

EARLY EVENTS IN NEUROMUSCULAR JUNCTION FORMATION IN VITRO

Induction of Acetylcholine Receptor Clusters in the Postsynaptic Membrane and Morphology of Newly Formed Synapses

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ABSTRACT

The development of clusters of acetylcholine (ACh) receptors at newly formed synapses between embryonic chick spinal cord and muscle cells grown in vitro has been studied by iontophoretic mapping with ACh. A semi-automated technique using on-line computer analysis of ACh responses and a photographic system to record the position of each ACh application permit the rapid construction of extensive and detailed maps of ACh sensitivity. Clusters of receptors, evident as peaks of ACh sensitivity, are present on many uninnervated myotubes. The distribution of ACh sensitivity closely parallels the distribution of ^{125}I - α -bungarotoxin binding sites on the same muscle cell.

In all cases where individual myotubes were adequately mapped before and after synapse formation, ingrowing axons induced new clusters of receptors rather than seeking out preexisting clusters. Synapses can form at active growth cones within 3 h of nerve-muscle contact. New receptor clusters can appear beneath neurites within a few hours.

Many of the uninnervated clusters on innervated myotubes disappear with time. In contrast, receptor clusters on uninnervated myotubes remain in the same location for many hours. Synaptic clusters and clusters on uninnervated myotubes are stable even though individual receptors are metabolized rapidly.

The morphology of several identified sites of transmitter release was examined. At the scanning EM level, synapses appeared as small, rough-surfaced varicosities with filopodia that radiated outwards over the muscle surface. One synapse was studied by transmission EM. Acetylcholinesterase and a basement lamina were present within the synaptic cleft.

KEY WORDS acetylcholine receptor induction
embryonic synapses

adult vertebrate neuromuscular junctions. The
sensitivity to iontophoretically applied ACh and
the density of ^{125}I - α -bungarotoxin (^{125}I - α -BuTx)

Acetylcholine (ACh) receptors are concentrated at

binding sites in the immediate postsynaptic mem-

brane are 500- to 1,000-fold greater than at nearby, extrasynaptic sites (5, 18, 20, 32, 43). We have investigated synapses that form in culture between embryonic chick spinal cord neurons and muscle cells to determine how this clustering comes about during early development.

The function of nerve-muscle synapses that form in vitro is similar in many respects to that of mature junctions in adult animals (21). Most important for this study is the fact that innervated myotubes are extremely sensitive to ACh in the immediate vicinity of sites of transmitter release (13). One explanation for this precise spatial correlation is that motor axons induce receptor clusters at newly formed synapses. However, an alternative explanation became evident in early studies of the distribution of receptors on uninnervated myotubes.

Chick myotubes that form in vitro by fusion of mononucleated myoblasts are sensitive to ACh over their entire length, but the distribution of sensitivity is not uniform. Small patches of membrane 5–10 μm across that are severalfold more sensitive to ACh than surrounding regions can be found on most cells (22). Such "hot spots" are also evident as clusters of grains in autoradiographs of myotubes labeled with ^{125}I - α -BuTx (22, 49, 50). Thus, it is possible that sites of transmitter release on innervated myotubes correspond to peaks of ACh sensitivity because receptor clusters serve as recognition sites for ingrowing cholinergic axons.

To decide whether cholinergic neurites seek out preexisting hot spots or induce new ones, we mapped the surface of individual myotubes by microiontophoresis of ACh before and then again after innervation. One of the great advantages of cell cultures is that myotubes and fine neurites can be visualized, synapses can be precisely located, and the same functional contacts can be studied over a period of several days. We also examined the structure of several identified synapses by scanning and transmission electron microscopy. Preliminary accounts of some of this work have already appeared (24, 25). Experiments in which the distribution of ACh receptors on *Xenopus* myocytes was mapped with fluorescent α -BuTx conjugates have led to a conclusion similar to our own (1, 2).

MATERIALS AND METHODS

Cultures

Muscle cultures nearly devoid of fibroblasts were

prepared as previously described (21). Pectoral muscles were removed from 11-d chick embryos, diced, incubated in a Ca^{++} - and Mg^{++} -free salt solution (Pucks D₁G) for 30 min, resuspended in complete medium (see below), and then triturated by repeated passage through a fire-polished Pasteur pipette. The resulting suspension of mononucleated cells was depleted of fibroblasts by allowing them to settle on an uncoated Falcon tissue culture dish (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) for 30 min at 37°C (51). Cells remaining in suspension were plated on collagen-coated, 22-mm glass coverslips (Gold Seal No. 0, Clay-Adams, Div., Becton, Dickinson & Co. Parsippany, N. J.) at a density of 200/mm². Each cover slip was placed in a 35-mm tissue culture dish. The cells were fed every 2–3 d with 1.5 ml of Eagle's Minimum Essential Medium (MEM) made up in Earle's Balanced Salt Solution (BSS) and supplemented with glutamine (2.4 mM), horse serum (10% vol/vol), chick embryo extract (2% vol/vol), penicillin (50 U/ml), and streptomycin (50 $\mu\text{g}/\text{ml}$). Nearly all fibroblasts that remained after the preplating were eliminated by adding cytosine arabinoside (ara C; 10^{-5} M) to the medium for 24–48 h, beginning on the 2nd d after plating.

One or two spinal cord explants were placed on each muscle culture on the 3rd or 4th d after plating. Transverse sections, 100–200 μm thick, were sliced from the brachial enlargement of 13- to 14-d embryonic cords that had been stripped of sensory ganglia and meninges. The sections attached to the culture surface in a thin film of medium, and after several hours the normal volume (1.5 ml) was restored. In general, the neuritic outgrowth from 13-d spinal cord explants appeared later and was less prolific than that from 4- or 7-d explants used in previous studies. Growth cones first emerged ~24 h after the explants attached to the substrate. The relatively sparse outgrowth facilitated localization of sites of transmitter release (see below) but, even in these cultures, the overwhelming majority of nerve-muscle contacts were not functional synapses.

Electrophysiology

Electrophysiological experiments were performed on the stage of an inverted microscope equipped with interference-contrast optics. 1- to 2-wk-old cover slip cultures were placed in a shallow chamber fixed to the mechanical stage of the microscope. The bottom of the chamber was formed by a 25-mm No. 0 glass cover slip. Most observations were made with a $\times 40$ dry objective and a 5-mm working distance, dry condenser. The cells were perfused at ~5 ml/h with MEM supplemented with glutamine and antibiotics, but with only 1% horse serum and no embryo extract. The entire stage was warmed to 30°–35°C and the pH was maintained near 7.4 by blowing sterile, moist CO_2 over the chamber. The cells remained healthy and continued to grow in this situation, and we could observe the same culture for several hours on each of several successive days.

STIMULATION AND RECORDING: Fine-tipped glass microelectrodes (omega-dot tubing) filled with 3 M KCl or 4 M KAc were used for intracellular recording. Electrode resistances ranged between 80 and 120 M Ω . Electrodes with steep tapers broken to an internal tip diameter of 2–3 μ m and filled with perfusion medium were used for extra-cellular stimulation or recording. The tips were fire-polished and the shanks bent so that the electrodes could be placed perpendicular to the nerve or muscle surface. Motor (cholinergic) axons were identified by stimulating individual axons or bundles of axons some distance away from the innervated myotube. Sites of transmitter release were located by stimulating neurites overlying myotubes with 1-ms-long, 0.5- to 2.0- μ A, negative pulses. Tetrodotoxin (TTX, 10^{-7} M) was added to the bath to block propagation of action potentials so that only that segment of the nerve beneath the electrode was depolarized. The resolution of this technique is \sim 3–5 μ m. Synapses were also located by focal extracellular recording of synaptic currents. The extracellular fields associated with spontaneously occurring synaptic potentials are steep in this system and the resolution of this technique is also 3–5 μ m (see Fig. 5). Focal depolarization often damaged the underlying synapse, whereas focal recording did not.

ACh SENSITIVITY: The ACh sensitivity of small membrane patches was assayed by recording the change in membrane potential with an intracellular microelectrode while applying ACh iontophoretically from a microelectrode filled with 1 M ACh. The positive iontophoretic current pulses were 0.5–5.0 ms in duration and 0.5–20 nA in amplitude. High resistance electrodes (>100 M Ω) were used to apply ACh so negative braking currents of only 1–2 nA were sufficient to prevent receptor desensitization. Such small pulses of ACh probably affect membrane patches of only a few square micrometers (32).

A rapid, semi-automated procedure was devised to facilitate the construction of detailed, extensive maps of ACh sensitivity. The ACh current pulse (measured with an operational amplifier) and the membrane voltage response were sampled on-line with a PDP8E computer, and the position of the ACh pipette at each test site was photographed on a frame of 16-mm cine film. The pulses were sampled every 50 μ s and the digitized record was integrated numerically. The integral of the positive ACh current, rather than the algebraic sum of the positive current plus the backing current, was used.¹ An additional 2 ms at the end of the current pulse was included

¹ In several experiments we measured the response to varying ACh pulses at a single location on a muscle cell. The relationship was approximately linear once a "threshold" amount of charge had been ejected from the pipette (cf. Fig. 4 in reference 32). Empirically, for the high-resistance pipettes and short pulses used in these experiments, this threshold charge was very close to the backing current times the pulse duration.

in the integral to correct for capacitative artifacts. The muscle membrane potential was sampled every 0.5 ms; the average potential during the 4 ms preceding each pulse was taken as the zero or "resting" membrane potential. ACh sensitivity was then computed as the peak voltage response (the average of three consecutive voltage samples centered on the maximum value) divided by the charge delivered from the ACh pipette (mV/nC). ACh pulses were adjusted to give a small but easily measurable response (\sim 1 mV) at relatively insensitive areas; the same pulse could then be used to map most of the muscle surface. Only when the response exceeded 8–10 mV was the ACh pulse reduced. TTX was added to the bath in some experiments to block muscle twitches.

Pulses were repeated at 0.5 Hz and, at each test site, the ACh pipette was lowered gradually while observation was made on each digitized current and voltage record on a monitor oscilloscope. When the latency and rise time of the response reached a minimum and the calculated sensitivity reached a maximum (usually just as the pipette touched the muscle surface), a foot switch was depressed to activate simultaneously the movie camera and signal the computer to print the estimated sensitivity. In this manner we could test \sim 100 different sites in 30 min without damaging the myotube. Measurements at two sites, each consisting of a photomicrograph showing the muscle cell and the ACh pipette together with the digitized display of the ACh current, membrane potential, and computed sensitivity, are shown in Fig. 1.

Maps were constructed by projecting the movie film at a total magnification of \sim 1,000 with a stop-frame movie projector. The outline of the muscle cell was traced along with positions of nerve processes, cell nuclei, and the intracellular recording electrode. The film was then stepped through the series of measurements and the position of the ACh pipette in each frame was marked on the drawing with an integer that was proportional to the logarithm of the sensitivity at that location. The key for translating integer values into mV/nC is given in the legend to Fig. 2.

Autoradiography

The α -BuTx used in these experiments was purified and iodinated by S. J. Burden (9, 10). The sp act of the labeled toxin was \sim 100 cpm/fmol. Cultures were incubated for 1 h at 37°C in 10^{-8} M 125 I- α -BuTx in complete medium, washed several times in culture medium, and then either returned to the incubator (or microscope stage as in Fig. 6) or fixed immediately in 1% paraformaldehyde made up in 0.1 M phosphate buffer, pH 7.3, for $\frac{1}{2}$ h at room temperature. After a rinse in distilled water, the cover slip culture was glued to a microscope slide and dipped into NTB3 emulsion (Kodak) diluted 1:1 with water. The emulsion was gelled on a cold plate and then exposed at 4°C for several days in a dessicated box. Grains were developed in D-19 (Kodak) for 2 min at 20°C.

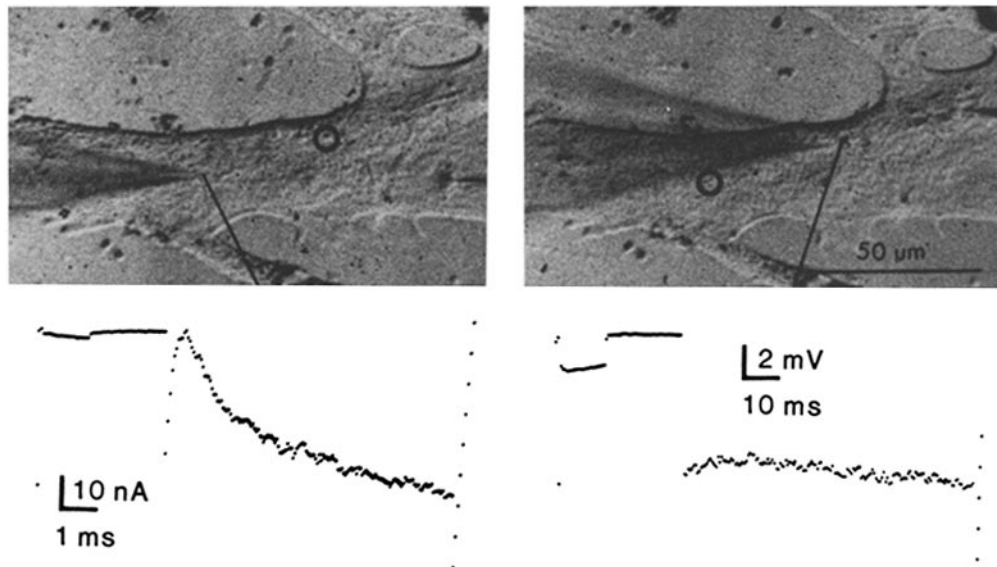


FIGURE 1 Intracellularly recorded responses to ACh applied iontophoretically at two sites on the same myotube. The position of the ACh pipette (shadow on the left in each interference contrast micrograph) and the digitalized voltage response (below) were recorded simultaneously. Digitalized ACh current pulses were displayed on faster time scale. Each dot in the columns to the right of the oscilloscope traces represents a 2.2-fold increase in ACh sensitivity. The sensitivity of the test site on the left was 3,730 mV/nC; on the right it was 190 mV/nC. The circle in each micrograph indicates the position of the test site in the other photograph.

Electron Microscopy

For scanning EM, cover slip cultures were fixed for 1 h in glutaraldehyde (2% in 0.12 M phosphate buffer, pH 7.2), dried by the CO₂ critical point method, and coated with gold. For thin section electron microscopy, the culture, fixed for 30 min and stained for acetylcholinesterase (AChE) according to the method of Karnovsky (30), was fixed for an additional hour in 2% glutaraldehyde, postfixated in 1% OsO₄, dehydrated, and embedded in Epon. The cover slip was removed by dipping the complex in liquid nitrogen. Thin sections were examined in a Philips 200 microscope. Photographs of physiologically identified synapses taken before fixation were used to relocate the same contacts in dried specimens and in the plastic disk.

RESULTS

Distribution of ACh Receptors on Uninnervated Myotubes

On-line computer analysis of ACh sensitivity, along with photographs of the ACh pipette at each test site, permitted more complete and precise mapping of the muscle surface than has been possible in the past. In the experiment shown in

Fig. 2, the response to iontophoretically applied ACh was tested at 64 locations. The value of each integer indicates the ACh sensitivity at that point. One area on the muscle surface was 10 to 20 times more sensitive than the surrounding membrane. In this study we define a hot spot as a circumscribed area at which the ACh sensitivity was at least three to five times greater than the surround. Single points of high sensitivity were not included. Discrete hot spots ~10 μm across were the rule, but on occasion larger areas of high sensitivity were encountered. Although most myotubes had at least one hot spot within the field of view (~250 μm across), a detailed search was usually necessary to find one; the sensitivity over most areas tested was relatively uniform.

There was a precise correlation between the distribution of ACh sensitivity and the distribution of α-BuTx binding sites. The autoradiograph in Fig. 2, prepared after the culture was incubated in ¹²⁵I-α-BuTx, shows a cluster of silver grains that corresponds to the locus of high ACh sensitivity. Every hot spot (on other myotubes) tested in this way corresponded to a cluster of α-BuTx binding sites and, conversely, regions devoid of hot spots

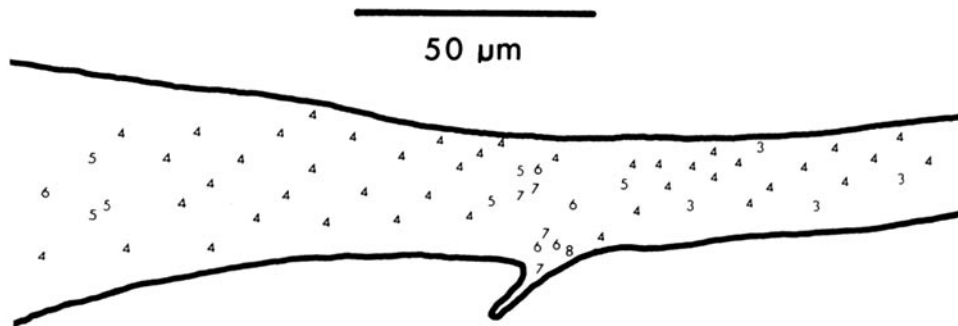
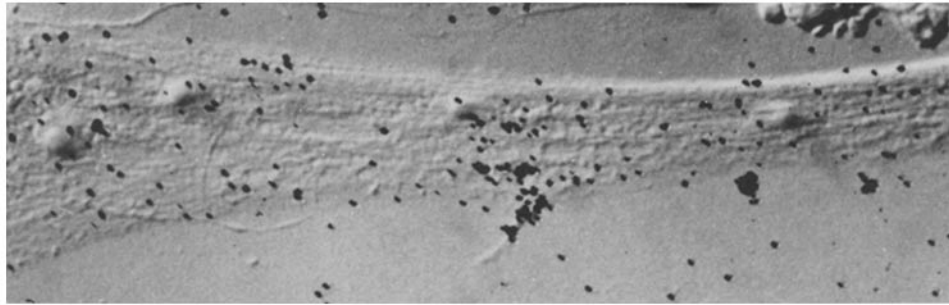


FIGURE 2 Correlation between ACh sensitivity and ^{125}I - α -BuTx binding sites. Each number represents the sensitivity of the myotube to ACh at that point according to the following scale: 1, 10–22 mV/nC; 2, 22–40 mV/nC; 3, 46–100 mV/nC; 4, 100–220 mV/nC; 5, 220–460 mV/nC; 6, 460–1,000 mV/nC; 7, 1,000–2,200 mV/nC; 8, 2,200–4,600 mV/nC; 9, 4,600–10,000 mV/nC. Some patches of membrane are 5–10 times more sensitive than surrounding regions. After the fiber was mapped, the culture was incubated in 10^{-8} M ^{125}I - α -BuTx and processed for autoradiography. Areas of high sensitivity correspond to clusters of silver grains in the autoradiogram.

exhibited low and relatively uniform levels of toxin binding.²

Many hot spots were found near muscle nuclei, but this was not an invariant rule (22). For example, some of the hot spots in Figs. 4, 11, and 12 were adjacent to muscle nuclei, but those in Fig. 3 and the two synaptic hot spots in Fig. 11 were not. Nuclei migrate through the myotube's cytoplasm, whereas hot spots on the surface are relatively stable (see below) so a more precise correlation between the locations of the two would be unexpected. Thin, triangular extensions along the edges of myotubes were frequently extremely sensitive to ACh (Figs. 2 and 4).

² The precise relation between toxin binding and ACh sensitivity is not known. In two myotubes, log-log plots of ACh sensitivity vs. grain density were approximately linear over a 40-fold range of ACh sensitivities. The slope was 0.6 in one case (the cell shown in Fig. 2) and 0.86 in the other (19, 33).

Distribution of Receptors on Uninnervated Myotubes Does Not Change with Time

Repeated maps of uninnervated myotubes showed that hot spots remain in approximately the same position. In the example shown in Fig. 3, the second map was constructed 36 h after the first. The overall level of sensitivity and the locations of two hot spots were the same. In all, 27 hot spots were relocated within 10 μm of their initial positions on 22 different uninnervated myotubes after periods of time ranging from 3 to 48 h; 10 clusters were followed for >20 h. No new hot spots appeared and none disappeared on myotubes that did not significantly grow or change in shape.

The distribution of receptors remained stable even when nearly all of the receptors were blocked with α -BuTx. Two hot spots were located on the fiber shown in Fig. 4 (left), and then the culture was exposed to toxin for 60 min. After only 30 min in toxin, the ACh sensitivity was virtually abol-

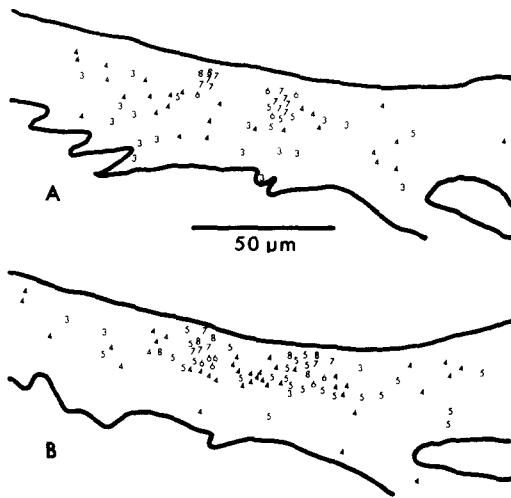


FIGURE 3 Stability of ACh sensitivity distribution with time on an uninervated myotube. The map shown in the lower panel of the figure was made 36 h after the one in the upper panel, yet the magnitude and distribution of sensitivities at the two times were similar. See Fig. 2 for relation between integers and mV/nC.

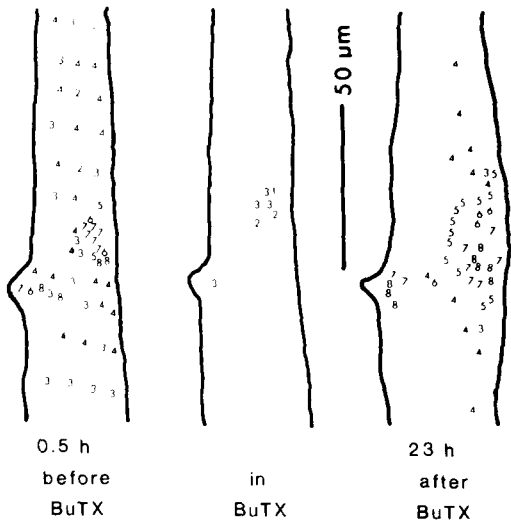


FIGURE 4 Stability of ACh sensitivity distribution after blockade of receptors with α -BuTx. *Left*: control ACh map. *Center*: ACh sensitivity after 30 min of a 60 min incubation in 10^{-8} M α -BuTx. The sensitivity is already decreased more than 20-fold. *Right*: ACh map 24 h after the culture was washed and returned to the incubator. The distribution and magnitude of sensitivity are comparable to the control (preblock) map. This myotube was not innervated. See Fig. 2 for relation between integers and mV/nC.

ished (Fig. 4, *center*) but 24 h later the sensitivity had returned to control levels and the hot spots had reappeared at the same sites (Fig. 4, *right*). The recovery of ACh sensitivity after α -BuTx blockade is due to synthesis and incorporation of new receptors rather than to dissociation of the toxin-receptor complex (15). Thus, the stable distribution of receptors does not depend on the metabolic stability of individual receptors.

Sites of Transmitter Release and Associated Hot Spots

As previously reported (13), synapses located by focal depolarization of nerve processes overlying innervated myotubes were restricted to short segments along motor axons. The same result was obtained by focal extracellular recording. As illus-

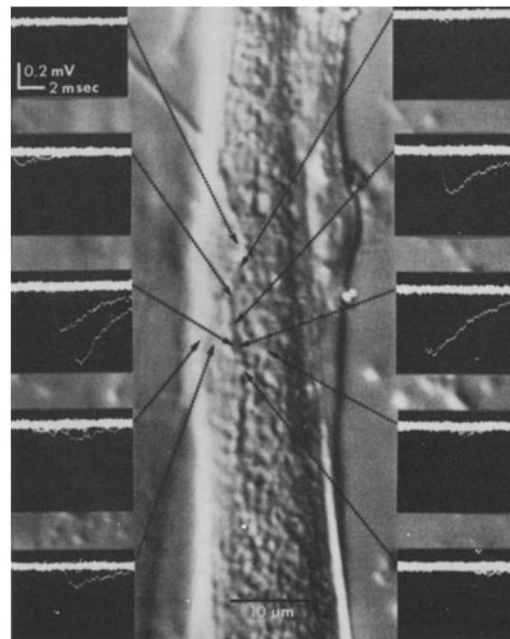


FIGURE 5 Localization of a nerve-muscle synapse by focal extracellular recording. The nerve process, visualized with interference contrast optics, contracts the myotube at the upper left, and then continues along the left side of the muscle cell. Each inset shows the largest potentials recorded at the position indicated by the arrowhead. The steep decline in amplitude of potentials when the pipette was moved 3–4 μ m to either side of the nerve illustrates the spatial resolution of the technique. The decline in response amplitude along the nerve was only slightly more gradual. Note the swelling in the nerve axon at the site of transmitter release.

trated in Fig. 5, the amplitude of extracellularly recorded synaptic potentials declined to less than half when the electrode was removed only a few micrometers along the length of a neurite away from the site of transmitter release.

Small, apparently smooth, oval dilatations, like that shown in Fig. 5, were observed at many functional synapses. Scanning electron microscopy, performed in collaboration with Dr. Gunther Albrecht-Buehler, showed that these varicosities were not so simple as they appeared. The varicosity labeled *a* in Fig. 6 was functionally identified as a synapse, but no synaptic potentials were recorded at the dilatations marked *b* and *c*. Several fine filopodia project from the synaptic

varicosity, and its surface membrane appears rough. Six other identified synapses relocated in the scanning microscope appeared similar to the one shown in Fig. 6. In contrast, the dilatations at *b* and *c* were smooth, and only one filopodium was evident. Most varicosities observed along the length of neurites were smooth and had no filopodia. Informed by the scanning microscopy, we reexamined identified synapses in unfixed cultures with an oil immersion ($\times 100$, numerical aperture = 1.3) interference contrast objective lens. Fine processes could be resolved at some sites of release (Fig. 7).

ACh receptors are clustered at sites of transmitter release on innervated myotubes (13). Fig. 8

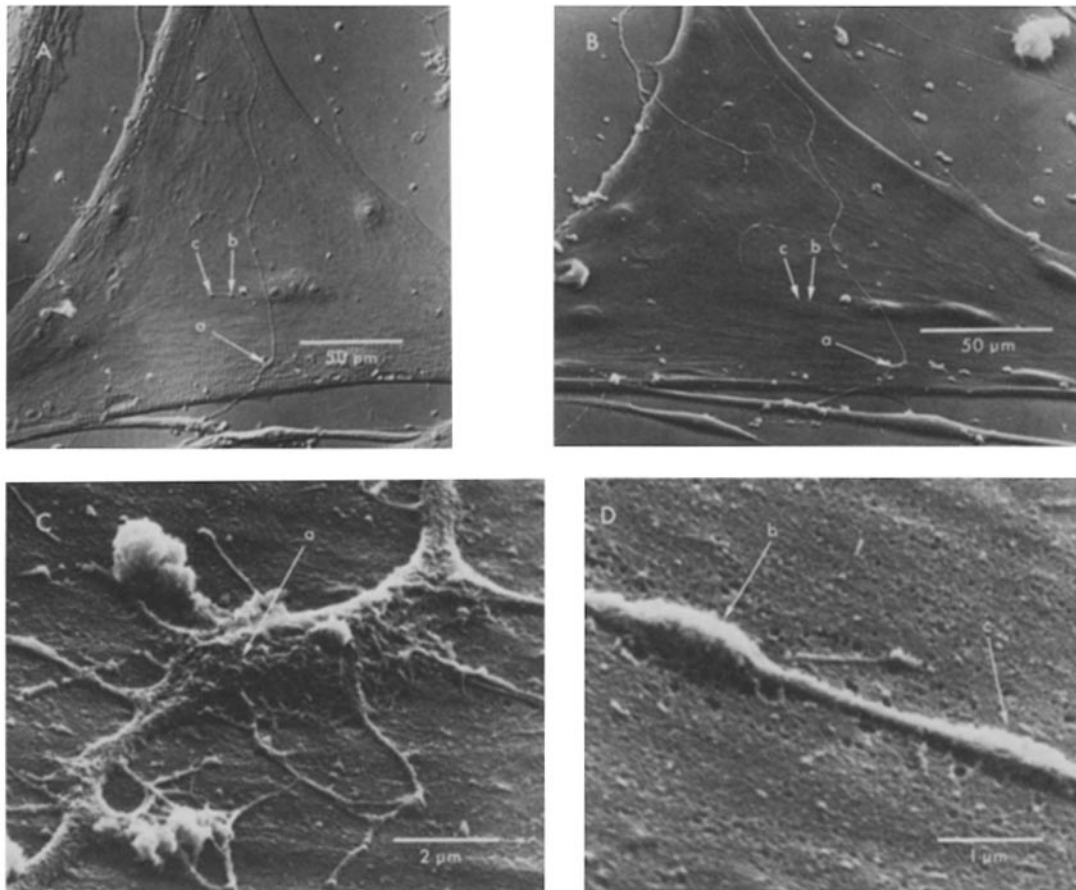


FIGURE 6 Scanning electron microscopy of identified sites of transmitter release. (A) Unfixed culture, viewed with interference-contrast optics. (B) The same area viewed by SEM (60° viewing angle). Synaptic currents were recorded at the varicosity labeled *a*, but not at varicosities *b* and *c*. At higher magnification (C and D), the synaptic varicosity is characterized by a rough surface texture and several long filopodia that project over the muscle surface, whereas the swellings at *b* and *c* are smooth and only one filopodium is evident. The photograph in Fig. 6 D was taken after the culture was rotated 180°.

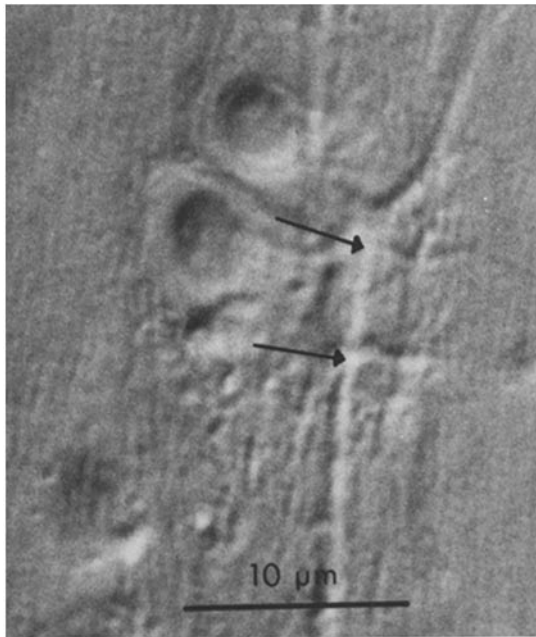


FIGURE 7 Two sites of transmitter release (arrows) in an unfixed culture viewed with interference contrast optics ($\times 100$ oil immersion objective). Note the fine filopodia that radiate outward from the synaptic varicosities.

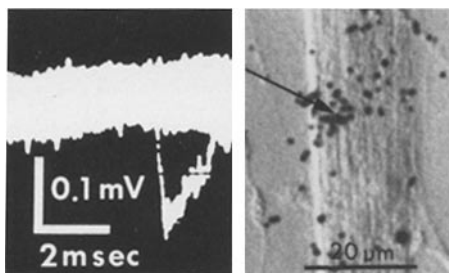


FIGURE 8 A cluster of ACh receptors at an identified nerve-muscle synapse. The culture was incubated in ^{125}I - α -BuTx and processed for autoradiography after the synapse was located by focal extracellular recording. A synaptic response is shown on the left. The density of toxin binding sites at synapses is 10 to 20 times greater than the density in nerve-free areas.

shows a cluster of ^{125}I - α -BuTx binding sites at an identified synapse. The grain density at synaptic hot spots was not obviously greater than that at uninnervated clusters. The geometric mean sensitivity of hot spots at identified synapses (3,300 mV/nC, $n = 16$) was slightly greater than that of hot spots not under neurites (1,820 mV/nC; $n =$

27), but the distributions overlapped extensively (Fig. 9). Estimates of absolute sensitivity depend on membrane potential and input resistance as well as receptor density, so we also calculated the ratio of hot spot to extra-hot spot sensitivity measured on the same fiber. Here again, the geometric mean ratio was slightly greater at innervated (12.7) than uninnervated (9.6) hot spots, but the distributions were similar. When all subneural hot spots (including those not identified as subsynaptic) were compared with non-neural hot spots, the histograms were virtually identical (Fig. 9).

New Hot Spots Appear during Synapse Formation

In contrast to the stable distribution of ACh receptors on uninnervated myotubes, we documented the appearance of 22 new hot spots beneath ingrowing spinal cord nerve processes. Synaptic potentials were evoked by focal stimulation at six newly formed hot spots. Six other new hot spots were beneath motor axons (synaptic potentials were recorded with an intracellular microelectrode after stimulation of the axon at some distance from the myotube), but the exact site of transmitter release was not determined; in two of these cases we were unable to evoke release by

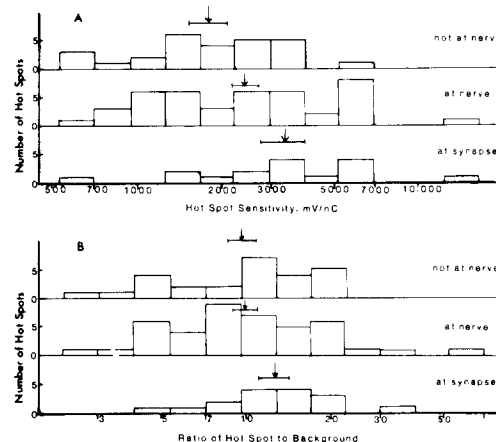


FIGURE 9 ACh sensitivities of hot spots at identified synapses (at synapse) of all hot spots beneath neurites (at nerve), and of hot spots that were not associated with neurites (not at nerve). Absolute sensitivities are shown in Fig. 9A and hot spot/background sensitivity ratios are shown in Fig. 9B. Four hot spots only two to four times more sensitive than the background are included, to illustrate the entire range encountered. The geometric mean of each distribution is marked by an arrow. Bars below each arrow indicate \pm SE.

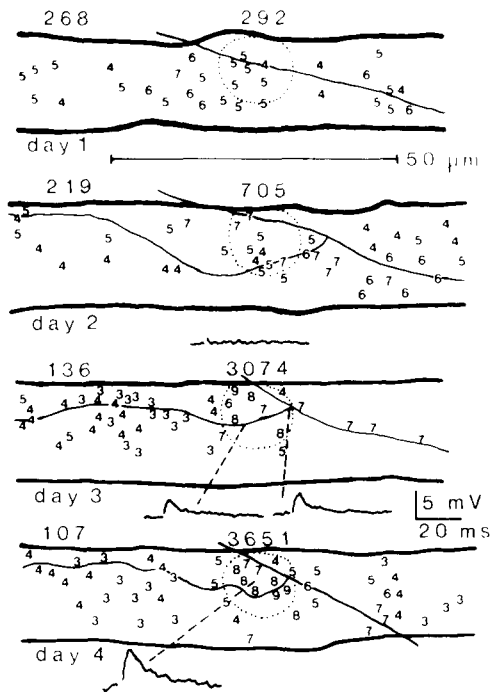


FIGURE 10 Formation of a new hot spot during synapse formation. The same myotube was mapped on 4 consecutive days beginning 3 d after addition of a spinal cord explant. For ease of reference a dotted circle delineates the region of synapse formation. Numbers above each circle are the mean sensitivities (mV/nC) of all points within the circle. Numbers to the left are the mean sensitivities of adjacent extrasynaptic areas. On day 1, the sensitivity within the circle was not significantly greater than background. By day 2, foci of high sensitivity within the future synaptic region had appeared, but synaptic potentials could not be elicited by focal stimulation at several locations in this area (a representative negative oscilloscope trace is shown below the map). Synaptic potentials were evoked on day 3 at positions indicated by the dashed lines, and at this time the ACh sensitivity had increased to 22 times the background level. On day 4, the sensitivity was 34 times higher than background. The ultrastructure of one part of this synaptic complex is shown in Fig. 16.

focal depolarization of the neurite over the hot spot. In the remaining 10 cases, the functional identity of the overlying neurite was not tested.

Our most completely documented sequence of hot spot and synapse formation is shown in Fig. 10. The mean ACh sensitivity of points within the dotted circle increased from a value that was indistinguishable from that of the sensitivity of the surrounding area to 34 times the background,

extrasynaptic level over a period of 4 d. Synaptic potentials were evoked at the indicated points within the circle on the 3rd d, but could not be evoked 1 d earlier. Synapses were still present within the circle on the 4th d, and 1 d later, the culture was fixed, stained for AChE, and prepared for electron microscopy (see below). The background sensitivity decreased about twofold during the 4-d period, but this was not observed during the formation of other synapses.

In the experiment shown in Fig. 11, synapses and hot spots formed at sites of relatively low sensitivity within 30 μm of a preexisting hot spot. A growth cone palpated the edge of the previously uncontacted myotube while the measurements in Fig. 11 (top) were being made; 12 h later, at least two synapses had formed (broken lines in Fig. 11, bottom) and the membrane at these sites had become 8–12 times more sensitive to ACh.

The sequence shown in Fig. 12 suggests that the

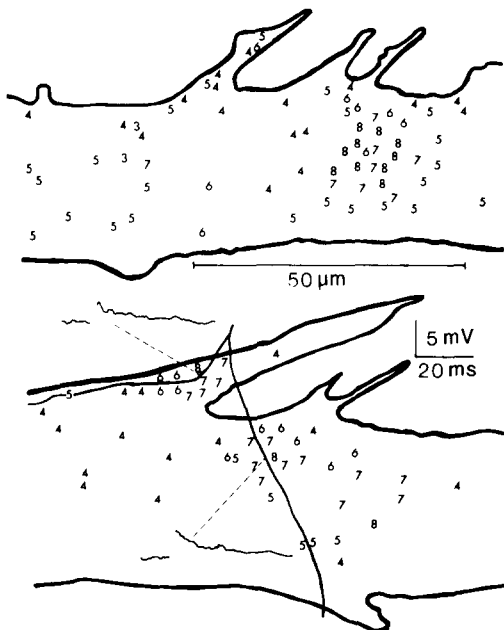
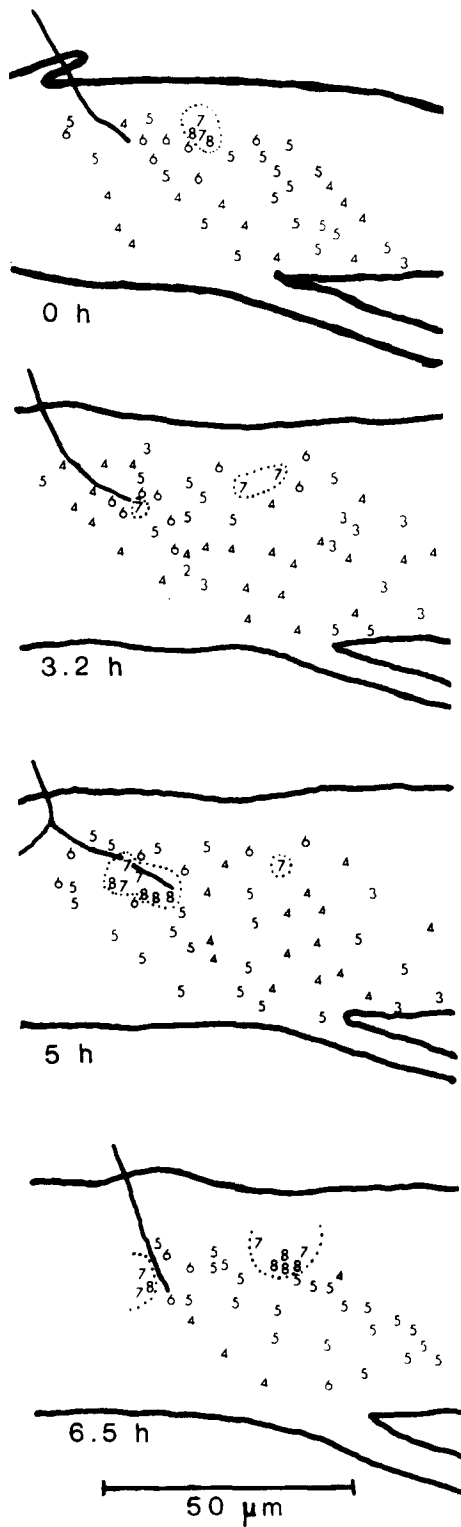


FIGURE 11 Synapse and hot spot formation within 30 μm of a preexisting hot spot. Growth cones palpated the myotube while the map shown in the upper panel was constructed. 12 h later, one branch of the axon had grown across the muscle cell and another had advanced along the upper edge. Synaptic potentials were evoked at the locations indicated by dashed lines and the ACh sensitivity at these sites had increased 8- to 12-fold. The sensitivity of the preexisting hot spot (to the right of the synapses) which was never innervated, decreased to background levels 1–2 d later.



distribution of ACh sensitivity can change within 3 h. When first examined, this nerve process ended in a stationary growth cone. The tip did not advance, but active microspikes (not drawn) were extended and retracted during the entire 6.5-h observation period. At this time, a single hot spot was evident $\sim 15 \mu\text{m}$ to the right of the nerve ending. About 3 h later, two hot spots were evident, one in close proximity to the nerve. Both hot spots then remained in the same location during the final 3.5 h of the experiment. Of the 22 new subneural hot spots, at least seven were created within 5 h.

We do not know whether the contact shown in Fig. 12 was destined to become a synapse, but in two other cases we were able to evoke transmitter release from active growth cones soon after they contacted a myotube. One example is shown in Fig. 13. Time-lapse cinematography showed that the growth cone migrated across a muscle fiber (on the right) before pausing for 2 h at the edge of the tested myotube. Synaptic potentials were evoked by focal depolarization of the tip at that time. Shortly thereafter, the growth cone advanced along the myotube, but synaptic potentials could still be evoked at the same site 18 h later. The other growth cone tested had also paused at the edge of a myotube before synaptic potentials appeared. In both cases, the muscle membrane immediately around the synapsing growth cone was extremely sensitive to ACh. Unfortunately, the same areas were not tested before the growth cones arrived.

Disappearance of Uninnervated Hot Spots

The hot spot shown in Fig. 11 that was present before the motor axon arrived was never innervated and it disappeared 2 d later. In contrast to

FIGURE 12 Rapid appearance of a subneural hot spot. When first examined this nerve process ended in a growth cone (not diagrammed) near a small hot spot. The ACh sensitivity beneath the growth cone was increased within 3.2 h. It increased further over the next 2 h and then stabilized. The nearby hot spot remained in place over the final 3 h. This myotube was innervated at 0 h, but it was contacted elsewhere by other neurites and the site of transmitter release was not determined. Between 5.0 and 6.5 h the neurite moved laterally but the growth cone remained fixed on the muscle surface. Dotted lines delineate regions with a sensitivity of 7 or higher.

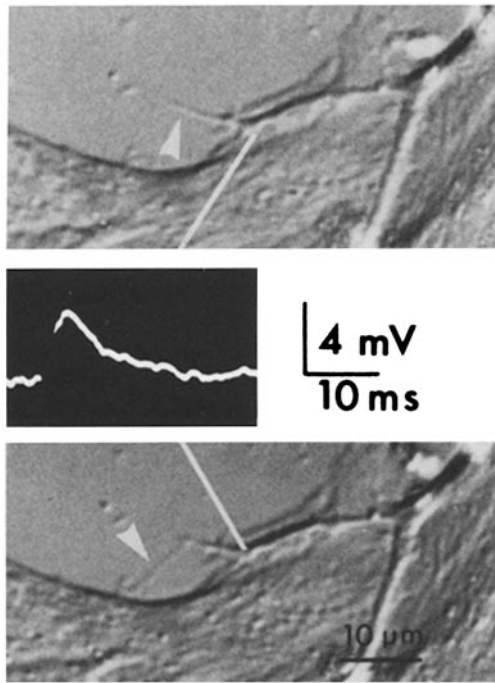


FIGURE 13 Synaptic potentials evoked by focal depolarization of an active growth cone. The neurite had grown rapidly over the myotube on the right, and had then paused for 2 h at the location shown. Synaptic potentials were evoked by focal stimulation at the broad base of the growth cone (white line). Stimulation at a site 10 μm more proximal failed to evoke transmitter release. The photographs were taken 2 min apart. In this interval a microspike (arrowheads) extended further and bent toward the myotube.

our findings on myotubes not contacted by nerve processes, 12 uninnervated hot spots on muscle fibers that were contacted disappeared with time. At least eight of these hot spots were on myotubes that were innervated at some other site.

Innervated and subsequently denervated hot spots also disappear when the myotube is reinnervated at another site. In the experiment shown in Fig. 14, three synapses were located physiologically, and each one was associated with an area of high sensitivity. All nerve processes were then pulled away from the myotube with a micropipette. By the next day, new neurites had contacted and innervated the myotube at another location (synaptic potentials were recorded with an intracellular microelectrode). At this time, the ACh sensitivity at the previously identified synaptic hot spots had decreased to background levels.

AChE Accumulates at Newly Formed Synapses

The synaptic complex studied in the experiment of Fig. 10 was fixed and stained for AChE 3 d after the onset of synaptic transmission. The enzyme was present (Fig. 15) and the reaction product was organized in round zones that resemble stained synaptic boutons at certain adult neuromuscular junctions (31, 38). Transmission electron microscopy of sections cut through this synaptic region (Fig. 16), prepared in collaboration with Dr. U. J. McMahan, revealed that the reaction product was

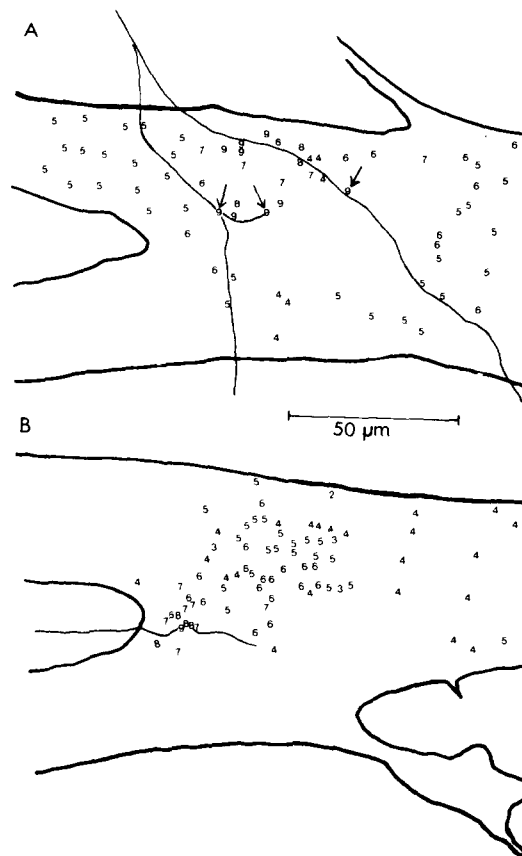


FIGURE 14 Disappearance of hot spots on an innervated myotube. (A) Three synapses were located by focal extracellular recording (arrows). Each synapse was associated with a region of high ACh sensitivity; a third hot spot was also found under a neurite but was not identified as a synapse. All neurites were then stripped away from the myotube with a micropipette, and receptors were blocked with $\alpha\text{-BuTx}$. (B) 21 h later, the myotube was reinnervated at another location, and all three areas of high sensitivity had disappeared.

located in the synaptic cleft and also within membrane-bounded cisternae in the myoplasm near the muscle surface. Some of these cisternae opened to the extracellular space. A definite basement membrane was present within the cleft and it

extended into some of the invaginations of the muscle surface. The nerve terminal contained many clear, 50-nm vesicles, and one region of the presynaptic membrane appeared thickened. The synaptic complex was not covered by a Schwann cell.

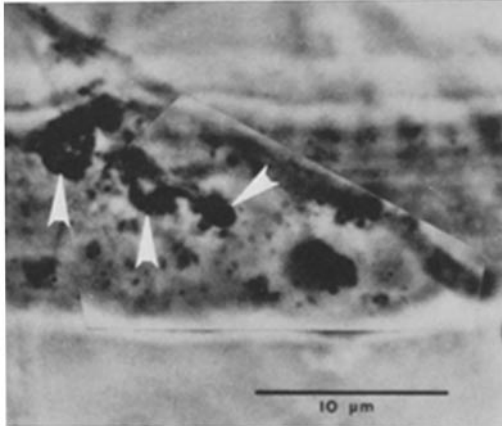


FIGURE 15 AChE at an identified synapse. The same nerve-muscle contact studied in Fig. 10 was fixed and stained for AChE 3 d after the onset of synaptic transmission. Three synaptic boutons, outlined with the esterase stain (arrows), appear to the left of the nerve bundle lying diagonally across the muscle fiber. A preterminal axon, also outlined with stain, connects two of these boutons. A nucleolus along the lower edge of the fiber appears dark in this phase-contrast micrograph, but is unstained.

DISCUSSION

Hot Spot Formation

The hypothesis that embryonic motor axons might seek out preexisting clusters of ACh receptors was based on their very existence, and also on the general observation that regenerating adult axons reinnervate vacated end plates (35). A high density of receptors persists at denervated end plates in vertebrate muscle (26, 36, 39). However, our data show that receptor clusters are not required for synapse formation. Spinal cord neurons can induce new clusters at discrete sites along their length. Since new hot spots rarely, if ever, appear on mature myotubes that are not contacted by nerve processes, our observation of 22 new hot spots beneath spinal cord neurites cannot be due to chance.

Only a small fraction of the nerve processes that emerge from spinal cord explants and contact myotubes in fact form functional synapses. However, all 12 of the neurites that induced receptor clusters and that were also electrically stimulated

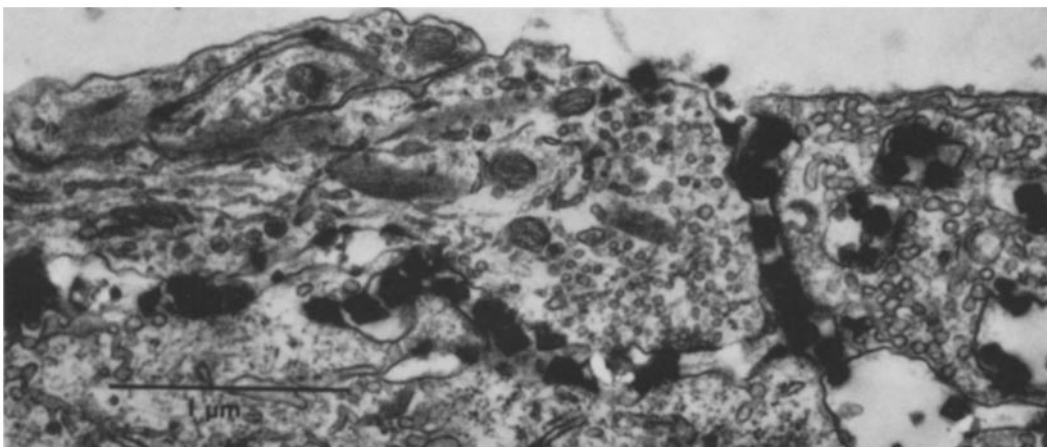


FIGURE 16 Ultrastructure of the middle synaptic varicosity shown in Fig. 15. The preterminal axon, filled with microtubules, enters from the left, and the terminal lies in a depression in the muscle surface. Synaptic vesicles are present within the terminal and appear clustered against the presynaptic membrane. AChE reaction product and a distinct basement membrane are present within the synaptic cleft, but not elsewhere on the muscle surface. Subsurface cisternae in the myotube also contain AChE reaction product and basement membrane. There is no Schwann cell associated with the synapse.

had innervated the underlying myotube at some point. Thus, the ability to induce hot spots may be unique to cholinergic neurons. Synaptic potentials were evoked at six of eight adequately tested new hot spots by focal depolarization of overlying neurites. The two failures might simply reflect the difficulty of the technique. Depolarization of fine neurites in the presence of TTX requires intense local currents, and there is a narrow margin between effective stimuli and those that damage the nerve. Another, more interesting possibility is that receptor clusters appear in the postsynaptic membrane before the development of mechanisms required for transmitter release. We found that subneural hot spots can appear within 3 h and, considering our method of assay, this probably reflects an upper limit. Finally, the possibility that hot spots can appear beneath cholinergic nerves at sites that are not destined to release ACh or beneath noncholinergic nerves must be considered. Hot spots have been detected on rat L-6 myotubes near N-18 neuroblastoma nerve processes even though functional contacts do not form between these cells (28). The distribution of receptors on L-6 myotubes grown without N-18 cells is more or less uniform (33).

Anderson et al. (2) have also concluded that spinal cord neurons are capable of inducing new clusters of ACh receptors. They cultured mononucleated *Xenopus* myocytes with and without neurons dissociated from the neural tube and mapped the distribution of ACh receptors with fluorescent α -BuTx conjugates. Streaks of fluorescence were found along the course of nerve processes overlying muscle cells. These streaks were longer than the small ovoid hot spots found on myocytes not contacted by spinal cord nerve processes, and this suggests that nerve processes caused a change in receptor distribution. Some of the *Xenopus* myocytes were innervated; they twitched when nearby neurons were stimulated. However, the same cells examined with fluorescent toxin conjugates were not also tested physiologically, so it is not certain whether the subneural streaks of fluorescence were unique to synaptic contacts.

In principle, subneural clusters might form by aggregation of receptors within the surface membrane or by the local insertion of newly synthesized receptors. Anderson and Cohen (1) observed characteristic streaks of fluorescence along neurites even when the myocytes were labeled with toxin conjugates before plating neural tube cells. Thus, at least some of the *Xenopus* receptors in

subneural clusters arrive by migration within the membrane. Migration of ACh receptors is not unique to amphibian muscle cells. Axelrod et al. (3) demonstrated, with fluorescence-bleaching techniques, that nonclustered receptors in uninnervated rat myotubes are mobile. They estimated that 75% of the receptors move with an effective diffusion coefficient of $7 \times 10^{-11} \text{ cm}^2\text{s}^{-1}$. It is probable, therefore, that migrating receptors contribute to new subsynaptic hot spots on chick myotubes, but this must be determined directly. The fact that uninnervated hot spots located near newly formed synapses disappear with time (Figs. 11 and 14; cf. reference 1) is consistent with the notion that nerve processes cause a migration of receptors already exposed on the surface.

An argument can be made that local insertion of receptors also plays a role in hot spot formation and maintenance. Receptors are lost from innervated hot spots on cultured myotubes and on intact embryonic muscle fibers with a half time of 30–40 h (9, 10, 47). Yet, hot spots do not disappear with time (and they reappear after blockade with α -BuTx [Fig. 4; see reference 3]), so they must contain newly synthesized receptors. Fluorescence bleaching experiments suggest that receptors within uninnervated hot spots do not exchange with receptors elsewhere in the muscle membrane (3). If the same finding applies to innervated clusters, then their stability must depend on local insertion and removal of receptor molecules.

The rapid turnover of receptors at newly formed embryonic junctions is in marked contrast to the situation at adult end plates where the half life of junctional receptors is longer than 5 d (6). Thus, neither the clustering of receptors nor the fact of innervation is sufficient to modify the metabolic fate of individual receptors. The rate of degradation of innervated chick receptors does eventually decrease but this occurs 2–3 wk after hatching (10).

The mechanism by which spinal cord nerve processes induce receptor clusters remains to be determined. In chick cultures, hot spots are restricted to discrete sites along the length of motor axons. Thus, simple proximity between a competent (cholinergic) neurite and a receptive myotube is not sufficient to induce a receptor aggregate. However, scanning and transmission electron microscopy indicate that the contact between nerve and muscle is specialized at sites of transmitter release and it may be that a unique, receptor-aggregating, physical interaction occurs at these

sites. Alternatively, a chemical factor that influences receptor number and/or distribution may be released in relatively high concentration at synapses. Recent studies have shown that extracts of spinal cord or brain and media conditioned by cultured neurons can increase the total number of receptors (29, 44) and the number of receptor clusters (11, 29, 44) on uninnervated myotubes.

The physiological significance of hot spots on uninnervated myotubes remains obscure. Hot spots are not unique to uninnervated chick myotubes *in vitro*: they are also found on cultured rat myotubes (3, 33) and on mononucleated *Xenopus* myocytes (2). Clusters of receptors also appear in extrajunctional regions of adult denervated amphibian (A. Michler, personal communication) and mammalian (16, 31) muscle fibers, so they may be common to skeletal muscle of all ages deprived of innervation. Extrajunctional hot spots have not been found in intact embryonic muscles (8, 9) but it is likely that all of the muscle fibers examined were already innervated. Receptors in both uninnervated and innervated clusters are similar in terms of their density, mean channel open time, and metabolic stability (47). Thus, the nerve may simply trigger the same mechanism that results in the formation of uninnervated hot spots. It is interesting that about one-third of the new subneural hot spots formed within 100 μm of a preexisting one. The significance of this observation is unclear.

Synapse Formation

Synapses form soon after nerve-muscle contact. Synaptic potentials can be evoked by stimulation at or near stationary growth cones (12). These functional contacts were not transient: the growth cones eventually moved on, but stable, *en passant* synapses were left behind. This result is not inconsistent with Dennis and Miledi's (14) finding of a "nontransmitting" stage during nerve-muscle synapse formation. This stage is apparently caused by block of impulse conduction in fine, regenerated nerve terminals. We evoked transmitter release by focal depolarization in the absence of action potentials. Synaptic vesicles have been recently found in growth cones of cultured adrenergic sympathetic ganglion neurons (34). It remains to be determined whether synaptic vesicles are present in cholinergic growth cones and whether transmitter release is quantal at the earliest times.

The appearance of thin cytoplasmic extensions of the nerve at some established synapses is strik-

ing. They are not artifacts of fixation or critical point drying since similar processes were observed at unfixated synapses. They resemble growth cone filopodia and raise the possibility that newly formed synapses continue to grow or are continually renewed (4). Growth cones are more firmly attached to the culture substrate than the remainder of the nerve process and, by analogy, synaptic filopodia may serve to anchor the newly formed release site in place.

Synapses mature rapidly in this system. Previous reports based on semi-serial thin sections of "nerve-muscle contacts" in young cultures have stressed the simplicity of the structures (19, 41, 42, 48). However, our sections through an identified synapse show that several specializations characteristic of adult junctions are present only 3 d after the onset of synaptic transmission. The presence of a definite basement membrane in the synaptic cleft is significant because myotubes in ara C-treated, fibroblast-free cultures are not encased in an external lamina (23). The basement membrane at adult end plates may be specialized. The entire basement membrane remains as a scaffold after the underlying muscle fiber is destroyed, yet regenerating motor axons seek out and terminate at sites on the external lamina that correspond to the original end plate (37, 46). Tufts of extracellular amorphous material are present on uninnervated myotubes (23) and it will be important to determine whether the basement membrane at newly formed synapses is present before or only after the nerve arrives.

AChE is a peripheral protein at adult end plates apparently associated with the basement membrane in the synaptic cleft (7, 27, 46). Therefore, it is perhaps not surprising that AChE accumulates at newly formed junctions. However, our finding that AChE was present at a synapse 3 d after the onset of synaptic transmission is in contrast with earlier histochemical studies (40, 42) in which foci of AChE did not appear in spinal cord-muscle cocultures until after 3-5 wk *in vitro*. Subsequent work in our lab has shown that AChE can be detected at the majority of synapses in 3- to 4-d cocultures and that when present, it serves to limit the duration of transmitter action (45).

In sum, synapses form and mature soon after receptive myotubes are contacted by appropriate spinal cord axons. Further studies *in vitro* should allow precise definition of the temporal relation between the onset of synaptic transmission, clustering of ACh receptors in the postsynaptic mem-

brane, and accumulation of AChE in the synaptic cleft. In vitro assays should also be useful in studies of the nature of the neural influence on the organization of the postsynaptic membrane and of the detailed mechanism of the muscle response.

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