plating. When the myocytes formed dense or multiple layers, impalements were usually made easily and were often maintained for hours; to reduce the probability that the microelectrode would be dislodged by contractions, impalements were made where movement was minimal. When the myocytes formed a sparse monolayer, impalements were more difficult, and it was usually necessary to re-impale the myocytes several times during an assay.

Because of the electrical coupling between myocytes (and between myocytes and fibroblasts), the myocytes usually beat in synchrony, and recordings from one myocyte were representative of the electrical activity of all the myocytes in this relatively small population. The similarity in the potential changes recorded from myocytes at two widely separated points in a microculture, when currents were passed through a microelectrode in a third myocyte, is shown in Figure 2. The two recording microelectrodes were on opposite sides of the microculture and the third microelectrode, for passing current pulses, was close to one of the recording electrodes (ca. 75 μm away). The electrical responses at the two recording sites were identical within about 5%. In all other experiments illustrated in this series of papers, only a single recording electrode was put into the myocytes (although during the course of an experiment this electrode might be moved from one point to another, as reimpalements were required). One of the important advantages of the microculture procedure is the summing of the synaptic effects at many release sites; the sensitivity for detection of weak synaptic effects is correspondingly enhanced.

The proportion of atrial to ventricular cells on an island varied among platings, and in most cases we did not know the relative contribution to the recordings of responses of the two types of cells. The durations of the myocyte action potentials at half-amplitude varied widely but were usually 60–200 msec (Fig. 2). The recorded amplitudes of the cardiac action potentials were usually 100–120 mV. The resting potentials (usually 50–60 mV) and action potentials (80–110 mV) of both the neonate- and adult-derived neurons were similar to those we reported for neonate-derived neurons in mass culture (O'Lague et al., 1978a, b).

Fine-structure of the neurons in microculture

Neonate-derived neurons in microculture, as viewed by phase microscopy, frequently possessed one to six thick dendrite-like processes (Figs. 1, 4) that closely resembled those revealed by silver stains of neonatal human sympathetic ganglia (de Castro, 1932). The processes tapered from approximately 5 μm near the soma to 2 μm or less near the tip. They sometimes branched and they often appeared to end in a blunt tip or in finger-like extensions. In order to characterize the processes more extensively, three neonate-derived neurons, each in a single-neuron microculture, were serially thin-sectioned in the plane perpendicular to the collagen substrate. Care was taken to orient the neuron so that the majority of the processes arising from the soma would be cut in cross section and, therefore, easier to identify and trace. Major processes were traced until they either ended or acquired distinctly axonal properties.

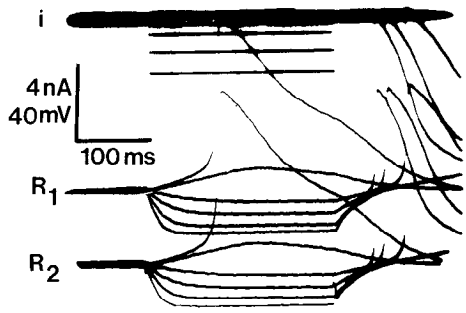


Figure 2. The uniformity of electrical activity in the cardiac myocytes of a microculture (ca. 600 μm in diameter; no neuron was present). Three microelectrodes impaled myocytes; two, at opposite sides of the island (550 μm apart), recorded the electrical responses (R_1, R_2) to current pulses (i) passed by the third placed about 75 μm from recording electrode R_1. The responses evoked by two depolarizing and four hyperpolarizing pulses are superimposed; only the three largest of the latter are clearly visible on the current-monitor traces. The corresponding responses at R_1 and R_2 are almost identical. (See further description in text.)

Based on ultrastructural properties, the neurons possessed two clearly distinct classes of processes and possibly a third. One was dendrite-like, extended from the cell body, and characteristically tapered. These processes were always postsynaptic. Near the cell body, they contained abundant rough endoplasmic reticulum, free polysomes, numerous mitochondria, and smooth endoplasmic reticulum; further from the cell body, only smooth endoplasmic reticulum and mitochondria were present (Fig. 4*c*). Typically, there were many neurofilaments organized into a central core or divided among several smaller bundles. The number of these processes was smaller than the mean number of primary dendrites (8.5) reported for principal neurons in the superior cervical ganglion of adult rats (Purves and Lichtman, 1985). The second distinct class of processes was the distal axon. These contained no rough endoplasmic reticulum or polysomes, but had some smooth endoplasmic reticulum and mitochondria. Their most striking characteristics were the preponderance of microtubules, the paucity of neurofilaments, and the presence of varicosities that contained aggregates of synaptic vesicles (Fig. 3). At synaptic junctions, distal axonal processes were always presynaptic. No specialized axoaxonic contacts were observed even in the dense plexuses formed by the distal axons (Fig. 3). A third class of processes has been identified as proximal axon. These processes were thicker than distal axons and contained more smooth endoplasmic reticulum and a greater number of mitochondria and neurofilaments. They arose from either the cell body or the distal regions of dendrites. In the reconstructions we were able to identify these structures with reasonable confidence, since they gave rise to distal axons and did not appear to participate in synaptic junctions as either the pre- or postsynaptic element. It is less clear that they can be reliably identified in random thin sections.

The range of morphologies exhibited by these neurons is indicated by the reconstructions in Figures 4 and 5. The neuron illustrated in Figure 5*a* had a simple geometry. A single thick process (proximal axon) extended from the cell body and gradually tapered to give rise to the distal axon. In contrast, the neuron of Figure 5*b* extended three thick, tapering dendrite-like processes and a fourth thin process that traveled approximately 50 μm from the cell body, reversed direction, and then bifurcated. The third cell (Fig. 4) possessed the most complicated geometry. Four major dendrites, three of which branched at least once, arose from the cell soma and ended at some distance from the cell body either bluntly, in a tuft of fine neurofilament-filled processes, or in fine finger-like extensions. A fifth, dendrite-like process extended from the cell body and gave rise to the

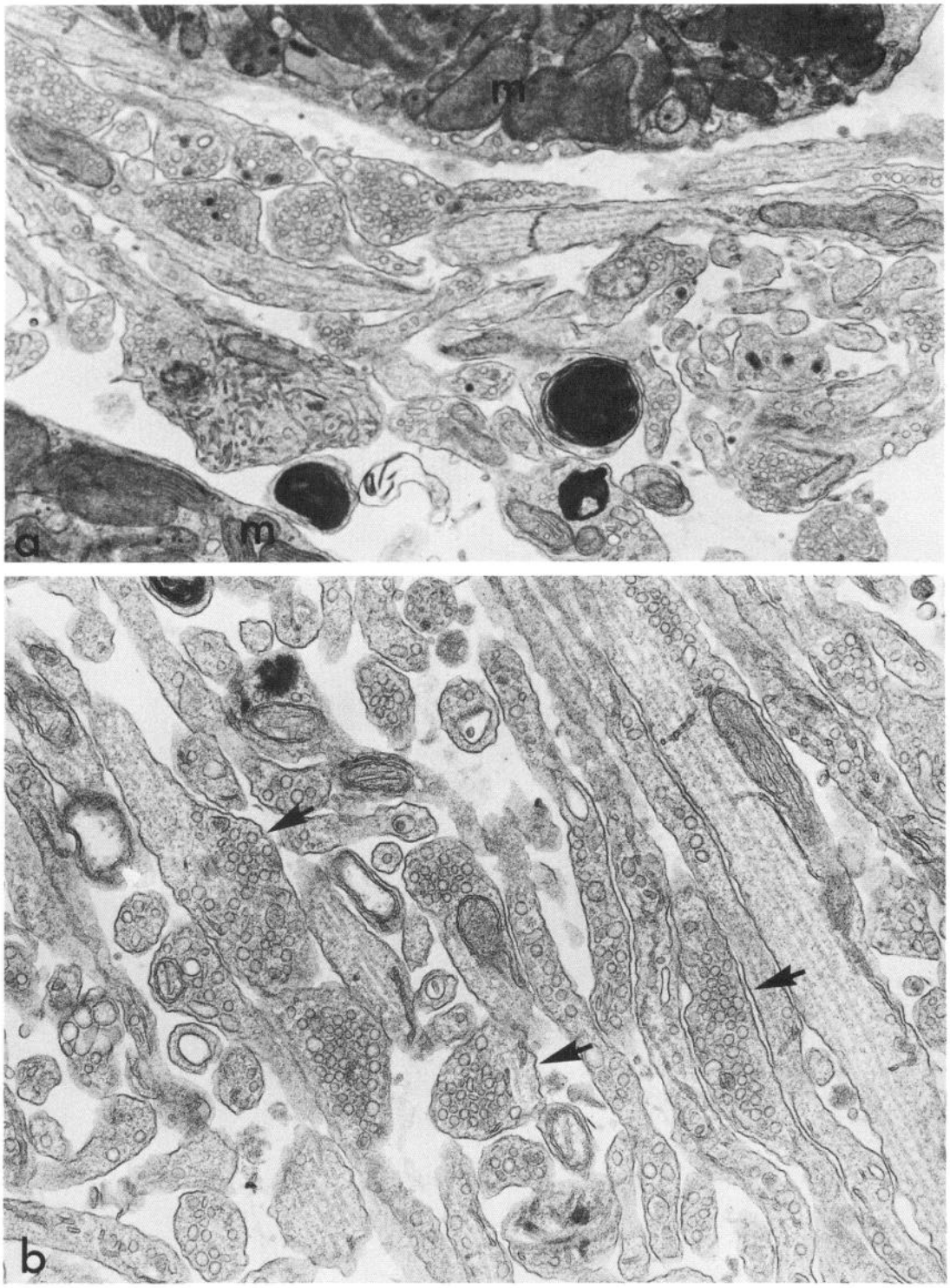
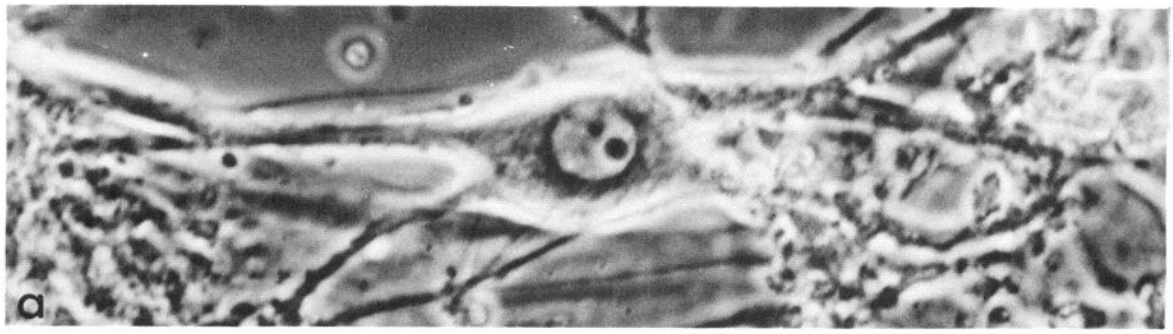
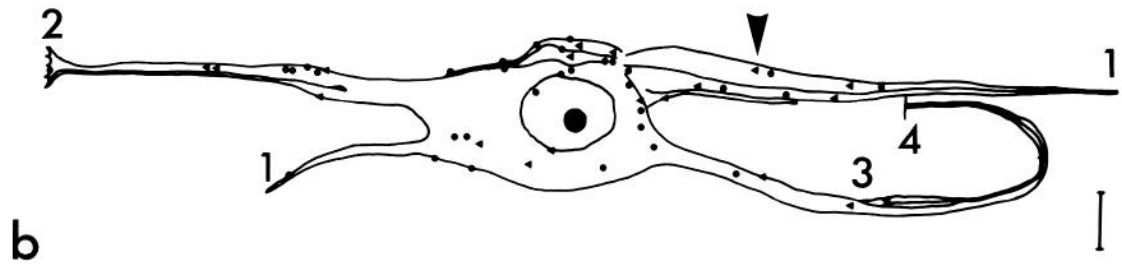


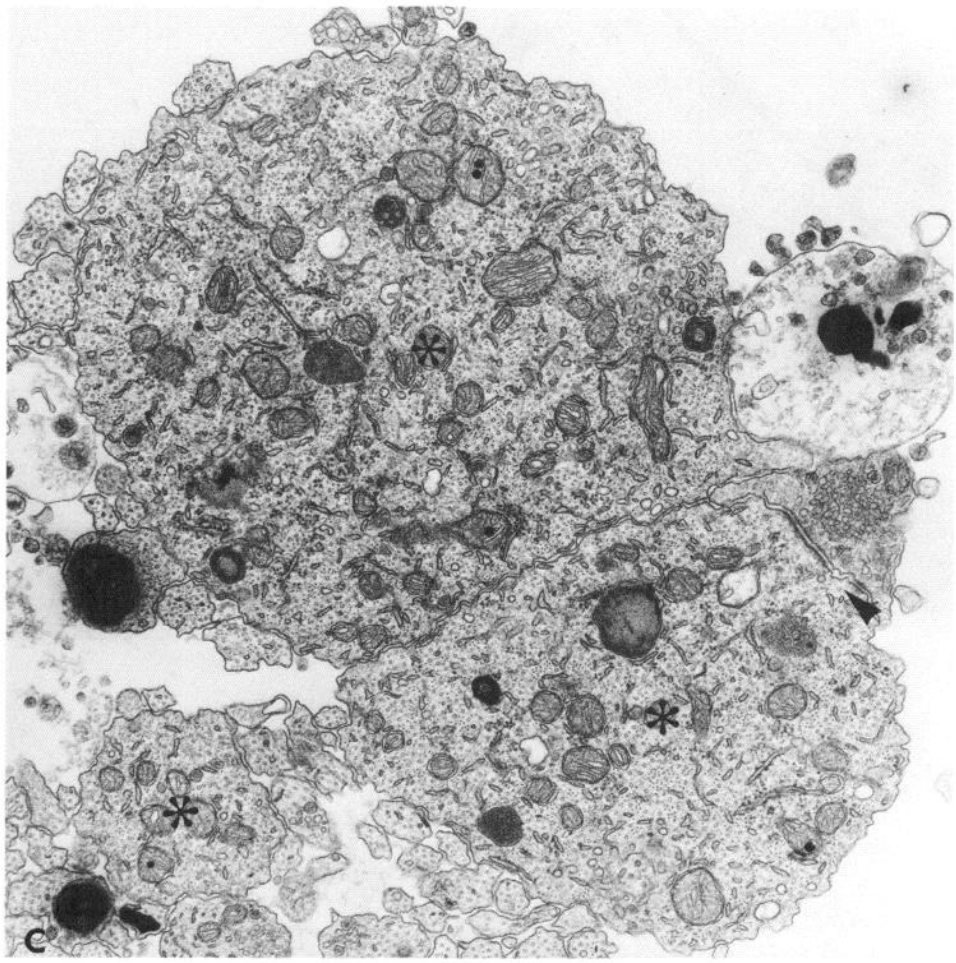
Figure 3. Varicosities in bundles of axon branches in a single-neuron microculture (neuron 110 d in culture). *a,* Bundle of axonal processes courses between two myocytes (*m*). The varicosities, or vesicle-containing profiles, lie as close as 0.2 μm from a myocyte, although most are more distant. ×20,000; aldehyde–osmium fixation. *b,* Axonal bundles also lie above the myocytes. These bundles contain tens of axon branches, each of which has numerous varicosities (*arrows*) along its length. Nine such varicosities, intersected by the plane of section at various angles, are evident in this field, which shows only a miniscule fraction of the whole axonal arbor. ×35,000.

→

Figure 4. Phase-contrast micrograph (*a*), reconstruction (*b*), and sample electron micrograph (*c*) from a solitary neuron 21 d in microculture. The electron micrograph is from the level marked with an *arrowhead* in the drawing (*b*); the neuron was sectioned from the left of the reconstruction to the right. The dendrites and soma received synapses from distal segments of the axon. Synapses on the upper surface are indicated by *filled*







circles; on the lower surface by *filled triangles*. Calibration bar, 10 μm. This neuron had a relatively complex shape. Four major dendrites, three of which branched at least once, arose from the cell soma and ended either bluntly (*b, 1*) or in a tuft of fine neurofilament-filled finger-like extensions (*b, 2*). The proximal axon arose from a dendrite-like process (*b, 3*) which branched and joined a bundle of axons as it became more axon-like (*b, 4*). In *c*, the culture substrate is to the *right*. Three dendritic processes are present (*asterisks*). Approximately 40 axon branches run along these dendrites. One synapse is marked by an *arrowhead*. The smallest caliber dendrite did not contain endoplasmic reticulum or polysomes. These dendrites were typical in that they contained more neurofilaments than microtubules but were atypical in that the neurofilaments were not segregated into a central bundle. Distal axonal segments such as those in *c* were similar to such segments in other neurons. ×17,100.

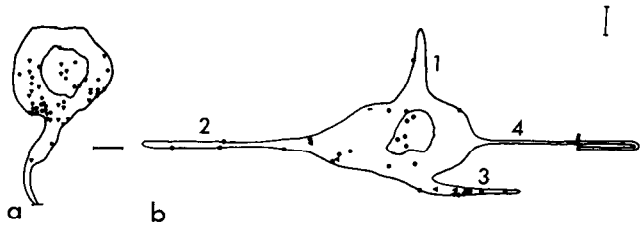


Figure 5. *a,* This reconstruction shows a relatively simple neuron. A single process extends from the rounded cell body, receives two synapses, and then acquires the properties of a proximal axon. Bar, 10 μm. *Filled circles* indicate synapses formed on the upper surface; *filled triangles,* synapses on the lower surface. *b,* This neuron possessed a more complicated geometry. Its dendrites and axon exhibited distinctly different properties throughout their lengths. The dendrites (*1, 2,* and *3*) each contained a central core of neurofilaments surrounded by microtubules in electron-dense cytoplasm. Distal axonal segments traveling with the dendrites contained predominantly microtubules with occasional neurofilaments in the electron-lucent cytoplasm. The proximal axon (*4*) arose directly from the cell soma and resembled the distal segment of the axon in its complement of organelles.

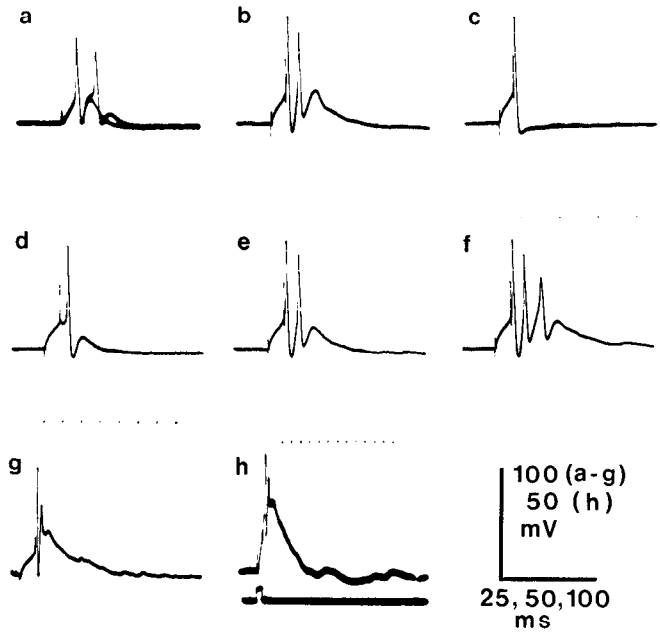


Figure 6. Autaptic EPSPs in neonate-derived neurons and their block by hexamethonium. Record *a* was from a solitary neuron 42 d in microculture; records *b–f,* from another solitary neuron also 42 d in microculture; records *g* and *h,* from two other solitary neurons 46 and 13 d in microculture. *c,* In the presence of 1 mM hexamethonium chloride, the autaptic responses were blocked. *d–f,* During washout of the drug, the responses become progressively more complex as the EPSP, which was initially subthreshold (*d*), exceeded threshold; the neuron then re-excited itself once (*e*) or twice (*f*). Records *g* and *h* show small delayed EPSPs that occurred for prolonged periods after the stimulus (for more than 150 msec in *h*). Time calibration, 25 msec for *a–f*; 50 msec for *g*; 100 msec for *h*.

proximal axon, which branched and joined a bundle of axons as it became more axon-like in its appearance. Under these culture conditions, neuronal processes, both axonal and dendritic, tended to bundle together.

Each of the reconstructed neurons formed numerous morphologically specialized synapses on its own cell body and dendrites (autapses; e.g., Fig. 4*c*). There were 52, 32, and 54 autapses on the neurons of Figures 4 and 5*a, b*. The appearances of the pre- and postsynaptic membrane specializations are identical to those described in mass cultures (Buckley and Landis, 1983a; Rees and Bunge, 1974). These synapses were formed with approximately equal frequency on the under and upper sides of the cell soma and dendrites; they covered only a small fraction of the surface. Numerous axonal branches, which coursed along the cell body and dendrites, displayed many vesicle-filled varicosities, most of which did not form morphologically specialized contacts. The proportion of varicosities that participated in synapses (<1%) appears to be similar to that observed in mass cultures (Buckley and Landis, 1983a). There are numerous reports that mammalian sympathetic principal neurons form direct contacts and synapses with each other *in vivo* (e.g., Elfvin, 1963, 1971; Grillo, 1965; Kondo et al., 1980; Matthews, 1974; Tamarind and Quilliam, 1971; Yokota and Yamauchi, 1974). However, we know of no clear evidence for autapses on these neurons *in vivo*; presumably, confinement of the growing neurites to the microculture favored formation of autapses.

Close synaptic contacts were only rarely observed on the myocytes; in fact, the varicosities were seldom closer to the myocytes than about 0.2 μm, whether the axonal bundles coursed within the myocyte layer (Fig. 3*a*), or, as was more common, directly above it (Fig. 3*b*). It is likely that all varicosities in microcultures, both synaptic and nonsynaptic, were able to release neurotransmitter, based on the evidence obtained by Buckley and Landis (1983b) for similar varicosities in mass cultures.

Synaptic transmission at autapses

Observations on neonate-derived neurons will be considered first. As described above and previously (Landis, 1976), when single-neuron microcultures were examined with the EM, synapses were almost always observed (on the neuronal cell body and dendrites). Neonate-derived neurons identified as cholinergic from their effect on the cocultured cardiac myocytes (see below) usually produced a cholinergic excitatory effect on themselves as well (see Furshpan et al., 1976). In the example shown in Figure 6*a*, the solitary neuron was stimulated with a pulse of current delivered through the intracellular recording electrode in its cell body. The directly evoked action potential was followed by an EPSP that, in turn, gave rise to another action potential; on a second superimposed sweep, the first EPSP failed to re-excite the neuron. These autaptic EPSPs were blocked by hexamethonium, as were the previously studied EPSPs evoked by cholinergic neurons in their neighbors in mass culture (O'Lague et al., 1978c). The sequence of records shown in Figure 6, *b–f,* was taken from another neuron before (*b*) and during (*c*) exposure to 0.5 mM hexamethonium chloride and during washout of the drug (*d–f*). We have not observed more than two impulses following the directly evoked one in any of our experiments; that is, the third EPSP in the sequence of self-excitation was always below threshold. The large compound response (EPSPs plus action potentials) was often followed by intermittent smaller EPSPs for over 100 msec (Fig. 6, *g* and *h*); these were probably produced by single quanta of ACh (delayed release), but we have not excluded the possibility of delayed impulses partially propagated in the complex axonal tree.

In a few cases, neonate-derived neurons that produced cholinergic responses in the cardiac myocytes failed to generate autaptic EPSPs. The presence of such EPSPs was therefore a sufficient, but not a necessary, indication that a neuron had (at least) cholinergic function. We did not study any of these cases further to determine whether these neurons were sensitive to ACh or whether autapses could be observed by electron microscopy.

Some neurons did not produce a detectable cholinergic effect either on themselves or on the cardiac myocytes. Almost all of these apparently noncholinergic neurons produced at least one

other synaptic effect on the myocytes (adrenergic, purinergic, or nonadrenergic excitatory, as described below and in the other papers of this series). Even when the noncholinergic effects on the myocytes were pronounced, the neurons did not produce detectable autaptic potential changes in response to one or a few stimuli. In six such cases, neonate-derived neurons were examined with the electron microscope and were found to have synapses on their own cell bodies and dendrites. The lack of postsynaptic potential change at such noncholinergic autapses is considered in the Discussion.

In contrast to the high incidence of autaptic EPSPs in neonate-derived cholinergic neurons, adult-derived cholinergic neurons only infrequently produced autaptic EPSPs (one of these cases is illustrated in Fig. 8*g*, discussed below) or EPSPs in neighboring neurons in multineuron microcultures. Observations with the EM of the cell bodies and dendrites of adult-derived neurons in several microcultures revealed the presence of some autapses. The incidence of autapses in randomly selected thin sections appeared to be significantly lower than in comparable sections of neonate-derived neurons, but examination of serial thin sections of an entire adult-derived neuron, to determine the total number of its autapses, was not made. The infrequent occurrence of autaptic EPSPs in the adult-derived neurons may have been due to several factors, including a lower incidence of synapses and a lower level of cholinergic induction.

Cholinergic and adrenergic responses of the cardiac myocytes

In this section, we describe two of the pharmacologically distinct responses of the myocytes evoked by activity in the neonate- and adult-derived neurons: an inhibitory response attributable to secretion of ACh and an excitatory response attributable to NE. In this paper, for simplicity, cholinergic and adrenergic effects on the myocytes are illustrated by cases in which the neuron detectably secreted only ACh or NE, although most neurons secreted more than one transmitter, as described in the later papers of this series.

Cholinergic inhibition of the myocytes

The release of ACh by a solitary neuron in microculture was signaled not only by the hexamethonium-sensitive autaptic EPSPs, but also by an atropine-sensitive inhibition of the myocytes. The myocyte response was the more sensitive and reliable indicator of cholinergic function, as it was evoked by some neurons that failed to generate autaptic EPSPs.

When the myocytes were beating spontaneously, stimulation of a cholinergic neuron caused a decrease in frequency or, more usually, an interruption in the train of myocyte action potentials (and contractions), often accompanied by a conspicuous hyperpolarization. In microcultures in which the myocytes were quiescent, only the hyperpolarization was observed in most cases. In some microcultures, in which the neuron appeared to be only cholinergic, the myocyte hyperpolarization was followed by a weak excitation (depolarization and/or beating); this may have been due to activation of voltage-sensitive channels ("rebound excitation") or to the local accumulation of K^+. In Figure 7, *a–c*, each vertical stroke in the myocyte recordings (upper traces) is an action potential; in *a* the myocytes were initially beating at about 1 Hz. Stimulation of the neuron caused a small hyperpolarization and cessation of beating for almost the entire period of stimulation (ca. 20 sec). The block of this inhibition by atropine is shown in *b*, and the reversal of the block, after atropine was washed out, in *c*. This inhibition of beating and its block by atropine resemble the responses of intact myocardium to stimulation of the vagus nerve (e.g., Hutter and Trautwein, 1956). An example of the response of nonbeating myocytes, in another microculture, to stimulation of a solitary cholinergic neuron is shown in Figure 7*d*; the reversible block of the response by atropine (1 μM) is shown in Figure 7, *e* and *f*. An autaptic EPSP (Fig. 7*i*) provided additional evidence for cholinergic function in this neuron.

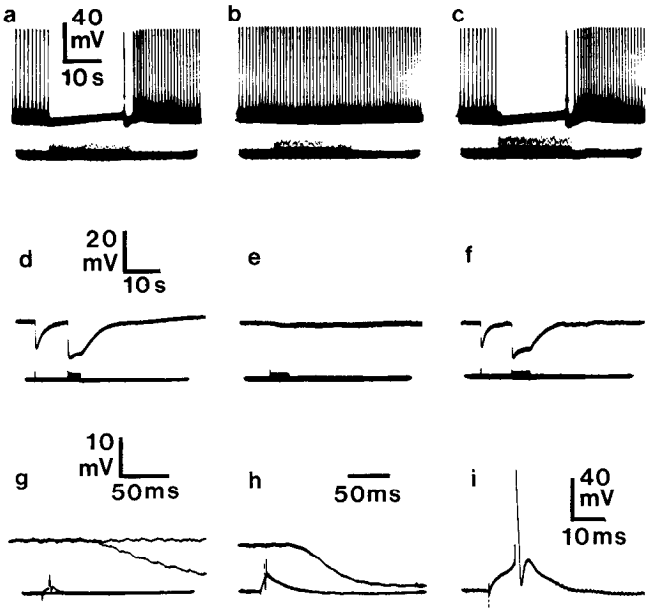


Figure 7. Cholinergic effects evoked by solitary neonate-derived neurons in three different microcultures. In *a–h*, as in almost all of the electrophysiological records in succeeding figures, myocyte activity recorded with an intracellular microelectrode is shown on the *upper trace* of each pair; neuronal activity, recorded with a second intracellular electrode, is shown on the *lower trace* at low gain. Records in *a–c* were taken from a microculture in which the neuron was 42 d *in vitro*. *a*, Train of neuronal impulses at 3.3 Hz (*lower trace*) produced an inhibition of the spontaneous impulse activity of the myocytes (*upper trace*) and of their contractions (not shown). *b*, Inhibition was blocked by the addition of 0.5 μM atropine sulfate to the perfusion fluid. *c*, After washout of the atropine, the inhibition was restored. Records in *d–f*, *g*, and *i* were taken from another microculture (neuron 46 d *in vitro*). *d*, First myocyte response (ca. 12 mV) was evoked by a single neuronal impulse (retouched), and the second myocyte response (ca. 17 mV) by a train of neuronal impulses (16 Hz for 3.5 sec). *e*, Inhibition evoked by a train of impulses (16 Hz for 5 sec) was blocked in the presence of 1 μM atropine sulfate. *f*, After washout of the atropine, the myocyte responses were restored. The autaptic EPSP recorded in this neuron is shown in *i*. *g* and *h*, Myocyte responses to single neuronal stimuli are shown on an expanded time base: *g*, sweeps with and without a neuronal impulse superimposed (same microculture as in *d–f*); *h*, neuron re-excited itself at autapses and fired twice (neuron 110 d *in vitro*). *Voltage calibrations* in *a*, *d*, and *g* apply only to the myocyte traces in *a–h*; these are 40 mV for *a–c*, 20 mV for *d–f* and *h*, and 10 mV for *g*. Time calibrations in *a* and *d* apply to *a–f*.

In many neurons exhibiting cholinergic function, especially in microcultures more than 4–5 weeks old, even a single neuronal impulse caused intense inhibition of the myocytes; the youngest neuron that produced such an effect was 11 d in culture. In the trial shown in Figure 7*d*, the myocytes were hyperpolarized by about 12 mV by a single neuronal impulse and by about 17 mV by a brief train of neuronal impulses. The intensity of such responses is presumably a reflection of the dense innervation of the myocyte layer and the large number of release sites formed by the neuron.

A similar sequence from an experiment on an adult-derived neuron is shown in Figure 8, *a–c*. This was one of the few adult neurons that displayed an autaptic effect (*g*), and it was the only one that produced only a cholinergic (atropine-sensitive) effect on the myocytes. Figure 8*a* shows the marked hyperpolarization of the myocytes produced by a train of neuronal impulses; repolarization was accompanied by a cardiac impulse, apparently as an aftereffect of the hyperpolarization. A longer train of neu-

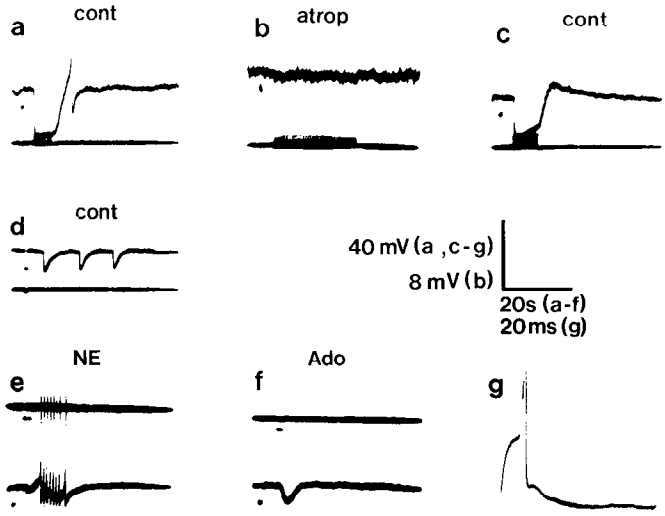


Figure 8. Effects evoked by a solitary adult-derived neuron (56 d *in vitro*; taken from a 350 gm rat) and by puffs of NE and of adenosine. *a,* Train of neuronal impulses (20 Hz for 5 sec; *lower trace*) produced a hyperpolarization of the myocytes of about 26 mV (*upper trace*) followed by a myocyte impulse. *b,* Response was abolished by perfusion with 0.2 μM atropine sulfate (note 5-fold increase in voltage gain) and was restored (*c*) after washout of the atropine. The myocyte depolarization that followed the hyperpolarization in *a* and *c* might have been due to changes in the transmembrane K^+ gradient or to effects on voltage-sensitive membrane channels. *d,* Three single stimuli to the neuron (at about 10 sec intervals) produced marked hyperpolarizations (ca. 10 mV). *e* and *f,* Myocyte responses were recorded (*lower traces*) to two puffs (500 msec duration) of 10 μM NE (*e*) or 10 μM adenosine (*f*) (timing of puffs signaled by *downward deflections of upper traces*). *g,* An impulse in the neuronal cell body was followed by an autaptic EPSP. In *a–f* the first downward deflections of the myocyte traces were calibration pulses (10 mV for *a* and *c–f*; 2 mV for *b*).

ronal impulses in the presence of atropine (*b*) produced no consistent effect on the myocytes; washout of the atropine restored the initial sensitivity to neuronal activity (*c*). Most adult-derived neurons, like neonate-derived neurons, secreted more than one transmitter. In this experiment, to check whether the absence of a myocyte response to neuronal activity in the presence of atropine (*b*) was due to insensitivity of the myocytes to NE or adenosine, these agents were puffed onto the myocytes (see Materials and Methods); the excitatory response to 10 μM NE (*e*) and the inhibitory response to 10 μM adenosine (*f*) indicate that the absence of any myocyte response to neuronal stimulation in *b* was not due to insensitivity to these agents but to a lack of their secretion in significant amounts.

Figures 7, *d, f,* and *g,* and Figure 8*d* show cases in which single neuronal impulses resulted in conspicuous hyperpolarization of the myocytes (in Fig. 7*h* the myocyte response was evoked by a single stimulus, but because of its autapses, the neuron fired again after an interval of about 3 msec). In a few experiments, these especially intense neuronal effects were recorded at faster sweep speed to observe the latency of the muscarinic effect, for comparison with the latency of the fastest adrenergic responses (see below). Figure 7, *g* and *h,* shows two such observations; the latencies of the myocyte responses were about 50 and 35 msec, with uncertainty due to the gradual onset of the responses. In three other experiments (not illustrated), the latencies were also about 35 msec; this was the shortest we encountered. In seven other experiments, the latencies were 45–60 msec, and in several further experiments, the latencies were up to 100 msec. These estimates include the unknown conduction time for the impulse(s) to reach a sufficient number of axonal release sites to produce the earliest visible response (see Discussion).

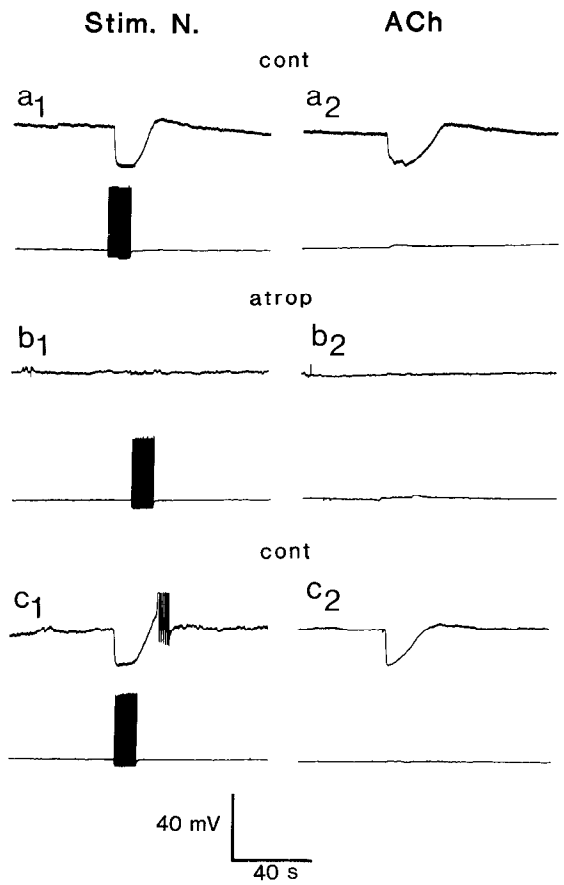


Figure 9. Comparison of the responses of the myocytes to activity in an apparently purely cholinergic neuron and to puffs of ACh. The neonate-derived solitary neuron was about 40 d in microculture. In the left-hand column (a_1–c_1) trains of neuronal impulses (20 Hz for about 10 sec; *lower traces*) evoked large hyperpolarizations (20–25 mV) of the myocytes followed by smaller depolarizations (*upper traces*) in control solution (*cont*). The amplitudes of the neuronal action potentials were not faithfully recorded by the chart recorder. In the right-hand columns (a_2–c_2), similar myocyte responses were evoked by brief puffs of ACh (2 μM; time of application indicated by *dots* above the myocyte traces). Both the neuron-induced and ACh-induced responses were abolished by atropine sulfate (0.2 μM; b_1, b_2). After washout of the atropine (c_1, c_2), the neuronal effects were restored; the after-depolarization produced by the neuronally evoked hyperpolarization (c_1) now led to a brief train of cardiac impulses. Voltage calibration applies only to the myocyte responses.

The rise times of the several fastest cholinergic responses, measured from the first apparent deviation from the baseline to the peak of the response, were in the range 150–250 msec; the durations of these responses to single stimuli, measured at half-amplitude, were about 1.5–2 sec.

We used relatively high concentrations of atropine (0.1–1 μM) in most experiments to reduce cholinergic responses to minimal levels and increase the chance of detecting the underlying effects of other transmitters. However, in two experiments we used atropine at low concentration (1 nM) and observed partial block of neuronally evoked myocyte inhibition. In one of these experiments, a more complete dose–response relationship was determined. The solitary neuron in this case was strongly cholinergic; in response to a single stimulus it evoked a large hyperpolarization (11 mV) in the myocytes. This response was reduced in amplitude by 20% with 1 nM, by 55% with 10 nM, and by 93% with 0.1 μM atropine. In many other experiments, the cholinergic inhibition appeared to be abolished by 0.1 μM atropine (e.g., Fig. 7, *d–f*). More intense cholinergic responses

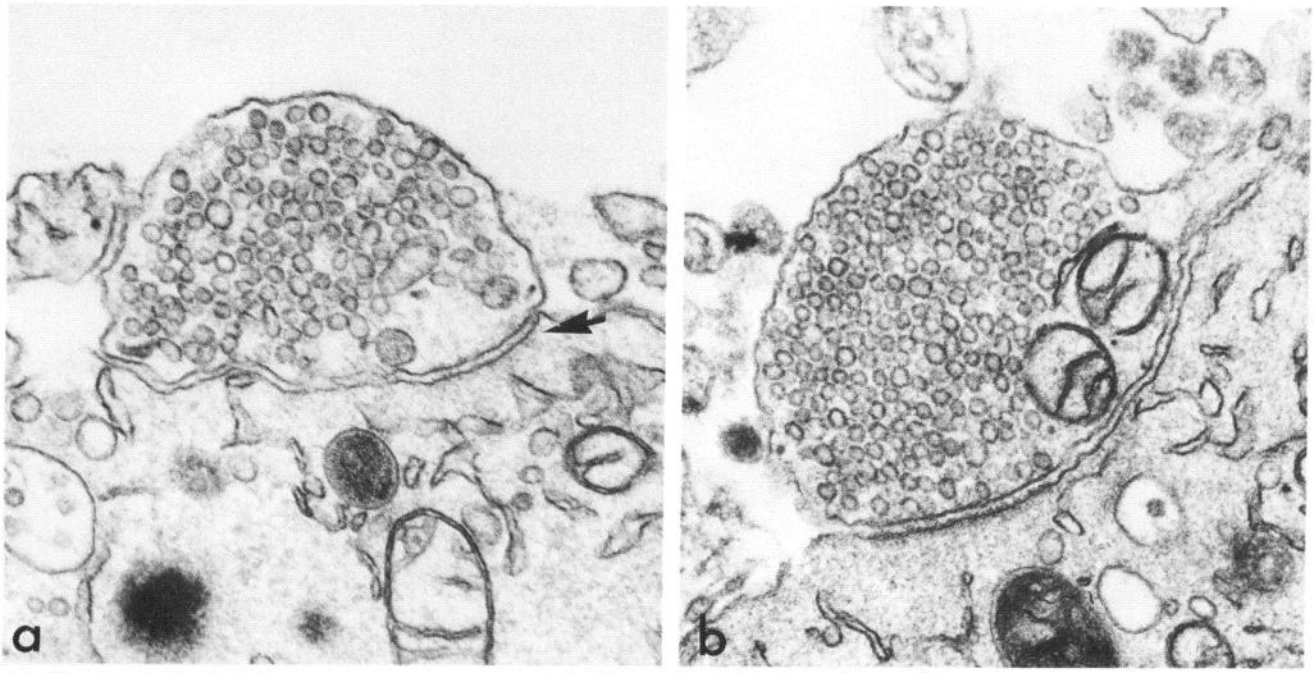


Figure 10. Terminals of cholinergic neurons; permanganate fixation. *a,* Varicosity found in apposition to a neuronal cell body in a microculture containing two cholinergic neurons. No SGV are detectable. *Arrow* indicates site of membrane specialization. Neurons 19 d in culture. ×44,000. *b,* Autapse in a microculture containing two neurons, one cholinergic, the other dual-function and strongly adrenergic. No SGV are present in this synaptic bouton or in many of the others sampled from this microculture; the remainder (not shown) contained on average 38% SGV. Neurons 44 d in culture. ×44,000.

required higher concentrations of atropine for apparently complete block. The atropine-sensitive responses were not abolished by hexamethonium. We often recorded large cholinergic hyperpolarizations, and their reversible block by atropine, in the continuous presence of hexamethonium in concentrations up to 1 mM (however, see Glitsch and Pott, 1978); examples are shown in Potter et al. (1986, Figs. 5 and 8).

The neuronally evoked hyperpolarizations described in this section could be mimicked by "puffing" ACh onto the myocytes from a micropipette (see Methods). An example is shown in Figure 9. When this neonate-derived solitary neuron was stimulated (a_1), the myocytes were hyperpolarized by about 23 mV and then depolarized; both effects were fairly well mimicked by a single puff of 2 μM ACh (a_2). Atropine completely blocked the effects of neuronal stimulation and of the puff. As no other neuronal influence on the myocytes was unmasked by atropine (b_1), the neuron was apparently purely cholinergic. The reversibility of the effects of atropine is shown in Figure 9, c_1 and c_2.

As described in Potter et al. (1986), many neurons exhibited adrenergic as well as cholinergic function (dual-function neurons). In such cases, we often applied brief puffs of a mixture of ACh (2–10 μM) and NE (2–100 μM) to the myocytes to see whether simultaneous application of these two agents could mimic the dual effect produced by the neuron. The characteristic response of the myocytes to such puffs of ACh/NE was an initial hyperpolarization evoked by the ACh, followed by a more prolonged excitation evoked by the NE (see Fig. 11*e*). In 19 such experiments we tested the effect of atropine; in each case the hyperpolarizations, whether evoked by puffed ACh or by neuronal stimulation, were largely or completely blocked by atropine in the concentration range 0.1–1 μM (see Fig. 1 in Potter et al., 1986). In five experiments, neuronally evoked hyperpolarizations were mimicked roughly by puffs of the muscarinic agonist bethanechol, 20–30 μM (e.g., Fig. 6 of Potter et al., 1986); in one of these cases, the effect of 30 μM bethanechol was blocked by 0.2 μM atropine sulfate.

Five microcultures in which neurons had been characterized as functionally cholinergic were fixed with $KMnO_4$ and the terminals examined with the EM; one of these cases is shown in Figure 10*a*. In all cases, the small synaptic vesicles, both in the terminals at morphologically specialized synapses and in simple varicosities, contained only electron-lucent centers and resembled the SCV present in parasympathetic endings in the iris (Hökfelt, 1967; Nishida and Sears, 1969; Richardson, 1966) and in cholinergic sympathetic endings in rat sweat glands (Landis and Keefe, 1983).

In a microculture with two neurons—one apparently purely cholinergic, the other of mixed function with a strong adrenergic component—the presumptive endings of the cholinergic neuron were readily identified: In many of the endings and varicosities in this microculture, no SGV were present (e.g., Fig. 10*b*); the remainder (not shown) contained an average of 38% SGV.

In summary, several lines of evidence indicate that the neuronally evoked hyperpolarizations described in this section were produced by ACh acting via muscarinic receptors on the myocytes. (1) Moderate concentrations of atropine substantially or completely blocked the hyperpolarizations evoked either by the neuronally released agent or authentic ACh or bethanechol applied in quantities that roughly mimicked the neuronal effect. (2) Many of the solitary neurons that produced this effect also produced a hexamethonium-sensitive excitation at autapses; thus, there was parallel evidence for secretion of ACh by the same neuron at another site. (3) When fixed in permanganate the synaptic endings and varicosities made by such neurons possessed the SCV characteristic of cholinergic junctions *in vivo.* (4) There is biochemical evidence for synthesis and storage of ACh by sympathetic neurons (neonate- or adult-derived) cocultured with cardiac cells or grown in medium conditioned by such cells (Patterson and Chun, 1974, 1977a; R. Nishi, personal communication).

Adrenergic excitation of the myocytes

In many microcultures, stimulation of the neuron evoked (as at least one of its effects) myocyte excitation that was blocked

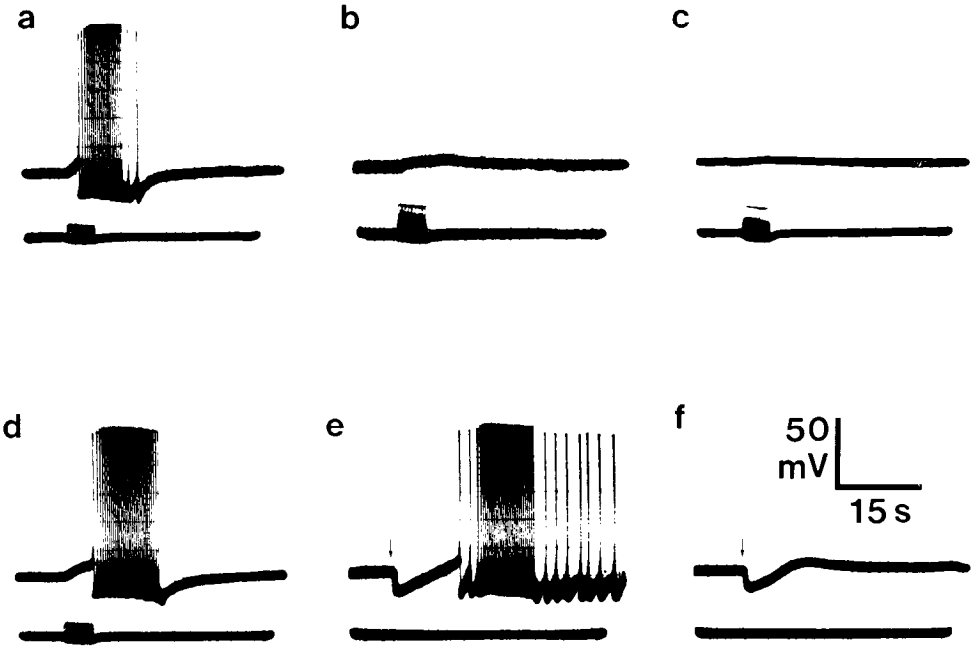


Figure 11. Adrenergic effect on cardiac myocytes of a neonate-derived solitary neuron 26 d in microculture. In *a–d* the neuron was stimulated (16 Hz for about 5 sec; *lower traces*); in *e* and *f*, a puff of perfusion fluid containing both ACh (5 μM) and NE (20 μM) was applied at the *arrows* above the myocyte (*upper*) traces. The excitation produced by neuronal activity (*a*) was partly blocked by 10 μM atenolol added to the perfusion fluid (*b*), and more completely blocked by both 10 μM atenolol and 0.1 μM phentolamine (*c*). The excitatory effect was restored after washout of the adrenergic blockers (*d*). The excitatory effect of the puffed NE (*e*) was blocked (*f*) in the presence of the two blockers (atenolol, 10 μM; phentolamine, 0.1 μM); the inhibitory effect of the puffed ACh persisted.

by adrenergic antagonists. When the myocytes were already beating, stimulation of such neurons caused an increase in beating rate; when the myocytes were not beating, stimulation evoked a depolarization in the myocytes and often initiated beating. Several β-adrenergic receptor antagonists (propranolol, atenolol, alprenolol, and sotalol) strongly blocked this excitation; a more complete block was often obtained by addition of the α-adrenergic receptor blocker phentolamine, and this agent was routinely added to the β-blocker in later experiments (see Flavahan and McGrath, 1981). [It has recently been reported by Morris and Woodcock (1983) that expression of α_1- and β-adrenergic receptors by neonatal and perinatal rat heart cells, at ages similar to those of the microcultured myocytes, is equal to or greater than that of mature heart cells.]

This effect and the action of the adrenergic blockers is shown in Figure 11. Stimulation of the solitary neuron caused a depolarization and onset of beating in the myocytes (*a*) that was strongly attenuated but not abolished by atenolol (*b*). Addition of phentolamine to the atenolol solution enhanced the block, although a small depolarization still remained (*c*). The blocking action of the two agents was reversible (*d*). The cause of the small neuronally evoked depolarization that persisted in the presence of adrenergic blockers in *c* is not known. It may have been due to incomplete block of the adrenergic effect or to secretion of a second excitatory transmitter (e.g., nonadrenergic excitatory effect described by Matsumoto et al., in press).

To check the sensitivity of the myocytes to NE and ACh, a brief puff of perfusion fluid containing ACh and NE was applied to the microculture (Fig. 11*e*). The initial hyperpolarization was the response to ACh; the delayed excitation, the response to NE. In the presence of atenolol and phentolamine, a puff of ACh/NE evoked the hyperpolarization but not the onset of beating. The sensitivity of the myocytes to applied NE, and the block of both the NE response and the neuronally induced excitation by moderate concentrations of adrenergic antagonists, suggest that the neuron released NE. Although the myocytes were sensitive to moderate concentrations of ACh (*e, f*), neuronal activation failed to elicit detectable hyperpolarization, even after substantial block of the excitatory effect (*c*). This indicates that the neuron did not release appreciable amounts of ACh. By the pharmacological criteria illustrated in Figure 11, we conclude that this neuron was at least predominantly adrenergic; these tests do not rule out the presence of weak nonadrenergic effects (e. g., Fig. 11*c*) or the release of transmitters to which the heart cells were insensitive. A summary of the proportion of neurons exhibiting adrenergic function is given in a later section (Incidence of cholinergic and adrenergic function).

Three other β-adrenergic blockers were found to be effective in reducing or eliminating this excitatory effect of the neurons: alprenolol (0.2–1.0 μM, 5 trials), sotalol (5–20 μM, 5 trials), and propranolol (many trials; e.g., Figs. 1, 2, 6, and 10 of Potter et al., 1986). Prolonged washing with drug-free perfusion fluid (usually 20–40 min) was required to reverse the block caused by propranolol or, especially, by alprenolol. In most of our experiments we used atenolol (5–50 μM; usually 10 μM) because the β-receptor block caused by atenolol was more rapidly reversible than that caused by propranolol or alprenolol and because atenolol is reported to have minimal local-anesthetic action (Higgins et al., 1979).

Some adult-derived neurons were also found to produce purely excitatory effects on the myocytes. An example is shown in Figure 12. In *a*, a train of neuronal impulses produced a depolarization of the previously quiescent myocytes and a train of cardiac impulses; even a single neuronal impulse evoked a depolarization of about 3.5 mV (*c*). Addition of atenolol and phentolamine to the perfusion fluid at 5× the usual concentrations sharply reduced the cardiac response but did not eliminate it entirely (*b*). After 45 min of perfusion with 10 μM reserpine phosphate, the neuronally evoked depolarization was completely eliminated. Given the well-known action of reserpine in unloading catecholamines from adrenergic vesicles, the result shown in Figure 12*d* is consistent with the idea that this neuron was purely adrenergic (no further action on the myocytes was unmasked by reserpine). However, it is also possible that the reserpine-sensitive effect was serotonergic (see Matsumoto et al., in press; D. Sah and S. G. Matsumoto, unpublished observations). Moreover, it has recently been reported that reserpine (0.09–9 μM) blocks secretion of prolactin from anterior pituitary cells (Login and MacLeod, 1981), the release of dopamine from the rat corpus striatum (Dyck and Boulton, 1980), and the entry of Ca^{2+} into certain mammalian smooth muscle cells (Casteels and Login, 1983); consequently, elimination of synaptic effects by reserpine must be interpreted cautiously.

Five microcultures in which a solitary, neonate-derived neu-

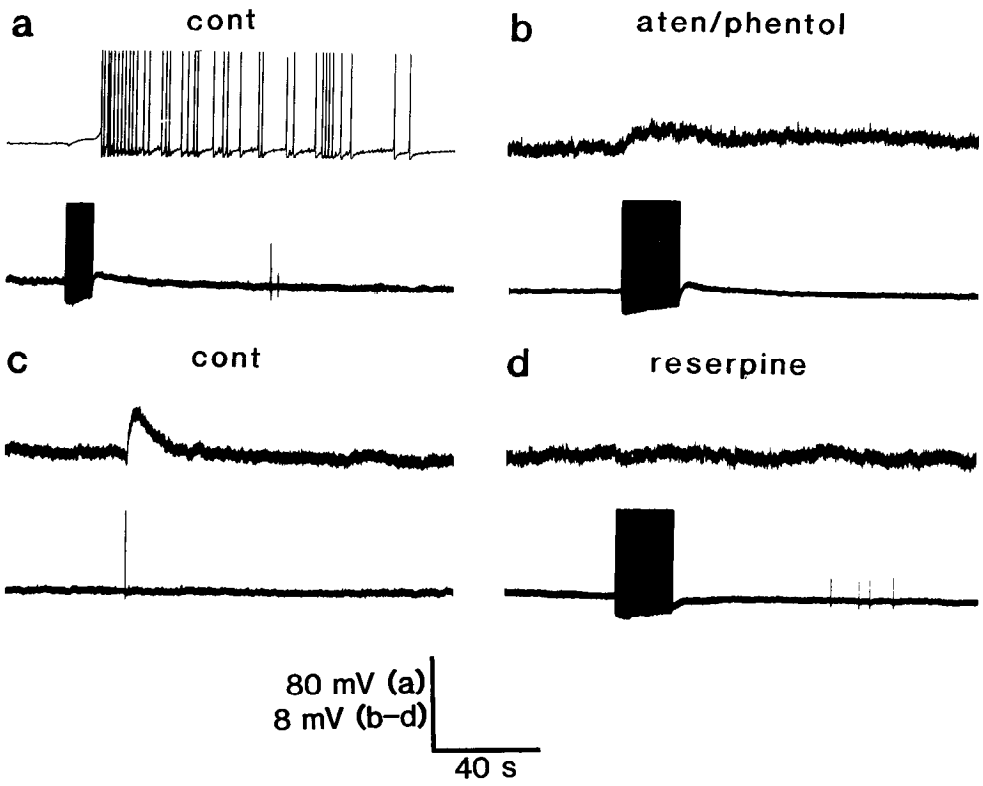


Figure 12. Excitatory myocyte responses evoked by an adult-derived neuron from a 350 gm rat, 82 d in microculture. *a*, Train of neuronal impulses (20 Hz for about 10 sec; *lower trace*) excited the myocytes (*upper trace*). *c*, Single neuronal impulse produced a depolarization of about 3.5 mV in the myocytes (time to peak, about 2.5 sec). The excitation of the myocytes was largely blocked by 50 μM atenolol and 0.5 μM phentolamine added to the perfusion fluid (*b*) and completely blocked by 10 μM reserpine phosphate perfused for 45 min (*d*). Voltage calibration applies only to the myocyte traces. Impulse amplitudes were attenuated by the chart recorder. See text for discussion.

ron was functionally identified as apparently purely adrenergic were fixed in $KMnO_4$ and examined with the EM. All of the synaptic terminals and varicosities contained numerous SGV; four examples are shown in Figure 13. The mean proportions of SGV in the five cultures were 59, 67, 72, 76, and 85%, similar to the proportions observed in terminals and varicosities of neurons in mass cultures grown under "adrenergic" conditions (Landis, 1978, 1980; Rees and Bunge, 1974). Treatment of two additional neurons with 10 μM reserpine phosphate for 120 min prior to fixation eliminated the granular precipitate. Other evidence for the presence of adrenergic function has been provided by observations that neonate-derived neurons in mass cultures (for references, see Bunge et al., 1978; Patterson, 1978) and microcultures (Reichardt and Patterson, 1977) can synthesize, store, and, in response to K^+-mediated depolarization, release NE.

Heart cells and rat serum are sources of a factor that induces cholinergic function in these neurons (O'Lague et al., 1978c; Patterson and Chun, 1974, 1977a; Wolinsky and Patterson, 1985); heart-cell factor(s) also can depress adrenergic function (Patterson and Chun, 1977a, b; Wolinsky and Patterson, 1983). The neurons used in the present experiments were all cocultured from the beginning with heart cells in the presence of rat serum; under these conditions neonate-derived neurons become more cholinergic and may lose adrenergic function with time in culture (Potter et al., 1980, 1981, 1986). To enhance the probability of obtaining neonate-derived neurons that produced vigorous adrenergic effects, we grew some microcultures in "high-K^+ medium" (see Walicke et al., 1977). After several weeks in high-K^+ medium, some neurons developed cholinergic function, but this induction usually appeared to be delayed and weak compared to microcultures fed normal medium. Moreover, the probability of finding neurons that lacked detectable cholinergic function was enhanced (Fig. 9 of Potter et al., 1986). A few of these neonate-derived neurons produced conspicuous adrenergic effects in the myocytes with one or a few neuronal impulses like the adult-derived neuron of Figure 12. One of these cases is illustrated in Figure 14, *a–f*. The myocyte responses shown in *a–c* were evoked, respectively, by one, two, and four neuronal action potentials. The effects of successive impulses summated; the response to four impulses (amplitude >6 mV) exceeded the myocyte threshold and initiated a brief burst of beating. The myocyte depolarizations evoked by this neuron were sharply diminished in 1 μM propranolol and were abolished in a mixture of 1 μM propranolol and 0.1 μM phentolamine (not shown). The amplitude of the largest depolarization we observed in response to a single impulse in a solitary neonate-derived neuron was 6 mV (Fig. 14*g*).

In the two experiments of Figure 14 the latencies of the adrenergic depolarizations evoked by one or a few stimuli were examined. Responses to one and to four neuronal impulses are shown in *d* and *e* (taken from the same experiment as those of *a–c* but at higher sweep speed). The gradual rise of the response made the measurement of latency uncertain, but in these and other records from the same experiment the latency from the peak of the neuronal impulse (or from the first neuronal impulse in a train) to the beginning of the myocyte depolarization was about 450–500 msec. In the experiment of *g* the latency was shorter (about 350–400 msec). In eight other experiments on strongly or exclusively adrenergic solitary neurons, the latencies were 400–500 msec. Most of these records were made at lower gain and sweep speed than those of *d* and *e*; the measurements were therefore less accurate but probably not in error by more than 20%. In several other cases in which the adrenergic depolarization rose more slowly, apparent latencies of 0.75–2.0 sec were observed. The shortest latencies (350–400 msec) were about 10× longer than the shortest latencies of the cholinergic responses (35–50 msec).

The rise times of the responses in the experiment of Figure 14, *a–f*, were 5–6 sec and in the experiment of *g*, 3–3.5 sec, more than an order of magnitude longer than the rise times of the fastest cholinergic responses. The duration, measured at half-amplitudes, of the adrenergic responses to one or a few stimuli was about 10 sec in both experiments, 3–5× the values

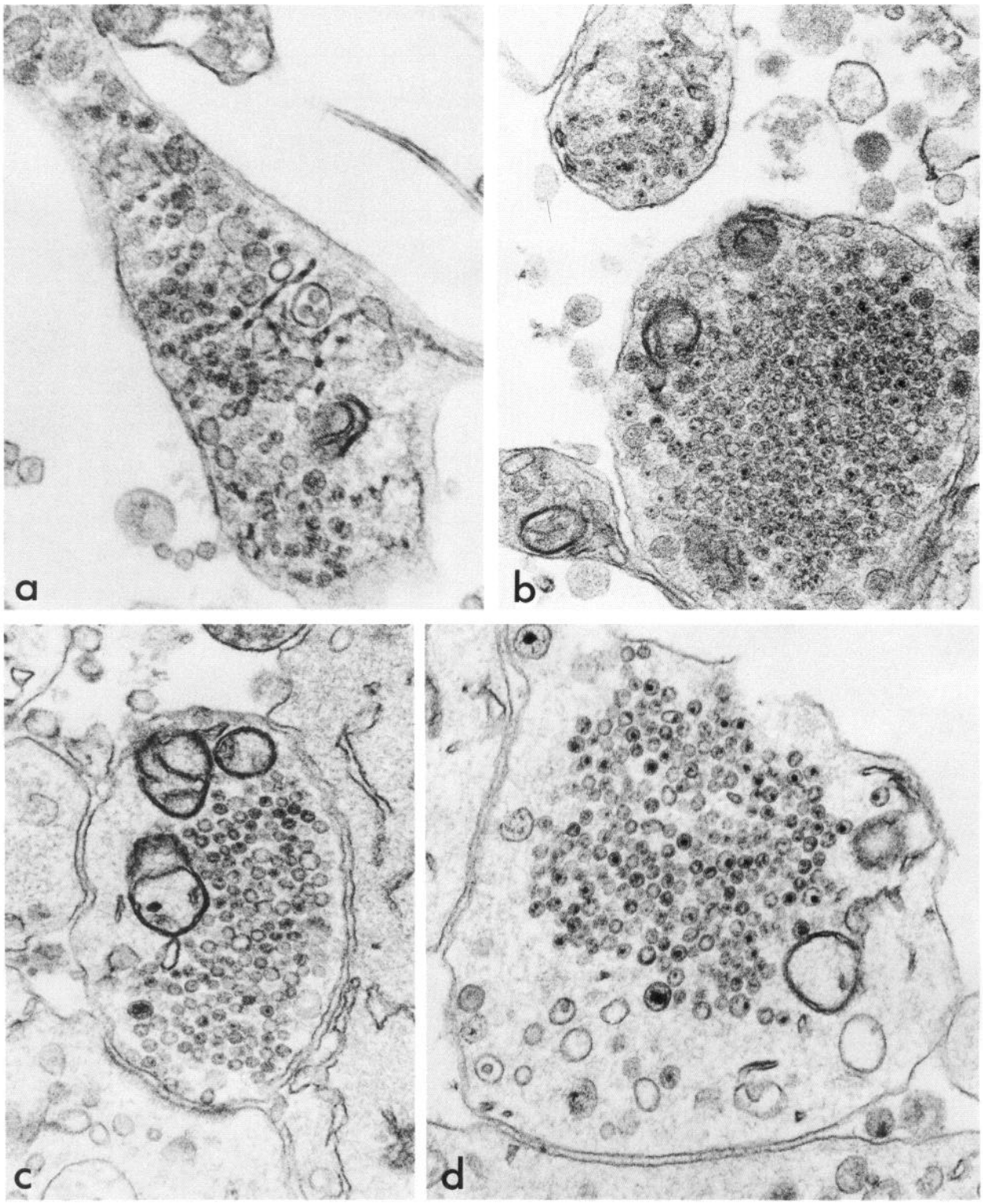


Figure 13. Terminals of adrenergic neurons; permanganate fixation. *a,* From a microculture containing a single neonate-derived 13-d-old neuron. Relatively few varicosities were evident in this young culture. On average, 59% of the vesicles contain precipitate. *b,* From a microculture containing a single adult-derived neuron 60 d in culture. Two varicosities containing numerous SGV (75% on average) are present. *c,* Synapse formed by a neonate-derived adrenergic neuron. Predominantly SGV (72% on average) are present. From a 21-d-old microculture containing two adrenergic neurons. *d,* Autapse formed by a solitary, neonate-derived, adrenergic neuron after 19 d in culture. This microculture had been exposed briefly to 1.0 and to 0.1 μM NE 2 hr before fixation. Note that the SGV are more numerous (81% on average), and the dense cores are larger.

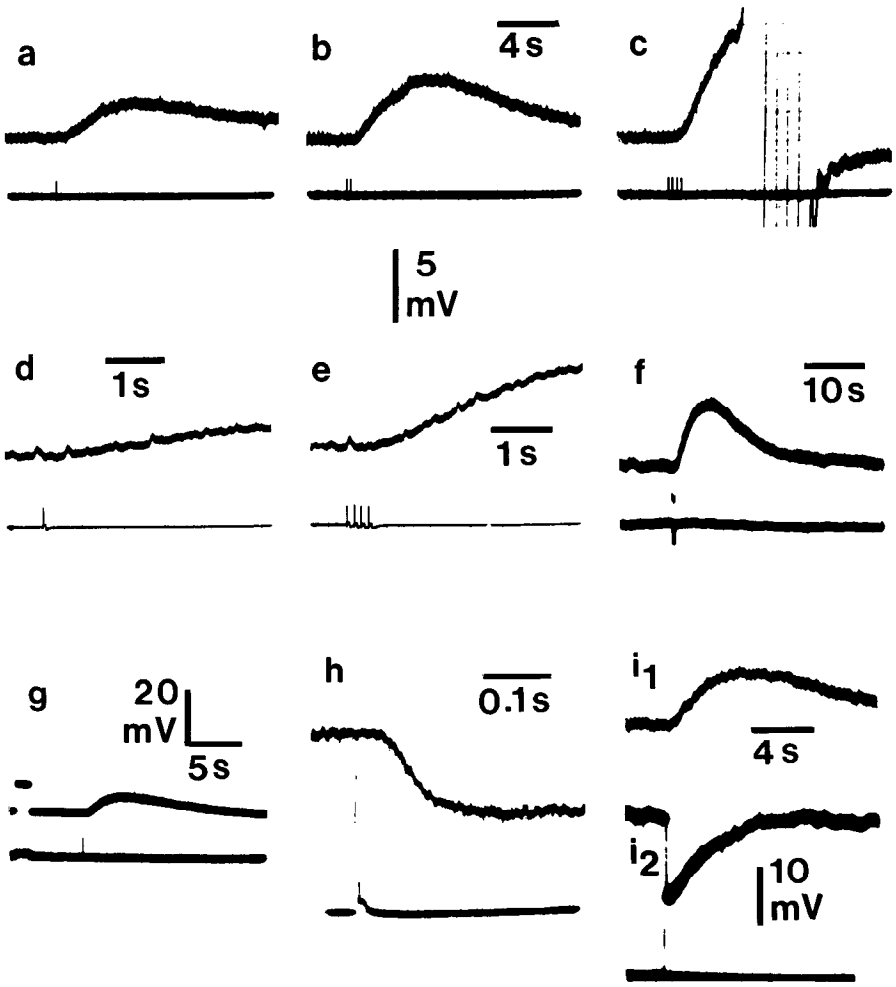


Figure 14. Time course of adrenergic effects on myocytes exerted by solitary neonate-derived neurons grown continuously in high-K^+ medium; comparison with the time course of cholinergic effects. The adrenergic myocyte responses (*upper traces*) in *a–f* and i_1 were all recorded from the same microculture (neuron 20 d *in vitro*). In *a, b,* and *c,* the neuron was stimulated once, twice, or four times (at 3.3 Hz); the myocyte depolarization evoked by four impulses exceeded threshold. In *d* and *e,* the myocyte responses to one and four impulses are shown on a 4× expanded time axis. In *f,* the whole response to three neuronal impulses (at 8 Hz) is shown. The record in *g* was taken from another microculture (neuron 24 d *in vitro*); the *upper trace* shows the largest and fastest adrenergic response observed following a single impulse in a neonate-derived neuron. The cholinergic myocyte responses in *h* (*upper trace*) and i_2 were evoked by a solitary neuron grown in normal (5.4 mM) K^+ for 108 d. The time courses of the adrenergic response in *e* and the cholinergic response in *h* can be compared; the time axis in *h* was expanded about 10-fold over that in *e.* In *i,* the adrenergic and cholinergic responses from these two experiments, recorded on the same time axis, are shown with the neuronal impulses aligned. The neuronal impulses in *a–e* and *g* (*lower traces*) were retouched. The time calibration in *b* applies to *a–c.* Voltage calibration above *e* applies to *a–f* and i_1; that in i_2 also applies to *h* (voltage calibrations apply to myocyte traces only).

for the cholingeric responses. Myocyte responses recorded from two different microcultures, in which the neuron was adrenergic in one and cholinergic in the other, are shown together at the same sweep speed in *i.* The cholinergic response (i_2) reached its peak in 200–250 msec, during the latency of the adrenergic response (i_1), and had fallen to about 25% before the adrenergic response reached its peak. A similar comparison is provided by the records in Figure 14*e* (an adrenergic response) and *h* (a cholinergic response, recorded at about 10-fold higher sweep speed).

This consistent difference in the time courses of the two responses, recorded separately, provides an explanation for the fact that when adrenergic and cholinergic effects were exerted simultaneously by puffs of a mixture of the two agents (Fig. 11, *e* and *f,* and Fig. 1 of Potter et al., 1986), the cholinergic effect consistently preceded the adrenergic effect (*cf.* the adrenergic/cholinergic dual-function neurons, described in Potter et al., 1986; also see Hill-Smith and Purves, 1978, for a similar effect following intramural nerve stimulation in intact rat atria or iontophoretic application of ACh and NE to isolated clusters of heart cells).

Neurons that produced adrenergic effects on the myocytes, but lacked cholinergic function, did not produce detectable autaptic effects in response to one or a few stimuli, as noted earlier (examples are given in Furshpan et al., 1976; Potter et al., 1981).

Incidence of cholinergic and adrenergic function

In a group of about 300 reasonably well characterized neurons (neonate- and adult-derived) of all culture ages, whose status is summarized by Matsumoto et al. (in press), about 17% (about 12% for neonate-derived neurons) were apparently purely adrenergic and about 10% (about 13% for neonate-derived neurons) were apparently purely cholinergic. More than two-thirds of the neonate-derived neurons that were apparently purely adrenergic had been grown in high-K^+ medium; one-third were studied during the first two weeks in culture and therefore were subjected to the cholinergic influence of the cocultured cardiac cells for a relatively short period. It must be emphasized that because of the diversity of culture conditions and ages represented in the neurons of this group, the exact percentages of neurons with adrenergic or cholinergic status are not significant. However, it is clear that only a minority of the microcultured neurons displayed only one or the other of these two states that, in the conventional view, characterize adult mammalian sympathetic principal neurons *in vivo.* An additional 23% of this sample (26% of the neonate-derived neurons) released both NE and ACh as their only detectable transmitters; the remaining 50% of the sample displayed other transmitter states (Matsumoto et al., in press).

Discussion

The microculture method was devised to investigate the transmitter status of individual sympathetic neurons. It has several significant advantages over the use of mass cultures or intact tissues for such studies. The first advantage is the reliability of synaptic interaction and the sensitivity of the assay. Under the influence of NGF and perhaps other factors produced by the cocultured cardiac cells, the sympathetic neurons develop an

extensive axonal arbor confined to a small group of defined target cells. The area of a mass culture (ca. 1 cm^2) is 500–1000× that of a microculture; if, in the presence of NGF, each neuron elaborates an axonal tree independent of target area, the ratio of target innervation density for an individual neuron in microculture to that in mass culture might approach 500–1000×. In practice, when MacLeish (1976) grew mass cultures of sympathetic neurons on heart cells, he rarely found an effect on the myocytes of stimulating individual neurons; in microcultures, the absence of a neuronal effect on the myocytes is rare.

The number of varicosities observed in older microcultures with the EM was very high (e.g., Fig. 3). It seems likely that the density of target innervation in such microcultures became much higher than that achieved by individual sympathetic neurons *in vivo*. For example, Malmfors and Sachs (1965) examined the branching patterns in the rat iris of the few sympathetic axons that remained 2–3 d after the SCG was crushed. The terminal arbor of a single axon was characteristically separated into several discrete regions, each about the size of a microculture (200–500 μm across); however, the density of terminal axon branches in each region (e.g., Fig. 2 of Malmfors and Sachs, 1965) was substantially less than that seen in older microcultures (e.g., Fig. 1*A* of this paper), and the proportion of the total target tissue contacted by terminal branches was also much less. In culture, where the availability of NGF is not limiting, the axon may continue to grow, branch, and elaborate varicosities for the lifetime of the culture.

Another indication of the high density of the functional innervation in microcultures was the large size of the postsynaptic responses, consistent with a high concentration of released neurotransmitter. For example, single impulses in solitary neurons that displayed cholinergic function often evoked myocyte hyperpolarizations of 5–20 mV. This can be compared with observations by Glitsch and Pott (1978), who made intracellular recordings from the isolated apex of the guinea pig atrium while stimulating intramuscular parasympathetic nerve fibers in the vicinity of the recording electrode; the number of nerve fibers activated was not known but was probably small. Trains of stimuli were usually required to produce visible hyperpolarizations of the atrium; in favorable cases, single stimuli evoked responses of about 0.2 mV.

In microcultures the apparent minimal latency for the cholinergic response of the myocytes to one or two neuronal impulses was comparatively short (about 35 msec). Pott (1979), in experiments similar to those described above (Glitsch and Pott, 1978), reduced the extracellular K^+ concentration to enhance the size of the atrial hyperpolarizations evoked by single parasympathetic nerve-fiber stimuli. He then found that the initial rising phase of the responses appeared to be proportional to t^3 (t = time) and had a large Q_{10}, consistent with the operation of several temperature-dependent processes in series (see Hartzell et al., 1977). Thus, the apparent latency might only measure the time at which a continuously increasing hyperpolarization becomes distinguishable from noise; in that case, the latency would decrease as the concentration of released ACh increased. The apparent minimal latencies observed by Pott (1979) were about 80–100 msec (at 35°C). Brown and Eccles (1934) applied single stimuli to cat vagus nerve and observed negative chronotropic effects on the heart within 100–160 msec. Hill-Smith and Purves (1978) examined the responses of clusters of neonatal rat ventricular cells to iontophoretically applied ACh and found minimal latencies of about 250 msec. This is about 7-fold longer than the apparent minimal latency in microculture. Among possible explanations for this difference are the presence of atrial cells in the microcultures and the likelihood that in microculture a high concentration of ACh is applied widely and quickly to the population of coupled target cells by a dense axonal arborization.

Another likely indication of the high density of innervation in microculture was the presence of appreciable myocyte responses to one or a few impulses in the most strongly adrenergic neurons (e.g., those grown in high-K^+ medium). The latencies of these responses were about 5-fold shorter than those reported for sympathetic effects in intact atria or for NE application to isolated clusters of heart cells (see Hill-Smith and Purves, 1978).

The high innervation density in microcultures enhances the probability of detecting weak synaptic effects. This is an important point, given the evidence that mammalian sympathetic neurons *in vivo* and in culture often express multiple transmitter status and may express transmitter functions in a graded way (see Furshpan et al., 1986; Matsumoto et al., in press; Potter et al., 1986, for evidence and discussion); as discussed in these same papers there is also growing evidence that sympathetic neurons are heterogeneous in transmitter status, even when their cell bodies are in the same ganglion or when they innervate the same target organ (e.g., Lundberg et al., 1982). While much has been learned by assaying whole ganglia biochemically or stimulating whole nerves, recent observations *in vivo* and in culture emphasize the importance of studying individual neurons.

Another advantage of single-neuron microcultures is the ease with which cytochemical or fine structural observations can be correlated with physiological observations, as all of the synaptic endings and varicosities observed by microscopy in the microculture belong to the assayed neuron. This type of correlation has been achieved more laboriously in mass cultures or in intact nervous systems by injecting a tracer substance into a physiologically characterized neuron (e.g., McGuire et al., 1984; Nelson et al., 1981; Purves and McMahan, 1972; Wakshull et al., 1979b). Immunocytochemical, histochemical, and autoradiographic methods offer the opportunity, in intact tissues, for assaying aspects of transmitter mechanisms in single neurons. These methods do not readily reveal if a product of interest is released, and some are subject to well-known concerns about false-negatives and false-positives. Clearly, a method that combines the advantages of electrophysiological and pharmacological assays with cytochemical and/or electron microscopical assays is stronger than any of the individual methods.

The microculture method also has several advantages in common with other cell culture methods. A specified neuron and its target cells can be stably impaled with microelectrodes under visual control. Pharmacological agents, applied to the culture in the perfusion stream or locally via a puffer pipette, have free access to the exposed cells at concentrations equal to or approaching those in the reservoir. Giga-ohm seal, patch-clamp, and whole-cell recording techniques, although not used in the current experiments, are particularly successful with cells in culture. A final advantage, exploited in the next paper (Potter et al., 1986), is that a physiologically characterized solitary neuron can be identified unambiguously for re-assay after an interval of days or weeks.

The microculture method also has several disadvantages. The success rate in the preparation of usable microcultures appears to be influenced by small variations in the plastic substrate, collagen, sera, and cell dissociation procedures; failed platings in which the myocytes are too sparse for stable impalements, in which the heart cells aggregate or roll up, or in which neighboring islands become interconnected by bridges of heart cells and/or neurites still occur unpredictably. After successful platings one must wait at least 10 d to make an assay with conventional microelectrodes, and the sensitivity of the assay generally increases with time in culture. The assay is time-consuming. To characterize the transmitter status of a neuron may take 3–6 hr if more than one transmitter is present and if repeated impalements of the myocytes must be made. Thus, while the method provides one of the most effective ways of investigating the transmitter profile of an individual neuron, it is a slow and

painstaking way of investigating the distribution of transmitter profiles in a population of neurons.

In order for the assay to detect each of the released transmitters, the target cells must have the appropriate receptors. By using only a single type of target cell one may well underestimate the transmitter repertoire of a neuron, or of a population of neurons. In principle, this problem could be minimized by using a variety of target cells; this would also increase the generality of the method, making it applicable to the study of neurons from a variety of sources. In practice we used only cardiac cells (and the sympathetic neurons themselves) as targets in the experiments reported here. Members of the laboratory are currently exploring the use of smooth muscle cells dissociated from the vas deferens as additional targets for sympathetic neurons.

The microculture technique shares with other experimental methods the possibility that the procedures may alter the mechanisms to be studied. Dissociation of the ganglia involves denervation and axotomy of the neurons, which are then grown in an unusual environment. It is not yet possible to assess fully the seriousness of this problem. One approach is to consider the ways in which behavior observed in microcultures resembles or differs from that already known or suggested for the cells *in vivo*; this is the subject of the next two sections.

Similarities of the microcultured neurons to their counterparts in vivo

As in mass cultures of neonate-derived neurons (O'Lague et al., 1978a–c), the microcultured neonate- and adult-derived neurons had resting and action potentials similar to those reported in adult rat sympathetic ganglia; they secreted transmitters by a Ca^{2+}-dependent mechanism (see Furshpan et al., 1986). The neurons expressed nicotinic ganglionic ACh receptors and the myocytes expressed muscarinic and α- and β-adrenergic receptors whose sensitivities to hexamethonium, atropine, phentolamine, and several β-blockers were similar to those of intact adult tissues. The physiological effects gated by these receptors (excitation or inhibition) were similar to those recorded *in vivo* in sign and in time course, with the exception noted above that intense neurally evoked effects on the myocytes were larger and had shorter latencies than those previously reported *in vivo* (e.g., Brown and Eccles, 1934; Glitsch and Pott, 1978). The transition in transmitter status, from adrenergic to cholinergic, observed in mass cultures (Patterson and Chun, 1977a, b) and in microcultures (Potter et al., 1981, 1986) also occurs in the innervation of certain sweat glands *in vivo,* and with a similar time course (Landis and Keefe, 1983; Yodlowski et al., 1984). The microcultured neurons form axons, dendrites, neuron–neuron synapses, and neuron–myocyte junctions with fine structures similar to those expressed by adrenergic or cholinergic peripheral neurons in adult animals *in vivo* (e.g., Gabella, 1976). Formation of these structures and operation of these functions presumably requires coordinated expression of many neuronal mechanisms. However, it seems likely that some control mechanisms exhibit altered function in the culture environment in which the concentrations of hormones and other regulatory molecules generally deviate from those *in vivo.* Discovering the effects of such factors has been one of the major goals of studying cell cultures.

A difference between microcultured neurons and their counterparts in vivo

A conspicuous difference between the microcultured neurons and their counterparts in adult sympathetic ganglia is the absence in culture of a satellite-cell investment of the cell bodies, dendrites, and axons (e.g., Figs. 3, 4, 10, and 13). Neonate ganglia were dissociated mechanically, and special precautions were taken to eliminate even small clumps of neurons; these are conditions that appear to minimize the plating of viable ganglionic nonneuronal cells (see O'Lague et al., 1978a). Although adult ganglia were dissociated by an enzymic procedure that might have been expected to release many ganglionic nonneuronal cells, the cell suspension was exposed to gamma-radiation to prevent proliferation of the nonneuronal cells. Nevertheless, it is likely that ensheathment of the neurons is not favored by the microculture conditions, which were similar to the mass-culture conditions (O'Lague et al., 1978a) in which ensheathment failed to occur even in the presence of large numbers of ganglionic nonneuronal cells (Claude, 1973; Landis, 1977). Bunge and Bunge (1978) and Cassel et al. (1982) have described the quite different culture conditions required to achieve a high incidence of myelination of dorsal root ganglion cells. It is notable that many important neuronal functions are expressed in culture in the absence of the normal relationship with supporting cells.

Electrically silent synapses?

A potentially interesting feature of the neonate-derived neurons was the lack of an autaptic effect attributable to secretion of NE. Does this indicate the presence of electrically silent synapses? In a large number of cases, in which the neuron secreted NE onto the myocytes, we observed no effect of one or a few neuronal action potentials on the neuron itself, provided the nicotinic, autaptic EPSPs were absent or had been blocked with hexamethonium. In six of these cases the presence of autapses of adrenergic appearance (numerous SGV in the presynaptic terminals) was confirmed by electron microscopy. Although single stimuli were usually effective in evoking cholinergic autaptic EPSPs, trains of stimuli might be required to evoke detectable adrenergic autaptic responses. In fact, we often observed slow depolarizations in the neurons during and after trains of impulses (10–50 Hz for 5–20 sec; e.g., N_2 in Fig. 8 of Potter et al., 1986; see also Suppes, 1984). The effects of adrenergic blocking agents were tested on these responses in 14 neurons that were shown to have at least adrenergic function in the myocyte assay. In each case the slow neuronal depolarization was unaltered by atenolol (10–20 μM) and phentolamine (0.1 μM). In mass cultures of these neurons we were unable to detect any reproducible effect of applying catecholamines in the perfusion stream, and we concluded that the neurons had low electrical responsiveness to these substances; the few responses that we did observe were hyperpolarizations (O'Lague et al., 1978c).

Thus, we have seen no autaptic responses plausibly attributable to adrenergic transmission, and it seems likely that the adrenergic autapses (or the adrenergic component of multifunction autapses) were electrically silent. It seems unlikely that the absence of an electrical response was due to failure of the adrenergic neurons to release NE at their autapses. Buckley and Landis (1983b) found that after these neurons in mass culture had been depolarized by an increase in extracellular K^+ concentration, the numbers of synaptic vesicles, both in synaptic endings and in nonsynaptic varicosities, were reduced by similar amounts, and many of the vesicles in both endings and varicosities had taken up an extracellular tracer (HRP). These observations suggest that vesicular release of transmitter had taken place, and this occurred whether the neurons had been grown in conditions that promoted expression of adrenergic or cholinergic function.

We do not know the origin of the slow neuronal depolarizations. Similar responses ("late delayed depolarizations") were observed by Suppes (1984) in all cultured neurons of the superior cervical ganglion of the rat exposed to high concentrations of tetraethylammoniun ions (20 mM or higher), and in a few neurons in the absence of this substance (but after low-frequency repetitive stimulation). It is unclear whether these responses or those observed in the current study were evoked by release of a transmitter (other than NE and ACh) or were due to nonsynaptic effects of the action potentials. Peters and Kreulen (1984)

have observed, in neurons of intact guinea pig myenteric ganglia, slow synaptic depolarizations that were mimicked by arginine-vasopressin (AVP); they found mutual occlusion of the synaptically evoked and AVP-evoked responses.

Properties of adult-derived neurons

Wakshull et al. (1979a, b) showed that some adult-derived neurons form functional cholinergic synapses with each other in mass cultures (15% of 960 neurons assayed) and with cocultured skeletal myotubes (21% of 42 tested). As NE is not appreciably electrogenic for the neurons or skeletal myotubes, these assays would not have revealed secretion of NE. With the microculture assay we have detected secretion of NE onto cardiac myocytes by most adult-derived neurons (77 of 82 assayed or about 94%; summarized in Matsumoto et al., in press), and we have confirmed the secretion of at least ACh by about 28% of adult-derived neurons (23 of the 82 assayed; see Fig. 11 in Potter et al., 1986).

The expression of cholinergic function appeared to be lower in neurons derived from adults than in neurons derived from neonates. Only one of the adult-derived neurons (<2%) was apparently purely cholinergic (Fig. 8 of this paper), and smaller proportions of adult-derived neurons expressed detectable cholinergic function compared to neonate-derived neurons at similar culture ages; this is illustrated and discussed in the next paper (Potter et al., 1986).

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