DEVELOPMENT AND SURVIVAL OF A POSTSYNAPTIC SPECIALIZATION IN CULTURES OF EMBRYONIC XENOPUS NERVE AND MUSCLE CELLS

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by

Peter L. Samuels

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Department of Physiology
McGill University
Montreal, Quebec, Canada

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Dedicated to Sandra, Ernest, Karen and Laura.

ABSTRACT

This study has focussed on the formation and survival of acetylcholine receptor (AChR) clusters at neuromuscular synapses formed in culture between myotomal muscle cells and spinal cord neurons derived from embryos of Xenopus laevis. AChRs were labelled with tetramethylrhodamine-conjugated α-bungarotoxin so that the neurite associated receptor patches (NARPs) on the muscle cells could be viewed by fluorescence microscopy. Reduced fluorescence excitation was used in combination with a low light level TV camera and a computer based image calculator to make daily observations on all NARPs throughout the entire neuritic arbor of individual neurons for up to 15 d of co-culture. Virtually all newly-formed NARPs (>90%) were detected at the same time as neurite-muscle contact and in the same proximal-distal sequence as neuritic growth. NARP formation was observed on all neurite-contacted muscle cells and continued for up to 13 d of co-culture even on muscle cells which already had substantial lengths of pre-existing NARPs. Newly-formed NARPs in 1-2 d old co-cultures and in 6-13 d old co-cultures had similar lengths, AChR densities and AChR numbers. On average, these parameters reached close to their ultimate values within a day of NARP formation but the behavior of individual NARPs, even on the same muscle cell was quite variable and many continued to exhibit positive and negative changes as long as they

survived. Virtually all NARPs (>95%) survived as long as their contacts. The survival rates decreased following spontaneous neurite withdrawal and spontaneous neuronal degeneration: poorly developed NARPs disappeared within a day whereas well developed ones survived for more than 4 days. Consistent decreases in NARP length, AChR density and AChR number were not detected prior to neurite withdrawal, neuronal degeneration or muscle cell degeneration. These observations suggest the following conclusions. Neurons retain the capacity to trigger NARP formation as long as they continue to grow. This capacity, as well as that of the muscle cells to respond, does not diminish as the cells age in culture. Changes in NARP shape as well as decreases (and increases) in NARP size can occur even in the absence of competitive interactions between neurons and these changes are locally regulated along the contact. The capacity of neurons to maintain NARPs in more proximal portions of their neuritic arbor persists even as growing distal portions continue to induce the formation of new NARPs.

RÉSUMÉ

La présente étude est axée sur la formation et la survie de grappes de récepteurs d'acétylcholine (AChR) sur les synapses neuromusculaires formées en milieu de culture entre des cellules musculaires myotomales et des neurones de la moelle épinière provenant d'embryons de Xenopus laevis. Les AChR ont été marqués au tétraméthylrhodamine conjugué à de l'α-bungarotoxine pour qu'il soit possible d'observer par microscopie en fluorescence les plaques de récepteurs associés aux neurites (NARP) sur les cellules musculaires. On s'est servi d'une baisse d'excitation en fluorescence conjointement à une caméra à intensification de lumière résiduelle et à une calculatrice vidéo informatique pour observer quotidiennement les NARP dans tout l'arbre névritique des neurones individuels pendant jusqu'à 15 jours de co-culture.

Pratiquement toutes les NARP nouvellement formées (>90 %) ont été détectées en même temps que le contact neurite-muscle et selon le même ordre proximal-distal que la croissance névritique. La formation de NARP a été observée sur toutes les cellules musculaires à contact névritique et s'est poursuivie pendant jusqu'à 13 jours de co-culture, même sur les cellules musculaires qui avaient déjà des NARP pré-existantes de longueur substantielle. Les NARP nouvellement formées après 1-2 jours et 6-13 jours de co-culture avaient une longueur, une densité d'AChR et un nombre d'AChR analogues. En moyenne, ces paramètres atteignent pratiquement leur valeur limite un jour après la formation des NARP même

si le comportement de chaque NARP, même sur la même cellule musculaire, est très variable et que beaucoup continuent à afficher des changements positifs et négatifs pendant toute leur survie. Pratiquement toutes les NARP (>95 %) survivent aussi longtemps que leur contact. Le taux de survie baisse après le retrait spontané des neurites et la dégénérescence spontanée des neurones: les NARP mal développées disparaissent au bout d'un jour tandis que celles qui sont bien développées survivent pendant plus de 4 jours. La diminution constante de la longueur des NARP, de la densité et du nombre d'AChR n'a pu être décelée avant le retrait des neurites, la dégénérescence des neurones ou la dégénérescence des cellules musculaires.

Ces constatations autorisent les conclusions suivantes. Les neurones sont capables de déclencher la formation de NARP aussi longtemps qu'ils poursuivent leur croissance. Ce potentiel de réaction, de même que celui des cellules musculaires, ne diminue pas pendant le vieillissement des cellules en culture. Des changements peuvent se produire au niveau de la forme des NARP et une diminution (et une augmentation) de leur taille peut également intervenir même en l'absence d'interactions concurentielles entre les neurones, ces changements étant régis localement le long du contact. La capacité des neurones à maintenir les NARP dans les portions plus proximales de leur arbre névritique persiste même pendant que les portions distales croissantes continuent de provoquer la formation de nouvelles NARP.

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LIST OF ABBREVIATIONS

A	area
ACh	acetylcholine
AChR	acetylcholine receptor
AxI	area x intensity
αBT	α-bungarotoxin
CNS	central nervous system
DHS	dialyzed horse serum
EBS	embryo bathing solution
EDTA	ethylenediaminetetraacetic acid
I	intensity
L	length
NARP	neurite associated receptor patch
NMJ	neuromuscular junction
RαBT	tetramethylrhodamine-conjugated α-bungarotoxin
TV	television

INTRODUCTION

The vertebrate neuromuscular junction (NMJ) is one of the best characterized chemical synapses (Kuffler et al., 1984). It consists of the unmyelinated terminal branches of a motor neuron which lie in shallow gutters on the surface of a skeletal muscle. Schwann cells form a cap over the nerve terminals and the entire structure is surrounded by basal lamina. The basal lamina also extends into the synaptic cleft, a 50 nm space between the nerve terminal and the muscle cell.

When an action potential reaches the nerve terminal, the associated influx of calcium ions triggers the release of acetylcholine (ACh). Synaptic vesicles filled with ACh fuse to the presynaptic plasma membrane and their contents are released into the synaptic cleft. The ACh diffuses across the synaptic cleft and binds to acetylcholine receptors (AChRs) present at high density in the postsynaptic membrane of the muscle cell. The binding of ACh to its receptor results in the opening the receptor's ion channel and the resulting ionic current depolarizes the muscle cell and leads to muscle contraction.

The efficiency of synaptic transmission is in large part due to the precise spatial alignment of the pre- and postsynaptic specializations. It is therfore of great interest to determine how this elegant spatial alignment comes about during synaptogenesis.

One of the best characterized postsynaptic specializations is the high density of AChRs present in the postsynaptic membrane of the vertebrate NMJ. The junctional sensitivity of the muscle cell to ACh is very much higher than the extrajunctional sensitivity (Feltz and Mallart, 1971; Peper and McMahan, 1972; Kuffler and Yoshikami, 1975). The snake venom derived α-bungarotoxin binds nearly irreversibly to the AChRs in skeletal muscle and when the toxin is conjugated with appropriate radioactive and fluorescent markers it is an effective probe for quantifying and staining the AChRs (Lee, 1972; Anderson and Cohen, 1974; Eldefrawi and Fertuck, 1974; Fertuck and Salpeter, 1974; Ravdin and Axelrod, 1977). Using electron microscopic autoradiography, Fertuck and Salpeter (1976) determined that the density of AChRs at the tops of the junctional folds in the postsynaptic membrane of the mouse was 2.6-3.0 x 10^3 AChRs per square μm. They also found that there was a very sharp gradient for AChRs with the density of AChRs decreasing 100-fold only 3 μm away from the NMJ.

The NMJ in the myotomal muscle of the frog Xenopus laevis has been particularly advantageous for studying the embryonic development of the high density of AChRs in the postsynaptic membrane. The adult animals are easy to maintain in the laboratory and can be reliably induced to mate. The development of the resulting embryos has been extensively characterized (Nieukoop and Faber, 1967). During their normal

development, the first skeletal muscle to form is the myotomal muscle and it has AChRs diffusely distributed on its surface as early as stage 19 (20.8 hr). Nerve fibers reach the ends of the myotomes 1-3 hr later and dense patches of AChRs appear at the contacts approximately 3 hr later (Chow and Cohen, 1983). While the contacts appear to have rudimentary synaptic function as soon as they form, the presumptive pre- and postsynaptic regions are initially undifferentiated as seen by electron microscopy but begin to develop synaptic specializations shortly afterwards (Kullberg et al., 1977). Many of the nerve fibers make "en passant" contacts with the ends of the myotomes. These synaptic contacts are much like those which form in cultures of embryonic spinal cord neurons and myotomal muscle cells derived from Xenopus laevis (Weldon and Cohen, 1979; Chow and Cohen, 1983; Anderson et al., 1977; 1984).

In cultures of aneural myotomal muscle cells, AChRs are distributed diffusely over the entire surface of the muscle cell and also in discrete patches with much higher densities (Anderson and Cohen, 1977; Anderson et al., 1977). Contact of muscle cells by growing neurites has local as well as remote effects on the distribution of AChRs. Dense patches of AChRs called neurite associted receptor patches (NARPs) form at sites of neurite-muscle contact, even in the absence of action potential activity in the muscle and nerve cells (Anderson and Cohen, 1977; Anderson et al.,1977; Davey and

Cohen, 1986). The NARPs emerge from the background at the sites of contact and are not the result of neurite contact with pre-existing patches of AChRs (Moody-Corbett and Cohen, 1982; Kuromi et al., 1985). Neurite contact also causes the disappearance of the pre-existing dense patches of AChRs remote from the site of contact (Anderson and Cohen, 1977; Moody-Corbett and Cohen, 1982; Kuromi and Kidokoro, 1984a). Pulse-labelling experiments indicate that many of the AChRs present in NARPs were present elsewhere in the muscle cell membrane prior to NARP formation and that they accumulate at contacts by lateral movement through the membrane (Anderson and Cohen, 1977). All of these findings indicate that nerve contact triggers the formation of postsynaptic specializations at the site of contact with the muscle cell.

The redistribution of AChRs can only be effected by cholinergic neurons. Dorsal root ganglia and other non-cholinergic neurons were found to be ineffective (Cohen and Weldon, 1980). NARP formation and extrajunctional patch dispersion are neural-specific phenomena and this type of specificity may play a role in the matching of growing axons to their appropriate targets. All of the neurons which were able to induce NARPs (competent neurons) also functionally innervated the muscle cell, while all incompetent neurons failed to form functional synapses (Cohen et al., 1987). NARP formation is therefore a good predictor of functional synaptogenesis not only in vivo but also in culture.

In order to gain insight into the development of the neuromuscular junction, investigators have tried following through time neurite-muscle contacts in culture using fluorescent conjugates of αBT as a stain for AChRs (Anderson and Cohen, 1977; Anderson et al., 1984). These studies have been unable to follow individual NARPs for more than 1-2 d because repeated exposure to the intense excitation light required for standard fluorescence microscopy and photography killed the neurons and tended to bleach the fluorescent stain. The advent of low light level television cameras for fluorescence microscopy overcame this technical limitation by permitting the use of reduced levels of excitation light. Kuromi et al., (1985), used a television camera which permitted a reduction of the intensity of the excitation light by a factor of 10 and made successive observations on developing NARPs over a period of less than 1 d. This approach has revealed that NARPs emerge from the background of the muscle cell membrane as small clusters of AChRs which then grow and/or fuse to form larger NARPs.

Neuromuscular junctions which form in cell culture differ somewhat from mature neuromuscular junctions in vivo. The neuromuscular junction in vivo has Schwann cells closely apposed to the nerve-muscle contact, whereas at neurite-muscle junctions formed in culture, these cells are absent (Weldon and Cohen, 1979). Another difference is in their relative stability following denervation. At adult neuromuscular

junctions the high density of AChRs in the postsynaptic membrane can persist for months following denervation (Frank et al., 1975; Steinbach, 1981b). However, when NARPs undergo neuronal degeneration in culture they tend to disappear rather quickly (Anderson et al., 1984). Furthermore, Kuromi and Kidokoro, (1984b) obtained indirect evidence that the survival time of NARPs following neuronal degeneration is related to their maturity; NARPs from 3 day old co-cultures tended to survive longer than NARPs from 1 day old co-cultures. This is analogous to the finding that following denervation in vivo the survival time of the high density of AChRs is much shorter at developing NMJs than at mature ones (Slater, 1982; Steinbach 1981a,b).

It is clear from this brief review that sequential observations of the formation of the postsynaptic membrane in cultures of Xenopus embryonic nerve and muscle cells have provided important information and new insights into synaptogenesis. These studies have also generated interesting new questions. For example, does the capacity of the neurite to induce the formation of NARPs, and the ability of the muscle to respond, change with time in culture? Also, it has been established that NARPs increase in size following their formation but the time course of this growth has yet to be described. Additionally, the role of the neurite is well established for NARP formation, but its role in NARP survival has not been fully assessed. Does the capacity of an isolated

neuron to maintain NARPs extend throughout the entire neuritic arbor? To what extent does the postsynaptic membrane at neve-muscle junctions in culture change in size and shape during the course of its survival, under conditions where competitive interactions between neurons are absent?

The goal of the current study was to attempt to answer some of these questions by characterizing the development and survival of all of the individual NARPs which formed at contacts along the entire neuritic arbor of neurons in co-cultures of Xenopus embryonic nerve and muscle. To that end, an extremely sensitive low light level television camera was employed. The TV camera permits a reduction in the intensity of the excitation light to 3% of that needed for photography and a 20-fold decrease in the required exposure time. The purpose of this 600-fold decrease in the exposure of the cultures to the excitation light was to prevent the bleaching of the stain and prolong the survival of the cultures.

In order to characterize the development and survival of NARPs quantitatively, a computer image analysis system was used to automatically make measurements of NARP length, relative AChR density and relative AChR number.

MATERIALS AND METHODS

Cultures

Mating between pairs of adult Xenopus laevis was induced by injecting chorionic gonadotropin (500 U, Ayerst) into the dorsal lymph sac of both the males and females. Immediately after the injections, each pair was placed in a separate tank overnight. The following morning, fertilized eggs were selected, cleaned and placed in a sterile embryo bathing solution (EBS) consisting of 0.1 mg/ml gentamycin and 100 U/ml nystatin in dechlorinated water and adjusted to neutral pH using NaOH and phenol red as an indicator. The embryos were allowed to develop at room temperature (22-24 C) as well as at lower temperatures (13-14 C and 19-20 C). The lower temperatures, by slowing development, extended the range of stages and insured that embryos of the desired stage were available for preparing cultures.

The method of preparing cultures was that of Cohen et al., (1987) with some modifications. Stage 22-28 (Nieukoop and Faber, 1967) embryos were selected and their jelly coats and vitelline membranes were removed. Dorsal segments which included the myotomes and spinal cord were dissected and placed in collagenase (0.5-1.0 mg/ml) for approximately 45 min to facilitate the removal of the skin and isolation of the myotomes and spinal cords. Myotomes and spinal cords were isolated under a dissecting microscope in a dissecting medium

consisting of 67% L15, 0.5% dialyzed horse serum (DHS), 0.05 mg/ml gentamycin and 50 U/ml nystatin. Isolated myotomes were placed for about 1 hr in a dissociating medium consisting of 1 mg/ml trypsin and 0.4 mg/ml EDTA in calcium/magnesium-free phosphate buffered saline. Subsequently, the tissue was triturated in a solution consisting of 67% L15 and 5% DHS in order to obtain dissociated muscle cells. The cells were plated in culture chambers (see Anderson et al., 1977) on a substrate of collagen and laminin (kindly provided by Dr. S. Carbonetto) in a medium consisting of Holmes alpha-1 protein (0.2 μg/ml) in 67% L15. The muscle cell cultures were kept overnight in the dark at room temperature. The next day, freshly isolated spinal cords (stage 33/34) were dissociated as described above and the dissociated spinal cord cells were added to the muscle cell cultures. The co-cultures were kept either constantly at room temperature or at 13 C for the first night and room temperature thereafter. The exposure of the cultures to light was kept to a minimum. All solutions without nystatin were sterilized by filtration.

Fluorescent staining of AChRs

Tetramethylrhodamine-conjugated α-bungarotoxin (RαBT) was prepared according to the method of Ravdin and Axelrod (1977). The stain was included at a dilution of 1:250 in the culture medium when the muscle cells were plated. At this dilution, RαBT was found to label 30% of the AChRs in freshly

dissected myotomal muscle in 1 hr, as judged with ^{125}I-αBT and 70% of the AChRs in 3 hr. Upon the addition of the spinal cord cells, the stain was further diluted by half in order to minimize background fluorescence while continuing to stain newly-inserted AChRs.

Two experiments were performed in order to determine whether the RαBT in the culture medium remained effective after several days in culture. In one experiment, the culture medium was removed from two 8-d-old cultures and was then used to stain live, aneural muscle cell cultures. Characteristic AChR patches (Anderson et al, 1977), although faint, were seen within 1 hr of adding the 8-d-old RαBT-containing culture medium and were brightly fluorescent when viewed the next day. In the second experiment, the culture medium was removed from a 16-d-old co-culture and was used to stain AChRs in freshly dissected myotomal muscle from stage 47 tadpoles (see Anderson and Cohen, 1974). The muscles were stained for 1, 2, 4 and 8 hr, then washed, processed and examined. Fluorescent sites at the ends of the myotomes, the major site of innervation, were apparent in muscle which was exposed to the reclaimed culture medium for only 1 hr and these synaptic sites were intensely fluorescent in muscle stained for 8 hr. Taken together, these results indicate that over the entire period of co-culture, RαBT was present in sufficient concentration to label most AChRs within a few hours after their insertion into the surface membrane and to reveal sites of high AChR

density.

Analysis of nerve-muscle fields

One day old co-cultures were examined with phase contrast optics in order to select appropriate fields for following. The microscope stage coordinates were recorded so that the selected fields could be re-examined. Images of the fields were recorded with low power objectives (x25 or x40) to provide a map of the field. The neurite-muscle cell contacts in these fields were then examined with a high power objective (x100; numerical aperture 1.3) using both fluorescence and phase contrast optics. In regions where the neurite was not in a single plane of focus, more than one pair of images was taken.

Images were obtained, stored and analyzed using the Image Calculator software developed by Dr. Harald Riml of McGill University. A Venus TV-3M Low Light Level television camera was used in conjunction with a Zeiss IM 35 inverted microscope. The television picture was digitized by a Coreco Occulus-200 real-time video digitizer board installed in an IBM Personal Computer AT. The digitized images were stored on the hard disk of the computer while fields were being examined and ultimately transfered to tape for long term storage.

The images were displayed on a Sony PVM-1271Q Video

Monitor and measurements of the length (L), area (A) and intensity (I) of NARPs were made on the screen using the Image Calculator. Only those AChR patches which were directly apposed by a neurite were considered to be NARPs. NARP L was measured by the image calculator along the axis of the neurite-muscle contact. Area was measured automatically by using the Image Calculator to trace the contour of the NARP (see Fig. 4). Measurements of I were also done automatically by the software. The video digitizer board converts the analog television picture from the TV camera into an array of 512 x 485 picture elements (pixels). Each pixel has a grey level value assigned to it from a 256 level grey scale. The Image Calcultator calculated NARP I as the mean grey level value of all of the pixels within a NARP outline. Values for NARP I are therefore relative and are reported in arbitrary units. NARP I is an index of the mean AChR density within a NARP. The product of NARP area and NARP intensity (AxI) provides an index of the number of AChRs within a NARP and is also reported in arbitrary units.

Images of a single region of neurite-muscle contact taken on successive days were naturally not in perfect register within the frame. For purposes of comparison, a Mitsubishi P70W Video Copy Processor was used to make hard copies of the images. The neurite and the outlines of the muscle cells as well as the corresponding NARPs were then traced from the hard copies onto acetate sheets. Tracings from different days were

then superimposed so that the identity of individual NARPs could be established with certainty.

In the course of following two adjacent NARPs, the gap between them sometimes "filled in" thereby resulting in a single NARP. In such cases, the adjacent NARPs preceding the "filling in" were also considered as a 'single NARP". Conversely, when a gap formed along a single NARP, the resulting two NARPs continued to be treated as a "single NARP". When, as in the above cases, a "single NARP" had a gap, the sums of the individual segment lengths and of the individual segment areas were used as the values for NARP L, and NARP A respectively. The sum of the AxI values for each segment was divided by the sum of the areas and this value was used for NARP I.

As indicated in Table 1, the results reported here are based on observations made on 6 fields. In 5 fields the muscle cells were contacted by a single, isolated neuron whose neuritic arbor was free of contact by any other neuron. In one field the neurites of a few neurons joined together. In the latter field, it was not possible to know whether all of the neurons were competent to induce NARPs (see Cohen et al, 1987) and where their growth cones were located along neurite fascicles. Accordingly, this field was excluded from analysis of a) the delay between contact and NARP formation and b) the proximal-distal sequence of NARP formation (see Figs. 7 and 8). On the other hand, comparison of newly-formed

NARPs from the single neuron fields with those from the multiple neuron field (Fig. 1) revealed no significant differences (Mann-Whitney U test) between these two groups with respect to the distributions of L ($P>0.8$), I ($P>0.2$) or AxI ($P>0.5$). The results for both groups were therefore pooled.

Daily observations were made for up to 15 d of co-culture. In some fields, however, the observations were terminated considerably earlier because of degeneration of the muscle cells and/or neurons (Table 1). In order to assess the validity of following NARPs until just before the onset of muscle cell degeneration or neuronal degeneration, NARP L, I and AxI were compared on the days preceding these events. Tables 2 and 3 summarize these results and indicate that NARPs did not undergo any progressive decreases in L, I or AxI prior to muscle cell degeneration or neuronal degeneration. Accordingly, in studying the behavior of NARPs at surviving neurite-muscle contacts, the NARPs were used until the last day before the onset of muscle cell or neuronal degeneration.

Sources of error

In order to evaluate the resolution of the television camera system, the same fluorescence images obtained by the television camera and standard fluorescence photomicroscopy were compared. As indicated by the example in Fig. 2 the television camera failed to resolve patches of fluorescent

stain as effectively as standard photomicroscopy. This loss of resolution included a decrease in the sharpness of borders and a loss of sub-structural detail within the patches and resulted in a slight over-estimate of patch size.

As expected, measurements of NARP L, I and A were sensitive to focus. With increasing deviation from optimum focus, the apparent L and A of NARPs increased, whereas the apparent NARP I decreased (Fig. 3). NARP I was found to be most sensitive to deviations from best focus, having a very narrow range of focus height over which the value was maximum. Measurements of NARP A were less sensitive to deviations from best focus and measurements of NARP L were least affected, having a range of 2-3 μm over which they were at their minimum value. Only those NARPs which were in focus were used for measuring NARP L, I and AxI. Of course, even when they were not in focus, they could still be used for analysis of NARP survival.

Repeated measurements of L, I and AxI on typical NARPs revealed errors of ±10%.

RESULTS

NARPs at surviving neurite-muscle contacts

NARP development. Previous studies have examined the formation of NARPs over the first 1 or 2 d following the addition of neurons to muscle cell cultures (Anderson and Cohen, 1977; Anderson et al., 1984; Kuromi et al., 1985; Role et al., 1987). The present study reports on the formation of NARPs and on their survival, in considerably older cultures as well.

Figure 4 shows an example of NARP formation between day 9-11 of co-culture. On day 9 the terminal portion of a growing neurite was seen in contact with a muscle cell. Located at the expanded portion of the neurite, less than 20 μm from its distal tip, was a rather broad NARP. By the next day (day 10) the NARP was larger and brighter. Also apparent on day 10 were fainter and smaller NARPs which had formed more distally along the path of new contact. By day 11 the NARP which was first detected on day 9 appeared much the same as it did on day 10 whereas the NARPs which were first detected on day 10 had undergone marked changes. For example on day 10 the distance between the distal edge of the broad, proximal NARP and the distal tip of the most distal NARP was 23 μm. On day 11 this distance had increased to 28 μm thereby indicating that the most distal NARP had grown 5 μm at its distal end. In addition, whereas the distal NARP appeared

continuous on day 10, the same location on day 11 revealed that the NARP had developed a gap of 2 μm. This sequence indicates that NARPs can undergo significant changes, with some portions disappearing while others survive or grow. The sequence in Fig. 5 (day 5-day 10) likewise illustrates the development of a gap along a NARP. Alternatively, as illustrated in Fig. 6 (day 1-day 2), gaps of about 2 μm between a group of newly-formed NARPs can acquire a high density of AChRs over a 1 d period, thereby converting the group into a single NARP.

In the examples of Figs. 4 and 6, the NARPs were detected at the same time as the new contacts, on day 9 and on day 1 respectvely, whereas in Fig. 5 the NARP was first observed a day later. In fact, in the single-neuron fields where the onset of neurite-muscle contact could be determined without ambiguity, (see Materials and Methods), 92% of the NARPs were first detected on the same day that the contact was first seen and the remainder were detected one day later (Fig. 7). These results indicate that, independant of the age of the culture, most if not all NARPs begin to form in less than a day after neurite-muscle contact is established. In addition, as in the examples shown in Figs. 4 and 6, the sequence of NARP formation almost always occurred in the same proximal-distal sequence as neurite growth. Only 2% of NARPs formed proximal to pre-existing NARPs on the same neurite (Fig. 8).

Of 181 NARPs which formed during this study, 90 formed

in the single neuron fields and the remainder formed in the multiple neuron field (Table 1). NARP formation occurred on all 27 muscle cells that were contacted by neurites. It also occurred as neurites continued to grow on muscle cells which already had NARPs. Figure 9A shows an example where NARP formation occurred on a single muscle cell on day 2, 4, 6, 7 and 13 of co-culture. The length of pre-existing NARPs on the muscle cell was about 30 μm when NARP formation occurred on day 4 and had increased to more than 50 μm by the time that NARP formation occurred on day 13. It is apparent that even relatively long lengths of pre-existing NARP do not make the muscle cell refractory to NARP formation.

Most neurite growth and NARP formation occurred early in co-culture but since NARPs formed as late as day 13, the question arises whether NARPs differed according to when they formed. As indicated in Fig. 10, this did not appear to be the case. For each of the parameters (L, I and AxI) the distributions in day 1-2 co-cultures were not significantly different from those in day 6-13 co-cultures ($p>0.35$ for L, $p>0.06$ for I and $p>0.95$ for AxI).

It is apparent from Fig. 9 as well as from the examples in Figs. 4 and 6 that individual NARPs, even on the same muscle cell, can undergo different changes in the days following their initial detection. Some can increase in length, others may change little and others may even decrease in length. Positive as well as negative changes also occurred

in the I and AxI values of NARPs following their initial detection. On average, however, the changes during the first day or two were positive. As shown in Fig. 11A, NARPs tended to increase in length, approximately doubling in length over a period of 2 d and reaching close to their ultimate length after 1 d. By contrast, NARP intensity was, on average, close to its ultimate value when the NARPs were first detected (Fig. 11B). Clearly, the mean density of AChRs in a newly-forming NARP can increase rapidly and reach close to its ultimate value within a day (see also Fig. 9). The relative number of AChRs in a NARP as measured by AxI also tended to increase rapidly, virtually tripling in the first day after NARP detection and changing relatively little thereafter (Fig. 11C). Taken together, these results indicate that although NARPS exhibit considerable individual variability, they tend, on average, to reach close to their ultimate length, mean AChR density and AChR number within a day after they are first detected and within 1-2 d after the onset of neurite-muscle contact.

NARP survival. Even after reaching relative stability most NARPs still underwent some changes in L, I, and AxI. Figure 12 compares the daily, absolute changes of "mature" NARPs (3-9 d old) with those changes which occurred between the first two daily observations of newly-formed NARPs. It can be seen that there are some differences between the distributions of the two populations. For example, for newly

formed NARPs there were more positive than negative changes and the positive changes tended to be larger. Overall, there was a net positive change for the population of newly-formed NARPs. For "mature" NARPs the number and size of positive and negative changes were about the same and the fraction of NARPs which did not change was higher than for the newly-formed NARPs. The overall net change for the "mature" population of NARPs was close to zero. Summation of the daily decreases in NARP length revealed that these decreases were 8.5±1.1% (n=13; range: 2%-14%) of the total pre-existing NARP length and were not dependant on the age of the co-culture.

Even NARPs on the same muscle cell underwent changes in the opposite direction. For example the length of some of the individual NARPs on the muscle cell of Fig. 9 changed in opposite directions between day 6-7, day 7-8, day 10-11 and day 13-14. Conversely, not during any daily interval did all the NARPs on the same muscle cell change length in the same direction (Fig. 9A). Even when the analysis was limited to "mature" NARPs (at least 2 d old), in only one of ten cases did all the NARPs on individual muscle cells change length in the same direction. The incidence of all mature NARPs on the same muscle cell changing area in the same direction was equally low. On the other hand, in 60% of the cases, the I values of all mature NARPs on individual muscle cells did change in the same direction (see also Fig. 9B). Taken together, these results suggest that the sizes of NARPs on an

individual muscle cell are locally regulated whereas their AChR density may be under more global regulation.

The results of Fig. 9 also reveal that the formation of new NARPs did not result in corresponding losses in other pre-existing NARPs on the same muscle cell. For example, when NARPs e and f in Fig. 9 first appeared on day 7, totalling 9 μm in length, only two of the pre-existing NARPs (a, b) exhibited small decreases between day 6-7 whereas the other two (c, d) actually increased in length during this period. Further analysis also revealed a poor correlation ($r = -0.3$) between the change in length of newly-formed NARPs and the change in length of "mature" NARPs on the same muscle cell. Likewise, the formation of new NARPs was not associated with any consistent decrease in the I or AxI values of the pre-existing NARPs on the same muscle cell. If NARP formation involves competitive interactions with pre-existing NARPs on the same muscle cell, the effects are too subtle to be detected by the techniques used in this study.

Even though many NARPs did undergo some decreases in L, I and AxI, almost all of them survived as long as their neurite-muscle contact survived. As shown in Fig. 13, the daily survival rates were always close to 100% and the lowest daily survival rate was 96%. Only 5 NARPs at surviving contacts disappeared. Three of these NARPs were less than 1 d old when last seen and the other two were 1 and 3 d old. They were all located on the upper surface of the muscle cell

and were last seen as early as day 2 of co-culture and as late as day 10. Their lengths just prior to disappearance ranged from 2-4 μm and their AxI values ranged from 32-80. Since these values lie at the lower end of the distributions for newly-formed NARPs (Fig. 10) it is apparent these NARPs were not well developed before their disappearance. In addition, the decreases in L and AxI associated with their disappearance were within the range of decreases observed for surviving NARPs (Fig. 12A, C) and were not progressive.

NARP survival following neurite withdrawal

Neurites which formed contacts with muscle cells sometimes withdrew from the muscle cell or shifted their position on the muscle cell, particularly during the first few days of co-culture when most of the neurite growth and retraction occurred. Unlike NARPs at surviving contacts which had a survival rate close to 100%, NARPs from which neurites withdrew tended to disappear rapidly. Figure 14 shows the survival rate of NARPs following neurite withdrawal. Of the 31 NARPs from which neurites withdrew, 26 failed to survive even one day and only one survived for as long as 4 d.

An example of a field in which neurite withdrawal occurred from NARPs is shown in Fig. 15. On day 7 of co-culture the NARPs and contacts were observed for the first time. Both NARPs (X and Y) consisted of a large and small portion separated by a gap of about 1 μm. On day 8 the gap

was no longer apparent on NARP Y. NARP X was more intense and longer but only its smaller portion was still contacted. By day 9 the neuritic branches were no longer seen in contact with either NARP site. At this time, NARP Y had virtually disappeared. By contrast, NARP X was essentially unchanged except that the 1 μm gap between its two segments appeared to have filled in. That this filling in occurred in the complete absence of neuritic contact cannot be concluded with certainty because it is possible that that site may have been transiently contacted between day 8 and 9. By day 10 portions of NARP X had clearly disappeared whereas others were still present. By the next day the muscle cell had degenerated so day 10 represents the final observation. Considered altogether, this example illustrates that survival of NARPs following neurite withdrawal can vary even on the same muscle cell. In most cases, NARPs disappeared completely within a day. Alternatively, some survived for a day and then disappeared completely and others, such as NARP X in Fig. 15, disappeared in a segmental fashion.

That NARP disappearance following neurite withdrawal is relatively abrupt is further suggested by the comparison in Fig. 16 between the maximum daily decreases in the length of individual NARPs at surviving contacts and following neurite withdrawal. It can be seen that at surviving contacts many NARPs exhibited no decreases in length and when decreases were observed, for most NARPs they were not larger than 1-2

μm/d. By contrast, following neurite withdrawal the distribution of the maximum daily decreases was significantly different ($P < 0.001$). The distribution was less skewed and the range of decreases was larger. Taken altogether, the results emphasize that the neurite plays a role not only in inducing NARPs but also in maintaining them.

Most NARPs (27) were 1 d old or less just before the onset of neurite withdrawal. Of these, almost all (24) disappeared within 1 d and none survived for more than 2 d. Conversely, 1 of the 4 NARPs which were 3-4 d old just before the neurite withdrew survived for at least 4 d, thereby suggesting that older NARPs are more likely to survive longer following neurite withdrawal. Interestingly, the one NARP which survived at least 4 d after neurite withdrawal was longer, more intense and had a higher AxI value than the NARPs which survived for 2 d or less (Table 4). This suggests that in order for a NARP to survive for a long time after neurite withdrawal it must be mature in terms of age as well as size and AChR density.

Previous work on chick nerve-muscle cultures has suggested that NARPs begin to disappear prior to neurite withdrawal (Role et al., 1987). In the present study, no decrease in L, I or AxI was seen on the three days preceding neurite withdrawal (Table 5).

NARP survival following neuronal degeneration

As indicated in Table 1, some neurons degenerated during the course of the experiments. In two of these fields, the individual NARPs were followed for up to six days after degeneration. In one of the fields, neuronal degeneration occurred on day 4 (field B) whereas in the other field it occurred on day 10 (field C). In contrast to the rapid disappearance of NARPs following neurite withdrawal, most of the NARPs involved in neuronal degeneration tended to survive as long as the muscle cell survived (Fig. 17).

Figure 5 shows an example of a NARP which underwent neuronal degeneration. On day 9 of co-culture when the NARP was 4 d old it was still in contact with the terminal portion of a neurite which was continuous with the neuronal soma. By day 10 neuronal degeneration was apparent; the neurite had become discontinuous proximal to the NARP, although neuritic fragments were still apparent in the region of the NARP. On day 14 of co-culture, 4 d after the onset of neuronal degeneration, the neuritic fragments were still present as was the NARP which exhibited small losses at its proximal segment. It is interesting that the site where loss of NARP occurred was associated with a much smaller neuritic fragment than the site where the NARP survived more completely. This, however, was not always the case. In fact, the two NARPs which failed to survive even one day following neuronal degeneration (see Fig. 17) were both contacted by neuritic fragments. Two other

NARPs exhibited neuritic fragments only on the first day following neuronal degeneration, yet they survived for 5 and 6 d, respectively. It is possible that following neuronal degeneration, some material which is necessary for NARP survival was left behind by the neuron and was not visible using the techniques employed in this study.

It is clear that NARPs can survive for several days following neuronal degeneration but NARPs do not always survive intact. Figure 18 shows the relative L, I and AxI values of individual NARPs which survived for at least 4 d following neuronal degeneration. It is evident that some NARPs survived without undergoing losses in L or AxI while others survived in a less complete manner (Fig. 18A, C). None of the NARPs which survived for at least 4 d exhibited any substantial decline in I (Fig. 18B).

In the preceding section, it was suggested that in order for a NARP to survive for at least 4 d following withdrawal, it had to be mature in terms of age as well as L, I and AxI (see Table 4). A similar situation seems to apply to NARPs which underwent neuronal degeneration. For example, the 2 NARPs which failed to survive even 1 d were both less than 1 d old whereas the 12 NARPs which survived for at least 4 d were older. Likewise, the NARPs which survived at least 4 d following neuronal degeneration were more mature in terms of the NARP parameters than the NARPs which failed to survive even 1 d after neuronal degeneration (Table 6). In addition,

just as with neurite withdrawal, NARPs did not show any consistent decrease in L, I or AxI on the days prior to neuronal degeneration (Table 3).

DISCUSSION

In previous studies, NARPs were followed at most for 1-2 d and were observed no more than 4 times during that period (Anderson and Cohen, 1977; Cohen et al, 1979; Anderson, et al., 1984; Kuromi et al., 1985). A limiting factor was that neurons rarely survived for more than 1-2 d in culture when exposed to the intense excitation light required for standard fluorescence microscopy and photography (Moody-Corbett and Cohen, 1982). In the present study, the use of a low light level television camera permitted a reduction in the intensity of the excitation light to 3% of that normally used for viewing and photography. This experimental modification made it possible to follow the formation and survival of NARPs for up to 15 d, although some neurons and muscle cells degenerated considerably earlier. The latter degeneration is probably a reflection of limitations in the culture technique rather than a result of exposure to the excitation light because some early degeneration is seen even when the cultures are viewed only with low intensity phase contrast optics (unpublished observations). The combination of low light level television cameras and reduced fluorescence excitation has also permitted much longer periods of observation of AChRs at identified neuromuscular junctions in vivo (Lichtman et al., 1987; Rich and Lichtman, 1989) but so far, in vivo studies have not dealt with sequential observations during the embryonic

formation of the postsynaptic membrane.

In the current study, NARPs were followed at daily intervals from the onset of their formation and were measured in terms of length (L), relative AChR density (I) and relative AChR number (AxI). In addition, all of the NARPs which formed along the entire neuritic arbor of individual neurons were followed and in all but one field there was no possibility of competitive interactions between neurons because the muscle cells were contacted by only a single neuron which itself was free of contact by all other neurons in the culture (see Cohen et al., 1987). As a result of these experimental modifications it has been possible to obtain new quantitative information concerning the formation, growth and survival of NARPs.

NARP formation

The observation that almost all NARPs were first detected at the same time as their neurite-muscle contacts were first seen suggests that most NARPs form in less than 1 d after contact is established. This is consistent with previous reports of NARP formation within a few hours after the onset of neurite-muscle contact (Cohen et al., 1979; Role et al., 1987; see also Peng and Phelan, 1984). Preferential accumulation of AChRs at sites of synapse formation has also been detected within 3 hr of the onset of innervation of Xenopus myotomal muscle during normal embryonic development

in vivo (Chow and Cohen, 1983). The finding in the current study that 8% of NARPs were detected on the day following the onset of neurite-muscle contact could be the result of having, by chance, first examined the contacted region very shortly after the contact was established. By making sequential observations at shorter intervals it should be possible to determine with more precision how large the variability is in the delay between contact and NARP formation.

Virtually all NARPs formed in a proximal to distal sequence along the path of neurite-muscle contact in the same direction as neurite growth. The fact that NARPs rarely formed proximal to pre-existing NARPs along the same neurite branch is probably a consequence of the short latency of NARP formation following the onset of contact. The findings are also consistent with the conclusion that neurites lose their capacity to trigger new NARP formation proximal to pre-existing NARPs (Rodriguez-Marin and Cohen, 1988).

In the present study, all of the nerve-contacted muscle cells generated NARPs. This finding supports the conclusion that as long as they continue to grow, neurites retain the capacity to induce NARPs and that all muscle cells are able to respond (Cohen et al., 1987). That the distribution of NARP L, I and AxI were similar for newly-formed NARPs in 1-2 d old co-cultures and in 6-13 d old co-cultures (Fig. 10) further suggests that the neurite and muscle capacities relating to NARP formation undergo little change as the

neurons and muscle cells age in culture.

Even when the muscle cells had several pre-existing NARPs whose total length and AChR number were relatively high, they still retained the capacity to develop additional NARPs (Fig. 9). However, the total length of NARP on individual muscle cells did not exceed the apparent maximum limit of approximately 80 μm that was estimated previously (see Cohen et al., 1987). Studies on mature rodent muscle have indicated that a prerequisite for the formation of new sites of postsynaptic membrane is an elevation of the density of extrajunctional AChRs (Lomo and Slater, 1978). Although measurements of extrajunctional AChRs have not been made for Xenopus myotomal muscle cells which have been innervated for 1-2 weeks in culture, it is known that in vivo these muscle cells continue to exhibit relatively high levels of extrajunctional AChRs even 2 weeks after they have been innervated (Chow and Cohen, 1983, Kullberg and Kasprzak, 1985).

Another relevant observation concerning NARP formation is that the width of NARPs was variable, even at contacts formed by the same neuritic arbor and on the same muscle cell. Some NARPs were only about as broad as the neurite while others extended a few micrometers beyond the visible borders of the neurite (Figs. 4-6). Electron microscopy has likewise indicated that aggregates of AChRs extend beyond the region of axon-muscle contact early in the development of the

neuromuscular junction in foetal mice (Matthews-Bellinger and Salpeter, 1983). That NARPs can extend beyond the borders of the neurite might be explained if the neuronal factor which triggers NARP formation is diffusible. However, if this were the only contributing factor then one would expect to see consistently a declining gradient of intensity with distance from the neurite. Such gradients have been observed at developing neuromuscular junctions in rodent foetuses and neonates (Steinbach, 1981a; Slater, 1982) but this was not the case for many young NARPs in the present study (Figs. 5, 6). Another possibility is that the triggering influence of the neurite is restricted to the region of neurite-muscle contact but that the response of the muscle cell extends beyond that region. Alternatively, it may be that the development of broad NARPs is associated with transient contact of the entire NARP site during the course of neuritic growth. It should be possible to test this last possibility by following NARP formation and neuritic growth at sufficiently short intervals.

NARP growth and survival

On average, NARP growth as judged by length (L) and relative AChR number (AxI) continued for about 1 d (Fig. 11). However, the developmental changes for individual NARPs were quite variable. Sometimes gaps between adjacent NARPs "filled in" thereby resulting in a larger single NARP (Fig. 6) and in some cases gaps developed along previously continuous NARPs

(Fig. 5). Kuromi et al., (1985) likewise observed that NARP growth can occur by "filling in" of gaps between adjacent NARPs. At present, it is not known whether the neuritic triggering action is initially discontinuous at such sites and later extends to the gap region as well or whether the muscle response is delayed in the gap region. It is also unclear whether the development of gaps along previously continuous NARPs originates from a local change in the neurite or in the muscle cell.

What is clear from the present observations is that NARPs can undergo changes in size and shape even in the absence of competitive interactions between neurons. By contrast, recent in vivo studies have emphasized that the postsynaptic membrane at the mature mouse neuromuscular junction normally undergoes little change in size or shape (Lichtman et al., 1987; Rich and Lichtman, 1989). It remains to be determined whether these differences in the stability of the postsynaptic membrane are related to (a) differences in the stage of development, (b) a species difference or (c) differences in the behavior of neurons and muscle cells in vitro and in vivo. In the latter regard it is worth considering that neurite-muscle contacts in cell culture may be subject to much greater dynamic forces than in vivo because the absence of spatially-organized tissues lessens the spatial constraints on neuritic and muscle cell growth. In addition, the contacts in culture are not enveloped by Schwann cells or surrounded by collagen

fibrils (Weldon and Cohen, 1979; Anderson et al., 1984) which presumably contribute to the stabilization of the mature neuromuscular junction in vivo.

The daily survival rate of NARPs at surviving contacts was high, never falling below 96%. In fact, only 5 of 181 NARPs failed to survive as long as the neurite contact survived and these were not well developed as judged by their length, relative AChR density and relative AChR number. Anderson et al., (1984), likewise observed that some poorly developed NARPs disappeared along surviving neurite-muscle contacts. The observation that all of the contacted NARPs which failed to survive were on the upper surface of the muscle cell raises the possibility that the disappearance may have been preceded by a small separation of the neurite from the muscle cell. Separations of 0.5-1.0 μm would not have been detected but may be sufficient to eliminate the neurite-muscle interaction(s) required for NARP survival (see below).

Although almost all NARPs survived at surviving contacts, many of them continued to undergo increases and decreases in length, relative AChR density and relative AChR number. Even NARPs on the same muscle cell often changed size in opposite directions, thereby suggesting that NARP size is locally regulated along the contact. Changes in the relative AChR density sometimes also occurred in opposite directions for NARPs on the same muscle cell but more often the changes were in the same direction, suggesting that the density of AChRs

at NARPs may be under a more global control at the level of the muscle cell.

Competitive interactions between NARPs on the same muscle cell were not apparent inasmuch as the formation and growth of new NARPs did not cause the disappearance of pre-existing NARPs on the same muscle cell. By contrast, previous work has shown that the patches of AChRs which are present on the surface of aneural muscle cells in culture are dispersed when a neurite contacts the muscle cell and induces NARP formation (Anderson and Cohen, 1977; Cohen and Weldon, 1980; Moody-Corbett and Cohen, 1982; Kuromi and Kidokoro, 1984a). It seems that NARPs, unlike non-junctional patches of AChRs, are protected from dispersal. The difference in the behavior of these two types of AChR clusters suggests that the neurite plays an important role in maintaining NARPs. This conclusion is supported by the additional observation that NARPs tended to disappear rapidly following spontaneous neurite withdrawal. Apparently, at surviving contacts the neurite continues to interact with the muscle cell in a way which promotes NARP survival. Whether this interaction is similar to that which triggers NARP formation remains to be determined.

Two additional points are worth noting in regard to the role of the neuron in NARP survival. Since neuromuscular transmission was abolished by the presence of RαBT in the culture medium, it is apparent that NARPs can survive at least 2 wk in the absence of nerve-induced muscle contraction.

It is well established that NARP formation can also occur in the absence of muscle cell contraction (Anderson et al., 1977; Anderson and Cohen, 1977; Rubin et al., 1980; Ziskind-Conhaim and Bennett, 1982; Davey and Cohen, 1986). Secondly, the fact that almost all NARPs survived as long as their contacts throughout the entire neuritic arbor of individual neurons suggests that the capacity of the neuron to maintain NARPs in more proximal portions of its neuritic arbor persists even as growing distal portions continue to induce the formation of new NARPs.

The survival rate of NARPs was considerably higher following neuronal degeneration than following neurite withdrawal. This difference may have been fortuitous inasmuch as the NARPs involved in neuronal degeneration tended to be better developed than those involved in neurite withdrawal. Previous studies on cell cultures (Kuromi and Kidokoro, 1984b) and on the developing neuromuscular junction in vivo (Slater, 1982; Steinbach, 1981b) have indicated that the survival of the postsynaptic membrane following neuronal degeneration increases with maturity. It may also be pertinent that following neuronal degeneration, neuritic fragments often remained behind at the NARPs, so these may have contributed as well to the higher survival rates. In vivo, degenerating nerve terminals are phagocytosed by their overlying Schwann cells (Birks et al., 1960) but as pointed out above, Schwann cells are not associated with the neuromuscular synapses that

form in the cell culture system used in this study.

In the current investigation, NARPs did not exhibit any consistent decrease in length, relative AChR density or relative AChR number prior to neurite withdrawal or neuronal degeneration. This observation contrasts with the findings of Role et al., (1987) who obtained indirect evidence that in cultures of embryonic chick myotubes and ciliary ganglion neurons, NARPs disappeared prior to the retraction of primary neurites. In the present investigation, the retraction of a primary neurite was never observed; rather, neurite withdrawal entailed the shifting of a neurite away from a NARP or the retraction of a distal neuritic branch. It is possible that the retraction of primary neurites and the associated loss of NARPs are preceded by an interruption of the neurite-muscle interaction which normally promotes NARP survival. It is also possible in the present study that the onset of NARP loss preceded neurite withdrawal but the interval between these two events was too short to permit detection of this sequence during daily observations.

This work has described the formation and survival of a postsynaptic specialization in the apparent absence of competitive interactions between neurons. The observations have provided new information and insights and have raised interesting new questions which should be possible to address by making sequential observations at sufficiently short intervals. The findings also provide a reference point for

future investigations concerning the formation and survival of the postsynaptic membrane under conditions where more than one neuron competes for a single muscle cell. Direct observations of competitive interactions have recently been made for the case of experimentally-induced multiple innervation of the mature mouse neuromuscular junction in vivo (Rich and Lichtman, 1989). Similar direct observations during competitive challenges in culture may help uncover mechanisms which can be used as models for understanding the development of the much more complex networks found in the CNS.

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TABLE 1. Description of the fields.

Field	Number of neurons	Number of muscle cells	Number of NARPs	Co-culture age at last observation (d)	Reason for ending observations
A	1	2	5	4	Neuron degenerated
B	1	3	22	5	Neuron degenerated
C	1	5	16	15	Neuron and most muscle cells degenerated
D	$\geq$2	12	90	15	Neuron and most muscle cells degenerated
E	1	3	36	2	Could not re-locate field
F	1	2	12	3	Neuron degenerated
		27	181		

TABLE 2. The behavior of NARPs on the 3 d preceding muscle cell degeneration. NARP L, I and AxI are given relative to their values on the day before (-1 d) degeneration was detected. The number of NARPs on which means and standard errors are based is indicated in parenthesis.

Time before muscle degeneration	-3d	-2d	-1d
Relative L	0.8 (1)	1.2 ± 0.1 (7)	1
Relative I	1.0	1.0 ± 0.1	1
Relative AxI	0.6	1.3 ± 0.3	1

Note that the NARPs did not exhibit any consistent decrease in L, I or AxI on the 3 d prior to muscle cell degeneration.

TABLE 3. The behavior of NARPs on the 3 d preceding neuronal degeneration. NARP L, I and AxI are given relative to their values on the day before (-1 d) the onset of neuronal degeneration.

Time before neuronal degeneration	-3d	-2d	-1d
Relative L	0.7 ± 0.2 (3)	0.9 ± 0.2	1
Relative I	1.0 ± 0.1	0.9 ± 0.1	1
Relative AxI	1.0 ± 0.3	1.0 ± 0.3	1

Note that the NARPs did not exhibit a progressive decrease in L, I or AxI on the 3 d prior to neuronal degeneration.

TABLE 4. The effect of NARP maturity on survival time following neurite withdrawal. Values of L, I and AxI are given as mean ± standard error for the observation immediately preceding neurite withdrawal.

Survival time after neurite withdrawal (d)	≤ 2	≥ 4
Number of NARPs	27	1
L (μm)	3.7 ± 0.6	12
I	13.1 ± 1.2	21
AxI	65 ± 14	588

Note that the NARP which survived for at least 4 d was more mature in terms of L, I and AxI than the NARPs which survived no more than 2 d after neurite withdrawal.

TABLE 5. The behavior of NARPs on the 3 d preceding neurite withdrawal. NARP L, I and AxI are given relative to their values on the day before neurite withdrawal (-1d). Values are given as mean ± standard error.

Time before neurite withdrawal	-3d	-2d	-1d
Relative L	0.6 ± 0.2 (3)	1.2 ± 0.3 (5)	1
Relative I	1.0 ± 0.2	1.2 ± 0.3	1
Relative AxI	0.5 ± 0.1	0.9 ± 0.3	1

Note that NARPs did not exhibit a progressive decrease in L, I or AxI on the 3 d prior to neurite withdrawal.

TABLE 6. The effect of NARP maturity on survival time following neuronal degeneration. Values of L, I and AxI are given as mean ± standard error for the observation immediately preceding neuronal degeneration.

Survival time after neuronal degeneration (d)	< 1	≥ 4
Number of NARPs	1	8
L(μm)	3	5.8 ± 1.1
I	18	20.5 ± 1.9
AxI	36	224 ± 75

Note that the NARP which survived less than 1 d was less mature in terms of L, I and AxI than the NARPs which survived at least 4 d after neuronal degeneration.

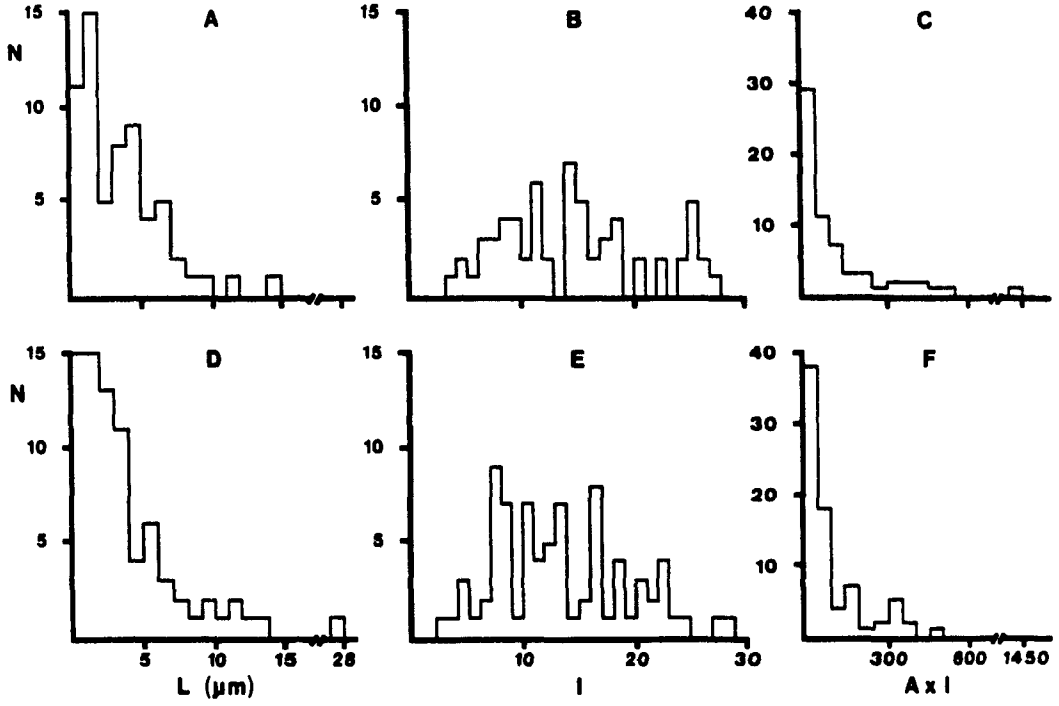

Fig. 1. Comparison between newly-formed NARPs in the single-neuron fields and the multiple-neuron field. A-C show the distribution of L, I and AxI for individual, newly-formed NARPs in the single-neuron fields. The corresponding distributions are shown for the multiple-neuron field in D-F. Values on the ordinate indicate the number of NARPs. Note that both groups of NARPs exhibited similar distributions for length (A,D), intensity (B,E) and area x intensity (C, F).

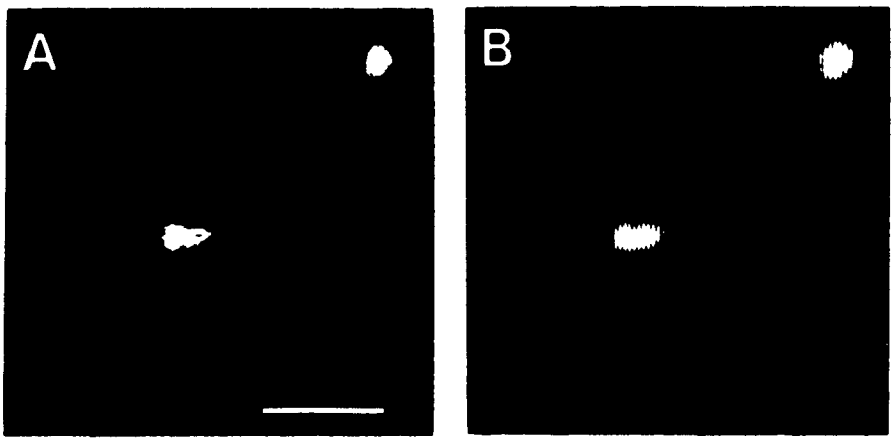

Fig. 2. Comparison of fluorescence images recorded by standard photomicroscopy (A) and by the television camera system (B). Note the loss of resolution with the television camera. Scale bar, 10 μm

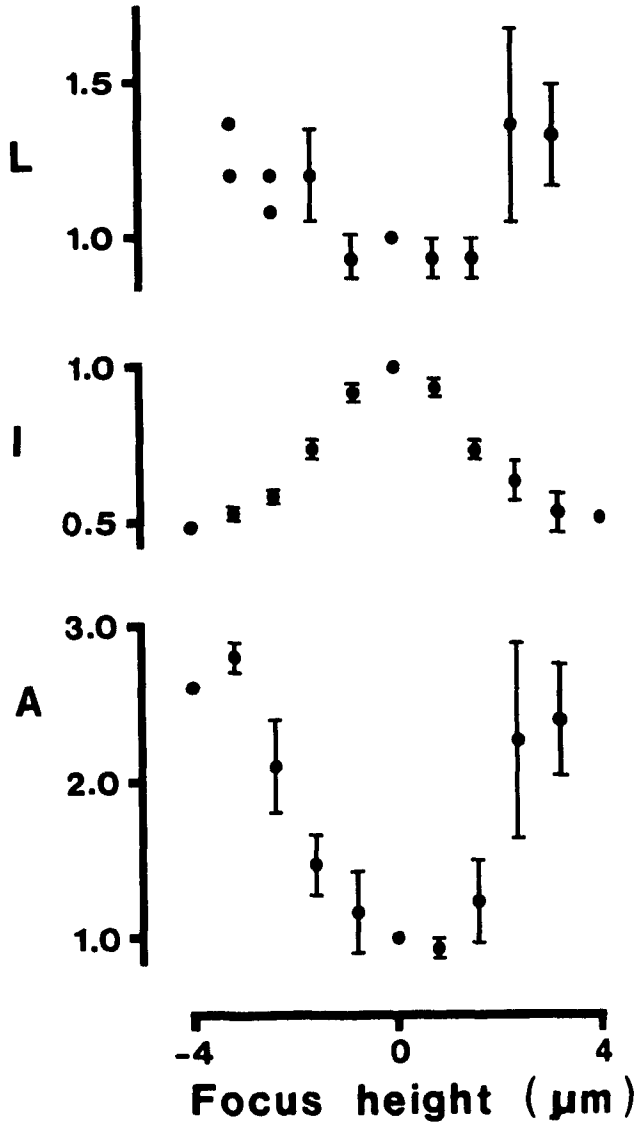

Fig. 3. The effect of focus on NARP length, intensity and area. Positive values on the abscissa indicate that the objective was farther away than the position of best focus, while negative values indicate that the objective was nearer. Values on the ordinate are relative to the value at best focus. Means and standard error bars are based on 3 NARPs. Note that deviations from best focus increased the apparent NARP length and area and decreased the apparent NARP intensity.

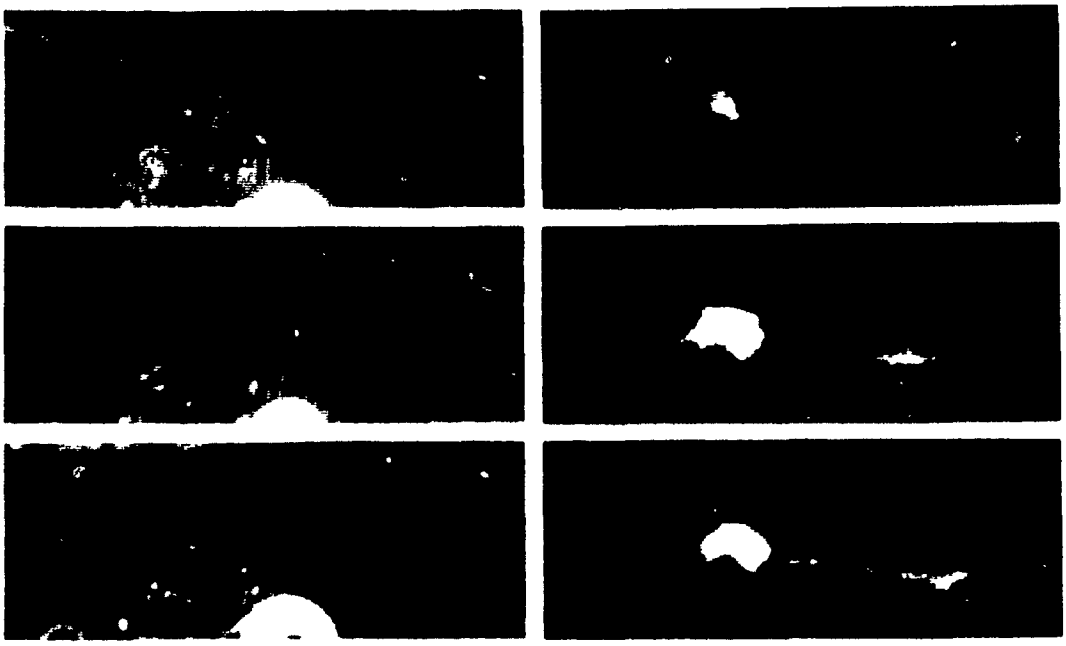

Fig. 4. Development of NARPs at a late-forming contact. Phase contrast and corresponding fluorescence views of the same field are shown on days 9, 10 and 11 of co-culture. To facilitate comparison of spatial relationships, the outlines of the NARPs have been superimposed at their exact positions along the neurite-muscle contact. For description, see text. Scale bar, 20 μm.

Fig. 5. Development and survival of a NARP whose neurite ultimately degenerated. Phase contrast and corresponding fluorescence views of the contact are shown on days 4, 5, 6, 9, 10 and 14. The NARP was detected on day 5, one day after the contact formed. Note that a gap developed along the NARP (day 9). By day 10 the neurite had degenerated but the NARP and neuritic fragments were still present 4 d later. As in this example, terminal neuritic varicosities sometimes formed by retraction of the distal portion of the neurite (day 4-day 6). Scale bar, 10 μm

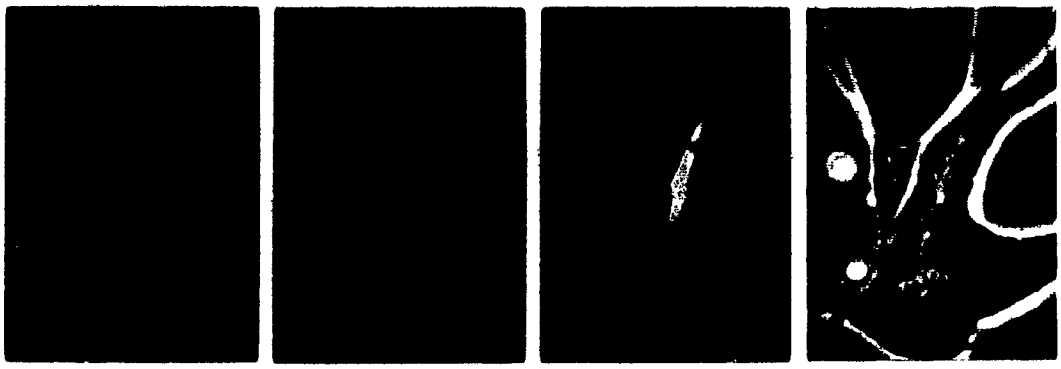

Fig. 6. The development and survival of an early-forming NARP. The NARP is shown on days 1, 2 and 9 of co-culture. A phase-contrast image of the contact on day 9 is also shown. As in this example, neurites which contact the lower surface of a muscle cell are more difficult to resolve than those which contact the upper surface (see Fig. 4). Comparison of day 1 and day 2 reveals that NARP growth occurred in part by "filling in" of gaps. Comparison of day 2 and day 9 reveals some change in the proximal portion of the NARP and new NARP formation distally (arrows). Scale bar, 20 μm

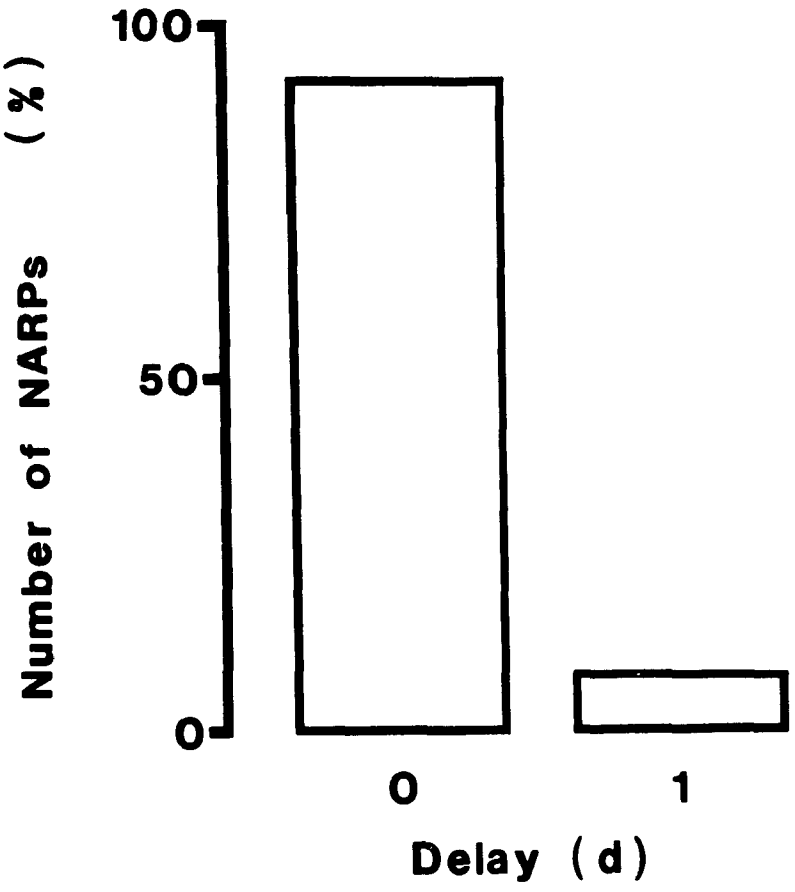

Fig. 7. The delay between contact and NARP formation. Values on the abscissa indicate the delay between the formation of a neurite-muscle cell contact and NARP formation. Note that almost all NARPs were first seen on the same day that the contact was first seen. Percentages based on 75 NARPs.

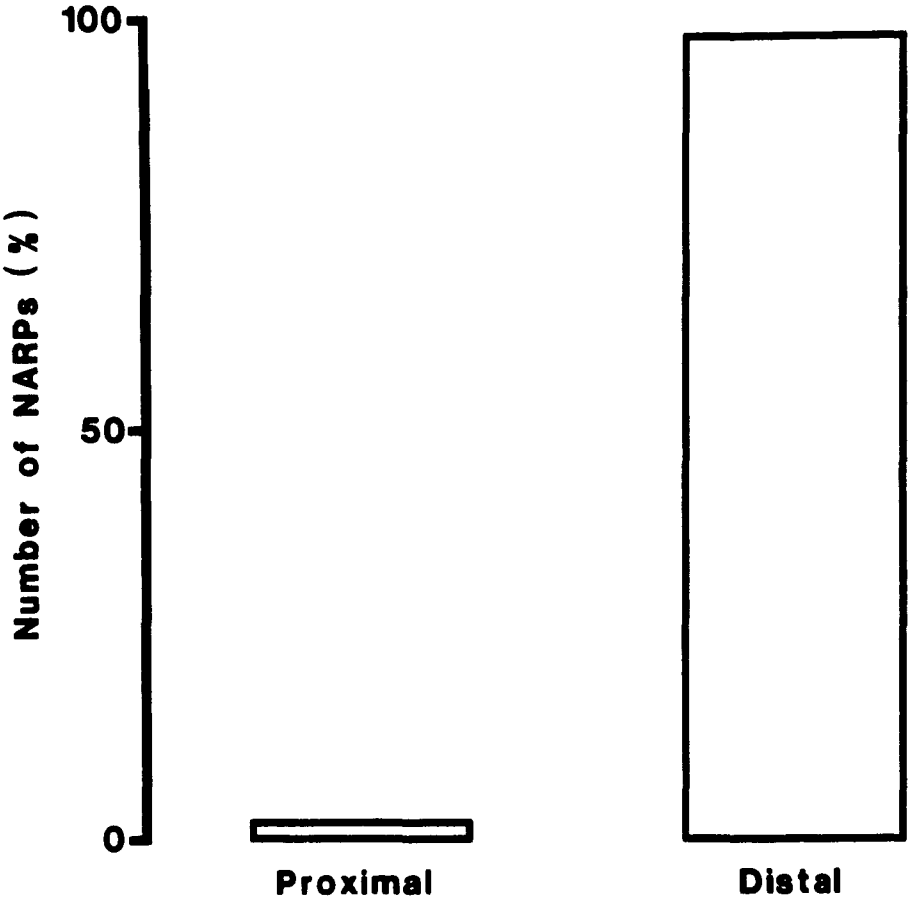

Fig. 8. The position of new NARP formation relative to pre-existing NARPs on the same neurite branch. Note that NARP formation almost always occurred in a proximal to distal sequence. Percentages based on 83 NARPs.

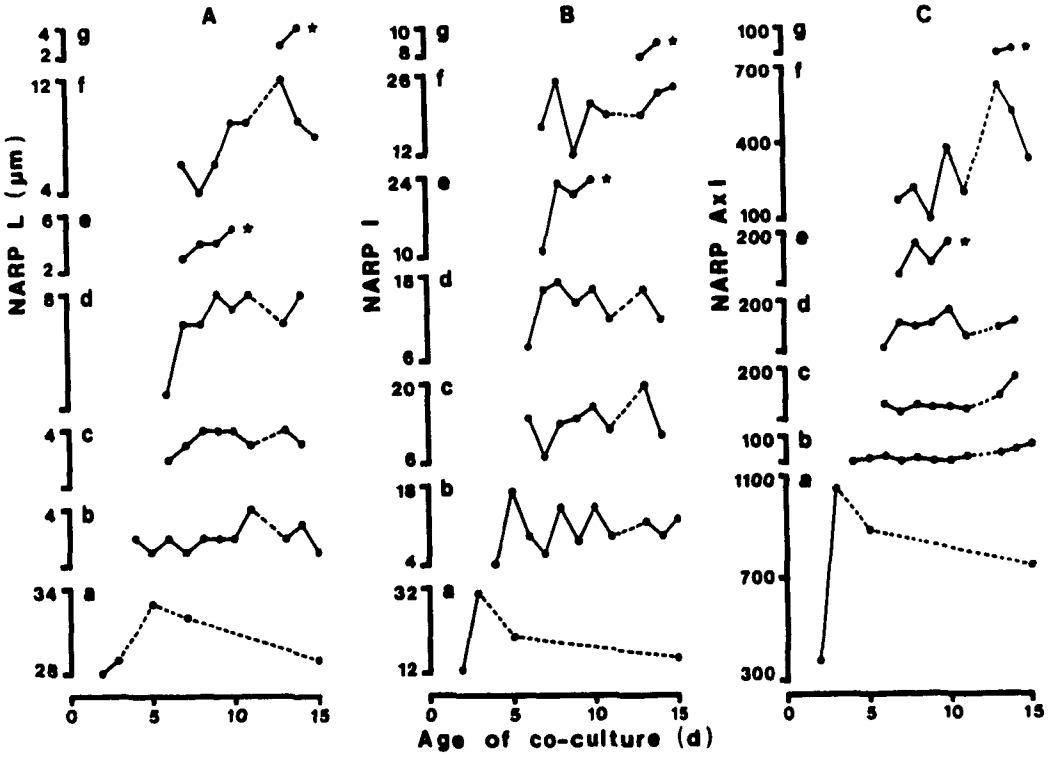

Fig. 9. Formation and survival of NARPs on a single muscle cell. NARP length (A), intensity (B) and area x intensity (C) are given for individual NARPs on the same muscle cell. Note that NARP formation occurred as late as day 13 of co-culture, when the muscle cell already had NARPs totalling more than 50 μm in length. Note also that L, I and AxI of individual NARPs fluctuated during the course of their survival. A star indicates that the neurite withdrew from the NARP. Values are plotted only for those cases where the NARP was in good focus (see Methods). Successive daily values are joined by solid lines whereas values interrupted by more than 1 d are joined by dashed lines.

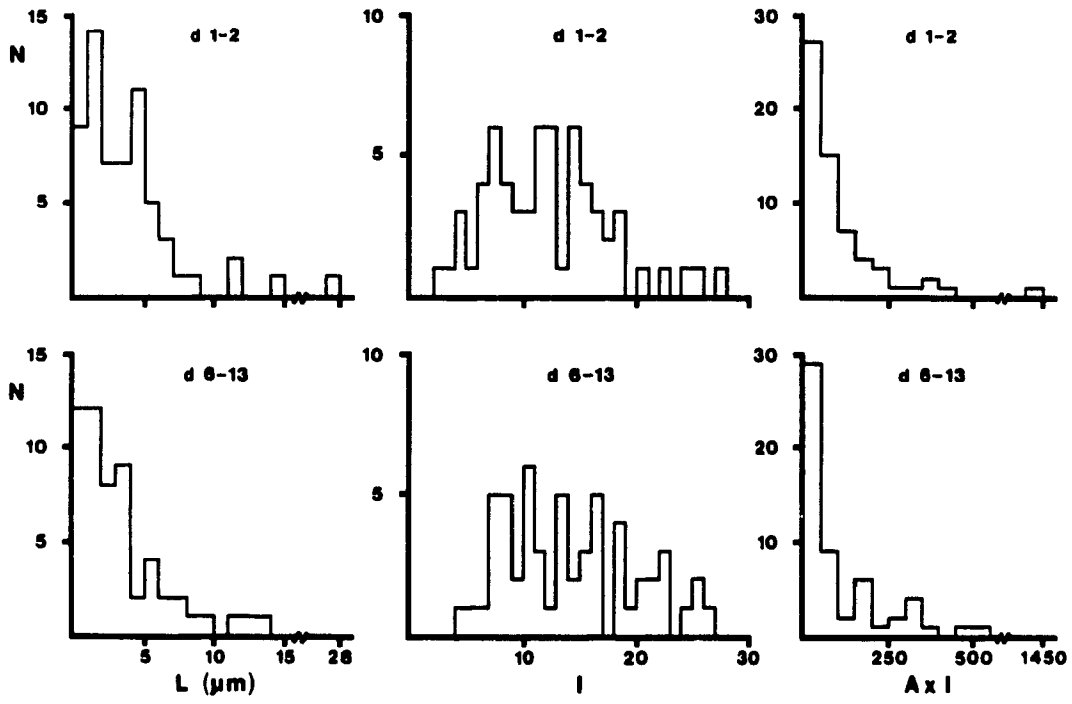

Fig. 10. Comparison between newly-formed NARPs in 1-2 day old co-cultures and in 6-13 day old co-cultures. Note that both groups of newly-formed NARPs exhibited similar distributions of L, I and AxI.

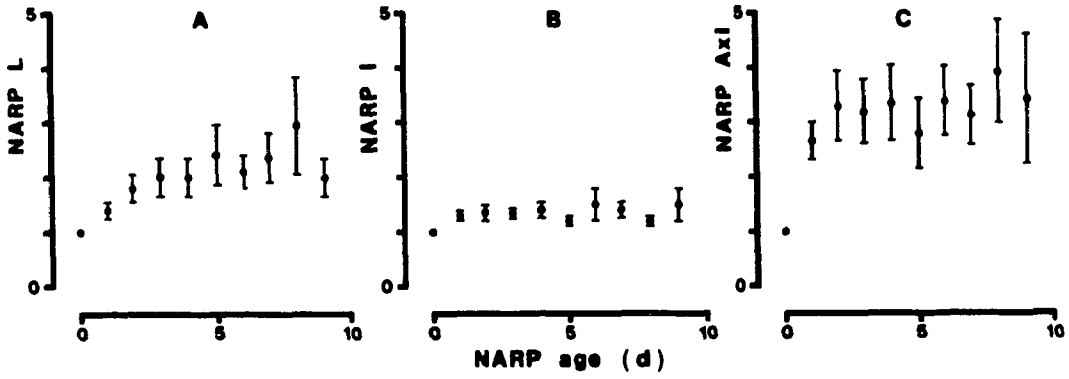

Fig. 11. Dependence of NARP length, intensity and area x intensity on NARP age. In each case the values are relative to those when the NARP was first detected (0 d old). Note that on average, NARPs attained close to their ultimate values within a day following their detection. The number of NARPs on which means and standard errors are based ranged from 56 for 1 d old NARPs to 7 for 9 d old NARPs.

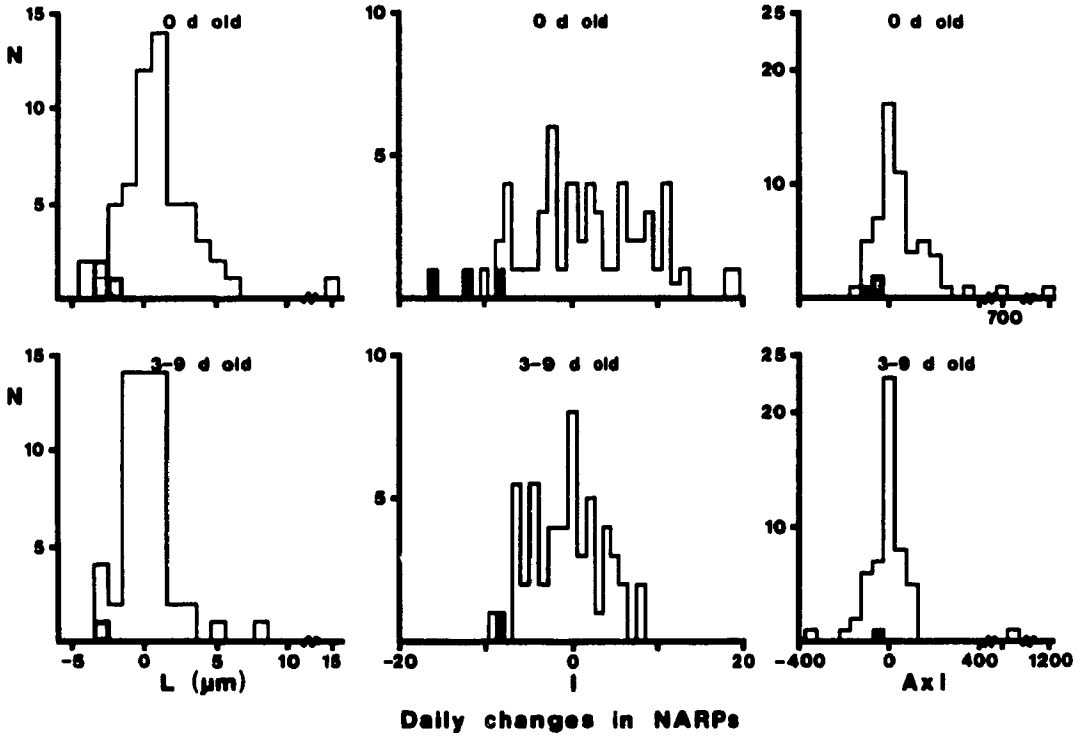

Fig. 12. Daily changes in the parameters of newly-formed (0 d old) and mature (3-9 d old), contacted NARPs. Values on the ordinate indicate the number of NARPs and values on the abscissa indicate the sign and magnitude of daily changes in NARP L, I and AxI. The shaded regions indicate NARPs which disappeared even though the contact survived.

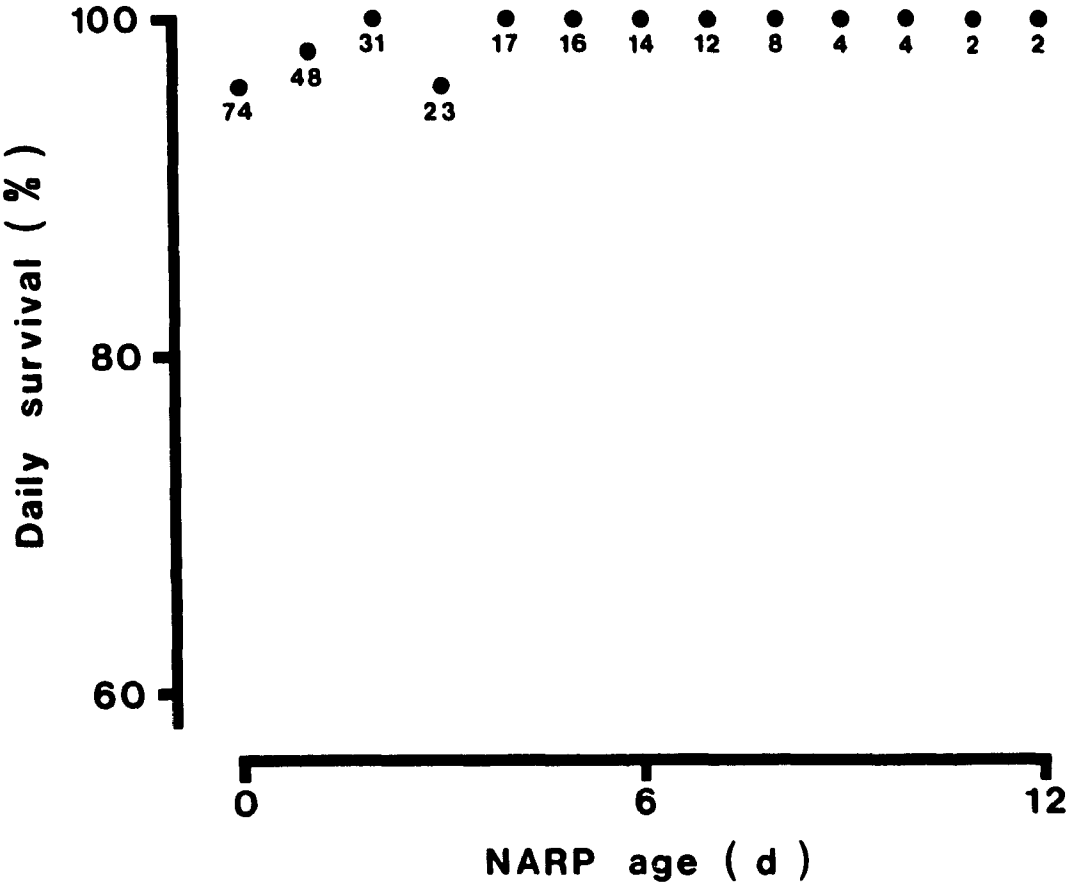

Fig. 13. Daily survival of NARPs at surviving contacts. Values on the ordinate indicate the percentage of NARPs of a specified age which survived at least until the next day. The numbers below the data points indicate the number of NARPs upon which the percentages are based. Note that NARPs tended to survive as long as their contacts survived.

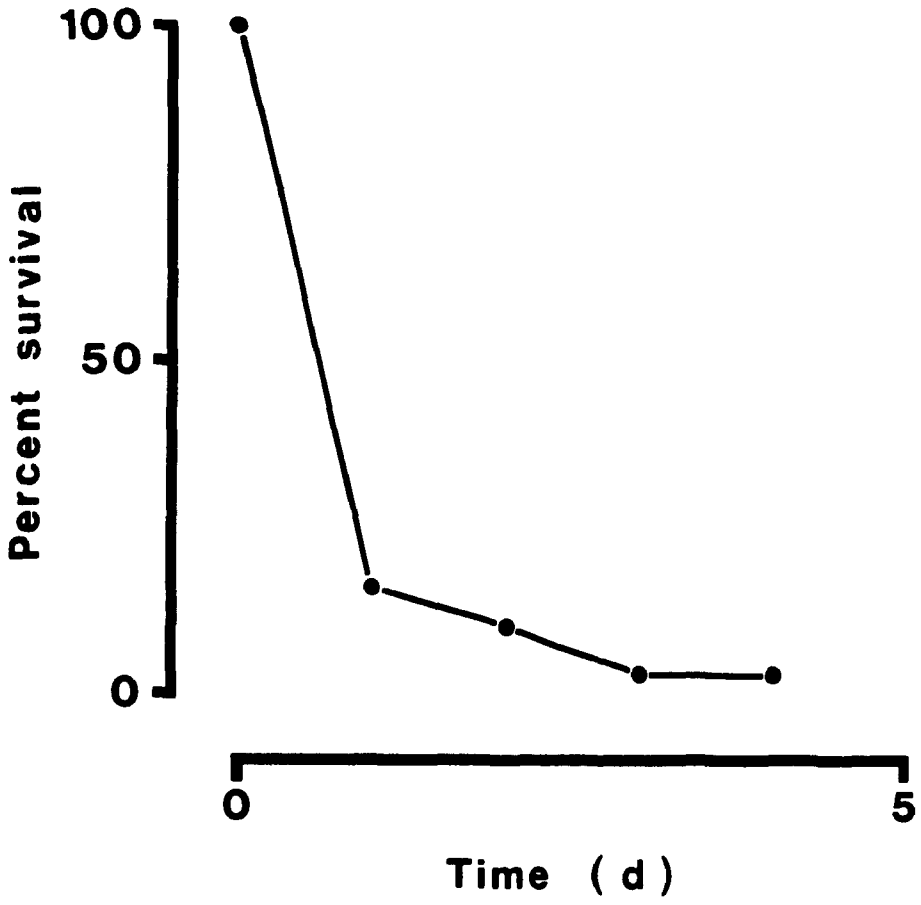

Fig. 14. The survival of NARPs following neurite withdrawal. The values are based on 31 NARPs at which neurite withdrawal occurred. Note that NARPs tended to disappear rapidly following withdrawal.

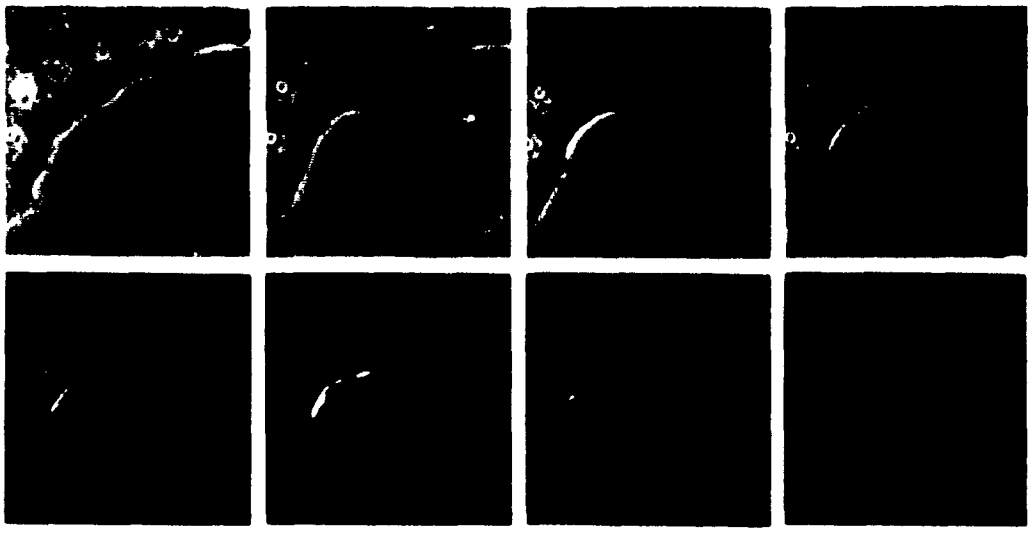

Fig. 15. NARP survival following neurite withdrawal. A region of neurite-muscle cell contact is shown on days 7, 8, 9 and 10 of co-culture. Images of the same region taken on day 7 at different planes of focus (not shown) indicated that both NARPs (X and Y) were completely contacted by the neurite at that time. See text for a detailed description. Scale bar, 20 μm

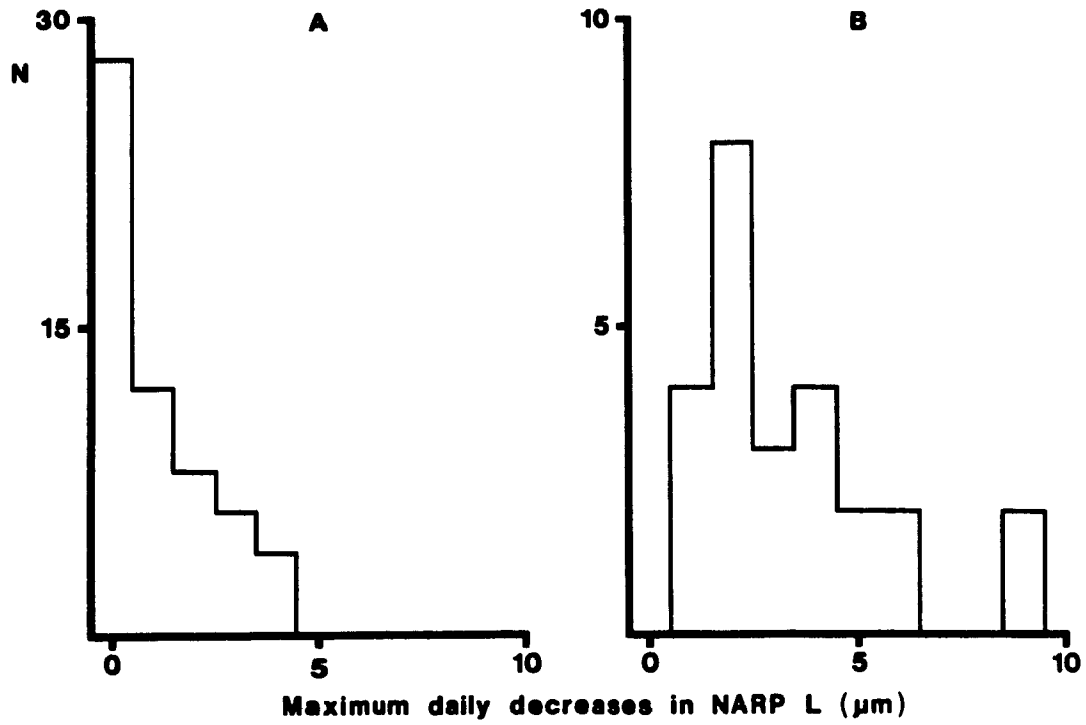

Fig. 16. The maximum daily decrease in NARP length for NARPs at surviving contacts (A) and for NARPs whose neurites withdrew (B). Note that following neurite withdrawal the decreases in length tended to be larger.

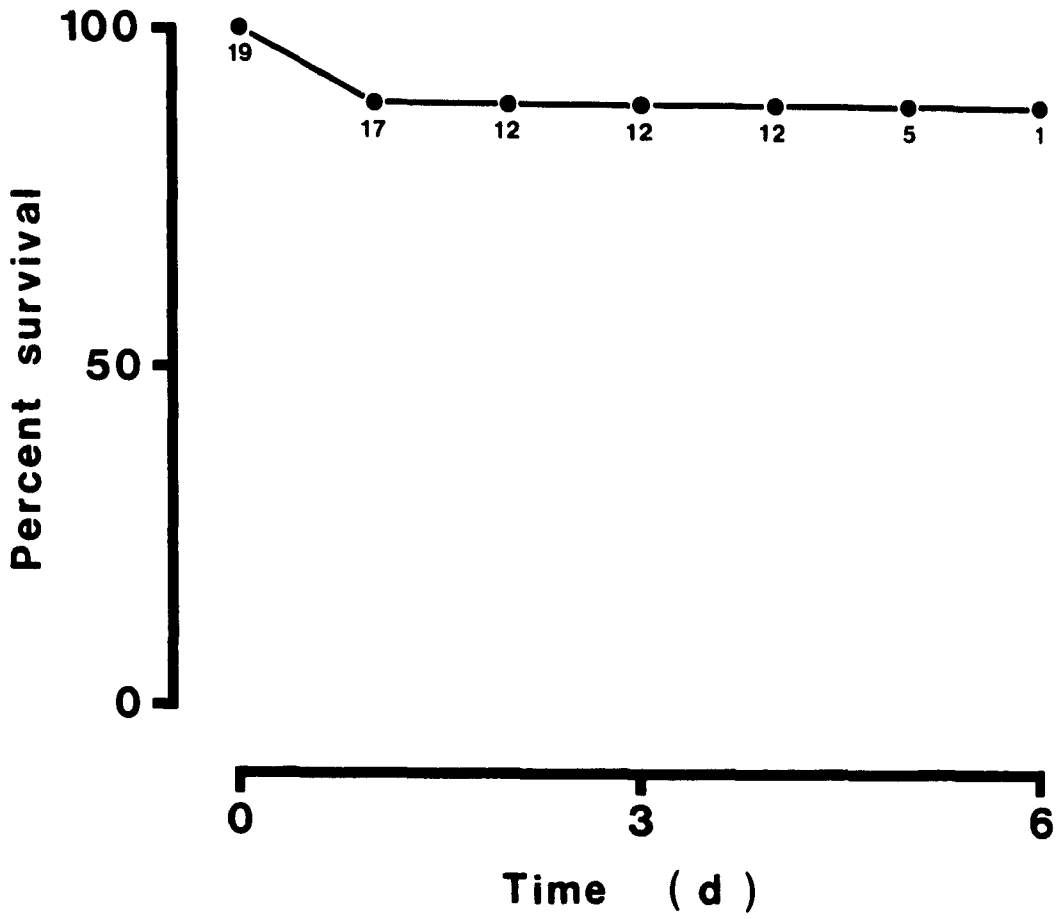

Fig. 17. The survival of NARPs following neuronal degeneration. The numbers below the data points indicate the number of NARPs upon which the percentages are based. This number decreases with time as a result of muscle cell degeneration and the termination of observations on cultures. Note that NARP survival was considerably higher following neuronal degeneration than following neurite withdrawal (see Fig. 14).

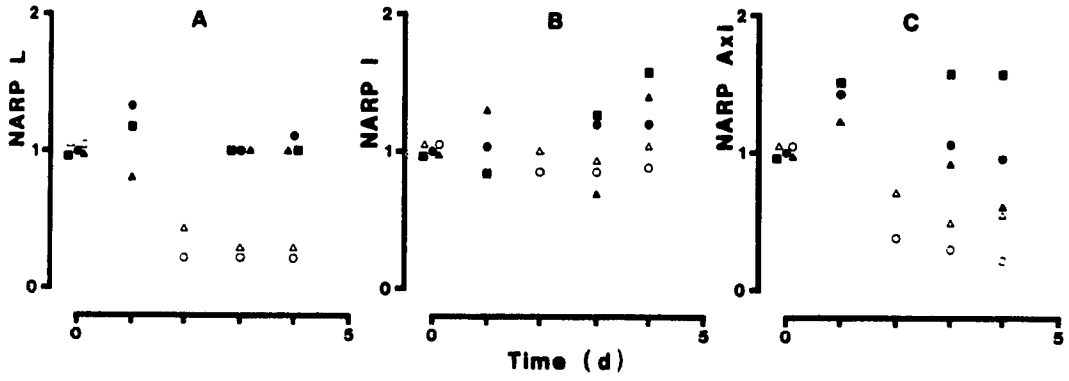

Fig. 18. NARP parameters following the onset of neuronal degeneration. The L, I and AxI of individual NARPs are expressed relative to their values at the onset of neuronal degeneration. Each symbol represents a different NARP. Note that some of the surviving NARPs underwent losses in L and in AxI whereas others did not.