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Evidence for Cholinergic Synapses Between Dissociated Rat Sympathetic Neurons in Cell Culture

(synaptic transmitters/neuronal development/cell interaction/ganglionic blocking drugs)

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ABSTRACT **Sympathetic principal neurons were dissociated from superior cervical ganglia of new-born rats, and grown in cell culture. In electrophysiological experiments two types of excitatory synapses were found. One, which was relatively rare, was shown to operate by electrical transmission. The other, the predominant type, had several characteristics of chemical transmission, and pharmacological evidence indicated it was cholinergic.**

Since the original demonstration of synapse formation in tissue culture by Crain and his colleagues (1), many laboratories have used culture methods to study neuronal development (2–4). Several recent papers have described the formation of neuromuscular and neuron–neuron synapses by primary excitable cells that were initially separated (e.g., 2, 4–7; see also ref. 8).

Levi-Montalcini and Angeletti (9) found that neurons dissociated from chick sympathetic ganglia survived and grew neurites in culture, provided nerve growth factor was present. In this laboratory we are culturing dissociated sympathetic neurons from superior cervical ganglia of newborn rats. Mains and Patterson (10) have described conditions for growing reliably long-term cultures of these cells either in the presence or near-absence of nonneuronal cells.

In this paper we report that these sympathetic neurons formed synapses with each other in a medium permissive for proliferation of nonneuronal cells. An unexpected result was that the synapses were excitatory, and pharmacological evidence indicated they were cholinergic. In an accompanying paper, Patterson and Chun (11) show that cultures grown under these conditions synthesized and accumulated appreciable quantities of acetylcholine (AcCh) in addition to catecholamines.

METHODS

Sympathetic neurons were dissociated by mechanical disruption of superior cervical ganglia from newborn rats (Charles River CD), as described by Bray (12). A suspension of isolated neurons and small clusters of cells from 40 ganglia was plated into 12 culture dishes (Falcon; 35 mm diameter) prepared as previously described (10, 12), giving several thousand nerve cells and a small number of accompanying nonneuronal cells per dish. Most of the cultures were grown, initially in L-15-CO_2 medium (10) containing 5% adult rat serum, in a humid atmosphere of 92% air–8% CO_2, at 35–36°. They were fed every 3–4 days; after day six the medium was supplemented with 2% fetal-calf serum (Microbiological Associates, Inc.). Nerve growth factor (10) was present throughout.

For electrophysiological recording, the culture was placed on the stage of an inverted phase microscope and continuously perfused. The perfusion fluid contained 100 ml/liter basal L-15 medium (10) and, in mM: NaCl, 140; KCl, 5.5; $CaCl_2$, 2.8; $MgCl_2$, 0.18; $NaHCO_3$, 26; NaH_2PO_4, 0.56; glucose, 5.6; choline chloride, 0.07, imidazole, 0.8; phenol red 0.03. Glutamine (2 mM) was added just before use and, typically, bovine-serum albumin (0.6 g/liter); the pH was kept at about 7.4 by bubbling with an O_2:CO_2 (95:5) or an air:CO_2 (95:5) mixture. In some drug experiments $NaHCO_3$ was replaced by NaCl (pH adjusted with NaOH). Perfusion fluid was supplied at a constant rate of about 1 ml/min into a volume of 0.1–0.2 ml surrounding the cells. The temperature was kept at 34–36° and in a given experiment usually varied within ±0.5°. A multi-channel valve allowed changing solutions without altering flow rate.

Microelectrodes filled with 3 M KCl (resistance: 100–200 MΩ) were used to measure membrane potential or to pass stimulating currents by means of conventional circuitry. The experimental arrangement (more fully described in a manuscript in preparation by P. H. O'Lague, K. Obata, D. D. Potter, and J. Furshpan) was sufficiently stable mechanically and thermally to permit continuous intracellular recording from single cells, during constant perfusion, for up to 7 hr.

Drugs were obtained from the following sources: *d*-tubocurarine, Mann Research Laboratories; atropine sulfate, Merck; hexamethonium chloride, Pfaltz and Bauer, Inc.; mecamylamine·HCl, Merck, Sharp and Dohme.

For electron microscopy cultures were fixed *in situ* in phosphate-buffered formaldehyde–glutaraldehyde, postfixed with OsO_4, stained *en bloc* with aqueous uranyl acetate, and embedded in Epon. In one case 5-hydroxydopamine (50 μM; Aldrich Chemical Corp.) was administered in the perfusion fluid for 1 hr before fixation.

RESULTS

Most experiments were made on cultures grown in L-15-CO_2 for 3 weeks or longer. During this period, the neuronal cell bodies increased in diameter from 10–15 μm at plating to 20–35 μm at 3 weeks, and a complex network of fiber bundles developed (see Figs. 1 and 3 in ref. 10), bringing neurons into morphological contact with many others. In contrast to the neurons, the nonneuronal cells proliferated, forming a carpet

Abbreviations: AcCh, acetylcholine; e.p.s.p., excitatory postsynaptic potential.

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interwoven with the neuronal network (10). Fig. 1 shows several neuronal cell bodies under phase contrast from a typical culture at 20 days; the plane of focus passes above the carpet of nonneuronal cells. The neurons resembled principal cells of the intact ganglion in microscopic appearance and dependence on nerve growth factor (10, 13).

The observed resting potentials were 50–70 mV; the higher values were associated with more stable penetrations by finer electrode tips. We suspect the resting potential in an undisturbed cell is 70 mV or greater. Action potentials similar in size (75–100 mV) and time course (about 2 msec at half-amplitude) to those reported in intact rat ganglia (14) were routinely observed.

Synaptic interactions

In tests for synaptic interaction, two neurons were impaled with microelectrodes and then each cell stimulated in turn, while recording from the other. Two distinct types of interaction were seen, the first electrical and the second almost certainly chemical and cholinergic.

Electrical interaction

In only 10 out of about 300 cases of interaction was there evidence for passive spread of potential from one neuron to another. In these cases sustained potential shifts of either polarity were transmitted in both directions, and synaptic potentials were produced without appreciable delay in either cell by action potentials in the other. These synaptic potentials were unaffected by *d*-tubocurarine or hexamethonium (50–100 μM; compare chemical synapses described below).

Chemical interaction

A different synaptic interaction, seen much more frequently and the main subject of this paper, is illustrated in the oscilloscope traces of Fig. 1. Action potentials (bottom trace) were evoked in cell D ("driver") by current pulses (top trace) passed through the recording electrode. Five consecutive sweeps were superimposed in the top and bottom traces. The remaining five traces show the resulting potential changes in cell F ("follower"), the beam being displaced between sweeps. About 1 msec after the peak of an action potential in cell D, a potential change occurred in cell F that resembled a conventional excitatory postsynaptic potential (e.p.s.p.). Impulses in cell F did not produce e.p.s.p.'s in cell D. In many pairs of interacting cells, the e.p.s.p.'s were suprathreshold. Often the delay between presynaptic action potential and e.p.s.p. was short (1–3 msec) as in Fig. 1, suggesting, with evidence not described here, a direct synaptic link between the neurons. In some cases the delay was 5–20 msec, indicating long conduction paths, sometimes presumably including interposed neurons.

Electrophysiological, electron microscopical, and pharmacological evidence has indicated that the interaction of the type illustrated in Fig. 1 arose at chemical synapses. These approaches will be considered in turn.

Electrophysiology. Consistent with chemical transmission are the observations that: the minimum delay was 1 msec; the transmission, with one exception, was not reciprocal; maintained depolarizations or hyperpolarizations were not detectably transmitted. More compelling is the finding that the e.p.s.p.'s were reduced in size when the ratio $[Mg^{++}]/[Ca^{++}]$ was increased, as at chemical synapses generally (15).

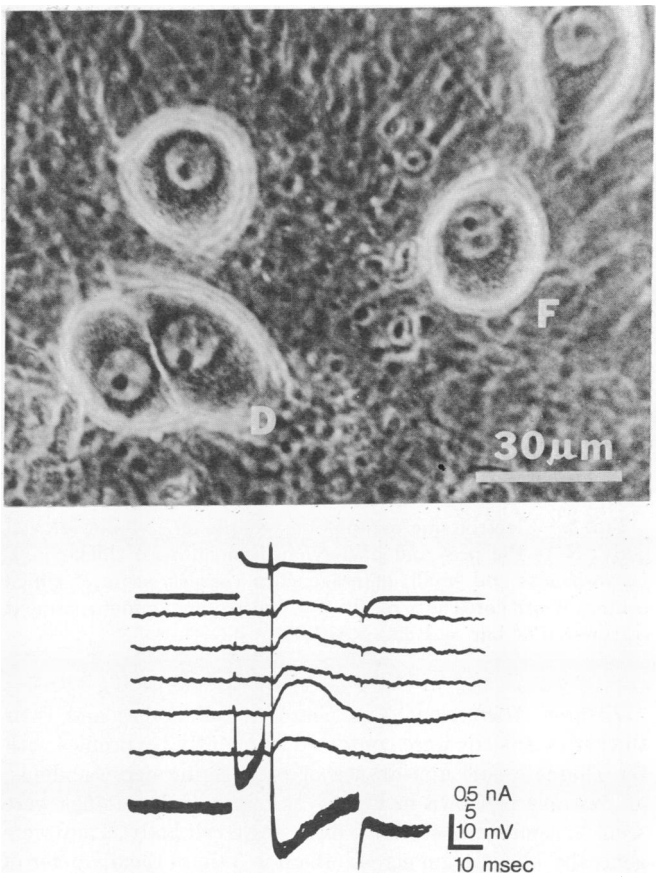

FIG. 1. Synaptic interaction between sympathetic neurons in culture. Upper: phase-contrast micrograph showing a "driver–follower" pair of neurons (D,F); culture, 20 days old. Lower: intracellular recordings from "driver" and "follower" neurons. Top oscilloscope trace: stimulating current supplied to cell D, five sweeps superimposed. Bottom trace: the resulting action potentials in D, five sweeps superimposed. Middle five traces: excitatory post-synaptic potentials (e.p.s.p.'s) evoked in cell F; beam displaced between sweeps.

This is illustrated in Fig. 2; *a* and *c* show the e.p.s.p.'s in the usual perfusion medium (Mg^{++}, 0.18 mM; Ca^{++}, 2.8 mM) before and after perfusing for 10 min with fluid containing Mg^{++}, 3.5 mM; Ca^{++}, 0.15 mM (Fig. 2b). The fall in $[Ca^{++}]$ and rise in $[Mg^{++}]$ markedly suppressed the e.p.s.p.'s. In other experiments reduced $[Ca^{++}]$ at constant $[Mg^{++}]$ also reduced the e.p.s.p.'s, but by a smaller amount.

Also consistent with chemical transmission is the apparently stepwise variation in amplitude of the e.p.s.p.'s (Fig. 4), suggestive of quantal transmitter release (15).

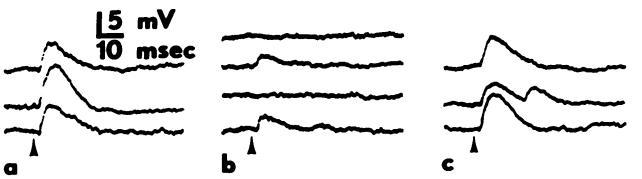

FIG. 2. Effect of Ca^{++} and Mg^{++} concentrations on synaptic transmission: (*a*) and (*c*), e.p.s.p.'s recorded in a 33-day-old culture perfused with 0.18 mM Mg^{++}, 2.8 mM Ca^{++} (standard concentrations), before and after 3.5 mM Mg^{++}, 0.15 mM Ca^{++} (*b*). Arrowheads indicate time of peak of the action potential in the "driver" cell.

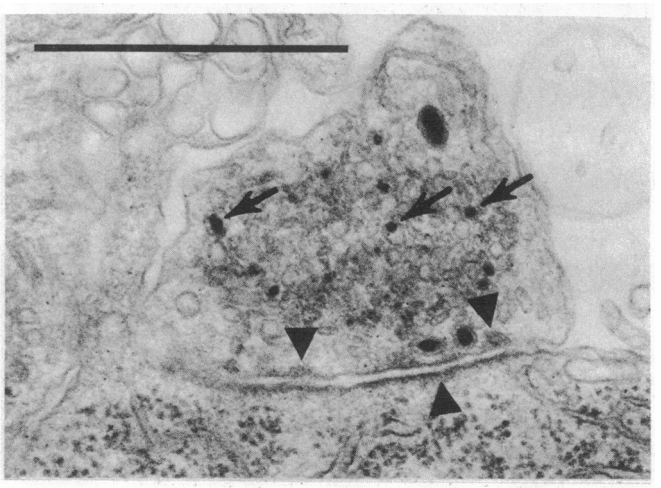

FIG. 3. Electron micrograph of a synapse on a "follower" cell body. Note the pre- and post-synaptic membrane thickenings, (arrowheads) and small, heterogeneous vesicles, some of which contain dense cores as a result of 5-hydroxydopamine treatment (arrows). The bar = 1 μm. ×41,000.

Electron Microscopy. The neuronal cell bodies and their thicker extensions were contacted frequently by profiles with the characteristic appearance of presynaptic nerve endings; an example is shown in Fig. 3. As many as 24 endings were seen, in semi-serial sections, on a single cell body. They were generally about 1 μm across (about 2–3 times the diameter of the cylindrical neurites) and contained small (50–70 nm), heterogeneously shaped clear vesicles and occasional large (120 nm) dense-cored vesicles. In many cases pre- and post-synaptic membrane thickenings were present.

Some of the small vesicles contained dense cores after exposing a culture to 5-hydroxydopamine (see *Methods*). This procedure has been used *in vivo* to identify adrenergic endings, which then show a high percentage of vesicles with dense cores (e.g., ref. 16). However, in the experiment of Fig. 3, only about 10% of the small vesicles became labeled (123 out of 1193 vesicles, in 24 endings; see *Discussion* and compare ref. 8).

Sensitivity of the e.p.s.p.'s to Ganglionic Blocking Drugs. Strong evidence that the e.p.s.p.'s arose at chemical synapses was obtained in pharmacological experiments which also indicated that the transmission was nicotinic-cholinergic. In all tests, the e.p.s.p.'s were strongly affected by the nicotinic blocking agents *d*-tubocurarine chloride (23 trials) or hexamethonium chloride (30 trials) added to the perfusion fluid. The e.p.s.p.'s were considerably reduced by either agent at about 10 μM and abolished by either at about 50 μM. The effects appeared rapidly and were reversed by perfusion with drug-free fluid. The e.p.s.p.'s were also diminished (at 2.5 μM) or abolished (at 5–10 μM) by the nicotinic blocker, mecamylamine·HCl (4 trials).

Atropine, characteristically a muscarinic blocker, affects nicotinic receptors at higher concentrations (17, 18). Fig. 4*b* and *d* shows the effects of atropine and hexamethonium on a single pair of cells. In this typical experiment hexamethonium, at 18 μM, reduced the e.p.s.p.'s to 27%, and atropine sulfate at 36 μM (equivalent to 72 μM atropine) reduced them to 18%; control amplitudes were means of responses recorded before and after drug perfusion.

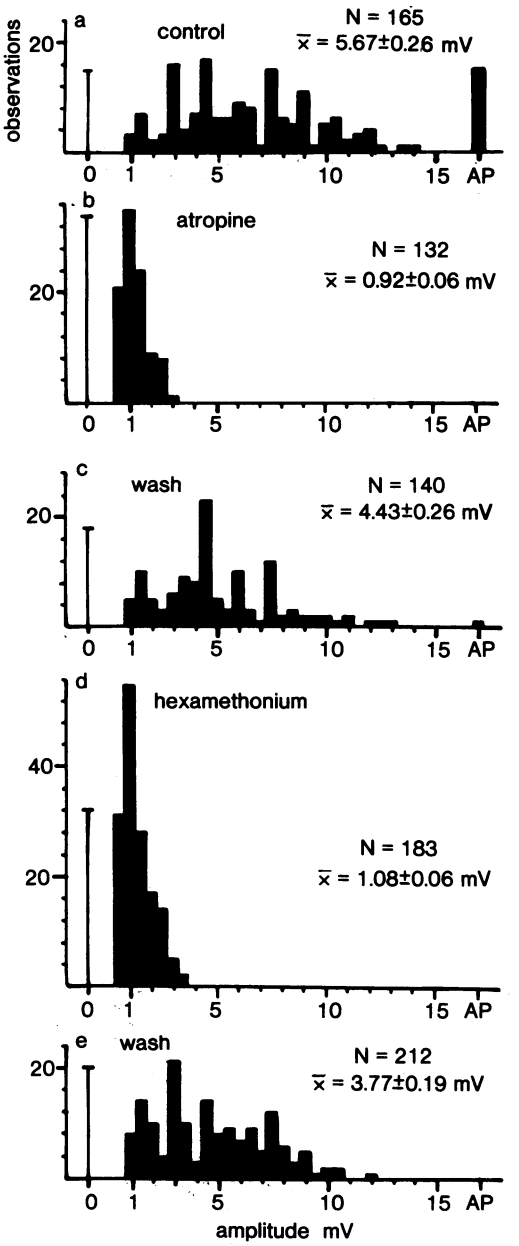

FIG. 4. The effects of atropine and hexamethonium on the e.p.s.p.'s. Continuous recording from a single "driver-follower" pair in a 47-day-old culture. Distribution of e.p.s.p. amplitudes during five consecutive periods of perfusion with bicarbonate-free fluid (see *Methods*); in *b*, with 36 μM atropine sulfate added; in *d*, with 18 μM hexamethonium chloride added. Consecutive e.p.s.p.'s were evoked at 0.5/sec for the last 5–6 min of each 10 to 40-min period. $\bar{x}$ = mean amplitude ± SEM; N = total number of responses; AP = action potentials evoked by e.p.s.p.'s.

The sensitivities to these blocking agents are comparable to those for nicotinic transmission from preganglionic nerve endings to principal cells in intact ganglia (see *Discussion*).

Sensitivity of the Neurons to Acetylcholine (AcCh). We have examined whether the neurons in culture exhibit AcCh-sensitivity which is expected of sympathetic nerve cells and which is a precondition for cholinergic transmission. Obata (19), working with younger cultures, found that the neurons were depolarized by AcCh applied either in the perfusion fluid or iontophoretically to the surface of a cell body; the level of

sensitivity was about 100 millivolts depolarization per nanocoulomb delivered from the AcCh pipette. In the older cultures used in the present experiments, higher sensitivities to AcCh, in the range 500–2500 mV/nC, were frequently observed. The regions of high sensitivity appeared to be sharply circumscribed and at such spots the AcCh-response could be made to mimic closely the appearance of an e.p.s.p.

These electrophysiological and pharmacological observations, taken with the demonstration by Patterson and Chun (11) that in L-15-CO_2 the cultures synthesize appreciable acetylcholine, leave little doubt that under these conditions principal cells form cholinergic synapses with each other.

The incidence of cholinergic "driver" cells

The probability that two impaled cells were linked by a cholinergic synapse increased with age in culture. When cells were grown as described in *Methods*, cholinergic e.p.s.p.'s were rarely seen earlier than 18 days after plating; their incidence was higher after 4–6 weeks than after 3 weeks. However, there was considerable variability between platings. In some, interaction was common at 3 weeks and by 4–6 weeks as many as one-quarter to one-half of neuron pairs tested showed cholinergic interaction. In others, no interaction was seen even at 6–10 weeks.

Our experience with cultures grown in HCO_3^--free medium (L-15-Air; ref. 10), in which few nonneuronal cells are present, is as yet limited. However, it is our impression that the incidence of cholinergic interaction in such cultures is much lower than in those grown in L-15-CO_2. This is consistent with the findings of Mains and Patterson (10) and Patterson and Chun (11) that net AcCh synthesis is relatively small or undetectable in L-15-Air cultures.

DISCUSSION

The chemical synapses formed between the sympathetic neurons in culture were excitatory. Pharmacological observations indicated they were cholinergic: The effective concentrations of the nicotinic blocking drugs were similar to, or less than, those reported for nicotinic-cholinergic synapses in intact sympathetic ganglia of adult mammals (20–23). The sensitivity to atropine was also comparable to that of nicotinic junctions (ref. 17; see also ref. 18) but was at least 100-fold lower than that of muscarinic AcCh receptors in intact ganglia (17, 24, 25).

The formation of excitatory, cholinergic synapses between sympathetic neurons was unexpected. Evidence for synapses between principal neurons in intact ganglia has been obtained with anatomical methods; however, these synapses appear to be adrenergic (26–30), and are presumed to be inhibitory because the effects of catecholamines on ganglionic transmission are inhibitory (e.g., refs. 20 and 31). We know of no evidence for the presence of cholinergic synapses between principal cells *in vivo* (21, 28, 32), nor did we see any indication of inhibitory synapses between the neurons in culture. The fact that the cholinergic blocking agents, in moderate concentrations, abolished transmission suggests that there was no substantial noncholinergic component of the e.p.s.p.'s; moreover, Obata (19) has reported that catecholamines in high concentrations cause no change in membrane potential of rat sympathetic neurons in culture.

Perhaps the most conservative explanation for the cholinergic synapses is that some of the neurons originally isolated from the ganglia were destined to be cholinergic *in vivo*, and that in culture they retained this characteristic and formed principal-cell synapses. There is strong evidence for a small population of cholinergic sympathetic neurons in certain cat ganglia (33, 34); only ambiguous histochemical evidence is available for rat superior cervical ganglion (35–37). Possible difficulties with this explanation are that: (*1*) the proportion of "driver" cells in some older cultures was much higher than the fraction of cholinergic neurons generally thought to be present in sympathetic ganglia and (*2*) the electron microscopical observations on the cultures provided no evidence for two separate classes of synapses; 5-hydroxydopamine, which provides a marker for adrenergic vesicles *in vivo*, labeled a small fraction (about 10%) of vesicles in nearly all putative synaptic endings examined.

A less conventional explanation for the synapses in culture is that they are formed by a single population of neurons that simultaneously synthesize catecholamines and AcCh. Such dual synthesis is implied by the controversial proposal by Burn and Rand that an adrenergic terminal releases AcCh which, in turn, controls secretion of catecholamine (38, 39). It will be useful to determine whether cholinergic "driver" cells also exhibit the formaldehyde-induced fluorescence specific for catecholamines.

Another obvious explanation for these synapses is that the neurons in culture are displaying unusual properties, perhaps responding to alterations of the developmental cues that *in vivo* control the character of synapses. If so, the cultures may be useful for investigating such cues. In a number of respects, however, the cultured neurons exhibit characteristics expected of sympathetic principal cells: their general cytology as seen in the electron microscope; resting and action potentials similar to those recorded in intact ganglia; a high level of AcCh sensitivity; the synthesis and accumulation of catecholamines (11).

Whatever the explanation for the presence of cholinergic cells in our cultures, a significant factor in the formation of the synapses was likely to have been the absence of the normal preganglionic input. After denervation sympathetic neurons are known to accept cholinergic endings from diverse sources (see ref. 40 for references). It is, therefore, natural to wonder whether cholinergic, principal-cell synapses are made in denervated ganglia *in vivo*, and whether such synapses contribute to the recovery of function that develops in partially denervated ganglia (e.g., ref. 41).

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