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Formation of Cholinergic Synapses Between Dissociated Sympathetic Neurons and Skeletal Myotubes of the Rat in Cell Culture

(neuromuscular junctions/synaptic transmitters/acetylcholine receptors/cholinergic blocking drugs)

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ABSTRACT Sympathetic principal neurons, dissociated from superior cervical ganglia of newborn rats, were plated into cultures containing rat skeletal myotubes formed from previously plated primary myoblasts. Electrophysiological evidence is presented that the neurons developed cholinergic synapses with the myotubes. In addition, the neurons developed cholinergic synapses with each other as previously reported [O'Lague *et al.* (1974) *Proc. Nat. Acad. Sci. USA* 71, 3602–3606]. The acetylcholine receptors of myotubes differed from those of the neurons in their sensitivities to curare and hexamethonium, in a manner expected of adult muscle and ganglionic receptors. α-Bungarotoxin blocked synaptic transmission from neuron to myotube, but not from neuron to neuron in the same culture.

In a series of papers on cross-union of vertebrate nerves published between 1887 and 1904, Langley and Anderson (see refs. 1 and 2) reported that denervated skeletal muscles can be reinnervated by a variety of motor nerves and autonomic preganglionic nerves, but not by postganglionic sympathetic nerves. Later, when it was shown that the effective axons were all cholinergic, and that the ineffective axons were largely or exclusively adrenergic, Dale (3) suggested that a neuron's ability to reinnervate the denervated skeletal muscle was determined by its transmitter and not by its anatomical origin. Subsequent reports have been consistent with Dale's suggestion (4–8, see also refs. 9 and 10).

Several investigations suggest that tissue culture offers opportunities to investigate this matter further. Neuromuscular junctions have been shown to form when neurons, derived from spinal cord, are cultured with muscle fragments (11) or myotubes, formed either from primary (12–15) or cloned (16) myoblasts. Further, Crain and Peterson (17) have reported junction formation between explants of sympathetic ganglia and muscle fragments. In all these cases, the junctions are reported to be cholinergic.

In this laboratory, sympathetic principal neurons dissociated from the superior cervical ganglia of newborn rats have been found to develop cholinergic synapses with each other in culture (ref 18, see also ref 19). In light of the above findings, it was of interest to determine whether these cholinergic sympathetic neurons would innervate developing skeletal muscle cells in culture.

In this paper we report that these neurons form cholinergic synapses with primary rat myotubes and with each other as found previously (18). In addition, the two types of synapses differ in their sensitivities to several cholinergic blocking drugs.

METHODS

Preparation of Muscle Cultures. Pectoral muscles from newborn rats (Charles River, CD) were dissected under sterile conditions and cut into pieces of approximately 1 mm^3. The pieces were gently stirred at 37° for successive periods of 15, 20, and 20 min, in a Ca-Mg-free, phosphate-buffered (pH 7.4) salt solution containing (mM): NaCl, 130; KCl, 5.4; $NaH_2PO_4 \cdot H_2O$, 14.5; glucose 4.2; phenol red, 0.03; and 1 mg/ml of collagenase (Worthington Biochemical Corp.). The 15-min supernatant was discarded. The two 20-min supernatants were centrifuged at 240 × g for 3 min and the enzyme was inactivated by resuspending the cell pellets in growth medium (see below). These cell suspensions were then pooled, pelleted, resuspended in growth medium, and passed through a 10 μm Nitex filter (Tobler, Ernst, and Traber, Inc.). The number of fibroblasts was reduced by pre-plating the resulting cell suspension at 37° for 15 min in plastic tissue culture dishes (20). The nonadhering cells were harvested, counted in a hemacytometer, and plated (10,000 cells per dish) into the collagen-coated central well (area about 1.5 cm^2) of culture dishes prepared as previously described (21, 22). The growth medium was a modified form of L-15-CO_2 medium (22) containing 10% fetal calf serum (Microbiological Assoc.) but lacking Methocel, "vitamin mix," bovine serum albumin, and adult rat serum. The cultures were kept in a humid atmosphere of 92% air–8% CO_2 at 35–36° and the medium was changed every 4–5 days. Growth of fibroblasts was reduced in either of two ways: (*a*) on about the fourth day the cultures were exposed to 10 μM arabinosylcytosine for 2–3 days (15); (*b*) after about 1 week and prior to addition of sympathetic neurons (see below) the muscle cultures were irradiated with 5000 rads at a dose rate of approximately 227 rads/sec from a cobalt-60 source (5000 Ci). These procedures reduced the division of fibroblasts, but did not noticeably hinder subsequent muscle differentiation.

Preparation of Mixed Cultures. Superior cervical ganglia from newborn rats (Charles River, CD) were mechanically dissociated as previously described (21, 22). The cell suspension, containing isolated neurons and small cell-clusters from 40 ganglia, was plated into 12 muscle cultures approximately 1 week old. These mixed cultures were grown as described above, except that nerve growth factor was present in the growth medium.

Electrophysiological Methods. These methods have been described elsewhere (18). Intracellular microelectrode record-

Abbreviations: AcCh, acetylcholine; e.j.p., excitatory junction potential; m.e.j.p., miniature excitatory junction potential; e.p.s.p., excitatory post-synaptic potential; α-BuTX, α-bungarotoxin.

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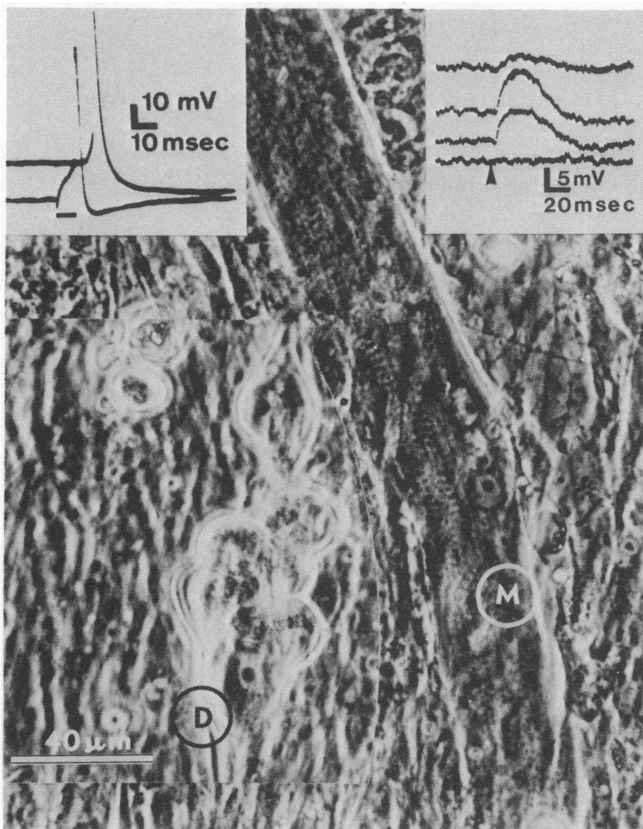

FIG. 1. Synaptic interaction between a sympathetic neuron and a myotube in culture. The phase-contrast micrograph (a montage) shows a "driver" neuron (D) within a small clump of neurons and a striated myotube (M) from a 16-day-old culture (neurons added at day 7). Upper right: excitatory junction potentials (e.j.p.'s) recorded in myotube M. Arrow indicates time of peak of action potential in neuron D; beam displaced between sweeps. Note variation in e.j.p. amplitude including occasional failure (bottom trace). Upper left: intracellular recording of an action potential (peak not shown) in myotube M (upper trace) evoked by an action potential in neuron D (lower trace). Lower black bar: Duration of depolarizing current pulse in neuron D.

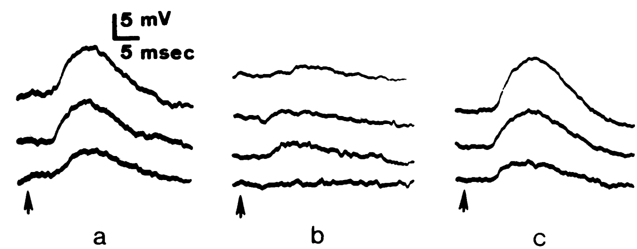

FIG. 2. Effect on synaptic transmission of changing the extracellular Ca^{++} and Mg^{++}. (a) and (c), e.j.p.'s recorded in a 21-day-old culture (neurons added at day 8) perfused with 0.18 mM Mg^{++}, 2.8 mM Ca^{++} (normal perfusion concentrations), before and after 2.2 mM Mg^{++}, 0.4 mM Ca^{++} (b). Arrowheads indicate time of peak of the presynaptic action potential.

ings were done at 34–36° during continuous perfusion of the culture. The perfusion fluid contained 100 ml/liter of basal L-15 medium (22) and (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) and bovine-serum albumin (0.6 g/liter) were added just before use; the pH was kept at about 7.4 by bubbling an air: CO_2 (95:5) mixture.

Acetylcholine (AcCh) was applied iontophoretically by the method of del Castillo and Katz (23); other drugs were applied in the perfusion fluid. Drugs were obtained from the following sources: *d*-tubocurarine chloride, Mann Research Laboratories; atropine sulfate, Merck; hexamethonium chloride, Pfaltz and Bauer, Inc.; tetrodotoxin, acetylcholine chloride, and arabinosylcytosine, Sigma Chemical Co.

RESULTS

Most experiments were performed on cultures 16 to 26-days old (9–19 days after adding neurons). Synaptic activity in cultures irradiated (see *Methods*) or exposed to arabinosylcytosine did not differ qualitatively from that in untreated cultures. However, irradiated cultures were used in experiments reported here, because they were easier to prepare and provided better control of cell proliferation.

The appearance of the myotubes was similar to that reported by others (15, 24, 25). By day 7, elongated, multinucleated myotubes had formed and during the second week many of these contracted spontaneously. Resting potentials of 55–80 mV and action potentials of 75–110 mV were routinely observed in intracellular recordings from myotubes. Action potentials were followed by a prolonged after-hyperpolarization, as seen in other skeletal muscle cultures (24, 26) and in denervated muscle fibers (27). Spontaneously occurring subthreshold potentials and rhythmic spikes (26, 28) similar to the fibrillation seen in denervated muscle fibers (28–30) were routinely recorded in many myotubes.

In mixed cultures, neuronal processes grew and formed a complex network, bringing the neurons into contact with each other and with the myotubes. The growth characteristics and electrophysiological properties of the neurons were similar to those reported in sympathetic neuron cultures (18).

Synaptic Interaction. Tests for synaptic interaction between neurons and myotubes or between one neuron and another were made by randomly impaling pairs of cells with microelectrodes, and stimulating a neuron while recording from the other cell. Synaptic interactions were seen with both cell combinations: the neuron–neuron interaction was indistinguishable from the cholinergic transmission previously described (18). The neuron–myotube interaction was chemical, and pharmacological evidence leaves little doubt that it was also cholinergic. The major purpose of this paper is to describe the electrophysiological and pharmacological properties of neuron–myotube junctions and certain differences between them and the neuron–neuron synapses.

Neuron–Myotube Interaction. In 42 of about 300 random tests, an evoked neuronal action potential gave rise to a depolarizing postsynaptic response in the myotube. These responses will be called excitatory junction potentials, e.j.p.'s. Examples are shown in the oscilloscope traces in the upper right corner of Fig. 1. The oscilloscope beam was displaced between sweeps to show individual e.j.p.'s of myotube M, evoked by consecutive action potentials in neuron D ("driver"). The peak of the action potential in neuron D occurred at the time shown by the arrowhead. These e.j.p.'s arose about 3 msec after the peak of the presynaptic spike and their amplitudes varied in a manner reminiscent of quantal

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transmission at chemical synapses (35). Occasional e.j.p.'s in myotube M were suprathreshold; in the example illustrated in the upper left corner of Fig. 1, an action potential evoked in neuron D (bottom trace) by a depolarizing current pulse (duration shown by black bar) gave rise to an action potential in M (top trace). A latency of 2–3 msec from the peak of the presynaptic action potential to the beginning of the e.j.p. was frequently observed. This, together with pharmacological evidence described later, suggests that the neuron and myotube were in direct synaptic contact. In some cases, latencies of 7–20 msec were observed, indicating longer conduction paths possibly involving intercalated neurons. The time to peak of the e.j.p.'s ranged from 2.5 to 10 msec and time to half decay from 4.5 to 20 msec. These values are comparable to those reported for developing neuromuscular junctions *in vivo* (31–34, see also ref. 8).

Three electrophysiological observations indicate that the e.j.p.'s arose at chemical synapses: (*i*) there was always a minimum latency of 2–3 msec, and passive transfer of hyperpolarizations and depolarizations did not occur; (*ii*) in the six cases tested, transmission was reduced as at chemical synapses generally (35) by lowering the extracellular Ca^{++}/Mg^{++} ratio. In Fig. 2 control responses in normal perfusion fluid (2.8 mM Ca^{++}; 0.18 mM Mg^{++}) are shown before (*a*) and after (*c*) changing to 0.4 mM Ca^{++} and 2.2 mM Mg^{++}, which greatly reduced the evoked responses (Fig. 2b); (*iii*) the evoked e.j.p.'s appeared to vary in a stepwise manner (Fig. 3b) as would be expected of quantal transmission (35).

Effect of Cholinergic Blocking Drugs on Neuron–Myotube Interaction. The sensitivities of e.j.p.'s to cholinergic antagonists that affect neuromuscular transmission *in vivo* (36, 37) were tested. *d*-Tubocurarine at 0.7 μM (2 trials) reduced the e.j.p. by about 75% and at 1.4 μM (10 trials) abolished it reversibly. This curare sensitivity is similar to that at neuromuscular junctions in culture (15, 16) but is less than that reported for adult rat neuromuscular junctions *in vivo* (36). Although hexamethonium is a classical ganglionic blocking agent, at higher concentrations it will block neuromuscular transmission (39, 40). As expected, hexamethonium was less effective than curare; 37 μM (4 trials) produced little or no effect, whereas 90 μM (3 trials) caused about a 40% reduction in the e.j.p. In this small number of trials, the sensitivity to hexamethonium was consistently higher than the values reported for the neuromuscular junctions of adult rat diaphragm (39, 40). Atropine, which affects nicotinic synapses at much higher concentrations (36) produced a 90% reduction at 270 μM (3 trials). In these drug experiments, the percentage reduction of e.j.p.'s was determined using the means of responses recorded before and after drug perfusion as control amplitudes. These results suggest that the neuron–myotube synapses were nicotinic-cholinergic and provide additional evidence for a chemical link between these cells.

α-Bungarotoxin (α-BuTX), which blocks endplate potentials in rat skeletal muscle (37, 38), abolished both the e.j.p.'s (6 trials) and the responses to iontophoretically applied acetylcholine (see later) at 0.2 μM. Blockage occurred 2–3 min after application and did not reverse after washing for 45 min, the longest period tested.

Neuron–Neuron Interaction. These synapses were pharmacologically similar to the cholinergic synapses studied previously (18). The excitatory postsynaptic potentials (e.p.s.p.'s) between the neurons were considerably reduced by hexamethonium and curare at 10 μM and blocked by either at about 50 μM. Unlike its effect on the e.j.p.'s, α-BuTX (7 trials) failed to affect the e.p.s.p.'s even after perfusion for several hours at concentrations up to 0.6 μM, three times higher than that which blocked e.j.p.'s in the same culture. In a previous study on cultured rat sympathetic neurons, α-BuTX, even at a much higher concentration (3 μM), failed to affect the acetylcholine sensitivity (26). We know of no other electrophysiological study on the effect of α-BuTX on sympathetic neurons *in vivo* or *in vitro*. In an autoradiographic study, dissociated chick sympathetic neurons were found to bind α-BuTX *in vitro* (41), but the acetylcholine sensitivity of these cells in the presence of α-BuTX was not reported.

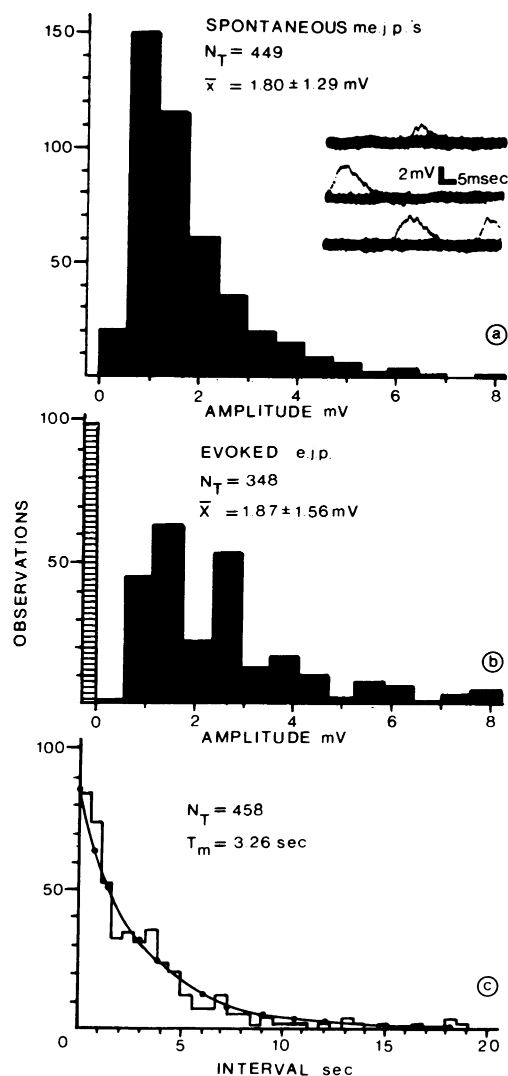

FIG. 3. Amplitude and interval histograms of synaptic potentials recorded from a single myotube in a 26-day-old culture (neurons added at day 7): (a) amplitude histogram of miniature excitatory junction potentials, m.e.j.p.'s, in the presence of 3 μM tetrodotoxin. Inset: three oscilloscope traces, each trace superimposed, showing typical m.e.j.p.'s. (b) amplitude histogram of the evoked e.j.p.'s. Height of crossed bar indicates number of failures; width represents baseline noise. (c) interval histogram of the m.e.j.p.'s shown in (a); continuous curve drawn for a stochastic process $n = N_T \Delta t / T_m \exp(-t/T_m)$. Mean interval T_m = 3.26 sec; class interval, Δt = 600 msec; N_T = total number of responses; $\bar{x}$, $\bar{X}$ = mean amplitudes ± standard deviation.

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Several driver neurons were found to interact with both myotubes and other neurons. For example, an action potential in the driver neuron of Fig. 1 also produced an e.p.s.p. in a neuron several hundred μm away (not shown; latency 2.6 msec). This response was practically abolished by 37 μM hexamethonium (a concentration which had little or no effect on the e.j.p.). In contrast, the e.p.s.p. was not noticeably reduced by curare at 1.4 μM, a concentration which abolished the e.j.p. recorded from the myotube M (see Fig. 1). The persistence of the e.j.p. in 37 μM hexamethonium is consistent with a monosynaptic connection between D and M, since at this concentration interposed neuron–neuron cholinergic synapses would presumably have been blocked (18); however, an interposed electrical neuron–neuron synapse, capable of transmitting one-to-one, was not ruled out.

Acetylcholine (AcCh) Sensitivity of the Myotubes. Myotubes were sensitive to iontophoretically applied AcCh as in other studies (42, 43). Measurements were usually made in the presence of 3 μM tetrodotoxin (TTX), to eliminate active responses (44). The sensitivity varied over the surface membrane of individual multinucleated myotubes from about 1700 to 5000 mV depolarization per nC of iontophoretic current (mV/nC) (30 myotubes in four cultures, ages 14–19 days), values somewhat higher than those reported in other studies of myotubes in culture (26, 42, 43). In most cases the AcCh sensitivity of innervated myotubes (15 cases) was similar to that in control cultures lacking neurons. However, in one older culture (22 days old) the four innervated myotubes tested were found to have high sensitivities (3000–4000 mV/nC) but restricted to two or three spots (about 10 μM diameter); neighboring membrane had sensitivities less than 100 mV/nC. In innervated myotubes the AcCh response could be made to mimic the time course and amplitude of the e.j.p.'s. In other experiments AcCh potentials were reversibly reduced to about half of control amplitudes by bath-applied *d*-tubocurarine chloride (0.7 μM, 2 trials) and blocked by 0.2 μM α-BuTX (2 trials).

AcCh sensitivity of the neurons was similar to that reported previously (18). In addition, α-BuTX failed to affect neuronal sensitivity at 0.6 μM, a concentration three times higher than that which blocked the myotube AcCh sensitivity in the same culture (see also ref. 26).

Spontaneous Activity in Myotubes. Curare-sensitive, spontaneous potentials resembling e.j.p.'s and often giving rise to action potentials were frequently recorded in myotubes from mixed cultures. The presence of more than one functional neuronal contact was suggested in some myotubes by the occurrence of spontaneous e.j.p.'s of similar amplitude but different rise times. Multiple innervation has been reported during development of neuromuscular junctions both *in vivo* (32, 33, 45) and *in vitro* (13, 15).

When tetrodotoxin (3 μM) was used to eliminate neuronal action potentials, spontaneous potentials sensitive to curare and resembling the miniature endplate potentials (m.e.p.p.'s) of adult muscle fibers were seen. The amplitudes of these potentials (m.e.j.p.'s) ranged from 0.5 to 8 mV and their distribution was skewed (see Fig. 3a). The mean frequency was from 1 to 20/min and in the few cases tested, the intervals between m.e.j.p.'s appeared to be randomly distributed (Fig. 3c), as is generally true of m.e.p.p.'s (46, 47). The presence of these potentials provided additional evidence for a chemical link between the sympathetic neurons and myotubes.

Incidence of Innervated Myotubes. In some cultures, each of 20 or more myotubes picked at random showed spontaneous synaptic potentials (e.j.p.'s and/or m.e.j.p.'s). Since electrical coupling between myotubes appeared to be rare (e.g., their contractions almost always occurred independently), it is likely that each myotube was separately innervated. On the other hand, only about one in seven neuron–myotube pairs picked at random was found to interact. Neurons that produced e.j.p.'s in myotubes could not be distinguished either electrophysiologically or visually from other neurons. Doubtless the fraction of neurons capable of forming junctions exceeds one in seven, but no attempts were made to sample many myotubes while stimulating a given neuron.

DISCUSSION

Pharmacological evidence presented here leaves little doubt that under our culture conditions the chemical synapses formed between the dissociated sympathetic neurons and skeletal myotubes were cholinergic; transmission, as at adult rat neuromuscular junctions, was more sensitive to curare than hexamethonium (39, 40) and was abolished by α-BuTX (37, 38). The effective concentration of atropine was similar to that at nicotinic junctions (36). The fact that these cholinergic blocking agents, in moderate concentrations, abolished transmission suggests that there was no appreciable non-cholinergic component of the e.j.p.'s.

The electrophysiological properties of synapses between these cholinergic sympathetic neurons and myotubes differed from adult neuromuscular junctions in several ways, e.g., slow time course of the synaptic potentials, low quantal content (unpublished observations), and low frequency of miniature synaptic potentials. However, these differences do not appear to be peculiar to sympathetic neurons, since the same differences are found for synapses formed between spinal cord neurons and myotubes in culture (15).

The formation of cholinergic junctions between the sympathetic principal neurons and skeletal myotubes reported here raises the question whether similar junctions occur *in vivo.* Adult postganglionic sympathetic neurons, largely or exclusively adrenergic, do not normally form synapses with skeletal muscle fibers (see refs. 48 and 49), nor, as far as we know, have such synapses been reported in developing vertebrates, including the rat (50, 51). Effects attributed to the release of acetylcholine from sympathetic nerves onto denervated skeletal muscle have been known for many years. In 1885, Rogowicz (52) showed that, in the dog, the buccofacial dilatation caused by stimulation of the cervical sympathetic trunk was associated with contracture of adjacent motor-denervated facial muscles. v. Euler and Gaddum (53) found that the contracture was potentiated by eserine and concluded that the effective axons were cholinergic and arose from neurons in the superior cervical ganglion. It is not clear whether this contracture was mediated by release of acetylcholine into the tissue fluid, or in part by direct synaptic contact between the cholinergic sympathetic axons and the denervated skeletal muscle fibers. However, in some cases the latency from stimulation to contracture was longer than expected for direct contact.

Attempts to reinnervate adult skeletal muscle with sympathetic postganglionic axons from the superior cervical

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ganglia of cats (1, 9) and rats (10) have not been successful. The above results suggest that functional contacts between sympathetic postganglionic neurons and skeletal muscle *in vivo*, if they occur at all, are rare. So far as we know, no one has attempted to reinnervate skeletal muscle with postganglionic sympathetic neurons in animals whose postnatal age is comparable to that of our cultures.

Dale suggested that a neuron's ability to secrete acetylcholine may be a prerequisite for reinnervation of denervated skeletal muscle regardless of the neuron's anatomical origin (3). Certainly our results are consistent with this. The presence of cholinergic neurons in cultures of dissociated sympathetic neurons was previously demonstrated, and their origin was discussed in some detail (18). The question arises whether the neurons that innervated the myotubes were a special class, in some ways different from those forming neuron–neuron synapses. Although no definitive answer can be given at present, we think this unlikely, since, in several instances, single neurons that formed neuronal synapses similar to the cholinergic synapses reported previously (18) also appeared to make direct synaptic contact with myotubes.

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