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Distribution of Synaptic Specializations Along Isolated Motor Units Formed in *Xenopus* Nerve–Muscle Cultures

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The capacity of individual muscle cells and neurons to establish synaptic specializations along the entire extent of their neurite–muscle contacts was assessed in culture. Spinal cord neurons derived from embryos of *Xenopus laevis* were plated at low density in cultures of *Xenopus* myotomal muscle cells in order to obtain isolated motor units whose neuron and muscle cells were not contacted by any other neuron. These isolated motor units were examined for localization of acetylcholine receptors (AChRs) after staining with fluorescent α-bungarotoxin and, in some cases, for localization of a synaptic vesicle antigen by immunofluorescence.

The neurite–muscle contacts formed by competent neurons exhibited discontinuous sites of AChR localization occupying about 25% of the contact length as compared with 2% for incompetent neurons. Competent neurons, unlike incompetent ones, established these neurite-associated receptor patches (NARPs) on virtually all the muscle cells they contacted and functionally innervated them. These and other observations on the distribution of NARPs throughout the isolated motor units indicate that the capacity of competent neurons to establish NARPs extends to the limits of growth of most if not all of their neurites, that this capacity is least in the most proximal portions of initial neuritic segments, and that the overall capacity of muscle cells to generate NARPs can be saturated by long lengths of neurite–muscle contact. The results also suggest that even in the absence of competitive interactions between neurons there are spatial discontinuities in neuritic action and/or muscle response during the establishment of NARPs.

Synaptic vesicle antigen patches (SVAPs) occurred along the neuritic arbor of all neurons, but their distribution in competent neurons (those which established NARPs) and in incompetent ones differed. For competent neurons the percentage of neurite length occupied by SVAPs was 4.8-fold greater on muscle cells than off, whereas the corresponding value for incompetent neurons was only 1.5-fold. This large preferential localization of SVAPs along neurite–muscle contacts of competent neurons was further associated with a colocalization of SVAPs and NARPs that was greater than predicted by chance. These results suggest that muscle cells are much more effective in influencing the distribution of synaptic vesicles along the neuritic arbor of competent neurons than along the arbor of incompetent neurons and that this influence is greatest at sites of postsynaptic differentiation. Spatial matching of pre- and postsynaptic specializations thus involves bidirectional triggering interactions that only competent neurons can participate in fully.

The establishment of a chemical synapse such as the neuromuscular junction involves a set of interactions that underlie the development and spatial alignment of the pre- and postsynaptic specializations. The nature of these interactions and the cellular processes that are brought into play have been studied most extensively in nerve–muscle cultures, particularly with respect to the development of the high density of acetylcholine receptors (AChRs) in the postsynaptic membrane. These studies have revealed that competent neurons, such as motor neurons and other cholinergic neurons, trigger profound changes in the distribution of AChRs on the surface of the muscle cells they innervate. Following neurite–muscle contact, AChRs accumulate in high density along the path of contact, while elsewhere on the muscle cell the survival of preexisting AChR patches is reduced and the formation of new AChR patches inhibited (Anderson et al., 1977; Frank and Fischbach, 1979; Moody-Corbett and Cohen, 1982; Kuromi and Kidokoro, 1984). As a result, AChRs become localized preferentially at sites of innervation, thereby permitting efficient synaptic transmission. Incompetent neurons, such as those of dorsal root ganglia and other noncholinergic neurons, are ineffective in this regard, thereby emphasizing the neural specificity of the triggering interaction (Cohen and Weldon, 1980; Role et al., 1985).

The neural agent(s) that initiates these remarkable changes in AChR distribution has not yet been identified, but it is known that it can act in the absence of activation of AChRs, as well as in the absence of muscle and neuronal action potentials (Anderson and Cohen, 1977; Anderson et al., 1977; Rubin et al., 1980; Davey and Cohen, 1986). The response of the muscle cell to the triggering event includes a process of receptor redistribution whereby preexisting mobile AChRs in neighboring regions of the surface membrane aggregate along the path of neurite–muscle contact where they become immobilized (Anderson and Cohen, 1977; Stya and Axelrod, 1984; Ziskind-Conhaim et al., 1984; Kidokoro et al., 1986). An increased incorporation of newly inserted AChRs can also contribute to the build-up of a high density of AChRs at sites of contact (Role et al., 1985).

The preferential localization of AChRs along the path of neurite–muscle contact is accompanied by the development of pre-

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synaptic specializations, including clusters of synaptic vesicles (Anderson et al., 1984). Initially, these 2 synaptic specializations may develop independently of each other, but with time the incidence of spatial alignment increases (Bixby and Reichardt, 1985). Components of the synaptic basal lamina have been implicated in the development of both these specializations (Sanes et al., 1978; Burden et al., 1979; Anderson and Fambrough, 1983; Vogel et al., 1983; McMahan and Slater, 1984; Fallon et al., 1985; Anderson, 1986).

In considering how synaptic sites become precisely organized, it is of interest to note that the distribution and extensiveness of these specializations can vary widely within an individual nerve–muscle culture. For example, some muscle cells develop sites of high AChR density along much of the length of neurite–muscle contact, whereas others exhibit these neurite-associated receptor patches (NARPs) along a very limited portion of the contact. How to account for such differences is not clear. Among the possibilities are factors such as variability in the overall capacity of individual neurons and muscle cells to establish synaptic specializations, gradients in the capacity of different portions of the neuritic arbor to trigger and maintain these specializations, and inhibitory and facilitatory interactions in response to 2 or more neurons contacting the same muscle cell or each other. In the present study we have sought to explore some of these possibilities in cultures of myotomal muscle cells and spinal cord cells derived from embryos of *Xenopus laevis*. We plated spinal cord cells 1–3 d after the muscle cells and analyzed only isolated motor units in which the neuron and its contacted muscle cells were free of contact from all other neurons in the culture. In this way we have attempted to minimize those interactions that might otherwise occur if 2 or more neurons contacted the same muscle cell or each other. Some motor units were examined not only for NARPs, but also for patches of synaptic vesicle antigen using an immunofluorescent approach developed by Bixby and Reichardt (1985).

Preliminary accounts of some of the findings have been reported in abstract form (Cohen and Wilson, 1985; Cohen et al., 1986).

Materials and Methods

Cultures. The method of preparing cultures was similar to that described previously (Anderson et al., 1977; Moody-Corbett and Cohen, 1982), with some modifications. Dorsal segments, including the spinal cord and myotomes, were removed from 1-d-old *X. laevis* embryos (stages 22–28; Nieuwkoop and Faber, 1967) and placed in a collagenase solution (0.1–0.2 mg/ml in 67% L15) for up to 1 hr to facilitate isolation of the myotomes and spinal cord. The isolated spinal cords of several embryos were stored in 67% L15/5% dialyzed horse serum (DHS) in the refrigerator for up to 3 d until required for plating. Isolated myotomes were placed for about 1 hr in a dissociating medium, consisting of trypsin (1 mg/ml) and EDTA (0.4 mg/ml) in calcium/magnesium-free PBS. To complete their dissociation the myotomal muscle cells were washed and triturated gently in 67% L15/5% DHS. They were then plated in culture chambers (see Anderson et al., 1977) containing 67% L15 and 0.2 μg/ml Holmes α-1 protein. One to three days later, the stored spinal cords and/or freshly isolated spinal cords were dissociated as described above for the myotomes and added to the established muscle cultures. Cultures were maintained either at room temperature (23–25°C) or in an incubator at 13–14°C. Culture solutions were obtained from GIBCO Laboratories.

Electrical stimulation of neurons. Cultures were transferred to a bath (volume, 0.5 ml) and perfused at a rate of ~0.5 ml/min with 67% L15 containing added $CaCl_2$ (5 mM). Neuronal cell bodies were stimulated extracellularly by passing short (0.5–1 msec) depolarizing currents (1–10 μA) through blunt fire-polished micropipettes (2–3 μm tips) filled with 67% L15. Twitch responses in the muscle cells contacted by the neuron were reversibly abolished by *d*-turbocurarine chloride (6 μg/ml). When neuronal stimulation did not elicit twitches in the contacted muscle cells, the stimulus duration and intensity were increased to 5 msec and 30 μA before concluding that neuromuscular transmission was absent.

Fluorescent staining. Tetramethylrhodamine-conjugated α-bungarotoxin was prepared according to the method of Ravdin and Axelrod (1977). In most cases it was added to the cultures at a dilution of 1:50–1:100 at the same time as the spinal cord cells. Tests on freshly dissected myotomal muscle revealed that, at these dilutions, 70–84% of the AChRs were labeled within 3 hr. When the neurons were to be electrically stimulated to test for neuromuscular transmission, AChRs were stained with rhodamine-toxin immediately afterwards. The stained cultures were examined alive or after overnight fixation in the refigerator with 4% formaldehyde in 90 mM phosphate buffer, pH 7.3. To avoid muscle contraction the fixative was first diluted and added to the cultures in small increments. Fixed cultures were cleared in a solution consisting of 1 mg/ml *p*-phenylenediamine, 10 mM sodium carbonate, and 90% glycerol (see Platt and Michael, 1983) and stored at −16°C until examined.

Monoclonal antibody 48 (Mab 48; kindly provided by L. Reichardt and J. Bixby) is directed against a 65 kDa protein associated with synaptic vesicle membrane and binds to synaptic vesicles in most, if not all, neurons (Matthew et al., 1981). It was employed in the present study according to the procedure described by Bixby and Reichardt (1985). After staining AChRs with rhodamine-toxin, the cultures were fixed for about 10 min with 4% formaldehyde, rinsed with 67% PBS/1% goat serum (GS), and then exposed for 1 hr to Mab 48, diluted 1:300 with 67% L15/1% GS/0.1% saponin. The cultures were rinsed again and then exposed for 1 hr to affinity-purified, fluorescein-labeled goat anti-mouse IgG (Cappel Laboratories), diluted 1:100 with 67% PBS/1% GS. After rinsing, the cultures were postfixed overnight in the refrigerator with 4% formaldehyde and cleared as described above.

Several control experiments were performed to assess the immunofluorescence obtained by staining with Mab 48. When used on freshly dissected myotomal muscle from *Xenopus* tadpoles, the pattern of immunofluorescence was the same as the pattern of motor innervation (see Chow and Cohen, 1983) and codistributed with sites of AChR localization. This immunofluorescence did not occur (1) if Mab 48 was omitted from the staining protocol, (2) if it was replaced by IgG2b in clarified mouse ascites fluid (Sigma Chemical Co.) at similar dilutions, (3) if permeabilization was avoided by omitting fixation and treatment with saponin, or (4) if the muscle was denervated. Considered together with previous studies employing Mab 48 and related antibodies (Matthew et al., 1981; Bixby and Reichardt, 1985; Peng et al., 1987), these results indicate that the immunofluorescence was associated with clusters of synaptic vesicles.

Analysis of isolated motor units. Cultures were surveyed for isolated motor units up to 7 d after plating the neurons. To be accepted for analysis the neuron and the muscle cells it contacted had to be free of contact from all other neurons in the culture. In most cases the neurites of other neurons were at least 500 μm away from the motor unit.

The isolated motor units were photographed with phase-contrast optics at low power (×16 or ×25 objective) and with phase-contrast and fluorescence optics at high power (×63 or ×100 objective). Phase-contrast photographs were usually taken before fixation, when the neurites were better resolved. Because neurites can grow over as well as under muscle cells, it was often necessary to photograph an individual field at several different levels of focus.

High-magnification photographs were enlarged so that 1 mm on the print was equal to 1 μm in culture. All photographs were printed on paper, and phase-contrast photographs were also printed as transparencies. By superimposing the phase-contrast transparency and its corresponding fluorescence print(s), the location of the fluorescent stain with respect to the position of the neuritic arbor could be readily assessed. A data back on the camera registered the day and time at a fixed position on each negative, thereby providing a precise reference point for properly aligning the superimposed prints. When both fluorescent stains were used in the same culture the rhodamine (AChR) fluorescence was traced onto the phase-contrast transparency, which in turn was superimposed over the fluorescein (synaptic vesicle antigen) fluorescence to reveal portions of colocalization.

Length measurements were made in the region of best focus. The rhodamine fluorescence along neurite–muscle contacts almost always had discrete borders, so that these measurements were subject to little

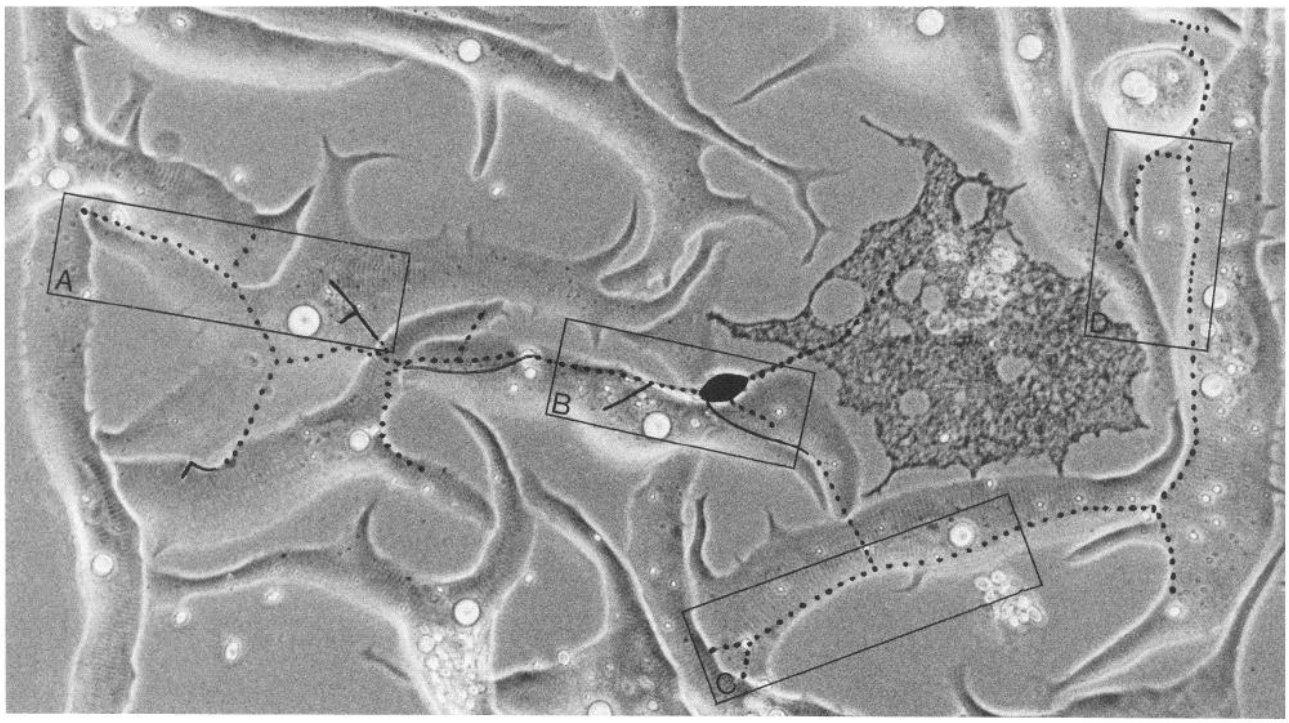

Figure 1. Low-power, phase-contrast view of muscle cells contacted by a single neuron. For the sake of clarity, the neuron has been blackened. *Solid lines* indicate portions of the neuritic arbor on the lower surface of the muscle cells (apposed to the floor of the culture dish); *dotted lines,* portions on the upper surface of the cells. Four neurites emerged from the soma and contacted 11 muscle cells and a pigment cell. The *framed areas* are shown at higher magnification in Figure 2. The length of rectangle *B* is 105 μm. Muscle cells cultured for 6 d and neuron for 5 d at 13°C.

if any error. The borders of the fluorescein fluorescence along neurites were also often discrete (see Fig. 9), but sometimes faint fluorescence was observed adjacent to brighter patches of fluorescence (see Fig. 10). This fainter, less discrete fluorescence was not included in the measurements. Although the subjectivity in excluding it gave rise to some error, repeated measurements on the same motor unit deviated by less than 15%.

Most of the neuritic arbor was readily resolved with phase-contrast optics, but some portions remained obscure. Neurites could not be seen in regions where they coursed over refractile portions of muscle cells, such as thick edges and vacuoles. If the neurites grew beyond the refractile region, their course in the refractile region could be inferred. Occasionally, however, they terminated in these refractile regions and their precise end point remained unresolvable. In such cases measurements were made only to the most distal portion that could be resolved. Another possible source of error arose in a few motor units in which a small portion of the neuritic arbor consisted of a closed loop. Such loops were measured but were not included in the analyses concerned with terminal neuritic segments. It is conceivable that one or more of the neuritic branches in these closed loops grew long distances along other portions of the neuritic arbor, thereby leading to large underestimates in our measurement of total neuritic length and contact length. However, our results were not substantially altered when these neurons were excluded.

Results

Neurite-associated receptor patches (NARPs)

Variability among neurons

Figure 1 shows an example of an isolated motor unit in culture. For the sake of clarity, the neuron has been inked in. Solid lines indicate portions of the neuritic arbor apposed to the floor of the culture dish, and dotted lines indicate portions on the upper surface of contacted cells. Eleven muscle cells and a pigment cell were contacted by the 4 neurites that emerged from the soma. The total neuritic length of this tetrapolar neuron was 1194 μm, of which 1034 μm was associated with muscle cells.

Four portions of the field, together with corresponding views of AChR staining, are shown at higher magnification in Figure 2. It can be seen that NARPs occurred along much of the neuritic arbor, at the most distal contacts and along lengthy portions of more proximal contacts. In fact, NARPs were observed on each of the 11 contacted muscle cells and occupied 24.7% of the total contact length. AChR stain was also present in the region of soma–muscle contact (Fig. 2*B*).

Altogether 56 neurons, cultured for 2–6 d at room temperature (23–25°C) or 5–7 d at 13–14°C, were analyzed. Forty-eight, including the one in Figures 1 and 2, were deemed competent with respect to the establishment of NARPs. Competent neurons had NARPs along 10% or more of their contact length, and the muscle cells they contacted had few if any AChR patches elsewhere on their surface. NARPs almost always occurred as narrow bands oriented in the same direction as the contact but were sometimes broader at the ends of neurites. By contrast, muscle cells contacted by incompetent neurons often had no NARPs at all along their contacts and usually had broad AChR patches elsewhere on their surface (see Fig. 10), similar to those seen typically on noncontacted muscle cells (e.g., Anderson et al., 1977; Moody-Corbett and Cohen, 1982). When some AChR stain was seen along the contacts made by incompetent neurons, it was either faint or appeared similar to the broad AChR patches associated with noncontacted muscle cells. Even when all forms and intensities of AChR stain were measured along neurite–muscle contacts of incompetent neurons, they never amounted to more than 6% of the contact length (mean ± SEM, 2.2 ± 0.7%). Upon electrical stimulation, some neurons elicited twitches in most (81%) of the muscle cells they contacted, and

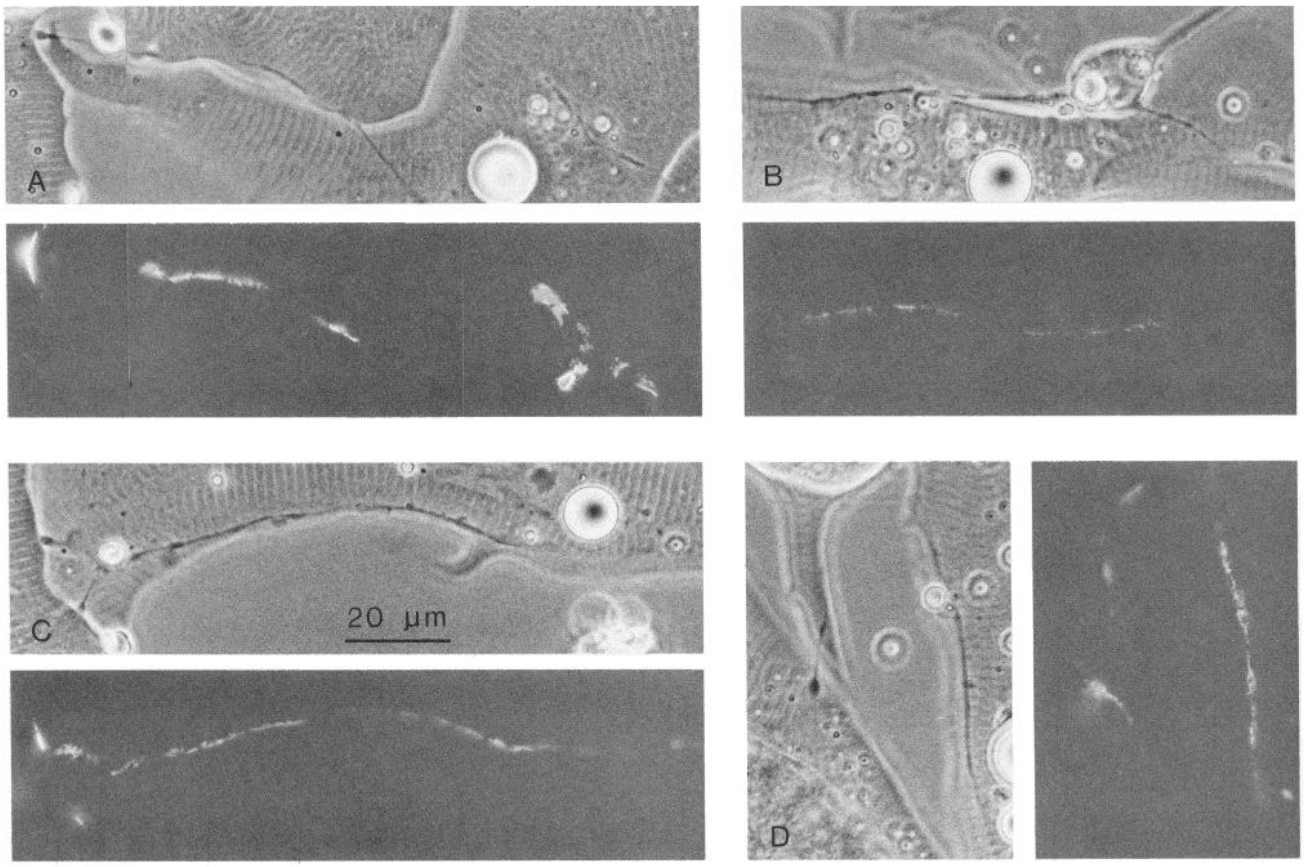

Figure 2. Framed areas in Figure 1 shown at higher magnification with phase-contrast and fluorescence optics. Bands of fluorescence, corresponding to sites of high AChR density, are associated with lengthy portions of neurite–muscle contact. These neurite-associated receptor patches (NARPs) can also be seen at the ends of 3 terminal neuritic segments in *A*, 2 in *C*, and 1 in *D*. The soma and 3 of its 4 neurites are seen in *B*. *A* is a composite at 3 different levels of focus. The scale in *C* applies to the entire figure.

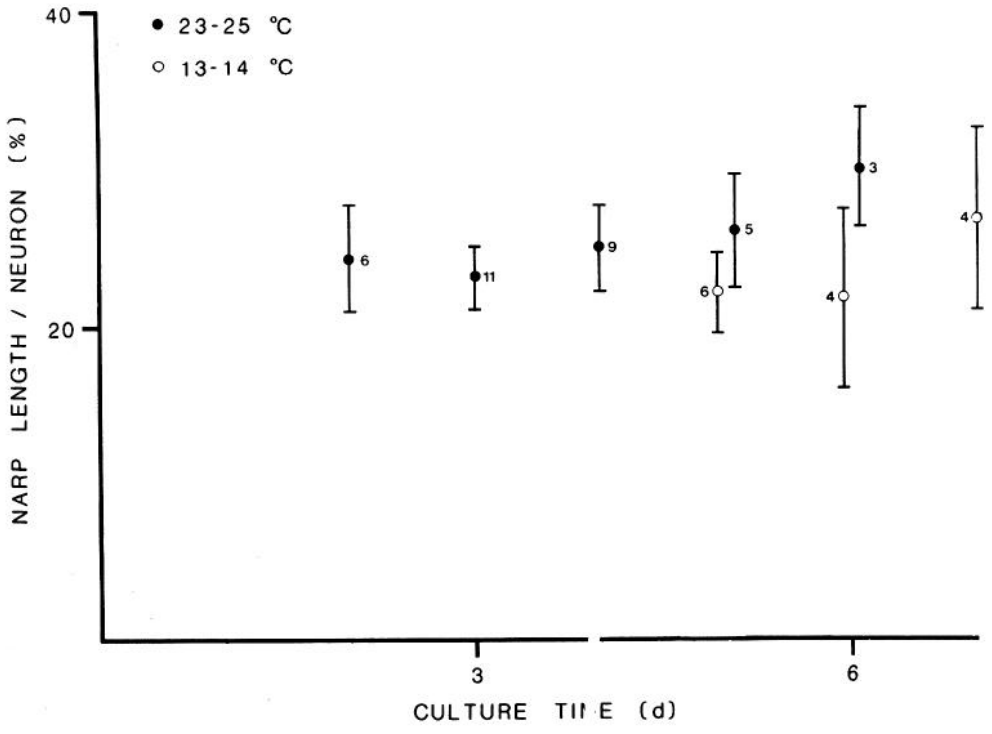

Figure 3. Relative lack of influence of culture time and temperature on the establishment of NARPs. NARP length per neuron, expressed as a percentage of contact length per neuron, is plotted against the number of days competent neurons were cultured together with muscle cells. Means and SE bars are shown together with the number of neurons on which they are based. Some cultures were maintained at room temperature (23–25°C) and others at 13–14°C. Note that culture time and temperature had little effect on the percentage of contact length occupied by NARPs.

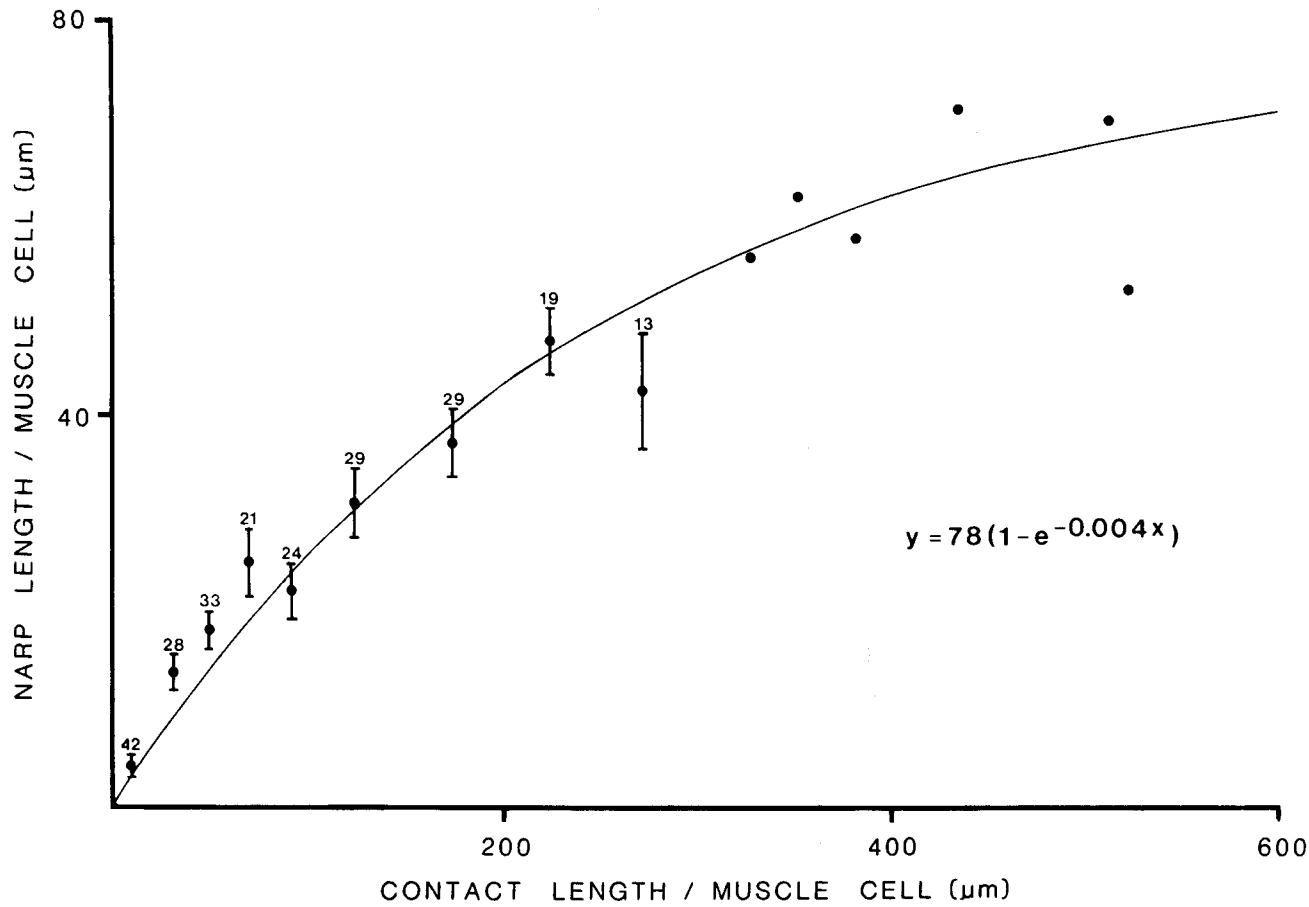

Figure 4. Relationship between NARP length and contact length on individual muscle cells. Absolute NARP length per muscle cell is plotted against contact length per muscle cell for all muscle cells contacted by competent neurons. Contact lengths have been grouped in 20 μm bins up to 100 μm and in 50 μm bins between 101 and 300 μm. Means and SE bars are plotted at the mean contact length for the muscle cells in each bin. The number of muscle cells in each bin is also indicated. Note that NARP lengths tend to plateau with increasing contact length. The smooth curve has been drawn according to the equation shown.

subsequent examination for NARPs revealed these neurons (18/18) to be competent according to the above criteria. Others did not elicit twitches in any of the muscle cells they contacted and proved to be incompetent in establishing NARPs (4/4). Since the neurons were derived from embryonic spinal cord, these results are most simply explained by assuming that the competent ones were motor neurons, whereas the incompetent ones were noncholinergic Rohon-Beard neurons and interneurons.

Competent neurons had NARPs along 24.6 ± 1.2% (mean ± SEM, n = 48) of their contact length, with a range of 10.6–38.6%. These values were little affected by the age of the cultures or the temperature at which the cultures were maintained (Fig. 3); nor were there any statistically significant differences between neurons of different polarity (26.2 ± 2.4, 23.3 ± 1.6, 27.4 ± 2.6, and 20.9 ± 3.8% for mono-, bi-, tri-, and tetrapolar neurons, respectively) or between neurons whose soma did (24.7 ± 1.2%) or did not (23.9 ± 3.1%) contact a muscle cell. The neurons varied considerably in total neuritic lengths (170–1448 μm) and contacted as few as one or as many as 12 muscle cells, but linear regression between these parameters and the percentage of contact length occupied by NARPs gave very poor correlations (r = 0.103 and 0.216, respectively). Thus, the morphological features of the competent neurons did not provide a simple guide for distinguishing between them in terms of the relative extensiveness of their NARPs. Likewise, we were unable to distinguish between competent and incompetent neurons simply on the basis of such morphological features as the number of neurites emerging from their soma or the extensiveness of their neuritic growth.

Sixty percent (23/38) of the competent neurons whose somata contacted muscle cells exhibited some AChR localization in this region of contact (Fig. 2*B*), suggesting that the capacity to induce the development of a high density of AChRs may reside not only in the neurites but also in the soma. However, the soma–muscle contacts were not examined at different levels of focus, so that the percentage of contact occupied by the AChR patches in this region could not be estimated. In addition, the resolution of the light microscope is insufficient to exclude the possibility that neurites were also present at the soma–muscle contacts. For the remaining analysis we considered only those contacts beyond the soma.

Distribution on muscle cells

Ninety-five percent (233/244) of the muscle cells contacted by competent neurons exhibited NARPs. The few muscle cells without NARPs either had relatively short contacts (≤33 μm for 10 of 11 cells) or were contacted by the most distal portions of the neuritic arbor and may not have had sufficient time to

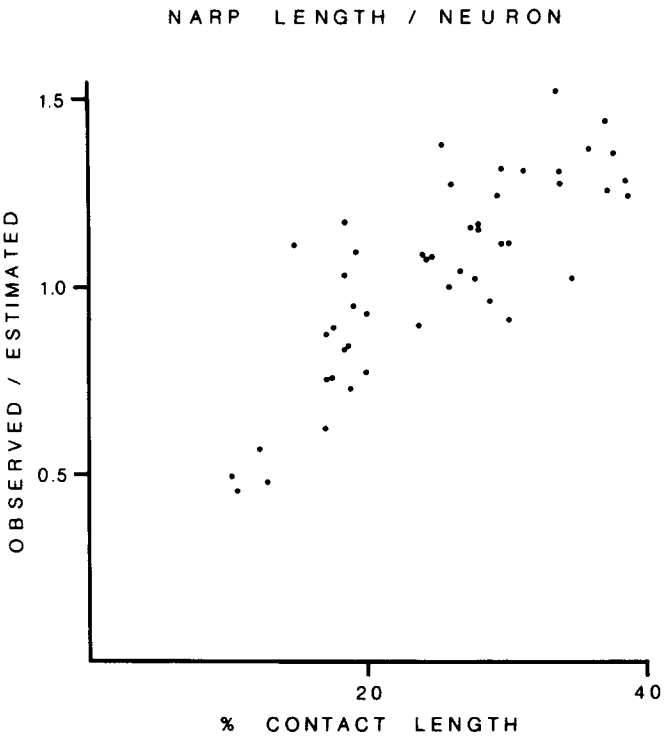

Figure 5. Variability among neurons in establishing NARPs is not eliminated by normalizing for the differences in contact length on individual muscle cells. Normalized values (Observed/Estimated) were obtained as follows. For each muscle cell contacted by a neuron an estimated NARP length was calculated on the basis of its contact length using the equation shown in Figure 4. The total observed NARP length for the neuron was then divided by the total estimated NARP length for the neuron, thereby normalizing for contact length. Note that the normalized values (Observed/Estimated) tend to be proportional to the observed values (% Contact Length). Their range (3.3-fold) and coefficient of variation (CV = 0.261) are almost as large as for the observed values (3.6-fold range; CV = 0.323). Thus, even after normalizing for contact length the neurons still exhibited large differences in the percentage of contact length occupied by NARPs.

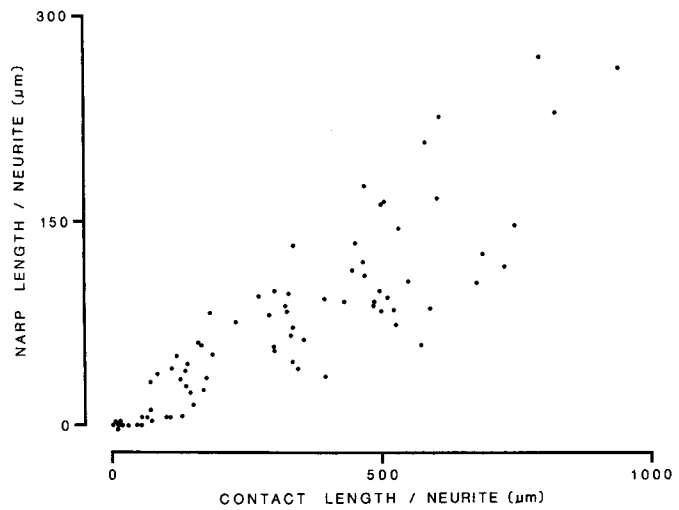

Figure 6. Relationship between NARP length and contact length for individual neurites. Absolute NARP length per neurite is plotted against contact length per neurite for each of the neurites that emerged from the somata of competent neurons and contacted muscle cells. Note that, on average, NARP length is directly proportional to contact length, and there is no apparent saturation even at the longest contact lengths. Conversely, only neurites with very short contacts did not establish NARPs.

generate detectable NARPs. It is apparent, therefore, that essentially all muscle cells in these cultures have the capacity to generate NARPs and that the capacity of the competent neurons to trigger the generation of NARPs extends over much of their neuritic arbor. As in previous studies, NARPs were usually distributed in a discontinuous pattern on individual muscle cells (Figs. 2, 9). Only on 10 cells did a NARP occupy essentially the entire contact length (>90%), and in each of these cases the contact lengths were relatively short (≤35 μm).

The relationship between absolute NARP length and contact length for all muscle cells contacted by competent neurons is shown in Figure 4. Although NARP lengths varied considerably for any particular contact length, on average they increased with increasing contact length but in a nonlinear fashion. The relationship tended towards a plateau at the greatest contact lengths and had the steepest slope at the shortest contact lengths. The smooth curve in Figure 4 was drawn according to the equation $y = 78\ (1 - e^{-0.004x})$. The reasonable fit supports the conclusion that the capacity of the muscle cells to generate NARPs is limited to a maximum length of about 78 μm and that this capacity can be saturated by a sufficient length of contact.

In view of the results of Figure 4, it was of interest to assess whether differences between competent neurons in establishing NARPs were related to how extensively the neurons contacted individual muscle cells. For example, if a neuron behaved according to the curve in Figure 4 and established a 500 μm contact on a single muscle cell, the NARP length would be about 67 μm, or 13.4% of the contact length. If the same neuron established contacts of 50 μm on each of 10 muscle cells, the NARP length would be 140 μm (10 × 14 μm), or 28.0%. To eliminate this source of variability we divided the "observed NARP length" by the "estimated NARP length," the latter value being obtained from Figure 4 on the basis of the contact lengths on each muscle cell contacted by the neuron. When the data were treated in this way, the resulting normalized values remained proportional to the observed values (Fig. 5), and their coefficients of variation were almost as large as those for the observed values (0.261 and 0.323, respectively). Thus, factors other than contact length per muscle cell must have contributed to the variability among competent neurons in establishing NARPs. Observed and normalized values for the percentage of contact occupied by NARPs also varied by as much as 2-fold or more even among muscle cells contacted successively by the same neuron or neurite. It may be that muscle cells as well as neurons differ from each other in their overall capacity to establish NARPs. Fluctuations in neuritic action and/or spatial discontinuities in muscle cell response may also have contributed to the variability (see Discussion).

Distribution along neurites

Figure 6 illustrates the relationship between absolute NARP length and contact length for the individual neurites that emerged from the somata of competent neurons. Although there was considerable variability, even among neurites of the same neuron, NARP length per neurite was, on average, directly proportional to contact length per neurite (r = 0.868, slope = 24.0%), and there was no indication of any saturation. This is consistent with the conclusion that the capacity to establish NARPs extends to the limits of neurite growth (see also below).

Neurites that did not establish NARPs had relatively short

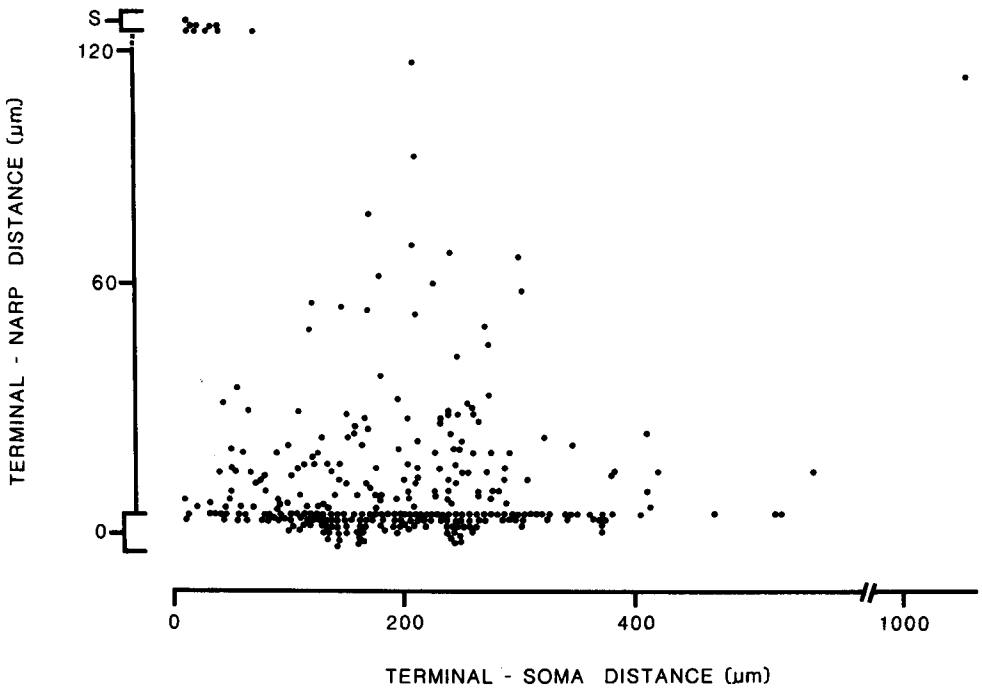

Figure 7. Distances between the ends of terminating neuritic segments and the closest NARPs. Values on the abscissa indicate the length of neurite between the soma and the ends of terminal neuritic segments contacting muscle cells. Values on the ordinate indicate the length of contact between the ends of terminal neuritic segments contacting muscle cells and their closest NARPs. Cases where there was no NARP between the soma and the end of a terminating neuritic segment are plotted opposite *S* on the ordinate. The majority (51%) of the terminating neuritic segments had NARPs at their very ends, and 90% had NARPs within 30 μm of their ends. The occurrence of NARPs at or close to the ends of terminating neuritic segments was seen over the full range of terminal–soma distances.

contacts (≤53 μm; Fig. 6) and were themselves relatively short and unbranched (Figs. 2*B* and 9*A*). All but one extended for less than 65 μm from the soma. Since the dendrites of developing *Xenopus* motor neurons *in vivo* commonly extend for similarly short distances (see Roberts and Clarke, 1982), the possibility arises that at least some of the short neurites were equivalent to dendrites and lacked the capacity to establish NARPs. Consistent with this possibility is the finding that, in each of 4 cases where it was tested, the short neurites that did not establish NARPs also lacked synaptic vesicle antigen (see Fig. 9*A*).

All but one of the muscle cells contacted by the short neurites were also contacted more extensively by a second neurite (range of contact lengths, 56–188 μm) that did establish NARPs. This suggested the possibility that when a single muscle cell is contacted by 2 different neurites, the neurite that contacts the muscle cell more extensively suppresses the establishment of NARPs by the other neurite. This possibility was assessed by examining 12 other muscle cells contacted by 2 different neurites belonging to the same neuron (range of contact lengths: short, 18–127 μm; long, 40–394 μm). On all but one of these muscle cells, both neurites established NARPs, and in 4 of the cases the percentage of contact occupied by NARPs was even greater for the shorter contact than for the longer one. Therefore, if suppression of NARP development does occur when 2 neurites contact the same muscle cell, then factors other than contact length must play a role.

The ineffectiveness of the short, unbranched neurites to establish NARPs also raised the question of whether the initial segments of all neurites lack the capacity to establish NARPs. That this is not the case is apparent from Table 1, which indicates that NARPs often did develop along the most proximal 25 μm of initial neuritic segments. On the other hand, when compared with the other portions of the same neurites, it was found that the percentage of contact occupied by NARPs was least in these most proximal neuritic segments and greatest in the most distal 25 μm of terminal neuritic segments (Table 1). Even when neurons that had AChR stain at soma–muscle contacts were excluded from this analysis, the values were still lowest for the most proximal segments (11.2 ± 3.0%, $n = 16$).

That the terminal portions of neurites are particularly effective in establishing NARPs is supported further by the results shown in Figure 7. NARPs were present at the very ends of more than 50% of the terminal segments, and in more than 90% of the cases they were located <30 μm from the ends of the terminal segments. This close association between the NARPs and the ends of terminal segments was seen over the full range of soma–terminal distances, even when the terminals were close to the soma. These latter terminals belonged to neurites with more distant terminals as well. Taken together, the results indicate that the capacity of competent neurons to establish NARPs extends to the very limits of growth of most of their neurites but is not distributed uniformly. On average, the capacity appears to be least in the most proximal portions of initial neuritic segments and greatest in the most distal portions of terminal neuritic segments.

Table 1. NARP lengths along different portions of neurites

Portion of neurite	Total contact length (μm)	NARP length (% contact length)	
		Total	Mean ± SE
Initial segment	976	16.5	17.0 ± 3.2
Terminal segment	3356	31.0	29.4 ± 3.0
All other portions	12,732	22.9	24.5 ± 1.7

NARPs were measured along contacts formed by (1) the most proximal 25 μm or less of initial neuritic segments, between the soma and first branch point; (2) the most distal 25 μm or less of terminal neuritic segments, from their ends to the preceding branch point; and (3) the remaining portions of the neurite. The values are based on 46 neurites for which all 3 measurements could be made. Neurites were excluded if their initial segments did not contact a muscle cell or extended for more than 25 μm before contacting a muscle cell. Also excluded were short neurites that did not establish any NARPs (see Fig. 6).

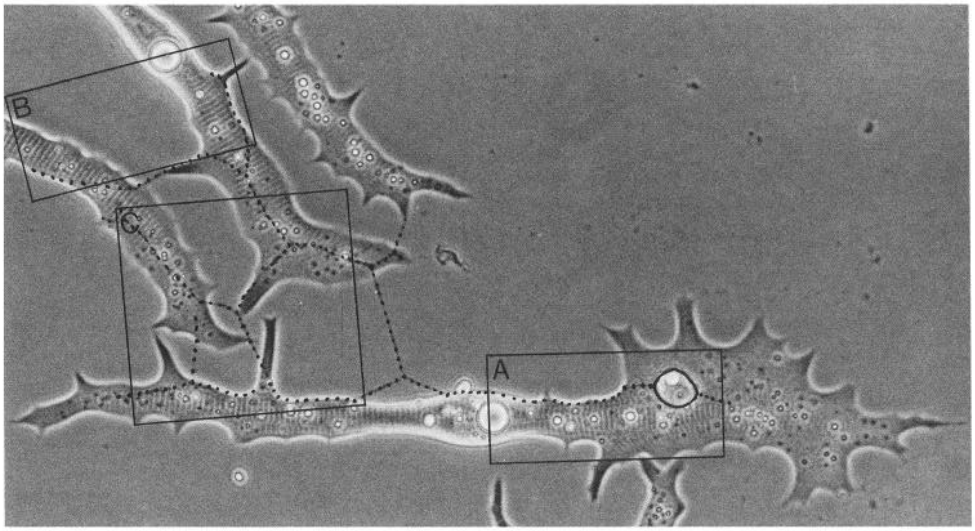

Figure 8. Low-power, phase-contrast view of muscle cells contacted by a single competent neuron. The soma and its neurites have been traced in ink. Two neurites, one short and unbranched, the other long and branched, emerged from the soma and contacted 4 muscle cells. The *framed areas* are shown at higher magnification in Figure 9. The length of rectangle *A* is 92 μm. Muscle cells cultured for 6 d and neuron for 4 d at room temperature.

Colocalization of NARPs and synaptic vesicle antigen patches

In view of the above findings on the distribution of a postsynaptic specialization (NARPs), it was of interest to determine as well the distribution of a presynaptic specialization. For this purpose we used Mab 48, a monoclonal antibody that is directed against a 65 kDa protein in synaptic vesicle membranes and binds to synaptic vesicles in most if not all neurons (Matthew et al., 1981). The distributions of synaptic vesicle antigen patches (SVAPs) and NARPs were examined in 15 isolated motor units 3–4 d after plating the neurons. Based on the occurrence of NARPs, 11 of these neurons were judged competent and 4 incompetent. SVAPs were associated with both types of neuron.

There was considerable colocalization of NARPs and SVAPs in competent neurons, but each specialization also occurred in the absence of the other (Figs. 8, 9). The colocalization was more extensive than would be expected simply on the basis of chance. For example, the competent neuron in Figures 8 and 9 had NARPs along 20.0% of its contact length and SVAPs along 15.6%. On the basis of chance, the length of colocalization should have been 3.1% (20.0% × 15.6%) of the contact length. The actual value was 12.4%. As summarized in Table 2, colocalization was greater than predicted by chance for each of the competent neurons, and the difference was statistically significant ($p < 0.001$). On average, the length of colocalization was about 13% of the total contact length. In view of this extensive colocalization it is not surprising that NARPs and SVAPs had the

Table 2. Colocalization of NARPs and SVAPs

Contact length (μm)	NARP length (%)	SVAP length (%)	Length of colocalization (%)	
			Predicted	Observed
Competent neurons				
186	28.0	42.5	11.9	17.2
291	27.8	24.7	6.9	16.2
300	19.3	18.0	3.5	6.0
321	17.4	52.6	9.2	15.6
485	18.6	18.6	3.5	6.4
540	20.0	15.6	3.1	12.4
578	18.5	25.1	4.6	10.5
648	17.1	23.1	4.0	10.8
711	18.4	14.3	2.6	10.7
810	33.5	28.8	9.6	19.6
1208	25.3	36.1	9.1	17.6
Mean ± SE	22.2 ± 1.7	27.2 ± 3.6	6.2 ± 1.0	13.0 ± 1.4
Incompetent neurons				
157	1.3	31.8	0.4	0
297	3.3	17.5	0.6	0.3
404	1.0	25.5	0.3	0.2
1153	3.4	17.6	0.6	1.1
Mean ± SE	2.3 ± 0.6	23.1 ± 3.5	0.5 ± 0.1	0.4 ± 0.2

Lengths of NARPs, SVAPs, and colocalization are expressed as a percentage of the contact length for each neuron. Predicted values assume colocalization occurred by chance.

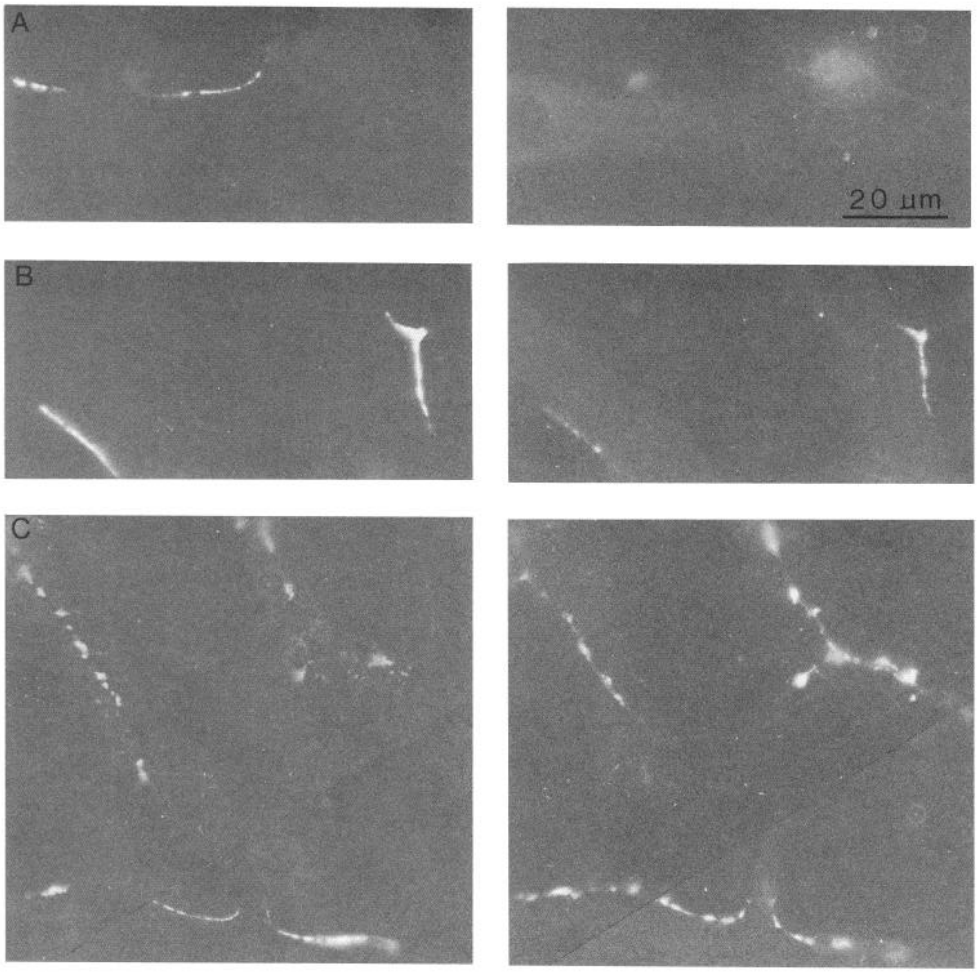

Figure 9. Framed areas in Figure 8 shown at higher magnification with rhodamine optics to reveal sites of high AChR density (*left*) and with fluorescein optics to reveal patches of synaptic vesicle antigen (*right*). *A,* NARPs are present along contact formed by long neurite but not short one. Synaptic vesicle antigen patches (SVAPs) are not present. *B* and *C,* Extensive colocalization of NARPs and SVAPs. *C* is a composite showing 2 different levels of focus. *Scale* applies to entire figure.

same general pattern of distribution and that, like NARPs, SVAPs occurred relatively infrequently along the most proximal 25 μm of initial neuritic segments (Table 3).

Among incompetent neurons, the percentage of contact length occupied by SVAPs was essentially the same as that for competent neurons (Fig. 10, Table 2). On the other hand, since by definition the incidence of NARPs was very low, the length of colocalization was also very low, averaging only 0.4% of the contact length, as compared with 13.0% for the competent neurons. Furthermore, the colocalization that did occur in incompetent neurons was not significantly different from that expected by chance (see Table 2). Taken together the results indicate that the local neurite–muscle interactions that lead to colocalization of SVAPs and NARPs are specific for competent neurons.

Besides occurring along neurite–muscle contacts, SVAPs were observed infrequently at soma–muscle contacts of competent (2/11) and incompetent neurons (1/4; Fig. 10*A*) but were never colocalized with the AChR patches that occurred at some of these sites (5/15). SVAPs were also observed along some portions of the neuritic arbor that were not in contact with muscle cells (Fig. 10*A*), but their occurrence in these regions was less extensive than along neurite–muscle contacts. Table 4 summarizes these results. For incompetent neurons, the percentage of neurite length occupied by SVAPs was 1.5-fold greater on muscle cells than off (20.3 vs 13.1%), suggesting that muscle cells may influence the distribution of SVAPs even in those neurons with little, if any, capacity to establish NARPs. However, preferential localization of SVAPs along neurite–muscle

Table 3. NARP and SVAP lengths along different portions of neurites

Portion of neurite	Total contact length (μm)	NARP length (% contact length)		SVAP length (% contact length)	
		Total	Mean ± SE	Total	Mean ± SE
Initial segment	290	6.9	7.7 ± 3.0	6.2	5.9 ± 2.7
Terminal segment	1133	25.5	22.6 ± 4.0	30.6	32.8 ± 4.0
All other portions	3742	22.2	22.9 ± 2.8	26.0	28.1 ± 3.9

Values are based on 14 neurites for which all measurements could be made. See footnote to Table 1 for further details.

Table 4. SVAP lengths on and off muscle cells

	Competent neurons (n = 11)			Incompetent neurons (n = 4)		
		SVAP length			SVAP length	
Portion of neuritic arbor	Neurite length (μm)	μm	% neurite length	Neurite length (μm)	μm	% neurite length
Contacting muscle (A)	6078	1614	26.6	2011	408	20.3
Not contacting muscle (B)	1411	77	5.5	510	67	13.1
A/B	4.3	21.0	4.8	3.9	6.1	1.5

contacts was much greater in competent neurons. For these, the percentage of neurite length occupied by SVAPs was 4.8-fold greater on muscle cells than off (26.6 vs 5.5%; Table 4). This greater influence of muscle cells on neurons with the capacity to establish NARPs presumably involves the local interactions that participate in the colocalization of NARPs and SVAPs.

Discussion

This study represents an attempt to determine the capacity of individual neurons and muscle cells to establish synaptic specializations. For this purpose we plated the neurons at low density and examined only isolated motor units whose neuron and muscle cells were not contacted by any other neuron. Whether they were never contacted by other neurons throughout the culture period cannot be stated with certainty, but some observations suggest that this was probably so. In most cases the neurites of other neurons were at least 500 μm away from the limits of motor units analyzed. Since the neurites in these cultures rarely extend for more than 500 μm from their soma (Fig. 7), it is unlikely that the isolated motor units had been transiently contacted by other surviving neurons. Furthermore, in each of 9 cases where the motor unit was followed daily beginning on the first day after plating, transient contacts by other neurons were not observed (data not shown). Accordingly, it seems likely that facilitatory and/or inhibitory interactions that might occur if the motor units were contacted by a second neuron were avoided in this study.

As expected from previous findings, the neurons of the isolated motor units fell into 2 main categories, competent and incompetent. The competent neurons established functional synapses and were presumably motor neurons. On average they exhibited NARPs along 25% of their contact length, whereas the corresponding value for incompetent neurons was 10-fold less. This is consistent with a study on chick nerve–muscle cultures, which indicated that the incidence of NARPs is 10-fold greater along contacts formed by motor neurons than those formed by interneurons (Role et al., 1985). In addition, muscle cells contacted by incompetent neurons, unlike those contacted by competent neurons, exhibited AChR patches elsewhere on their surface similar to those that normally occur on noncontacted muscle cells. It is therefore apparent that incompetent neurons derived from embryonic spinal cord, like those from other sources (Cohen and Weldon, 1980), have little influence on the distribution of AChRs. The fact that incompetent neurons develop SVAPs is also in agreement with previous electron microscopic observations of clusters of synaptic vesicles along the neurites of incompetent neurons (Cohen and Weldon, 1980). Clearly, the presence of synaptic vesicles per se does not provide an index of the competency of a neuron to influence AChR distribution in muscle. Rather, the findings are compatible with the notion that competency is linked to the transmitter status of the neuron and that competency to influence AChR distribution requires the cholinergic phenotype.

Our findings indicate that the capacity of competent neurons to establish NARPs extends throughout the length of most, if not all, of their neurites. There was no sign of saturation even among the neurites that established the longest contacts (Fig. 6). By contrast it was apparent that the capacity of individual muscle cells to generate NARPs can be saturated by a sufficient length of contact (Fig. 4). Besides AChRs, several muscle components have been implicated in the generation of NARPs, including components of the extracellular matrix (Burden et al., 1979; Anderson and Fambrough, 1983; Vogel et al., 1983; McMahan and Slater, 1984; Fallon et al., 1985) and the cytoskeletal system (Bloch, 1986; Froehner, 1986; Peng and Poo, 1986). The supply of any of these might limit the capacity of muscle cells to generate NARPs. This limited capacity is of interest inasmuch as it could provide a basis for competitive interactions when 2 or more neurons contact the same muscle cell.

In this study the possibility of such competitive interactions was reduced, if not eliminated, yet individual muscle cells usually exhibited a discontinuous pattern of NARPs. Such a pattern would arise if key muscle components that participate in NARP generation were distributed nonuniformly prior to neurite–muscle contact or were routed preferentially to only some sites along the contact. There could also be discontinuities in the supply of the neural agent(s) involved in triggering the development of NARPs and/or in maintaining them. The fact that short contact lengths ($\leq$35 μm) exhibited the largest variability in NARP length (0–100% of contact length) is also consistent with the notion that there are fluctuations in the action of the neurite and/or spatial nonuniformities in the response of the muscle cell. Fluctuations in neuritic action and/or spatial discontinuities in the response of individual muscle cells would also have contributed to the variability among competent neurons in establishing NARPs, as well as to the variability in NARP formation along successive muscle cells contacted by the same neuron. In addition, competent neurons and muscle cells may also differ from each other in their overall capacity to establish NARPs.

That there can be differences in the supply of key neural factors along the neuritic arbor is apparent from the finding that the percentage of contact occupied by NARPs was significantly less in the most proximal portions of initial neuritic segments and tended to be largest in the most distal portions of terminal neuritic segments (Table 1). A reduced supply of synaptogenic factors to the most proximal portions of initial neuritic segments

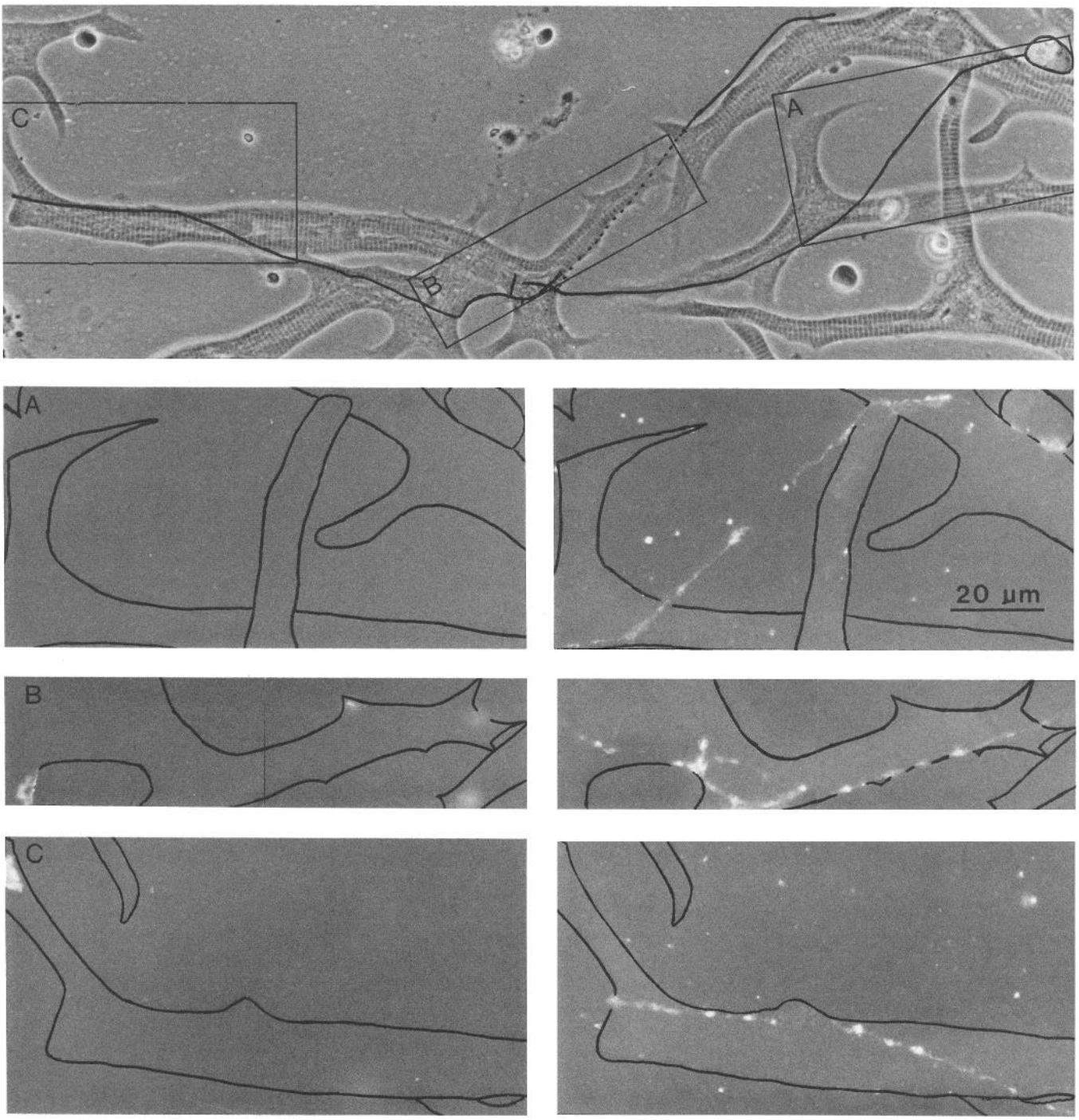

Figure 10. Muscle cells contacted by a single incompetent neuron. *Top,* Low-power, phase-contrast view. The soma and its neurites have been traced in ink. *Dotted lines* indicate portions of neuritic arbor on upper surface of muscle cells. A single neurite emerged from the soma and contacted 5 muscle cells. *Framed areas* are shown *below* at higher magnification with rhodamine optics to reveal sites of high AChR density (*left*) and with fluorescein optics to reveal SVAPs (*right*). Note the absence of NARPs in *A–C*. AChR patches, typical of those that occur on noncontacted muscle cells, are seen away from sites of neurite–muscle contact in *B* and *C*. Two of the patches in *B* are out of focus. Note also the occurrence of SVAPs along neurite–muscle contacts (*A–C*) and along portions of neurite not in contact with muscle (*A*). SVAPs are also seen in the region of soma–muscle contact (*A*). Scattered fluorescent dots on the floor of the culture dish were sometimes seen with fluorescein optics (*A, C*) but not rhodamine optics. *B* is a composite showing 2 different levels of focus. *Scale* applies to fluorescence micrographs. Length of rectangle *B* in phase-contrast photograph is 110 μm. Muscle cells cultured for 5 d and neuron for 3 d at room temperature.

is particularly intriguing in view of the fact that such portions do not normally encounter muscle cells *in vivo* (see Roberts and Clarke, 1982). The present findings indicate that even when they are given the opportunity to establish synaptic contact with muscle cells, they exhibit less capacity to do so than more distal portions of the neuritic arbor.

It is of interest to note another similarity between development *in vivo* and in culture. Motor axons in 2- to 3-d-old *Xen-*

opus embryos reach the myotomes within 50–200 μm from their somata (see Roberts and Clarke, 1982) and need extend only about 200–300 μm further in order to innervate all the muscle cells within a myotome (see Nieuwkoop and Faber, 1967). This suggests that *in vivo* the soma–terminal distances range from about 50 to 500 μm. A rather similar range was observed in the present study (Fig. 7). In addition, there was a high incidence of NARPs occurring at or near the ends of terminal neuritic segments over the full range of soma–terminal distances. These comparisons suggest that, under the present culture conditions, neuritic growth was limited to that which is normally achieved *in vivo* and that, as *in vivo,* the limits of growth were associated with the formation of synapses.

Except for their occurrence off muscle cells, SVAPs exhibited the same general pattern of distribution as NARPs. That they reflect clusters of synaptic vesicles is suggested by the following observations. The monoclonal antibody, Mab 48, used to detect SVAPs is directed against a 65 kDa synaptic vesicle membrane protein that is associated with the synaptic vesicles of most, if not all, neurons (Matthew et al., 1981). This antigen is colocalized with another synaptic vesicle antigen, synapsin 1, both in rat motor nerve terminals and in chick nerve–muscle cultures (Bixby and Reichardt, 1985). The immunofluorescence obtained with Mab 48 in freshly dissected myotomal muscle from *Xenopus* tadpoles is likewise located at synaptic sites, as revealed by AChR localization (unpublished observations). Clustering of vesicles, induced in *Xenopus* spinal cord neurons by contact with polyornithine-coated beads, can also be observed by immunofluorescent staining with Mab 48 (Peng et al., 1987). Taken together these findings make it seem likely that the SVAPs revealed by Mab 48 in *Xenopus* neurons mark sites of clustered synaptic vesicles.

In agreement with previous work (Bixby and Reichardt, 1985), we found that such clusters can occur not only along portions of neurites associated with muscle cells, but also along portions not in contact with muscle or any other cell type. However, contact with muscle clearly influenced their distribution. The influence of muscle on incompetent neurons was moderate: the percentage of neuritic length occupied by SVAPs was about 1.5 times greater for portions of neurites on muscle than for portions off muscle (see Table 4). This suggests that muscle has associated with it a factor that can cause some preferential localization of vesicles even in incompetent neurons. However, the influence of muscle on competent neurons was considerably greater: the percentage of neuritic length occupied by SVAPs was about 5-fold greater for portions of neurites on muscle than for portions off muscle. This result is in agreement with recent elegant studies indicating that contact with muscle regulates the distribution of ACh release sites in these neurons and causes an immediate (within seconds) local increase in ACh release (Chow and Poo, 1985; Xie and Poo, 1986). In addition, we found that about 50% of the SVAP length on muscle was located at NARPs. Since this colocalization cannot be explained on the basis of chance, it indicates that the influence of muscle in localizing synaptic vesicles is greatest at NARPs. Altogether our results support the notion that bidirectional triggering interactions subserve spatial matching of pre- and postsynaptic specializations during embryonic development of the neuromuscular junction and that only competent neurons can participate fully in these interactions.

In mature muscle, synaptic basal lamina contains a factor(s) that induces clustering of synaptic vesicles in regenerating axons (Sanes et al., 1978). Existing evidence suggests that a similar mechanism may operate during synaptogenesis in the embryo. For example, basal lamina can be detected at developing synapses on *Xenopus* myotomal muscle cells *in vivo* and in culture at about the same time that AChRs begin to accumulate at these sites, and shortly afterwards there is an increased appearance of clusters of synaptic vesicles (Kullberg et al., 1977; Chow and Cohen, 1983; Anderson et al., 1984). This development of basal lamina, like the localization of AChRs, is nerve induced (Anderson et al., 1984). In turn, some component(s) of the basal lamina could promote clustering of synaptic vesicles at that site, thereby contributing to the spatial matching of pre- and postsynaptic specializations.

Although this scheme of synaptogenic interactions between nerve and muscle may be generally correct, some of our observations suggest that it requires additional refinement. In the isolated motor unit of Figures 8 and 9, the most proximal NARPs did not have SVAPs associated with them (Fig. 9*A*). Does this mean that SVAPs never formed at these sites, even though there was pronounced colocalization with NARPs in more distal portions of the neuritic arbor (Fig. 9, *B, C*)? If so, this would suggest that NARP formation is not always accompanied by the development of sufficient levels of a synaptic vesicle clustering factor or growing neurites are not always responsive to it. Alternatively, it may be that SVAPs did form at the proximal NARPs shown in Figure 9*A* but later disappeared. If this was the case, it raises the question of whether the NARPs would also have disappeared in due course or whether the maintenance of NARPs and SVAPs is not linked by a common factor at neurite–muscle contacts. Following the formation and survival of these synaptic specializations in time should help to answer such questions.

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