

The Distribution of Acetylcholine Receptor Clusters and Sites of Transmitter Release along Chick Ciliary Ganglion Neurite-Myotube Contacts in Culture

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Abstract. Acetylcholine receptors accumulate along the length of cholinergic neuron-skeletal muscle contacts in vitro. The main purpose of this study was to describe, in a quantitative way, the distribution of acetylcholine receptor clusters induced by ciliary ganglion neurons over a period of time extending from hours to weeks after contacts are established. Neurites were filled with Lucifer Yellow and receptor clusters were identified with rhodamine-bungarotoxin. A cluster located within 5 μm of a nerve process or 10 μm of the base of a growth cone was considered to be a neurite-associated receptor patch (NARP). The first synaptic potentials were evoked 20 min after growth cone-myotube contact, and, after 24 h of co-culture, >60% of the nerve-muscle pairs tested were functionally connected. NARPs appear rapidly; the first clusters were detected \sim 6 h after the neurons were plated. They were composed of several small subclusters or speckles of rhodamine-bungarotoxin fluorescence. The initial accumulation of receptors may occur at the advancing tips of nerve processes because NARPs were

found at >80% of the growth cone-muscle contacts examined between 12 and 24 h of co-culture. Over the 3-wk period examined, the mean incidence of NARPs ranged between 1.0 and 2.6 per 100 μm of neurite-myotube contact, with the peak observed on the second day of co-culture. During the first 3 d in culture, when the neurons were multipolar, nearly all of the primary processes induced one or more clusters. With time, as the neurons become unipolar (Role and Fischbach, 1987) NARPs persisted along the remaining dominant process. Measurements made during the third day of co-culture suggest that NARPs disappear along shorter neurites before they retract. Synaptic currents were detected by focal extracellular recording at 55% of the NARPs. The fact that spontaneous or evoked responses were not recorded at 45% suggests that contacts with clusters exhibit two functional states. Two types of presynaptic specialization at identified NARPs observed by scanning electron microscopy appear to be correlated with the functional state.

EMBRYONIC motoneurons promote the accumulation of acetylcholine (ACh)¹ receptors (AChRs) at newly formed nerve-muscle junctions in vivo and in vitro (Fischbach et al., 1979; Cohen, 1980; Steinbach and Bloch, 1986; Schuetze and Role, 1987). In chick cultures, it is clear that receptors accumulate soon after nerve-muscle contact is established, and that each neuron is capable of inducing more than one receptor cluster (Frank and Fischbach, 1979; Role et al., 1985). However, little information is available

concerning the distribution of clusters along nerve-muscle contacts or their relation to sites of transmitter release after longer periods of time.

Cell cultures prepared from embryonic chick ciliary ganglia are particularly convenient for such studies. Neurons dissociated from embryonic day (E) 8-E9 ganglia are relatively large, easy to identify, electrically excitable soon after plating (Nishi and Berg, 1977; Tuttle et al., 1980; Bader, et al., 1982; Role and Fischbach, 1987), and they extend processes rapidly on a suitable substrate. All of the neurons within the ganglion are cholinergic, and many of them are capable of innervating skeletal myotubes in vitro (Margiotta and Berg, 1982). This is not surprising considering that \sim 50% of the cells in the intact ganglion innervate striated muscle fibers in the iris and ciliary body (Pilar and Vaughn, 1969a, b, 1971; Marwitt et al., 1971; Pilar et al., 1980). The remaining cells innervate smooth muscle cells in the choroid coat of the eye (Marwitt et al., 1971; Pilar et al., 1980). Most importantly for this study, neurite-associated receptor patches

1. *Abbreviations used in this paper:* ACh, acetylcholine; AChR, acetylcholine receptor; NARP(s), neurite-associated receptor patch(es); R-BTX, rhodamine-conjugated alpha-bungarotoxin.

Portions of this work have appeared in abstract form (1982. *Soc. Neurosci.* 8:129 and 1983. *Soc. Neurosci.* 9:1179).

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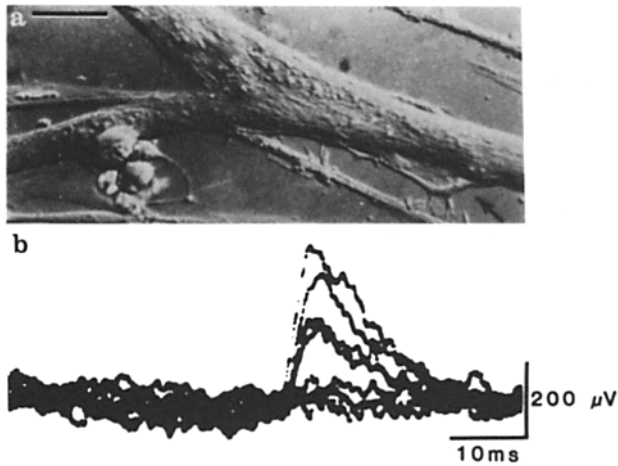


Figure 1. Synaptic potentials evoked after 10 h of neuron–muscle co-culture. The interference contrast micrograph (a) shows a ciliary ganglion neuron that had settled on a myotube. The process extends to the right, and the growth cone is indicated by an arrow. Bar, 20 μm . The synaptic potentials, (b), are typical of >90% of the contacts studied within the first 12 h of co-culture in that they are small, and they fluctuate in size.

(NARPs), appear at ciliary ganglion–myotube contacts with about the same incidence as they do along processes of spinal cord motoneurons (Role et al., 1985).

The neuritic arbor of ciliary ganglion neurons changes with time in vitro (Role and Fischbach, 1987) in much the same manner as it does in vivo (Landmesser and Pilar, 1974, 1976). During the first 3–4 d after plating, the cells are multipolar with several processes extending directly from the cell body. Over the next several days all but one of the processes retract, and the neurons remain unipolar for at least 3 wk, the longest time examined. We previously reported that more than one primary process was associated with receptor clusters (Role et al., 1985). In this paper we describe the distribution of NARPs at early times in more detail, the distribution during the period of process pruning, and the distribution at later times.

We have also attempted to determine if NARPs are located at functional synapses. Several lines of evidence suggest that NARPs mark sites of synapse formation in chick and *Xenopus* cultures. They are present on innervated muscle cells (Anderson et al., 1977; Fischbach and Cohen, 1973; Frank and Fischbach, 1979), and they are apparently uniquely associated with neurites of cholinergic neurons (Cohen and Weldon, 1980; Role et al., 1985). In *Xenopus* cultures, the amplitude and frequency of mepps are correlated with the extent of receptor accumulation (Anderson et al., 1979). In chick cultures, sites along neurites at which transmitter can be released by focal depolarization are associated with receptor clusters (Cohen and Fischbach, 1977; Frank and Fischbach, 1979). However, it is not known if all clusters are located at sites of transmitter release.

Materials and Methods

Cell Culture

Myogenic cells dissociated from 11-d-old chick embryo pectoral muscles were plated on collagen-coated glass coverslips. 5–7 d later, after the cells had fused to form multinucleated myotubes and fibroblasts were eliminated with cytosine arabinoside, the cultures were seeded with neurons dissociated

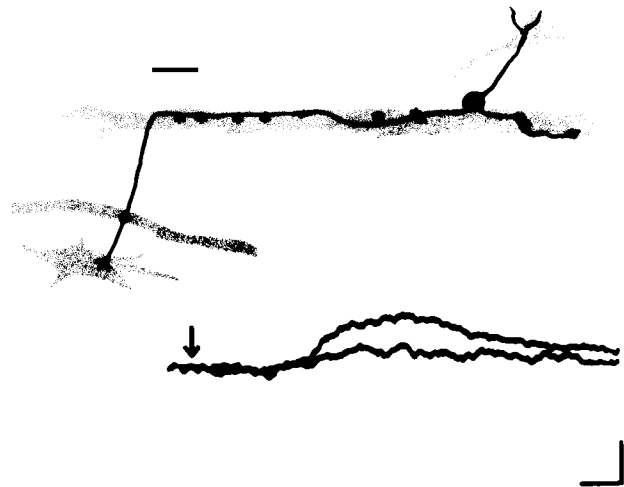


Figure 2. Slow, long latency synaptic potentials. The arrow marks the position of the neuron soma spike (not shown). No responses could be evoked after the second stimulus. Such responses were found at $\sim 10\%$ of the contacts studied during the first 12 h of co-culture. Calibration, 20 ms \times 1 mV. The tracing above the oscilloscope sweeps shows the corresponding nerve–muscle pair. Bar, 20 μm .

from E8 or E9 ciliary ganglia, as described in the preceding paper (Role and Fischbach, 1987). The coverslips formed the bottom of 18-mm holes cut in 60-mm culture plates.

Electrophysiology

Recording conditions were identical to those of the preceding study (Role and Fischbach, 1987) except that the cultures were warmed to 37°C and the chamber (volume ~ 0.2 ml) was perfused at a rate of 1 ml/min. The recording medium contained Hepes (12.5 mM), NaCl (137.5 mM), KCl (6 mM), MgCl_2 (0.8 mM), CaCl_2 (10 mM), glucose (5 mM); pH 7.3. Spontaneous and evoked synaptic potentials were recorded with fine-tipped intracellular electrodes that measured 80–100 M Ω when filled with 3 M KCl. Extracellular electrodes used to detect focal synaptic currents were pulled in two stages and fire-polished to an inner tip diameter of 2–3 μm . They measured 20–50 M Ω when filled with recording medium. Intracellular electrodes were connected to an amplifier (701; World Precision Instruments, New Haven, CT); extracellular electrodes were connected to an 8900 current amplifier (10 G Ω feedback resistor; DAGAN Corp., Minneapolis, MN). Neurons were stimulated through sealed-on extracellular patch electrodes filled with KCl (140 mM), MgCl_2 (2.0 mM), EGTA-K (11.0 mM), CaCl_2 (1.0 mM), Hepes (10.0 mM); pH 7.4 (Role and Fischbach, 1987).

AChR Clusters

Neurons were injected with Lucifer Yellow CH, and the distribution of dye-filled neurites was analyzed as previously described (Role and Fischbach, 1987). AChR clusters were visualized by labeling receptors with one of the following: (a) rhodamine-conjugated alpha-bungarotoxin R-BTX, (b) an immunoglobulin fraction of serum from a myasthenic patient (my-ab; isolated by Michael Lerner, Washington University), or (c) a monoclonal antibody raised in rats (mAb 35; provided by Dr. Jon Lindstrom, Salk Institute, San Diego, CA) that binds to an extracellular aspect of chick AChR but does not block ACh action or BTX binding. In the latter two cases, AChR clusters were visualized with fluorescein-coupled second antibody, either goat anti-human (Kirkegaard & Perry Labs, Inc., Gaithersburg, MD) or goat anti-rat (provided by J. Lindstrom), respectively. The correspondence between R-BTX staining and my-ab or mAb 35 staining was precise (Role et al., 1985). mAb 35 was used in most experiments because it produced lower background staining than the myasthenic immunoglobulins.

The following criteria were adopted to define a NARP. Any AChR aggregate within 5 μm of a nerve process was considered to be a NARP. AChR clusters are present on uninnervated myotubes, but they are few and far between so they were not a source of confusion in this regard. Many neurite endings in young cultures (24 h) had typical growth cone morphology with filopodia that extended 10–50 μm from the growth cone base. Because filopodia can extend and retract or rotate within a few seconds, the proximity

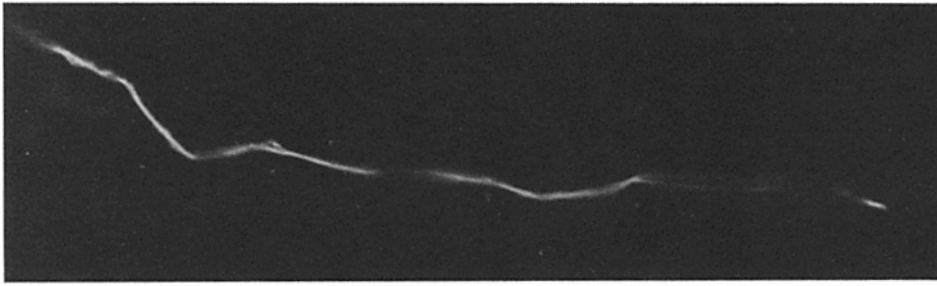


Figure 3. NARPs. The neurite (top) was filled with Lucifer Yellow, and the ACh receptors (bottom) were labeled with R-BTX. The underlying myotube is not visible in these fluorescence micrographs. Bar, 10 μ m.

criterion for NARPs was relaxed from 5 to 10 μ m near growth cones. AChR clusters had to have a minimum area of 10 μ m² and be separated from other clusters by a minimum of 5 μ m to be considered distinct NARPs. AChR clusters that were composed of several small aggregates that appeared as speckles of fluorescence were considered single NARPs.

To aid in the analysis of NARP distribution, images of injected neurons, previously recorded on video tape (one-half inch tape; Panasonic) were analyzed with a digitizing tablet (GTCO) connected to a computer (11/23; PDP). The computer program was written by Viken Matossian (Washington University).

Morphology

For scanning electron microscopy, the cells were fixed by slow replacement of the recording medium with 2.5% glutaraldehyde in a Sorenson's phosphate buffer (pH 7.0) containing 100 mM sucrose at 37°C. The cultures were maintained in this fixative overnight at 5°C. The coverslips were cut away from the culture dishes, and the cells were critical point dried and coated with gold using standard techniques. Specimens were viewed in a Phillips 501 scanning electron microscope. The following procedure was used to relocate NARPs in the scanning electron microscopy that were first identi-

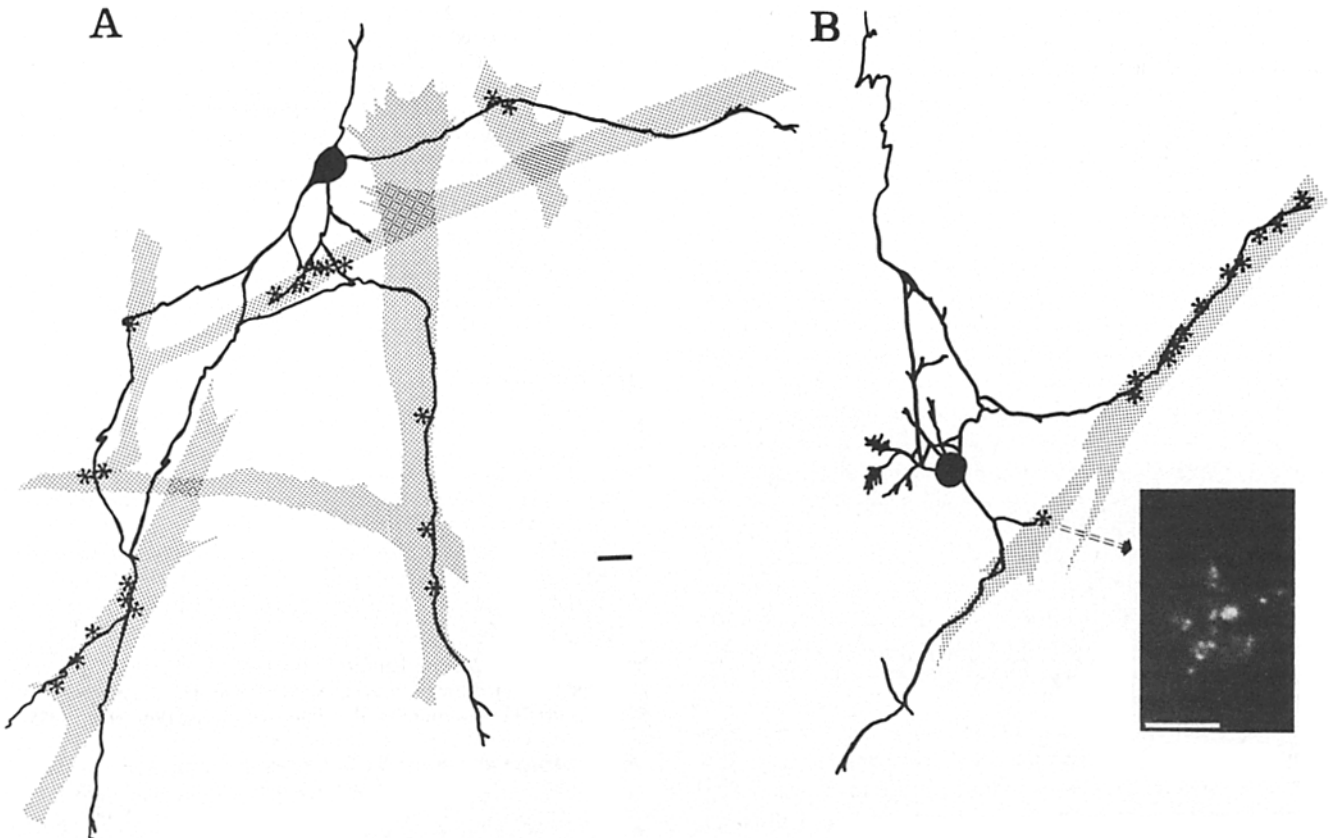


Figure 4. Two neurons (A and B) that illustrate the extent of NARP induction within the first 24 h of co-culture. The neurons (solid lines), myotubes (shaded areas), and NARPs (asterisks) were traced from videotape. Note that several NARPs are found along single neurites, and that more than one primary process is capable of inducing a NARP. Note also the speckled appearance of the growth cone-associated receptor cluster (inset in B). Bars: (A) 15 μ m; (inset in B) 10 μ m.

fied by fluorescence microscopy. Several high power (400–630 \times) fields of view were stored on video tape (see Role and Fischbach, 1987). NARPs tested for synaptic transmission were viewed with the extracellular electrode in place. Low power phase contrast micrographs were then taken to map the general area of interest, and the coverslip was scored from below with a diamond marker. After fixation, critical point drying, and gold coating, the etched circle viewed from above with a dissecting microscope was relocated and the cell-containing upper surface was scored with a fine wire. This rather complicated procedure allowed us to unambiguously relocate $\sim 60\%$ of the NARPs studied electrophysiologically.

Results

Early Synaptic Transmission

The first evoked responses were detected 20 min after nerve–muscle contact. Postsynaptic potentials could be evoked in 29% ($n = 97$) of the neuron–muscle pairs tested within the first 12 h of co-culture, and the proportion of connected pairs increased to 61% by 24 h ($n = 66$). Fig. 1 shows an example of a connected pair examined 10 h after the neurons were plated. The responses are typical of the majority of synaptic potentials recorded at this stage in that their rise time was < 10 ms and successive responses fluctuated in apparently discrete steps consistent with a quantal release mechanism. They differed from responses recorded after 24 h of co-culture in that their amplitude was small (200–500 μ V) and that they fatigued after ~ 20 stimuli delivered at 0.5 Hz. In five of the connected pairs studied within 12 h of co-culture, the evoked responses were quite different; their latency was relatively long (20–50 ms), they rose slowly (50 ms), and they lasted for > 150 ms (Fig. 2). Such long latency and slow potentials cannot be explained by release of transmitter at a site distant from the recording electrode because the longest neurites at this stage were < 300 μ m. More detailed analysis was not possible, as the slow responses fatigued rapidly, often disappearing after only two to three trials. A similarly rapid fatigue of neuromuscular transmission has been noted in the iris of 15-d-old chick embryos (Pilar et al., 1980).

AChR Clusters

The experiments described above show that transmitter release can be detected soon after nerve–muscle contact. NARPs were observed within 6 h of plating neurons on multinucleated myotubes, and by 24 h nearly all of the functionally connected nerve–muscle pairs were associated with at least one NARP. Since most ciliary ganglion neurons are multipolar during the first 2–3 d after plating (Role and Fischbach, 1987) it was important to determine if more than one of the processes were able to induce receptor accumulation. We charted the position of all receptor clusters along Lucifer Yellow–filled neurites by labeling the AChRs with R-BTX. Using a SIT camera and video tape recorder (see Role and Fischbach, 1987) we mapped ~ 100 ciliary ganglion neurons at different times ranging from 4 h to 3 wk after plating. The appearance of an injected neurite process and underlying NARPs is shown in Fig. 3.

Nearly all of the processes of multipolar neurons that contacted myotubes were associated with receptor clusters. Furthermore, several processes induced more than one NARP along a contacted myotube. The tracings in Fig. 4 illustrate both phenomena. Each of the three primary processes of the neuron shown in *A* that contacted adjacent myotubes were associated with receptor patches. The upper right hand process

12–36 h



Figure 5. Schematic representation of ciliary ganglion neurons and NARPs observed between 12 and 36 h after co-culture. Filled circles and the first vertical lines on the left represent cell bodies. Thin lines represent neurites lying on the collagen substrate. Thick lines represent neurites lying on myotubes. Dots represent NARPs. A majority of the neurites overlying myotubes are associated with more than one receptor cluster.

of the neuron shown in *B* traveled along a myotube for ~ 100 μ m and induced 11 discrete NARPs, including one at the growth cone ending. The NARP at the other neurite ending in *B* had a speckled appearance (inset).

Linear representations of ciliary ganglion neurons and associated NARPs that are typical of the 23 cells examined be-

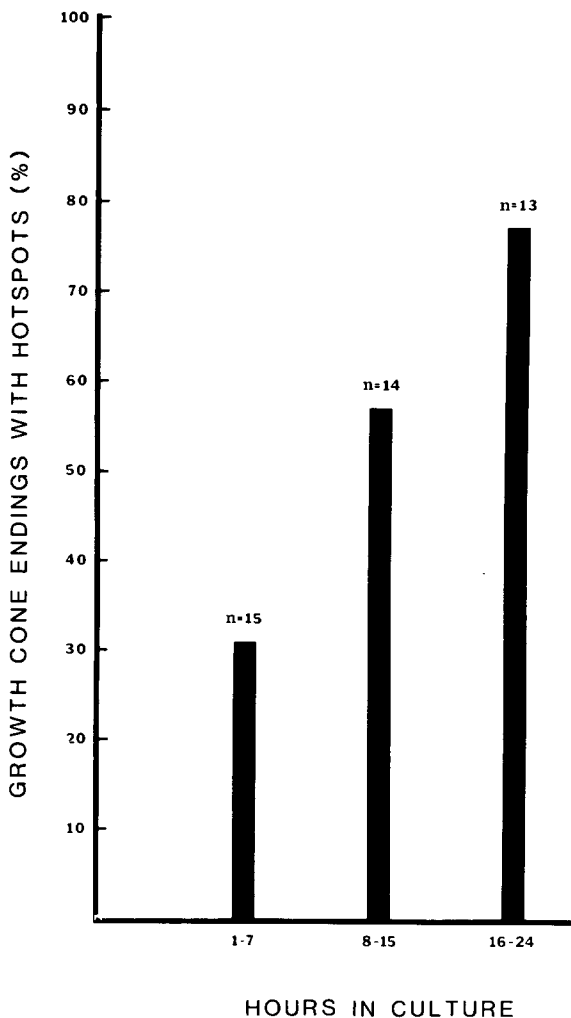


Figure 6. The relation between growth cones and AChR clusters during the first 24 h of co-culture. Because growth cones extend filopodia, any receptor (R-BTX) patch within 10 μm of the nerve ending instead of the usual 5 μm (along the neurite) was considered a NARP. The number of growth cone–myotube contacts examined in each interval is indicated above each bar.

tween 12 and 36 h after plating are shown in Fig. 5. At this stage, the number of clusters per neuron ranged between 9 and 30. Of the 82 processes that contacted myotubes examined during this interval, only 6 were NARPless.

Many of the neurite endings located on myotubes were associated with NARPs. Fig. 6 summarizes data from 42 growth cone–myotube contacts (33 neurons) examined during the first 24 h of co-culture. Within the first 7 h, NARPs were found at $\sim 30\%$ of the growth cones. The incidence increased to nearly 80% between 16–24 h.

The incidence of NARPs along neurites changed with time in vitro (Fig. 7). During the first 8 h after plating, when the processes were only 200–300 μm long, the mean NARP density was 1.4/100 μm of nerve muscle contact. Within the next 8 h the incidence nearly doubled to 2.6/100 μm , the peak value observed at any time during the 3-wk period examined. Between 16 and 24 h, the incidence of NARPs decreased. By 25–36 h, the mean was 1.0/100 μm . This level was maintained for the next 3 wk. During the first 36 h after plating, neurites grow rapidly ($\sim 40 \mu\text{m}/\text{h}$) but the rate slows con-

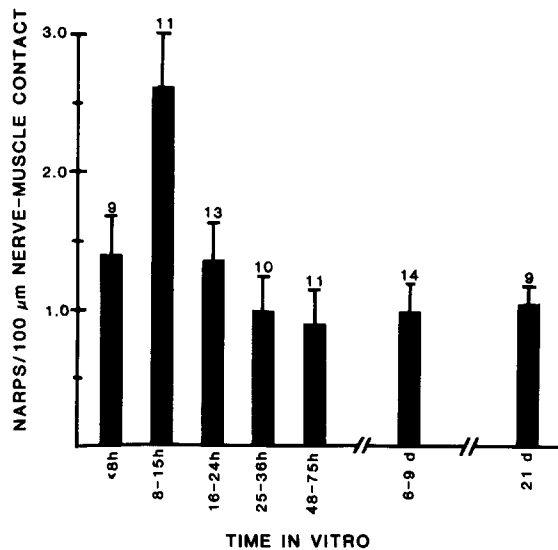


Figure 7. The incidence of NARPs along ciliary ganglion neurites as a function of time. The number of NARPs per 100 μm of neurite–myotube contact was determined for 77 neurons examined between 4 h and 21 d of co-culture.

siderably thereafter, and by 7 d there is no net increase in neurite length at all (Role and Fischbach, 1987). Therefore, the fixed NARP density implies that no new NARPs appear or that new NARPs replace old ones on a 1:1 basis.

Although the overall incidence of NARPs stabilized, their distribution among the several processes changed dramatically. As noted above, nearly all of the ciliary ganglion neurons change from a multipolar to a unipolar geometry during the first week after plating. The remaining, dominant process of all 23 neurons examined after 1 wk was associated with

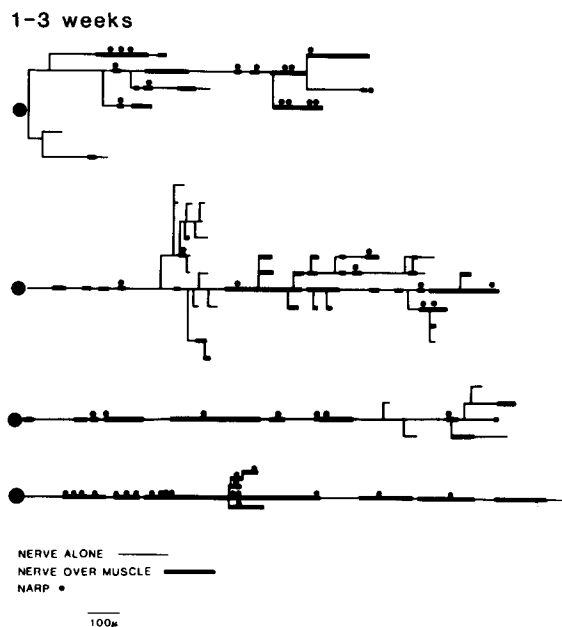


Figure 8. Schematic representation of ciliary ganglion neurons and NARPs examined between 1 and 3 wk of co-culture. At this time, nearly all of the neurons are unipolar; one process issues from the cell body (Role and Fischbach, 1985). The major process was always associated with several AChR clusters.

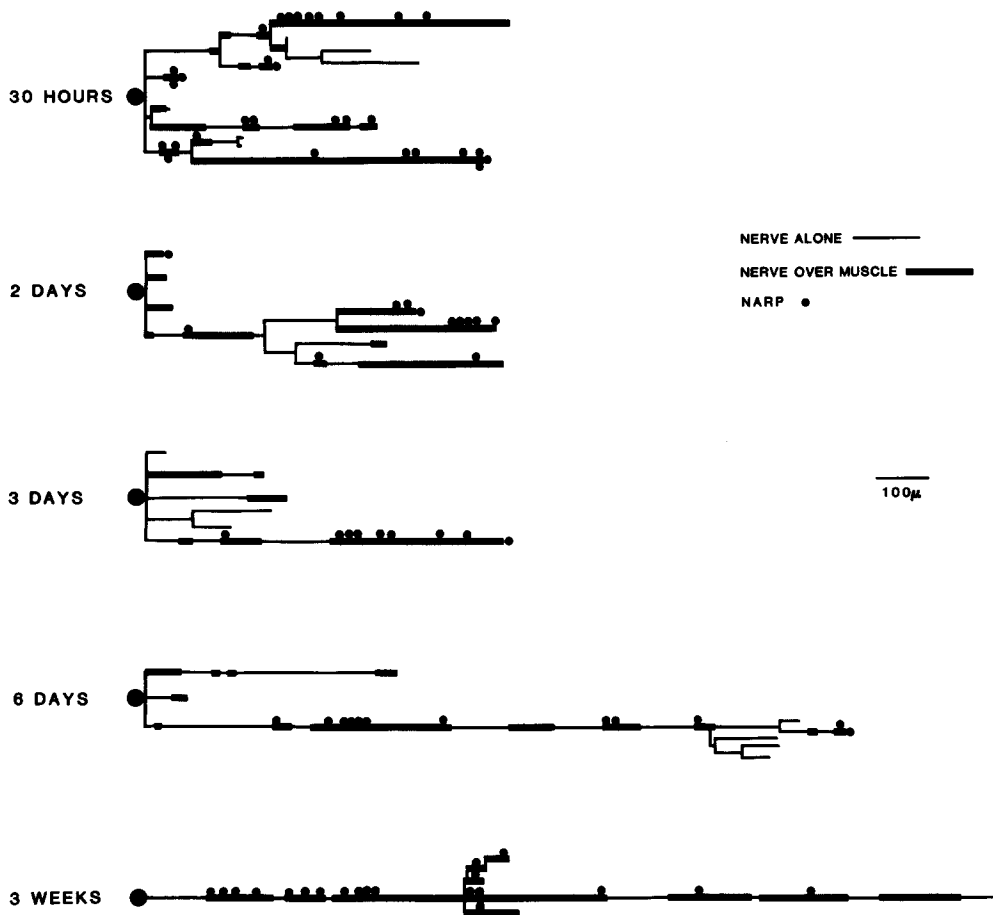


Figure 9. Diagrams of ciliary ganglion neurons that are representative of samples examined at the indicated times. At early times, nearly all of the neurites are associated with AChR clusters. At intermediate time (3 and 6 d) the shorter processes have few if any NARPs even though they are still in contact with myotubes.

several NARPs. Representative examples are shown in Fig. 8. Examination of cells at intermediate times suggest that NARPs disappear before processes retract. As shown in the representative tracings in Fig. 9, between 3 and 6 d after plating, when pruning is incomplete, >90% of the NARPs are associated with the dominant process, even though shorter neurites of the same cells are still in contact with myotubes. Results from all neurons studied are summarized in Fig. 10. They suggest that NARPs are reapportioned along the dominant process before there is significant elimination of neurites. The 48–75 h period is the critical interval.

Although an accumulation of AChRs is characteristic of all functionally connected nerve–muscle pairs, we do not know if all NARPs are sites of transmitter release. We sought evidence for spontaneous and/or stimulus-evoked transmitter release by focal extracellular recording. To facilitate electrode placement, NARPs were visualized with a myasthenic antiserum or with an anti-AChR monoclonal antibody, neither of which blocked synaptic transmission at the concentrations used. The presence of a synapse at some point along the nerve–muscle contact was documented by simultaneous intracellular recording. Stimulation was considered adequate if synaptic potentials were recorded with the intracellular electrode and/or if a conducted spike was detected with the extracellular electrode. In total, synaptic events were detected at 13 of the 24 NARPs examined. Examples of spontaneous synaptic potentials recorded at two NARPs separated by ~20 µm along the same neurite are shown in Fig. 11a, b. No synaptic currents were detected when the extracellular

electrode was placed midway between them. In several cases, stimulus-evoked responses but no spontaneous currents were recorded with the extracellular electrode. A recording from a negative NARP on an innervated myotube is shown in Fig. 11 c. Note that the extracellular electrode was placed close

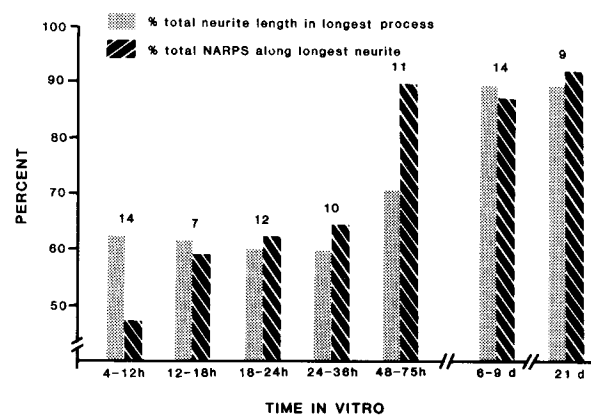


Figure 10. The distribution of NARPs along the longest neurite as a function of time of co-culture. This summary bar graph includes results from 77 ciliary ganglion neurons. Numerals above the bars indicate the number of cells examined in each interval. The number of NARPs along the longest neurite parallels the length of that neurite except during the 48–75-h interval. During this period, the longest process accounts for only 65–70% of the total arbor, but it is associated with 90% of the NARPs.

enough to detect the presynaptic spike (Fig. 11 c, bottom trace).

Scanning Electron Microscopy

Over 400 μm of nerve muscle contact without NARPs were examined by scanning electron microscopy, and in no case did these controls appear specialized in any obvious way. The cylindrical processes seemed to simply course over the myotube (Fig. 12 A). We examined 20 NARPs by scanning electron microscopy; 19 of them showed one of two distinct types of specialization. The first, observed at 12 NARPs, is illustrated in Fig. 12 B. The neurite appears flattened and partially embedded in the myotube surface. The second, seen at seven NARPs, was characterized by a profusion of fine filopodia-like processes, many of which contained small varicosities $<0.5 \mu\text{m}$ across (Fig. 12 C).

In another series, 10 NARPs in 6–7 d co-cultures were studied with focal extracellular microelectrodes and then successfully relocated by scanning electron microscopy. Synaptic currents were recorded at 7, and they all displayed fine neurites studded with varicosities. The three negative NARPs all resembled the flattened contact shown in Fig. 12 B.

Discussion

Clusters of AChR were detected beneath ciliary ganglion neuron processes within 6 h after the dissociated neurons were added to embryonic myotubes. Since it takes 2–3 h for the neurons to settle on the culture surface and extend a process of at least 100 μm , NARPs may form as soon as 3 h after the first nerve–muscle contact is established. Indeed, our data indicate that NARPs are induced at growth cone–myotube contacts. Between 16 and 24 h, 80% of the growth cones were associated with receptor clusters. Considering that the

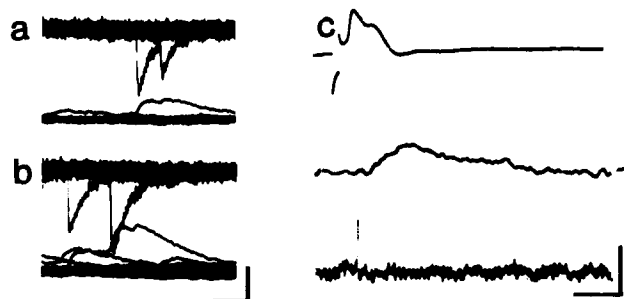


Figure 11. Focal extracellular microelectrode recordings at identified NARPs. The NARPs were labeled with antireceptor antibodies and visualized under fluorescence illumination. The electrodes were positioned under phase contrast illumination. *a* and *b* show recordings at two different NARPs located $\sim 20 \mu\text{m}$ apart along the same neurite. The extracellular potentials (*top traces*) coincide with the rising phase of responses recorded simultaneously with an intracellular electrode (*bottom traces*). Some intracellular responses are not associated with extracellular potentials, and thus, must have originated some distance away. Calibration, 10 pA \times 10 ms. Recordings from a third NARP associated with a different neuron are shown in *c*. The intracellular record (*middle trace*) shows that stimulation of the neuron (*top trace*) evoked a synaptic potential (*middle trace*). However, no synaptic response was detected by the extracellular electrode (*bottom trace*). The neurite spike appears in the extracellular record. Calibration, 20 mV, 2 mV, and 5 pA (from top to bottom) \times 10 ms.

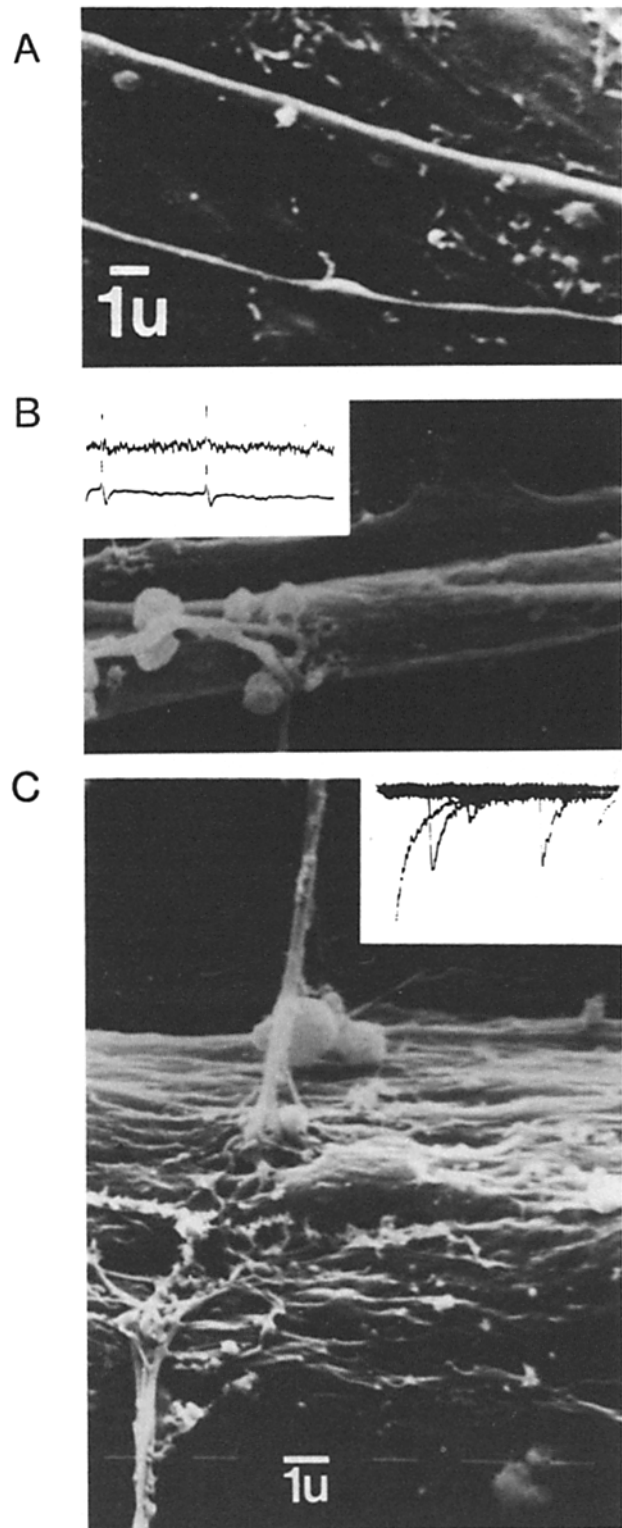


Figure 12. Scanning electron micrographs of neurite–myotube contacts. (A) Control; no NARPs in the vicinity. The cylindrical neurite passes over the muscle surface. NARPs exhibited two types of localization. In one (B), the neurite appeared flattened and embedded in the myotube. Extracellular recordings at such contacts failed to detect synaptic response (*inset*). In the other (C) the neurite appeared to break up into several fine branches, many of which exhibited small varicosities. All of the NARPs at which focal synaptic currents were detected and that were relocated by scanning electron microscopy were in this category. The inset shows spontaneous synaptic currents recorded at the contact shown in the micrograph.

mean incidence of NARPs along neurites at this time is 1.3/100 μm of neurite–myotube contact, this high percentage cannot be due to chance. According to the binomial law, the probability of this outcome is <0.0001 . A similarly high coincidence of nerve endings and receptor clusters has been noted in *Xenopus* neural tube–myocyte co-cultures (Cohen and Wilson, 1985). Other studies have shown that growth cones can release ACh even before they contact target myotubes (Hume et al., 1983; Young and Poo, 1983). It may be that they release other molecules as well, some of which are responsible for inducing postsynaptic receptor clusters.

Some of the earliest evoked responses were reminiscent of ACh release detected at growth cones before contact in their slow time course, long delay (>50 ms) and rapid fatigue (Hume et al., 1983). The fact that most responses evoked during the first 12 h after contact were rapidly rising, had a short latency, and were less subject to fatigue may indicate that transmitter release mechanisms mature rapidly.

The earliest NARPs on chick myotubes are composed of several miniclusters that appear as small speckles of fluorescence when labeled with R-BTX (Fig. 4, this paper; Fig. 3, Role et al., 1985). Similar observations have been made at *Xenopus* nerve–muscle contacts in vitro (Anderson et al., 1984), and at developing chick and rat junctions in vivo (Jacob and Lentz, 1979; Steinbach, 1981; Smith and Slater, 1983). At later times, NARPs appear as elongated streaks or as large condensed patches. Evidence that speckles coalesce to form the larger streaks was obtained by sequential observation of individual *Xenopus* nerve–muscle junctions (Anderson et al., 1984). Data from *Xenopus*, rat, and chick indicate that diffusely distributed receptors present in the surface membrane before growth cone–muscle contact migrate to the junction (Anderson and Cohen, 1977; Moody-Corbett and Cohen, 1982; Kuromi and Kidokoro, 1984; Ziskind-Conhaim et al., 1984; Role et al., 1985). Thus, speckles might reflect a process of microaggregation. In chick cultures, another mechanism contributes to the formation of NARPs. During the first 24 h, NARPs are composed predominantly of new receptors not exposed on the surface before contact is established (Role et al., 1985). Thus, in this species at least, speckles might represent packets of newly inserted, receptor-rich membrane (Bursztajn and Fischbach, 1984).

For reasons stated in the introduction, it seems reasonable to assume that NARP formation is a reliable index of synapse formation. The fact that we recorded synaptic currents at the majority of NARPs provides some evidence for this view. However, the fact that synaptic currents were not detected at 45% of the NARPs cannot be ignored. In each experiment the extracellular electrode was positioned under direct visual control, close enough to the nerve–muscle contact to detect the neurite spike, so it is unlikely that synaptic currents were simply missed. However, the conclusion that negative NARPs are not synapses is unwarranted. Transmitter release may occur, but at a lower level than observed at positive NARPs. At the adult neuromuscular junction, less than one quantum, on the average, is released per active zone per impulse (Katz and Miledi, 1979; Heuser et al., 1979). If the release at negative NARPs is only 20% of this level, and if only a few active zones are seen by the extracellular electrode, then according to the Poisson law, only 1 in 50 stimuli would be expected to evoke a quantal event. More precise experiments with long trains of stimuli are needed to rule out low levels of quantal

release. Although the distinction between a low output synapse and a truly silent one may be difficult to discern, it seems clear that at least two functional states exist at identified NARPs. The physiological significance of the different functional states remains to be determined. Silent NARPs might represent an early stage in synapse formation or an early stage in synapse elimination.

The strikingly different appearance of positive and negative NARPs under scanning electron microscopy provides additional support for the existence of two functional states. The fine, varicose processes found at NARPs that exhibited synaptic currents are reminiscent of the filopodia-like processes observed in an earlier study of spinal cord explant–muscle synapses in vitro (Frank and Fischbach, 1979). Several studies suggest that nerve terminals may extend, branch, and retract at mature junctions (Grinnell and Herrera, 1981). It may be that a similar remodeling process is evident and even exaggerated at embryonic contacts. Of course, the extension of fine branches does not in itself explain the onset of transmitter release. Some more proximate cause such as development of active zones, inward Ca^{++} current, or the accumulation of ACh in a releasable form may be correlated with the branched morphology.

It is surprising that nearly all of the primary processes that issue directly from multipolar ciliary ganglion neuron cell bodies are capable of inducing NARPs. Ciliary ganglion neurons extend small dendrites transiently during the course of development in vivo (Landmesser and Pilar, 1974, 1976; Dahm, L., and L. Landmesser, personal communication), so some of the neurites observed during the first 3–4 d in vitro might be considered homologous to the dendrites observed in vivo. Therefore, the ability to cluster ACh receptors cannot be used as a criterion for distinguishing axons from dendrites in culture. If a neuronal factor is responsible for inducing NARPs (Jessell et al., 1979; Buc-Caron et al., 1983) it must be transported throughout the neuritic arbor. The same conclusion was drawn in an earlier study of spinal cord motoneurons co-cultured with muscle (Role et al., 1985). The remarkable correspondence between neurite endings and NARPs suggests that inducing factors may be concentrated in or selectively released from growth cones.

NARPs were not found beneath relatively short neurites during the period when processes are pruned. It is clear that trophic communication at developing neuromuscular junctions is a two-way street, and it is unlikely that the two avenues are independent of one another. Our population study indicates that reduction in the number of NARPs precedes process resorption. At a critical interval, between 2 and 3 d after adding ciliary ganglion neurons to established myotubes, $\sim 90\%$ of the NARPs were associated with the dominant process, even though the longest process comprised $<70\%$. During the same interval, the shorter processes changed slightly from 43 to 37% of the total arbor, but their share of NARPs decreased from 37 to 10%. Thus, loss of NARPs may be an early sign of a dystrophic junction. In the preceding paper (Role and Fischbach, 1987), we showed that ciliary ganglion neurons prune all but one of their processes, and that this elimination of neurites is not prevented by co-cultured myotubes. The sequence described above, however, raises the possibility that myotubes might influence which process survives. Time-lapse studies of individual neurons are needed to more precisely determine the temporal relation

between transmitter release, stability of AChR clusters, and survival of individual neurites.

We are grateful to J. Lindstrom for providing monoclonal antibody (No. 35), to Viken Matossian for writing the computer program used to analyze receptor distribution, to Pia Nystrom for technical assistance, and to Jeff Lichtman for critical comments on the manuscript.

This work was supported by grants from the United States Public Health Service (NS-18458) and the Muscular Dystrophy Association of America (G. D. Fischbach), and by a National Institutes of Health postdoctoral fellowship (F32NS06710; L. W. Role).

Received for publication 25 June 1986, and in revised form 27 October 1986.

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