Localization of Acetylcholine Receptors and Synaptic Ultrastructure at Nerve-Muscle Contacts in Culture: Dependence on Nerve Type

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ABSTRACT In cultures of *Xenopus* myotomal muscle cells and spinal cord (SC) some of the nerve-muscle contacts exhibit a high density of acetylcholine receptors (AChRs [Anderson et al., 1977, *J. Physiol. (Lond.)*. 268:731–756, 757–773]) and synaptic ultrastructure (Weldon and Cohen, 1979, *J. Neurocytol.* 8:239–259). We have examined whether similarly specialized contacts are established when the muscle cells are cultured with explants of *Xenopus* dorsal root ganglia (DRG) or sympathetic ganglia (SG).

The outgrowth from the ganglionic explants contained neuronal and non-neuronal cell processes. Although both types of processes approached within 100 Å of the muscle cells, synaptic ultrastructure was rarely observed at these contacts. Because patches of postsynaptic ultrastructure also develop on noncontacted muscle cells, the very few examples of contacts with such specializations probably occurred by chance.

AChRs were stained with fluorescent α-bungarotoxin. More than 70% of the SC-contacted muscle cells exhibited a high receptor density along the path of contact. The corresponding values for DRG- and SG-contacted muscle cells were 10 and 6%. Similar values were obtained when the ganglionic and SC explants were cultured together in the same chamber. The few examples of high receptor density at ganglionic-muscle contacts resembled the characteristic receptor patches of noncontacted muscle cells rather than the narrow bands of high receptor density seen at SC-muscle contacts. In addition, $> 90\%$ of these ganglionic-contacted muscle cells had receptor patches elsewhere, compared to $< 40\%$ for the SC-contacted muscle cells.

These findings indicate that the SC neurites possess a specific property which is important for the establishment of synaptically specialized contacts with muscle and that this property is lacking in the DRG and SG neurites.

The postsynaptic membrane of the vertebrate neuromuscular junction contains a high density of acetylcholine receptors (AChRs). A localized high density of AChRs has also been observed repeatedly at some of the nerve-muscle contacts which form in culture (4, 5, 10, 18, 21, 23, 24, 32). In some of these studies it has been found that the contacts with high receptor density are located on functionally innervated muscle cells. For example, in cultures derived from SC and muscle of chick embryos, it has been demonstrated directly that the high receptor density at nerve-muscle contacts is situated at sites of transmitter release (18, 24). Such contacts also exhibit characteristic pre- and postsynaptic ultrastructural specializations (24). Likewise, in cultures derived from spinal cord (SC) and muscle of *Xenopus* embryos functionally innervated muscle cells almost always have sites of high receptor density along the path of nerve-muscle contact (4, 5); conversely, muscle cells with a high receptor density along the path of contact invariably exhibit synaptic activity (5). Some of the nerve-muscle contacts in these cultures also have typical pre- and postsynaptic ultrastructure (65). Studies employing cloned neuroblastoma and muscle cells have also revealed a high receptor density at the nerve-muscle contacts but in these cultures the

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contacts do not exhibit synaptic function (28, 33, 60) or typical synaptic ultrastructure (33).

The question of neural specificity in the localization of AChRs at nerve-muscle contacts therefore arises. For example, in cultures of *Xenopus* SC and muscle ~ 70% of the nerve-contacted muscle cells exhibits sites of high receptor density along the path of contact (5; also this paper). A simple explanation is that the SC contains different types of nerve cells and only some are competent to establish such contacts. An alternative possibility is that all nerve cells are competent in this regard but not all of the cultured muscle cells are responsive. To assess such possibilities we have cultured muscle cells together with explants of SC, dorsal root ganglia (DRG), and sympathetic ganglia (SG), and have examined the resulting nerve-muscle contacts for a high density of AChRs and for synaptic ultrastructure. Our results indicate that, unlike SC neurites, DRG and SG neurites have little if any capacity to form muscle contacts with such synaptic specializations. Brief accounts of this work have been reported elsewhere (16, 17).

MATERIALS AND METHODS

Preparation of Cultures

Myotomal muscle and SC were isolated from stage 22–26 *Xenopus laevis* embryos (47) and cultured as previously described (4). Briefly, the myotomes and SC were separated from each other and from adjacent tissues by treating with collagenase (1 mg/ml) and were then dissociated in a calcium-magnesium-free solution of trypsin (5 mg/ml) and EDTA (2 mg/ml) and added to the culture chambers. In some cases the SC were dissociated without the addition of trypsin or EDTA. The culture medium consisted of L-15 (67%) and Holmes' α-1 protein (~ 0.2 μg/ml). For the 1st d dialyzed horse serum (5%) was also included in the medium. In cultures containing SC D-tubocurarine chloride (5–10 μg/ml) was usually added to abolish spontaneous contractions of muscle cells contacted by the SC neurites.

DRG were isolated from stage 53-58 *X. laevis* tadpoles which had been kept overnight in a solution of gentamycin (100 μg/ml) and Mycostatin (100 U/ml; Grand Island Biological Co., Grand Island, N. Y.). Only the anterior-most 12 or so ganglia were isolated. At stages 53-58, the ganglia are ~ 3–6-wk old (47), there is considerable growth and turnover of the neuronal population (52) and the cells are electrically excitable (6). After treating the ganglia for ~ 20 min with collagenase, adhering tissue and associated nerves were removed and large ganglia were cut into smaller fragments. They were then placed in calcium-magnesium-free trypsin-EDTA for up to 1 h. This treatment did not dissociate the cells but appeared to be essential for good plating. Between two and five ganglionic fragments were added to single culture chamber.

SG were isolated from *Xenopus* juveniles weighing 0.5–2 g. At these stages the ganglia are at least 2-mo old (47) but they are still relatively small and difficult to isolate. In most cases ganglia 6–9 were isolated from both sympathetic chains but other ganglia, such as 3 and 4, were sometimes used for culture as well (61). The ganglia were treated in the same way as described above for DRG. In some cultures nerve growth factor (NGF; Collaborative Research Inc., Waltham, Mass.) was added to the culture medium.

Electron Microscopy

Cultures were prepared and maintained as described above except that the floor of the culture chamber was made from a sheet of Aclar (Allied Chemical Corp., Specialty Chemicals Div., Morristown, N.J.) rather than a glass coverslip to facilitate isolation of desired sections of the culture after embedding in plastic (42). All procedures, from fixation to examination in the electron microscope, were the same as described previously (65). The blocks were sectioned in semiserial fashion to sample their entire cellular content. The results for DRG-muscle cultures are based on examination of 10 selected blocks from two cultures in which the ages of muscle and nerve at the time of fixation were 3 and 7 d, respectively. The results for SG-muscle cultures are based on examination of ~ 20 blocks from three cultures in which the ages of muscle and nerve were 3 and 9 d, respectively, in two cultures, and 4 and 10 d in the third culture.

Fluorescent Staining of AChRs

Receptors were stained with tetramethylrhodamine-labeled α-bungarotoxin (~ 10 μg/ml), and the staining patterns were examined in living cultures as described previously (4). The labeled toxin had a potency of ~ 2% relative to unlabeled toxin, as judged by inhibition of carbachol-induced contraction of tadpole myotomal muscle (2). Staining was entirely prevented by pretreating cultures for 30 min with 1 μg/ml unlabeled toxin and was also eliminated by the presence of 300 μg/ml curare.

In cases where the same culture was to be examined on successive days the culture was stained and then maintained in a relatively low concentration of the fluorescent toxin (~ 0.1 μg/ml). This procedure appeared to be effective in staining all AChRs because restaining the cultures with our standard procedure did not increase the staining intensity or reveal any additional patches of stain on the muscle cells. Judging from the low background fluorescence of the muscle cells, nonspecific binding to the cell surface was not increased. However, some of the muscle cells did exhibit small dots of stain, < 2 μm in diameter, some of which appeared to be located intracellularly as judged by focusing at a magnification of ×500. Such dots of stain were much less frequent in freshly stained cultures and probably reflect internalization of stain or stained receptors. Accordingly, they were not considered in evaluating the staining patterns of muscle cells.

Muscle cells were judged to be "contacted" by a neurite only if the neurite appeared at the same level of focus as the surface of the muscle cell when examined with phase-contrast optics at a magnification of at least ×500. The cells were then examined in their entirety for localization of AChRs. In the great majority of cases the contact was made by a growing neurite after the muscle cells had plated rather than by muscle cells happening to fall on neurites during the plating procedure (e.g., Figs. 1 and 2). In fact, in cultures containing DRG or SG explants, muscle cells located in the region of the original neuritic outgrowth often remained poorly developed presumably because they did not attach to the floor of the culture dish. Such muscle cells usually exhibited little if any receptor staining and were not counted. Patterns of receptor staining on noncontacted muscle cells were examined on cells beyond the periphery of the neuritic outgrowth.

RESULTS

Description of Cultures

The growth and differentiation of SC explants and myotomal muscle cells in culture was similar to that described previously (4). Pertinent differences between the development of these cultures and cultures containing explants of DRG or SG are included in the following two subsections.

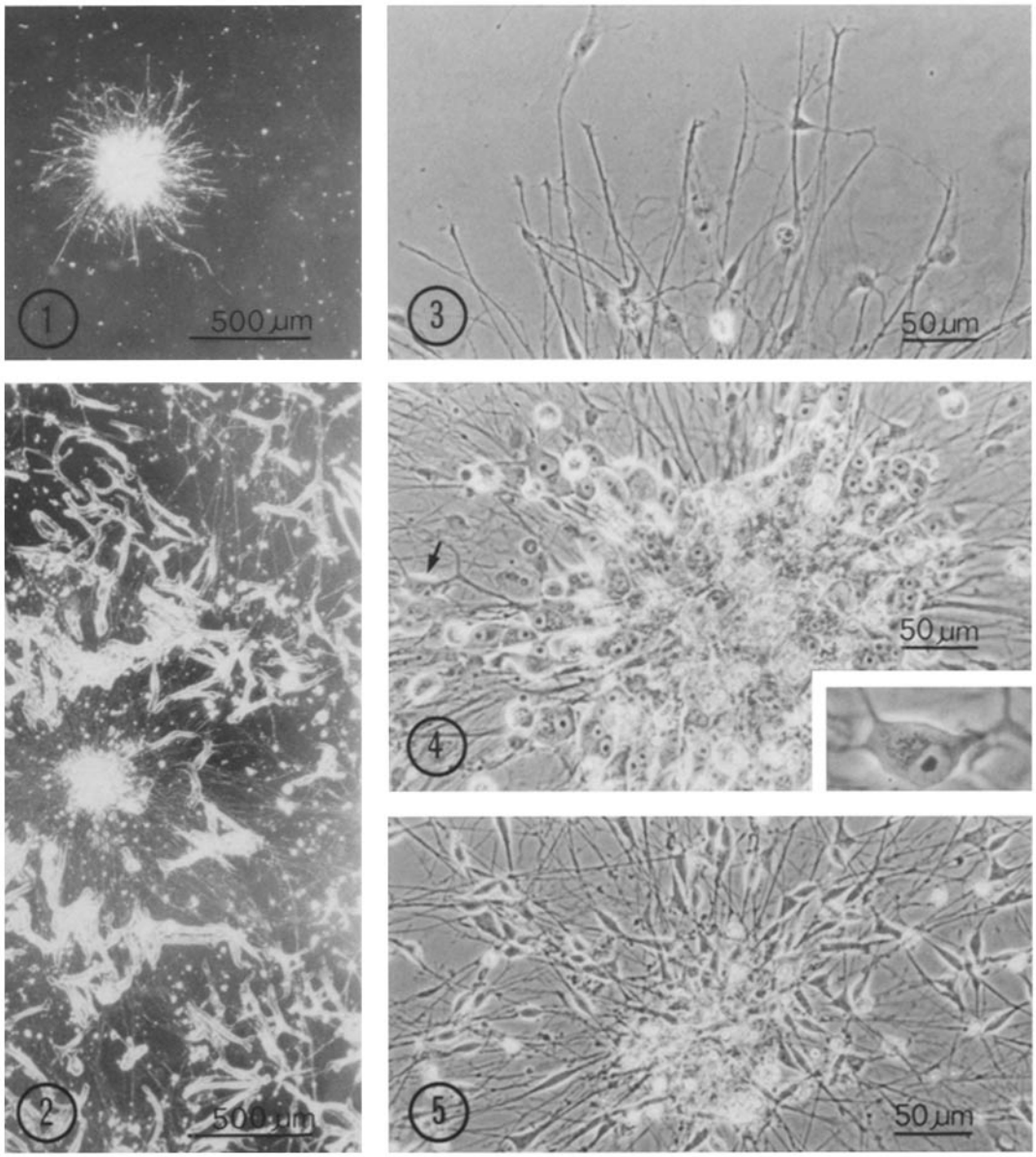
DRG-MUSCLE CULTURES: DRG explants became attached to the culture dish in 1–3 d. Within the next day or two a dense radial outgrowth of neurites emerged from the explants (Figs. 1 and 3). Muscle cells were usually added to the cultures before the neuritic outgrowth exceeded 500 μm. Unlike SC neurites, which begin to retract after 3–4 d in culture (4), the DRG neurites continued to grow for several days and encountered many muscle cells (Fig. 2). Some of the neurites were relatively uniform along their length (Fig. 3) whereas others exhibited scattered dilatations. Small spindle-shaped cells, presumed to be Schwann cells, could sometimes be recognized in association with the DRG neurites. Multipolar cells (see below) and fibroblasts were also seen scattered throughout the outgrowth region.

In young cultures characteristic DRG cell bodies were most readily resolved at the periphery of explants. After a few days, as the explants flattened and became less refractile, the DRG cell bodies were also clearly seen throughout the explant (Fig. 4). They had a pale spherical nucleus with a dark central nucleolus (Fig. 4, inset) typical of DRG neurons (39). Occasionally an explant was seen which lacked these characteristic DRG neurons but contained instead smaller multipolar and spindle-shaped cells (Fig. 5). These cells were similar in appearance to those seen at much lower density in the outgrowth region of the more typical DRG explants and were probably non-neuronal (39). Our studies of DRG nerve-muscle contacts refer to explants that contained a high density of typical DRG neurons.

Muscle cells beyond the borders of the original neuritic

outgrowth appeared to grow and differentiate as usual, at least for the first 4 d. From the 2nd d on they contracted when carbachol (10 μg/ml) was added to the culture chamber. However, in contrast to SC-contacted muscle cells (4), none of the DRG-contacted muscle cells contracted spontaneously. In addition, the DRG-contacted muscle cells did not contract in response to electrical stimulation of the DRG explants but we did not determine in these tests whether impulses were conducted along the neurites. After ~ 5 d in culture the muscle cells appeared to become less adherent to the culture dish and some began to detach especially in regions of dense neuritic outgrowth. Accordingly, we almost always limited our experiments to the first 4 d after plating the muscle cells.

SG-MUSCLE CULTURES: The growth of amphibian SG explants in culture has been previously described (29) and generally appeared similar in the present study. The explants became attached to the culture dish ~ 3–5 d after plating. Neuritic outgrowth then commenced and muscle cells were usually added before the outgrowth exceeded 500 μm. Over the next few days muscle cells beyond the borders of the original neuritic outgrowth differentiated as usual and many were contacted by the growing SG neurites (Fig. 6). The neuritic outgrowth from the SG explants (Figs. 6 and 7) was generally more fasciculated than that from the DRG explants (Figs. 1–3). In other respects the SG-muscle cultures were similar to DRG-muscle cultures: some of the SG neurites had dilatations



FIGURES 1–5 Growth and characteristics of DRG explants.

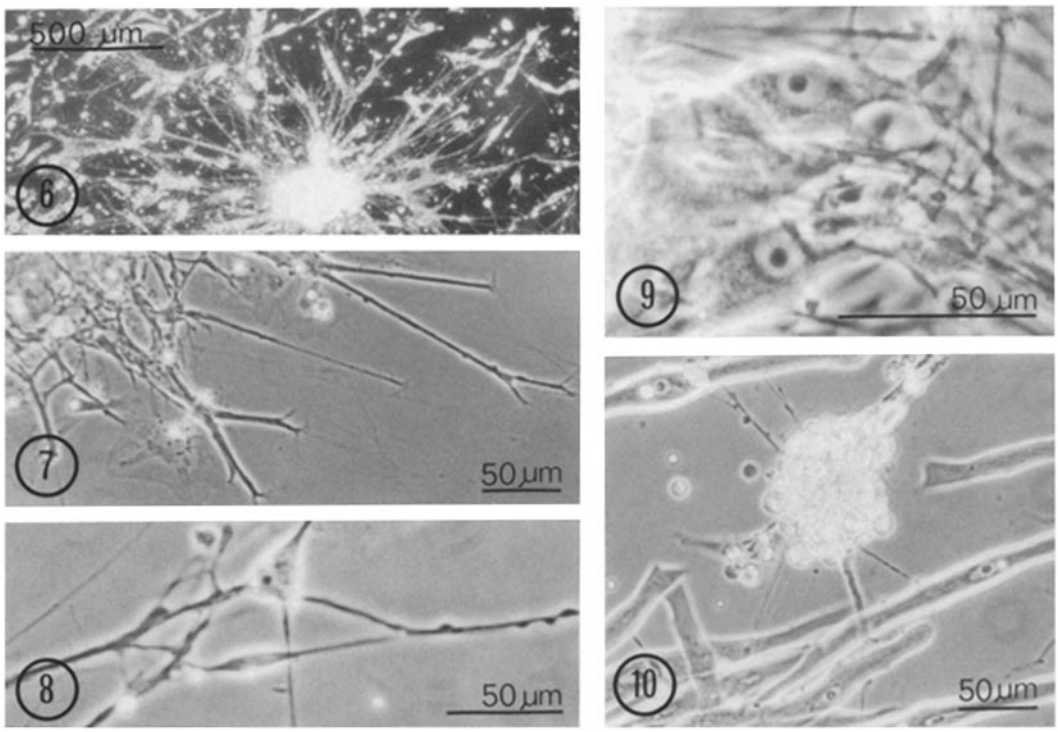
FIGURE 1 Dark field, low power, view of 3-d-old explant just before adding muscle cells.

FIGURE 2 Same field as Fig. 1, 6 d later. Neurites have grown extensively and cross many muscle cells.

FIGURE 3 Phase-contrast view of part of neuritic outgrowth from 3-d-old explant.

FIGURE 4 Cell bodies in 9-d-old explant. The arrow points to cell body shown at 2.5 times higher magnification in inset.

FIGURE 5 Atypical, 5-d-old, DRG explant. Cells are spindle-shaped and multipolar.



FIGURES 6–10 Growth and characteristics of SG explants.

FIGURE 6 Dark field, low power, view of explant and muscle cells in culture for 8 and 4 d, respectively. Neurites extend radially from explant and course across muscle cells.

FIGURE 7 Phase-contrast view of part of neuritic outgrowth of 4-d-old explant just before adding muscle cells.

FIGURE 8 Spindle-shaped and tripolar cells in association with neurites. 9-d-old explant.

FIGURE 9 Typical cell bodies at periphery of 7-d-old explant.

FIGURE 10 SC explant and muscle cells after 4 d in culture. Note the smallness of the SC explant and its sparse neuritic outgrowth.

(Figs. 7 and 8); small spindle-shaped and multipolar cells were observed, often in association with the growing neurites (Fig. 8); and characteristic SG cell bodies (51, 61) could be resolved at the periphery of the explant (Fig. 9). Some muscle cell detachment was also seen after ~ 5 d, especially in regions of dense neuritic outgrowth. At concentrations of 1–100 ng/ml NGF did not appear to enhance neuritic outgrowth (but see 29). A lack of dependence upon exogenous NGF has also been observed for cultures of embryonic mouse SG (19) and may vary among different species.

In some experiments, fragments of SC were added to cultures of SG explants (and DRG explants) at the same time as the muscle cells. The SC explants were placed some distance away from the SG explants and were readily identified by their smaller size and less extensive neuritic outgrowth (Fig. 10). In contrast to some of the muscle cells contacted by SC neurites, those contacted by SG neurites did not twitch either spontaneously or in response to electrical stimulation of the explant.

Electron Microscopy

Cultures containing SC explants and myotomal muscle cells from *Xenopus* embryos have recently been examined with the electron microscope (65). Pertinent ultrastructural differences between these cultures and cultures containing DRG and SG explants are included in the following subsections.

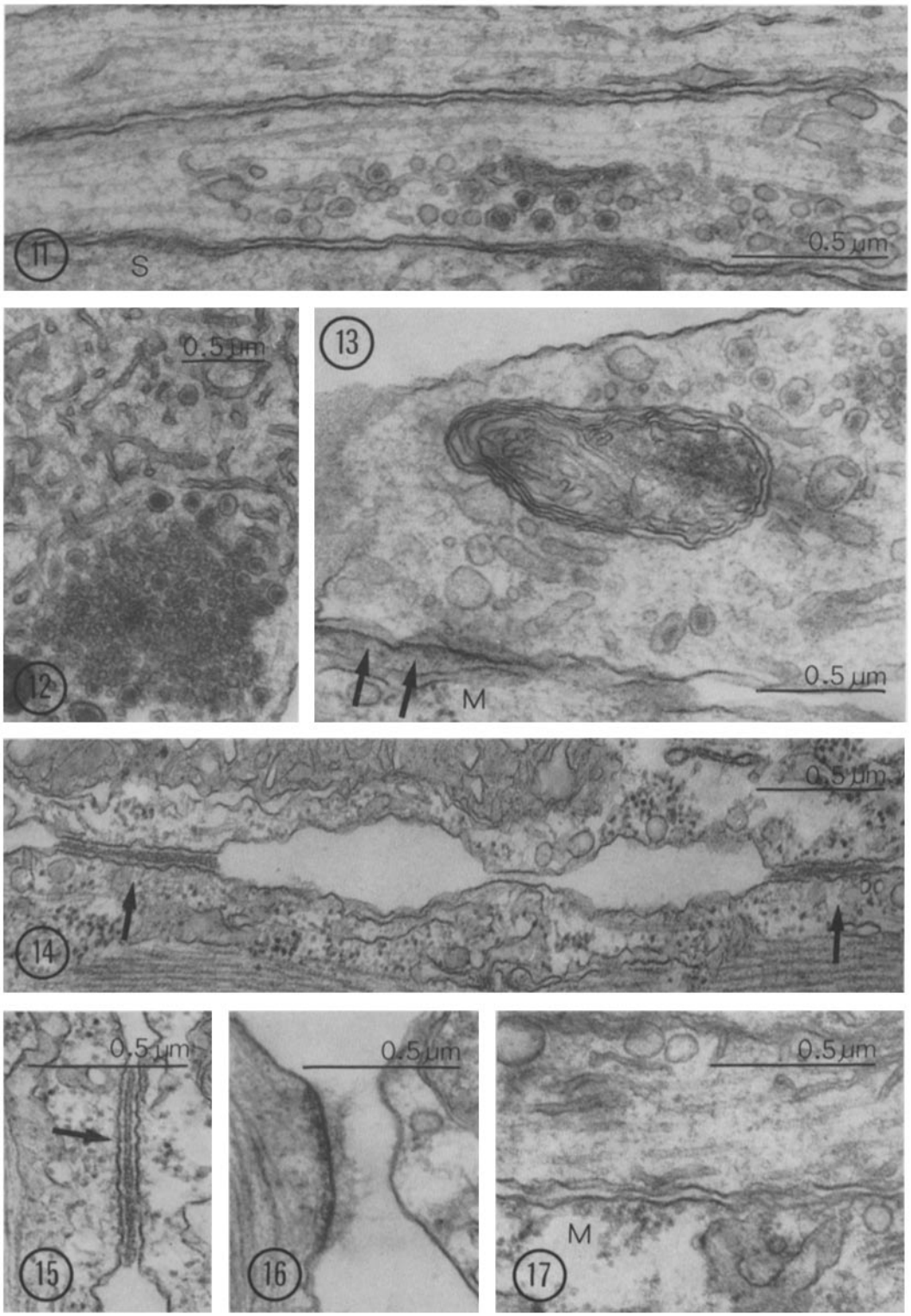
GANGLIONIC NEURITES: The neuritic outgrowth from both DRG and SG explants usually consisted of bundles of closely apposed nerve fibers and was often accompanied by non-neuronal (presumptive Schwann cell) processes as well (Figs. 11 and 18). This is in contrast to SC neurites which are not accompanied by Schwann cell processes (65). The nerve fibers were typically 0.5–1 µm in diameter and contained longitudinally oriented microtubules and neurofilaments (Figs. 11, 17, 18). Some regions of the nerve fibers also contained dense-core vesicles of variable size and aggregates of smaller (400–600 Å), electron-lucent vesicles (Figs. 11–13). Another common feature was the occurrence of dilatations of variable size and appearance. Some contained a heterogeneous population of organelles including vesicles, vacuoles, mitochondria, myelin bodies, and lysosomes whereas others contained highly branched tubules of smooth-surfaced reticulum (Fig. 12). Such dilatations are characteristic of DRG and SG nerve fibers in cultures derived from other species (11, 13, 64). Growth cones were also seen and contained characteristically large amounts of microfilamentous material (Fig. 18).

Occasionally cells containing large aggregates of membrane-bound granules were observed in SG-muscle cultures but not in DRG-muscle cultures. These cells appeared similar to the small, intensely fluorescent cells which have been observed in amphibian sympathetic ganglia in vivo (61, 62) and in culture (29, 30). They were sparsely distributed in our cultures and were not seen in contact with the muscle cells.

MUSCLE CELLS: Muscle cells in cultures containing ganglionic explants had the same ultrastructural characteristics as previously described for cultures with and without SC explants

(65). These characteristics include scattered surface patches of basal lamina associated with a local increase in the electron density of the sarcolemma and subjacent sarcoplasm (Fig. 16). Such patches closely resemble the postsynaptic specializations at sites of innervation on myotomal muscle cells in vivo (35) and in culture (65). A recent study of embryonic chick muscle has indicated that similar surface specializations are associated with a high density of AChRs (31).

An unusual association between adjacent muscle cells was seen in ganglionic-muscle cultures, but not in muscle cultures with or without SC explants (65). This intercellular relationship consisted of areas of close apposition (~ 600 Å wide), among neighboring muscle cells. Interposed between the muscle cells was a layer of electron-dense material ~ 300 Å thick (Fig. 14). In some instances this material had a trilaminar appearance suggesting two plasma membranes separated by a very thin layer of cytoplasm (Fig. 15). Such structures were not observed at appositions among any other cell types. From the regularity of the apposed sarcolemmas it would appear that such profiles represent areas of adhesion among the muscle cells and that



the interposed material plays a role in this cellular interaction. Although it has not been possible to identify the origin of this material one likely possibility would seem to be the presumptive Schwann cells which are prominent only in cultures containing DRG and SG explants.

CONTACTS BETWEEN GANGLIONIC NEURITES AND MUSCLE CELLS: In cultures with ganglionic explants, appositions between presumptive Schwann cell processes and muscle cells were at least as prevalent as nerve-muscle appositions. At both types the cleft width varied considerably, in many cases being as little as 100–200 Å, but in the great majority of examples, there was no evidence of either pre- or postsynaptic specializations (Figs. 17–19). However, some examples were occasionally observed which exhibited some morphological characteristics resembling synaptic specializations. At a few of the nerve-muscle appositions small accumulations of 400–600-Å clear vesicles and larger dense-core vesicles were seen in the nerve fibers but the vesicles were not localized against the axolemma, active zones were not present, and there was no specialization of the apposed muscle cell surface (Fig. 13). Alternatively, presumptive Schwann cell processes and nerve fibers, which contained no significant accumulation of either dense-core or electron-lucent vesicles, were occasionally observed in contact with regions of muscle cell surface that exhibited patches of flocculent extracellular material and a local increase in the electron density of the sarcolemma and underlying sarcoplasm (Fig. 20). Very rarely contacts were seen with some vesicle accumulation in the neuronal element, a basal lamina in the cleft, and a thickening of the muscle cell surface membrane. Fig. 21 illustrates such an example and is the most specialized contact observed in this study. Even this example stands in contrast to the more extensive synaptic specializations which were seen at much greater frequency in cultures containing SC explants (65).

Fluorescent Staining of AChRs

MUSCLE CELLS NOT CONTACTED BY NEURITES: As indicated in Table I virtually all noncontacted muscle cells exhibited one or more patches of stain, independent of the presence or type of nerve in the culture chamber or the age of the muscle cells in culture. These receptor patches have been described previously (4). They vary considerably in size but are usually <20 μm in their greatest dimension and never >40 μm. They also vary in location. Those on the bottom cell surface (facing the floor of the culture chamber) are most often located at the periphery of the cell whereas those on the top cell surface tend to be located in more central regions. When they do occur in the central regions of a cell they never extend across the entire cell breadth.

SC-CONTACTED MUSCLE CELLS: Altogether, 451 SC-contacted cells were examined in 11 SC-muscle cultures and 72.5% of these cells exhibited localization of stain along the paths of nerve-muscle contact. In individual cultures the values ranged from 50 to 90% (Fig. 22 *a*). The values tended to decline with the age of the SC explants and muscle cells (2–4 d) but there was also considerable overlap between different ages (Fig. 22 *a*). In addition, as indicated in Fig. 23 *a*, the percentage of SC-contacted muscle cells that had stain localized at sites of contact was not reduced when DRG or SG explants were also present in the cultures; the values ranged from 74 to 85% and the overall value for 289 SC-contacted cells was 76.8%. In these latter cultures there was no apparent decline with the age of the SC explants and muscle cells, and the values for 4-d-old SC explants and muscle cells (75 and 78%; see Fig. 23 *a*) were higher than for those in 4-d-old SC-muscle cultures without ganglionic explants (50 and 67%; see Fig. 22 *a*). Whether such differences reflect an enhancing effect by the ganglionic explants or whether they are fortuitous and due simply to variability among cultures is not clear at present. What is clear however is that the DRG and SG explants did not alter the culture medium in an adverse fashion with respect to the localization of receptors at SC-muscle contacts.

The patterns of receptor stain along paths of SC-muscle contact have been described previously (4) and proved to be the same in the present study. This was the case even when the cultures also contained ganglionic explants (Figs. 24 and 25). The stain along paths of contact usually occurred as narrow interrupted bands and sometimes extended entirely across central regions of the cells (Fig. 24). Often the same neurite contacted a number of successive muscle cells and stain was localized at sites of contact on each of the cells (Figs. 24 and 25). Occasionally, the stain along SC-muscle contacts extended with minor interruptions for distances of >40 μm. These characteristic staining patterns along SC-muscle contacts stand in

FIGURES 11–17 Ultrastructural features of SG-muscle cultures.

FIGURE 11 Part of a neurite bundle containing two nerve fibers and a presumptive Schwann cell process (*S*). One of the nerve fibers contains a loose aggregate of electron-lucent vesicles and larger dense-core vesicles.

FIGURE 12 Part of a dilation in a nerve fiber. The dilation contains branching cisternae and a dense aggregate of electron-lucent vesicles and larger dense-core vesicles.

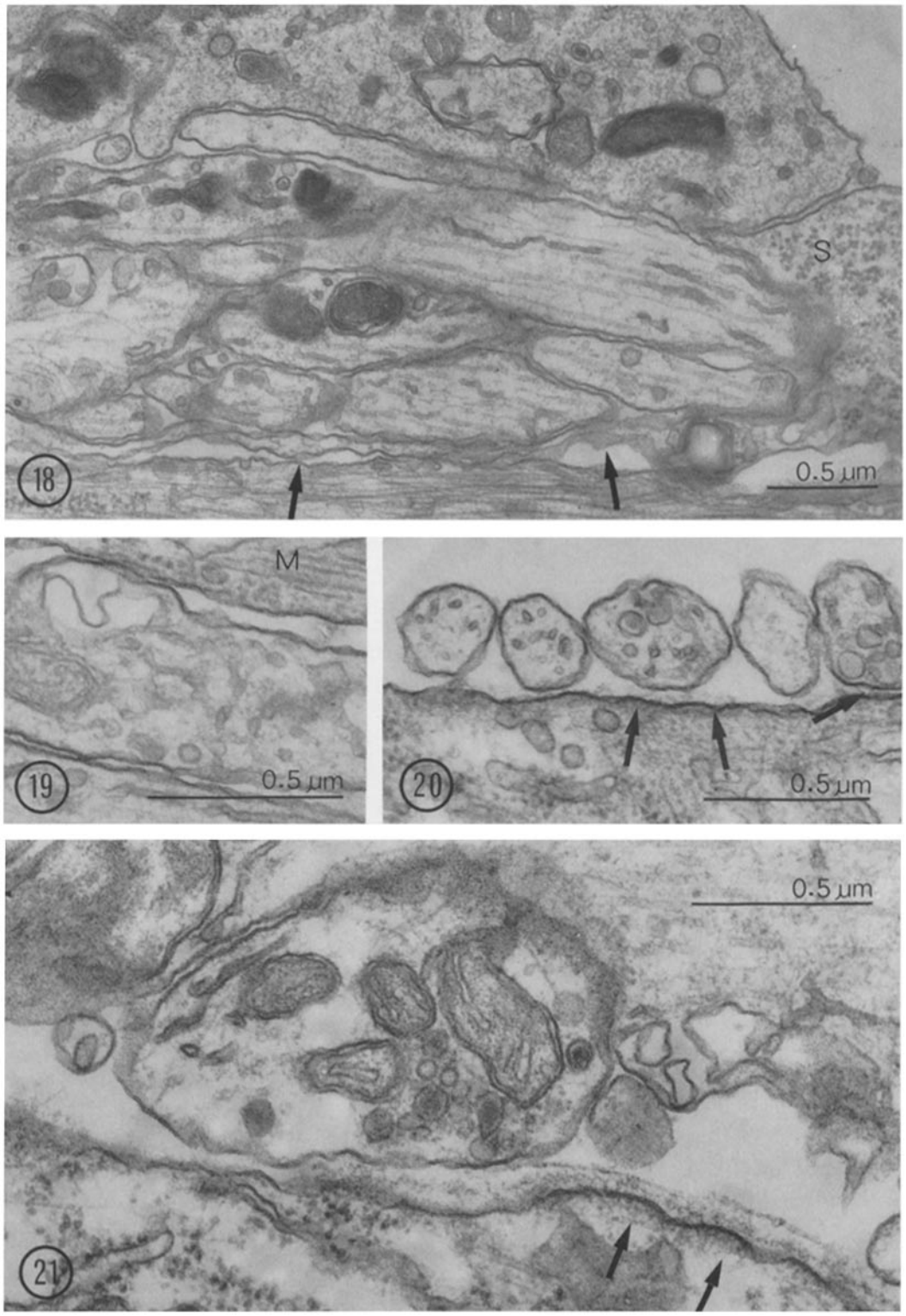
FIGURE 13 Enlarged portion of a nerve fiber apposed to a muscle cell (*M*). The sarcolemma (arrows) appears smooth and unspecialized. The nerve fiber contains a mitochondrion and a scattering of dense-core and electron-lucent vesicles.

FIGURE 14 Areas of close contact between adjacent muscle cells. Note the interposed layer of electron-dense material (arrows) at two of the contacts.

FIGURE 15 Area of close contact between muscle cells. The thin layer of interposed material (arrow) appears to be bounded by a cell membrane suggesting that it may be a flattened cell process.

FIGURE 16 Two muscle cells in close approximation. The cell at the left displays a protrusion that is covered by a patch of basal lamina. Note that the sarcolemma and subjacent cytoplasm exhibit increased electron density.

FIGURE 17 Close apposition of a nerve fiber and a muscle cell (*M*). The intercellular gap is ~ 200 Å, there is no accumulation of vesicles in the nerve fiber, and the muscle cell surface shows no specializations. This was typical of most nerve-muscle appositions in these cultures.



FIGURES 18–21 Ultrastructural features of DRG-muscle contacts.

FIGURE 18 Neurite bundle and muscle cell in close association. Note that a presumptive Schwann cell process (*S*) is interposed between the nerve fibers and the muscle cell surface (arrows). Part of a growth cone containing microfilamentous material occupies the upper portion of the field.

FIGURE 19 Nerve fiber in close approximation to a muscle cell (*M*). Although the nerve fiber and muscle membranes are closely apposed there are no pre- or postsynaptic specializations.

FIGURE 20 Five nerve fibers in close contact with a muscle cell. A small amount of flocculent material is associated with the muscle cell surface (arrows) and the sarcolemma and underlying sarcoplasm are moderately electron dense.

FIGURE 21 Nerve fiber in close approximation to a muscle cell. Basal lamina is present on the muscle cell surface and the sarcolemma is ridged and "thickened" (arrows). Note also the small cluster of electron-lucent and dense-core vesicles in the nerve fiber. Contacts displaying such ultrastructural specializations were extremely rare.

TABLE I

Patches of Stain on Muscle Cells Not Contacted by Neurites

Nerve type	Age of muscle in culture	Number of cultures examined	Number of cells examined	Cells with patches of stain
	d			%
None	2	3	180	99.4
	3	3	170	100
	4	3	170	100
			520	99.8
SC*	2	1	60	95.0
	3	2	80	100
			140	97.9
DRG*	2	1	60	100
	3	2	110	99.1
	4	2	110	97.3
			280	98.6
SG*	2	5	290	98.4
	3	4	220	100
	4	5	240	97.9
			750	98.9
SC and SG‡	2	3	180	97.8
	3	2	120	100
	4	1	60	100
			360	98.9

* From same cultures as in Fig. 22 and Table II.
‡ From same cultures as in Fig. 23 and Table III.

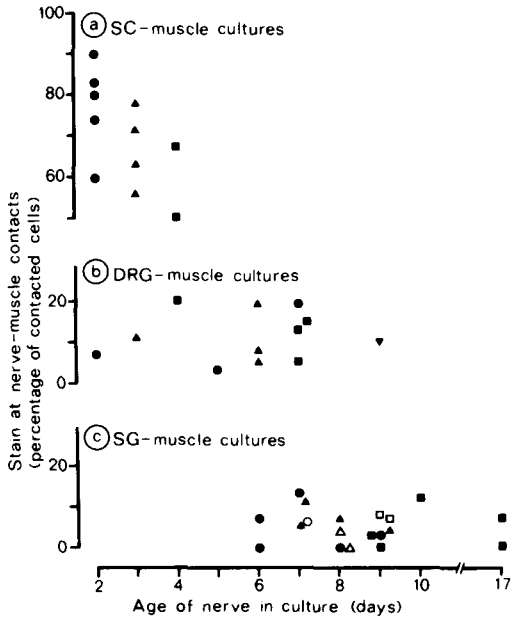


FIGURE 22 Percentage of nerve-contacted muscle cells which had stain localized at sites of contact in SC-muscle cultures (*a*), DRG-muscle cultures (*b*), and SG-muscle cultures (*c*). Each value indicates results from a single culture. Muscle cells were examined after 2 d (●), 3 d (▲), 4 d (■), and 6 d (▼) in culture. Open symbols are from cultures which contained NGF (50 ng/ml). Average sample sizes were 41 for SC-muscle cultures, 33 for DRG-muscle cultures, and 28 for SG-muscle cultures. The smallest sample size for each type of culture was 15.

contrast to the receptor patches seen on noncontacted muscle cells and support the conclusion that SC neurites can induce the development of a high receptor density at sites where they contact the muscle cells (3, 4).

Figs. 24 and 25 reveal another fairly common feature of the staining patterns on SC-contacted muscle cells: those cells that exhibited stain along the path of contact frequently had no receptor patches elsewhere. In SC-muscle cultures only 32% of cells with stain at sites of nerve-muscle contact had patches of stain elsewhere (Table II). By contrast 89% of cells with no stain at sites of nerve-muscle contact had patches of stain elsewhere (Table II). Similar results were also obtained for SC-contacted muscle cells in cultures which contained ganglionic explants as well (Table III). It appears therefore that SC neurites which form contacts that have a high receptor density also reduce the probability of receptor patch formation away from the sites of contact.

DRG-CONTACTED MUSCLE CELLS: Altogether, 394 DRG-contacted cells were examined in 12 cultures and only 10.2% had any stain localized at sites of contact. As indicated in Fig. 22 *b*, in individual cultures the values ranged from 3 to 20% and did not appear to be dependent on the age of the DRG explants (2–9 d) or the age of the muscle cells (2–6 d). The great majority (92.5%) of these DRG-contacted cells also had patches of stain elsewhere, as did almost all (97.5%) DRG-contacted cells with no stain at the sites of contact (Table II). Essentially similar results were obtained for DRG-contacted cells in one culture which also contained SC explants (Fig. 23 *b*, Table III).

The staining patterns on DRG-contacted cells (Figs. 26–29) were similar to those seen on noncontacted muscle cells. Even when stain was localized at sites of contact (Fig. 29) its ap-

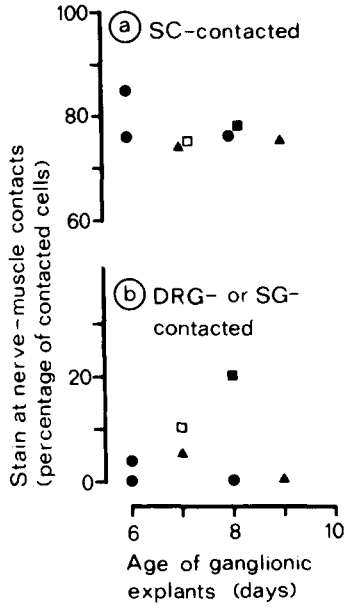
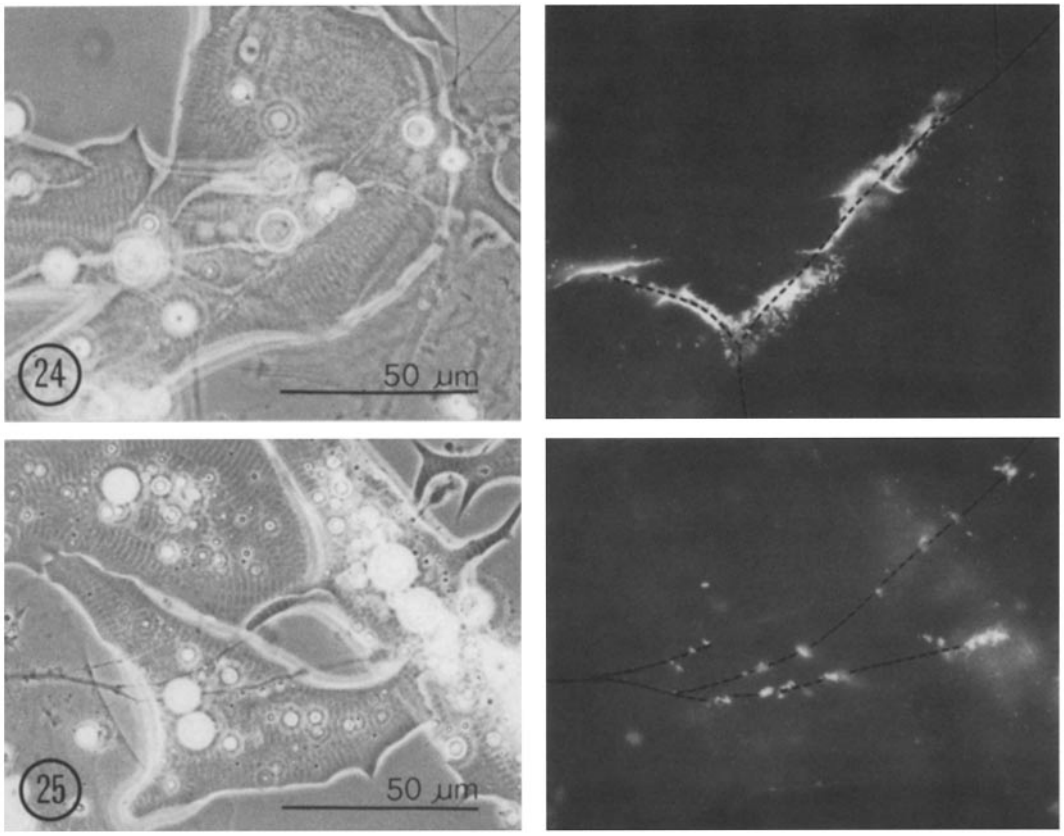


FIGURE 23 Percentage of nerve-contacted muscle cells that had stain localized at sites of contact. Each culture contained SC as well as SG (filled symbols) or DRG (open symbols) explants and was examined for localization of stain at SC-muscle contacts (*a*) and at ganglion-muscle contacts (*b*). The cultures were examined 2 d (●), 3 d (▲), and 4 d (■, □) after adding muscle cells. SC explants were plated at the same time as the muscle cells. For SC-contacted muscle cells the smallest sample was 23 and the average sample size was 41. For ganglion-contacted muscle cells the corresponding values were 15 and 23, respectively.



FIGURES 24 and 25 Phase-contrast and corresponding fluorescence views of SC-contacted muscle cells after staining with fluorescent α-bungaratoxin. Dashed lines on fluorescence micrographs indicate neurites. Note that almost all of the fluorescent staining is restricted to the paths of nerve-muscle contact and that successive muscle cells contacted by the same neurite also have stain along the path of contact.

FIGURE 24 4-d-old SC-contacted muscle cells. Culture also contained 7-d-old DRG explants. From same culture as Figs. 27 and 28.

FIGURE 25 3-d-old SC-contacted muscle cells. Culture also contained 7-d-old SG explants. From same culture as Fig. 32.

pearance was similar to the receptor patches seen on noncontacted cells and not like the narrow bands of stain seen at SC-muscle contacts. Thus, the stain at DRG-muscle contacts was never as much as 40 μm in length and never extended entirely across the central region of a cell. In addition, examples were not observed where the stain was localized along paths of contact on two or more successive muscle cells contacted by the same DRG neurite.

Previous studies have indicated that the development of a high receptor density at sites of SC-muscle contact can occur within 6 h after contact is made (16). It was therefore of interest to determine if the DRG-muscle contacts survive for this period of time, and if so, whether receptor accumulation would occur at surviving contacts. Accordingly, eight DRG-contacted muscle cells were examined twice, the second observation being made 1 d after the first. In every case the neurites were still in contact with the muscle cells at the time of the second observation yet no stain was seen along the path of contact, and patches of stain were present elsewhere on the cells. An example is shown in Figs. 27 and 28. Of particular interest in this example is that during the 1-d interval between observations, the muscle cell grew in the region between two sites of nerve contact and the distance between the neurites increased. This suggests that at least one of the neurites was in fact adhering to the muscle cell as it grew. Despite this relative stability no stain was seen at either contact.

SG-CONTACTED MUSCLE CELLS: In 19 SG-muscle cultures only 5.6% of 532 contacted cells examined had stain localized at sites of contact. As indicated in Fig. 22 *c*, in individual cultures, the values ranged from 0 to 13% and did not appear to be dependent on the age of the SG explants (6–17 d) or the age of the muscle cells (2–4 d). Nor were these values increased when NGF (50 ng/ml) was present in the culture medium (Fig. 22 *c*, open symbols). Almost all of the contacted cells had patches of stain elsewhere (Table II). Essentially similar results were obtained for SG-contacted muscle cells in six cultures which also contained SC explants. Thus, as before, almost all of the SG-contacted cells in these latter cultures had patches of stain away from the paths of contact (Table III). Furthermore, only 6.6% of 121 SG-contacted cells examined in these cultures had stain localized at sites of contact. The values for individual cultures ranged from 0 to 20% (Fig. 23 *b*). When contrasted with the much higher values for SC-contacted cells in the very same cultures (Fig. 23 *a*) it is apparent that the large differences in the frequency of receptor localization at SC-muscle contacts and at SG-muscle contacts cannot be explained on the basis of some alteration of the culture medium by the different types of nerve explant.

Examples of the staining patterns on SG-contacted muscle cells are shown in Figs. 30–32. These patterns were similar in all respects to those already described for DRG-contacted muscle cells. This was the case even when the cultures contained SC explants (Fig. 32) or NGF. No attempt was made to follow identified fields in SG-muscle cultures but some of the

TABLE II

Patches of Stain Away from Sites of Nerve-Muscle Contact

Nerve type	Age of muscle in culture	Cells with stain at sites of contact*: No. of cells examined	Cells with stain at sites of contact*: Cells with patches of stain elsewhere	Cells with no stain at sites of contact*: No. of cells examined	Cells with no stain at sites of contact*: Cells with patches of stain elsewhere
	d		%		%
SC	2	220	27.3	63	88.9
	3	84	42.9	43	100
	4	23	43.5	13	61.1
		327	32.4	124	88.7
DRG	2	5	80.0	66	95.5
	3	16	93.7	142	97.9
	4	15	100	110	97.3
	6	4	75.0	36	100
		40	92.5	354	97.5
SG	2	10	80.0	137	98.5
	3	10	90.0	148	100
	4	5	100	121	94.2
		25	88.0	406	97.8
SG‡	2	1	100	16	100
	3	1	100	43	100
	4	3	100	37	100
		5	100	96	100

* Same cells as in Fig. 22.

‡ Culture medium contained 50 ng/ml NGF.

cultures were examined at daily intervals. In doing so five cases were obtained where the same SG-contacted muscle cell was photographed twice. In each case the contacts survived during the 1-d interval but were not associated with any stain even at the second observation. Instead stable patches of stain were seen away from the sites of contact.

The location of contacts by SG neurites, and by DRG neurites, was highly variable but they appeared to occur most often on the bottom surface of the muscle cells. The frequency of stain at sites of contact was also higher on the bottom surface. 17 out of 22 examples of stain at sites of SG-muscle contact which were documented in this way were located on the bottom surface, and the corresponding values for DRG-muscle contacts were 24 out of 28.

DISCUSSION

Even though DRG and SG neurites had the same opportunity as SC neurites, to contact and interact with muscle cells, the frequency of occurrence of a high density of AChRs at sites of contact was much greater on muscle cells contacted by SC neurites than on those contacted by ganglionic neurites. This difference in frequency was not diminished when SC and ganglionic explants were cultured together in the same chamber thereby ruling out the possibility that the explants influenced the response of the muscle cells by altering the culture medium. Instead the present findings indicate that the high receptor density at sites of SC-muscle contact must have arisen from a specific, local interaction between the SC neurites and muscle cells. The much greater frequency of ultrastructural postsynaptic specializations at contacts between SC neurites and muscle (65) than at contacts between ganglionic neurites and muscle likewise indicates that their establishment is caused by a specific, local interaction.

In principle the interaction might be permissive or inductive. That is, because the muscle cells develop sites of high receptor density and of ultrastructural postsynaptic specializations in the absence of any contact by nerve it could be that the SC neurites have a special affinity for these sites. Alternatively, it could be that the SC neurites can induce the development of these synaptic specializations at sites where they contact the muscle cells. There is evidence for both possibilities. For example, it has been established that reinnervation of adult denervated muscle normally occurs at the original synaptic sites (38, 44) and that the regenerating motor nerve fibers interact with some component of the surviving basal lamina (41, 57). On the other hand, other studies indicate that under favorable conditions, the regenerating nerve fibers can induce the development of a high receptor density (40) and other postsynaptic specializations (1, 22, 45) at regions remote from the original synaptic sites. A nerve-induced development of high receptor density at sites of contact with muscle has also been demonstrated in cultures derived from chick embryos (24) as well as in the present culture system (3, 4). Our study reemphasizes this capacity of the SC neurites. For example, unlike receptor patches on noncontacted muscle cells, the narrow bands of high receptor density at sites of contact sometimes extended entirely across central regions of muscle cells, they often continued in aligned patterns over successively contacted muscle cells, and they occasionally extended for distances of >40 μm.

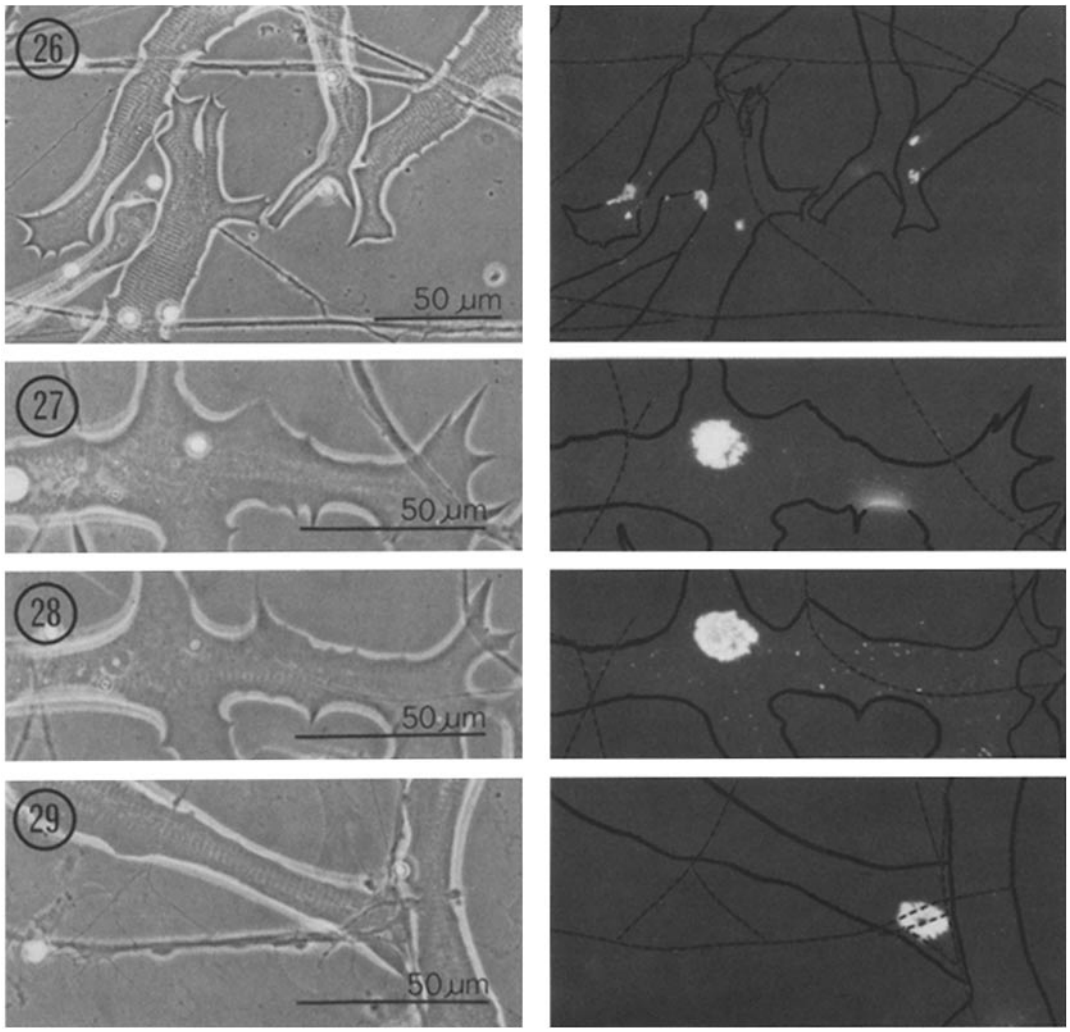
By contrast our findings provide no evidence that the few cases of high receptor density at sites of ganglionic nerve-muscle contact were caused by an induction process. The patterns of receptor distribution on these muscle cells did not have the unique features associated with SC-contacted muscle cells but instead were similar to the characteristic patterns seen on noncontacted muscle cells. It is therefore apparent that the neural factor responsible for inducing the development of a localized high receptor density in muscle is specific inasmuch as it is associated with SC neurites but not with ganglionic

TABLE III

Patches of Stain Away from Sites of Nerve-Muscle Contact in Cultures Containing Both SC and Ganglionic Explants

Nerve type	Age of muscle in culture	Cells with stain at sites of contact*: No. of cells examined	Cells with stain at sites of contact*: Cells with patches of stain elsewhere	Cell with no stain at sites of contact*: No. of cells examined	Cells with no stain at sites of contact*: Cells with patches of stain elsewhere
	d		%		%
SC	2	119	29.4	33	87.9
	3	55	34.5	19	94.7
	4	48	72.9	15	100
		222	40.1	67	92.5
DRG	4	2	100	38	94.7
SG	2	1	100	54	98.2
	3	1	100	35	100
	4	6	100	24	91.7
		8	100	113	97.3

* Same cells as in Fig. 23.



FIGURES 26–29 Phase-contrast and corresponding fluorescence views of DRG-contacted muscle cells after staining with fluorescent α-bungarotoxin. Dashed lines on fluorescence micrographs indicate neurites and solid lines outline muscle cells. Note that in all cases, except Fig. 29, patches of fluorescent stain are not associated with paths of nerve-muscle contact.

FIGURE 26 6-d-old muscle cells, contacted by neurites of a 9-d-old DRG explant.

FIGURE 27 3-d-old muscle cell contacted by neurites of a 6-d-old DRG explant. From same culture as Fig. 24.

FIGURE 28 Same field as Fig. 27, 1 d later. Except for appearance of scattered dots of stain the staining pattern changed only slightly. Note also that the muscle cell grew and that the distance between the neurites increased, suggesting that at least one of the neurites was adhering to the muscle cell surface.

FIGURE 29 4-d-old muscle cells contacted by neurites of a 7-d-old DRG explant. Note that the patch of stain at the site of nerve-muscle contact resembles those seen elsewhere on the muscle cells (Figs. 26–28) rather than the narrow bands of stain seen on SC-contacted muscle cells (Figs. 24 and 25). Also note that the neighboring muscle cell has no stain at sites of nerve-muscle contact.

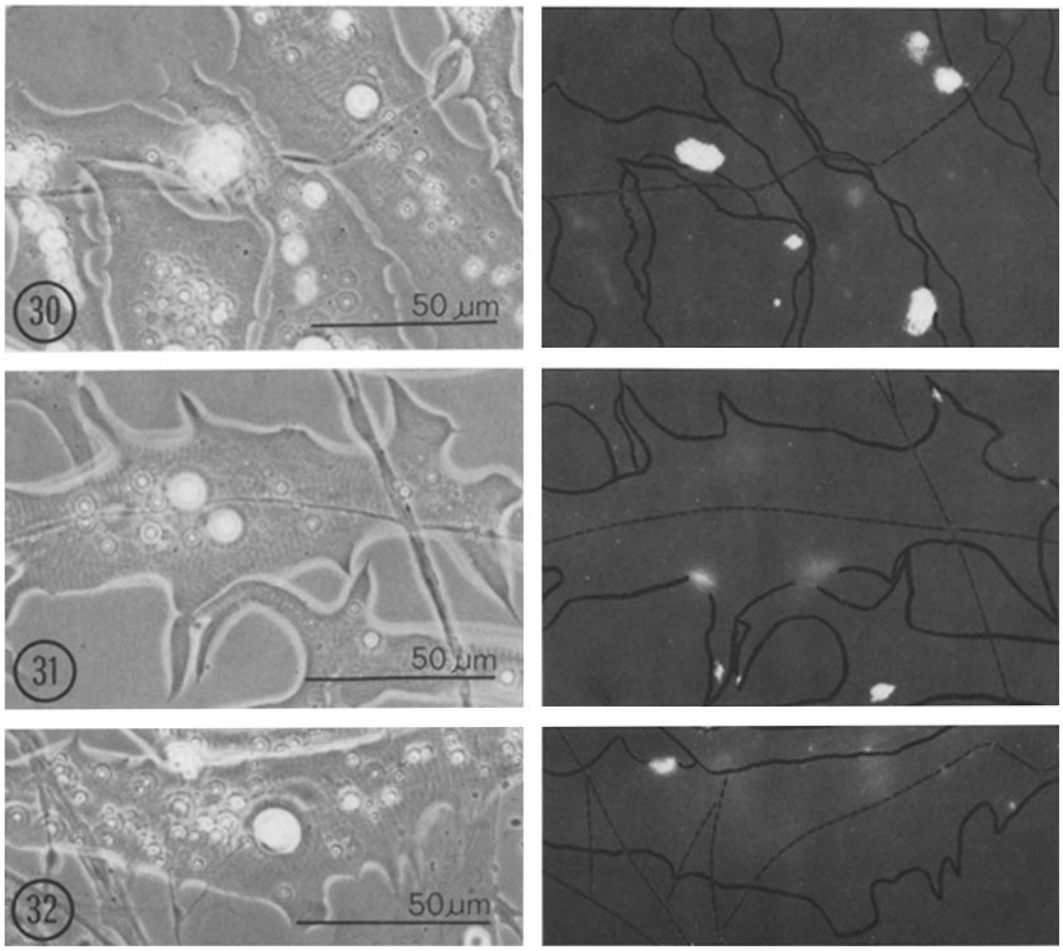
neurites. Furthermore the infrequent examples of receptor localization and of postsynaptic ultrastructure at sites of DRG- and SG-muscle contact may have occurred by chance rather than as a result of some special affinity of the neurites for such sites. For example receptor patches on noncontacted muscle cells occupy ~ 3% of the cell surface (4). Because some of the muscle cells were contacted by more than one DRG or SG neurite and because the neurites sometimes coursed across large portions of the muscle cells, the occurrence of receptor patches at sites of contact on 10% of the DRG-contacted cells and on 6% of the SG-contacted cells might well be within the expected range of probabilities for chance contacts. The neurites were often accompanied by non-neural processes which also entered into close apposition with the muscle cells. These non-neuronal cells apparently also lack the capacity to induce the development of, or to preferentially establish contact with, sites of high receptor density and postsynaptic ultrastructure.

In addition to postsynaptic specializations, contacts in SC-muscle cultures (65) also exhibit presynaptic specializations consisting of small clear vesicles clustered against the axolemma and an increased electron density of the axolemma (active zones). Such presynaptic specializations are not observed in other regions of the SC nerve fibers thereby indicating that they develop as a result of a local interaction between the muscle cell and nerve process (65). As noted above, aggregates of clear vesicles were observed in DRG and SG nerve fibers in the present study but they were rarely found at nerve-muscle contacts. Active zones were never observed. These findings

further emphasize the overall inability of the ganglionic processes to interact with, and establish synaptic contact with muscle. The only "specialized" contacts that were seen were curious "sandwich-like" contacts consisting of neighboring muscle cells separated by a narrow structure of unknown origin.

Synaptic function requires appropriate matching between the transmitter and the postsynaptic receptors and it is therefore tempting to suppose that the ability to induce or adhere to synaptic specializations on muscle may be linked to cholinergic metabolism. It is known from the original work of Langley and Anderson (37) and from more recent studies (9, 26, 36) that adult denervated muscle can be functionally reinnervated not only by skeletal motor neurons but also by other cholinergic neurons. Recent investigations in culture have provided additional examples of functional innervation by cholinergic neurons which do not normally innervate skeletal muscle (33, 46, 48, 49, 53, 59). Functional innervation however does not necessarily imply a localized high receptor density (5) or other characteristic postsynaptic specializations (35). Only for the case of the vagus nerve has additional evidence been obtained indicating that the nerve fibers preferentially establish contact at the original synaptic sites in adult denervated muscle (9, 36).

Many of the SC neurites in our culture system are indeed cholinergic and functionally innervate the muscle cells (4, 5). Conversely, DRG neurons are presumably noncholinergic and do not establish functional connections with muscle (26, 37, 49; but see 63). Amphibian SG do contain some cholinergic neurons but there is considerable variability between different ganglia along the SG chain and between different species (14) and information for *Xenopus* SG appears to be lacking. Experiments in frog (26) as well as in mammals (37, 43) have so far indicated a failure of SG nerve fibers to innervate adult denervated muscle. A similar result has also been reported for cultures of SG and muscle derived from chick (49). On the other hand, under appropriate conditions, cultured rat SG neurons can be converted from adrenergic to cholinergic metabolism and such neurons do functionally innervate cultured rat muscle cells (48). We do not know whether such conversion occurred in our cultures. Substantial aggregates of small electron-lucent vesicles were observed in SG (and DRG) nerve fibers but such aggregates were almost never found at nerve-muscle contacts and were never localized against the axolemma. Nor did we observe any evidence of synaptic function,



FIGURES 30–32 Phase-contrast and corresponding fluorescence views of SG-contacted muscle cells after staining with fluorescent α-bungarotoxin. Dashed lines on fluorescence micrographs indicate neurites and solid lines outline muscle cells. Note that patches of fluorescent stain are not associated with paths of nerve-muscle contact.

FIGURE 30 2-d-old muscle cells contacted by neurite of a 9-d-old SG explant.

FIGURE 31 4-d-old muscle cells contacted by neurites of a 10-d-old SG explant.

FIGURE 32 3-d-old muscle cell contacted by neurites of a 7-d-old SG explant. From same culture as Fig. 25.

but in the absence of intracellular recording the possibility of subthreshold synaptic activity cannot be excluded. Clearly further studies will be required to establish whether the ability to induce, or preferentially adhere to, synaptic specializations in muscle is a feature of all types of cholinergic neurons.

Another factor which may be considered is the developmental age of the neurons. In the present study SC explants were obtained from 1-d-old embryos whereas SG explants were derived from animals that were at least 2-mo old (see Materials and Methods). Perhaps SG neurites of younger ganglia would have exhibited a greater capacity to establish contacts having synaptic specializations. For example the shift to cholinergic metabolism in rat SG explants is dependent on the age of the ganglia and does not occur when adult ganglia are used (56). Such considerations might also apply to our DRG explants which were derived from ganglia which were 3–6-wk old (see Materials and Methods), although at these stages there is a high turnover of neurons (52) and many of the DRG neurons were probably much younger. Of course in vivo SC motor neurons (1, 22, 45) and other cholinergic neurons (9, 36) retain their capacity to establish synaptic contacts with muscle even in the adult.

Almost all experiments in the present study were limited to the first 4 d after plating muscle cells, and during this period there was no apparent increase with age in the frequency of receptor localization of ganglionic nerve-muscle contacts. Considering that SC-contacted muscle cells can develop synaptic ultrastructure (65) and a high receptor density (3, 4, 16) within less than a day after contact, the 4-d period is a relatively long time scale. The possibility can also be raised that the ganglionic neurites would have developed some capacity to interact with the muscle cells if they had been cultured for longer periods. For example, in cultures of rat SG neurons and explants the development of cholinergic metabolism increases progressively over a 3–4-wk period or more (50, 56) but some of the neurons are competent to functionally innervate muscle cells by 9 d (48). We usually examined SG-contacted muscle cells when the SG explants had been in culture for 10 d or less. However, in two cultures the SG explants were 17-d old and there was still no indication of an increase in the frequency of receptor localization at sites of contact. Whatever the reason for the lack of synaptic specializations at ganglionic-muscle contacts it is apparent that the occurrence of these specializations at SC-muscle contacts in our cultures involved a highly specific interaction.

The frequency of receptor patches on contacted muscle cells also deserves comment. About 90% of those SC-contacted muscle cells, that had no receptor localization along the path of contact, did exhibit receptor patches elsewhere. By contrast, receptor patches away from sites of contact were observed on <40% of those SC-contacted muscle cells which had a high receptor density along the path of contact. The reason for this relatively low frequency of receptor patches is not known. Muscle contraction is clearly not an important factor because in most cultures neuromuscular transmission was prevented by the presence of curare in the culture medium (see Materials and Methods). It could be argued that even though SC neurites can induce the development of a high receptor density they are also capable of seeking out receptor patches or precursor sites which are destined to develop into such patches. If that were the case then muscle cells contacted by such neurites would be less likely to have receptor patches away from the path of contact. Another possible explanation is that the inductive influence of some of the SC neurites is so great that it completely exhausts the limited capacity of the muscle cell to generate sites of high receptor density, or postsynaptic membrane generally. In this regard it is interesting to note that nerve fibers compete with each other for synaptic survival on muscle cells during normal development (7, 12, 54, 55) and also in experimental situations in the adult (8, 15, 20, 25, 27, 34, 58). In some cases the competitive interaction does not appear to involve a direct influence between the nerve fibers but rather appears to be mediated via the muscle (20, 25). The notion of individual nerve fibers and muscle cells having different capacities to interact with each other in the establishment of synaptic specializations may also be relevant in explaining such competition.

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