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DEVELOPMENT OF AN AMPHIBIAN NEUROMUSCULAR JUNCTION *IN VIVO* AND IN CULTURE

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SUMMARY

During normal development presumptive synaptic sites in the myotomes of *Xenopus laevis* begin to acquire a high density of ACh receptors within as little as 2 h after the arrival of the nerve fibres. Synaptic function also begins very shortly after the arrival of the nerve fibres. Initially synaptic currents are some eight times longer lasting than at maturity and are not prolonged by anticholinesterase. During the first day after nerve–muscle contact is made there is a considerable decrease in synaptic current duration, sensitivity to anticholinesterase develops, and synaptic ultrastructure becomes apparent. Schwann cells do not arrive until later. Synaptic development proceeds with a similar rapid time course in cultures of dissociated myotomes and spinal cord derived from *Xenopus* embryos. The cultured muscle cells also develop synaptic specializations in the absence of nerve including sites of high ACh receptor density, cholinesterase activity, and postsynaptic ultrastructure. Studies on mixed nerve and muscle cultures have further revealed that muscle impulse and contractile activity is unnecessary for the development of synaptic ultrastructure or for the localization of ACh receptors, that the localization of ACh receptors at nerve-muscle contacts is nerve-induced and involves a redistribution of surface receptors, and that the development of synaptic specializations does not occur at nerve-muscle contacts when the source of nerve is dorsal root ganglia or sympathetic ganglia rather than spinal cord.

INTRODUCTION

Because of its relative simplicity and amenability to precise experimental investigation more is known about synaptic transmission at the vertebrate neuromuscular junction than at any other chemical synapse, and the resulting information has often provided the impetus for subsequent investigations of more complex synapses (Kuffler & Nicholls, 1976). These features of the vertebrate neuromuscular junction also make it an attractive choice for studying the events and mechanisms of synaptogenesis. One key approach in this regard involves the use of cultures of dissociated spinal cord and muscle. Among the advantages offered by such cultures are the ability to visualize and follow individual cells and nerve–muscle contacts over a period of days and to experimentally control the fluid environment of the cells much more precisely than is possible *in vivo*. On the other hand conditions in culture are clearly quite different from those in the animal: the cells are grown on an artificial substrate and are often exposed to unnatural substances such as serum from other

animals, antibiotics and antimitotic agents. Therefore a description of the developmental sequence as it normally occurs in the animal is also important not only because of the insight it may provide but also because it establishes a basic reference point for assessing synaptogenesis in culture and in other situations where it might be modified.

This review focuses on the development of the myotomal neuromuscular junction of the South African frog, *Xenopus laevis*, *in vivo* and in culture. My introduction to research on this synapse dates back to the summer of 1967. I was a postdoctoral fellow in Steve Kuffler's lab and Steve decided that we should spend that summer assessing the feasibility of studying neuromuscular synaptogenesis in the type of culture system which Harrison (1910) had devised to demonstrate axonal growth. The cultures contained developing spinal cord and myotomes from frog embryos and appeared to be a promising choice because Harrison's original observations did in fact include evidence for the formation of nerve–muscle synapses. Our task was also simplified by Ed Furshpan and Dave Potter who generously provided invaluable instruction and advice on the subtleties of tissue-culture techniques. Needless to say the cultures were a success, and working at Steve's side on this venture was a most instructive and joyful experience. The summer passed quickly and because of other commitments it was necessary to put this work aside. But before we did we also recorded intracellularly in a few of the cultures and found that some of the muscle cells exhibited spontaneous potentials which proved to be synaptic. Equally important in becoming committed to carrying on this work were the many stimulating discussions concerning neuronal and synaptic development. I am deeply grateful to Steve for leading me into this area of research and for continued encouragement.

It quickly became apparent that the neuromuscular junction in *Xenopus* myotomes does offer several advantages for developmental studies. *Xenopus* embryos can be obtained in large numbers and inexpensively simply by inducing mating with chorionic gonadotrophin. Their overall development has been described in detail (Nieuwkoop & Faber, 1967) so that the developmental stage of any animal can be accurately determined. The myotomes are the first muscle to form and become innervated, and are readily accessible throughout development. They, as well as the spinal cord, can be isolated at early stages of development and then dissociated and cultured under relatively simple conditions (Anderson, Cohen & Zorychta, 1977; see also Cohen, 1972; Spitzer & Lamborghini, 1976). For example, the cells grow well at room temperature, antibiotics are not required, and since contamination by fibroblasts and other cell types is meagre treatment with antimitotic agents is unnecessary. Another advantage is that the myotomal muscle cells differentiate as mononucleated cells (Hamilton, 1969; Muntz, 1975), and because they are relatively small and can be plated at low density the full extent of each cell can be readily visualized in culture. It will also be apparent from this review that development in culture is rapid and in most respects appears to parallel development *in vivo*.

The studies on the normal development of the *Xenopus* myotomal neuromuscular junction that are considered below deal mainly with the temporal relationships between such events as the arrival of nerve fibres in the myotomes, the development of synaptic function and ultrastructure, and the localization of ACh receptors at presumptive sites of synaptic contact. The studies in culture have been aimed at some of the underlying nerve and muscle interactions, particularly with respect

to the localization of ACh receptors. Several collaborators have made important contributions to this research. Rick Kullberg investigated the onset and changes in synaptic activity during the normal development of the myotomal neuromuscular junction. John (M.J.) Anderson played a central role in developing fluorescent conjugates of α-bungarotoxin to stain ACh receptors and, along with Edy Zorychta, greatly extended our insights into the accumulation of ACh receptors at nerve–muscle contacts in culture. More recently Ida Chow has investigated the temporal relationship between the arrival of nerve fibres and the localization of ACh receptors during normal development and Frances Moody-Corbett has been examining the characteristics of sites of high receptor density on cultured myotomal muscle cells. Tom Lentz investigated ultrastructural development *in vivo* and Peter Weldon has kindly collaborated on ultrastructural development in culture. I am also indebted to Dick Birks for many helpful discussions and for generously making available facilities for microscopy and radioautography.

THE MYOTOMAL NEUROMUSCULAR JUNCTION IN *XENOPUS*

As a reference point for the developmental studies it is useful to provide a brief description of the myotomes and their neuromuscular junction at relatively mature stages. Each myotome consists of a few hundred muscle cells which run parallel to the long axis of the tail. The muscle cells are quite short and even by the age of 2 weeks when the myotomal neuromuscular junction is essentially mature the myotomes are only about 200–300 μm in length. Innervation occurs mainly at the ends of the myotomal muscle cells (Fig. 1A, B; see also Lewis & Hughes 1960; Filogamo & Gabella, 1967). Cholinesterase (Lewis & Hughes, 1960; Filogamo & Gabella, 1967) and high densities of ACh receptors (Anderson & Cohen, 1974) are likewise located mainly at the ends of the myotomal muscle cells. The sites of high receptor density occur as narrow bands, forming patterns at the ends of the individual muscle cells similar to those formed by the nerve fibres (Fig. 1C). On the other hand the pattern of cholinesterase distribution, as revealed histochemically, is characteristic of that seen at the myotendinous junction (Couteaux, 1963; Filogamo & Gabella, 1967) and tends to obscure any synaptic cholinesterase that might be present (Fig. 1D). That cholinesterase is localized at the myotomal neuromuscular junction is indicated by two other observations: the time course of transmitter action during synaptic potentials is brief, even briefer than at other frog neuromuscular junctions (Kullberg, Lentz & Cohen, 1977), and inhibitors of cholinesterase prolong the time course of transmitter action (Kullberg, Mikelberg & Cohen, 1980).

The myotomal neuromuscular junction has relatively shallow junctional folds but otherwise exhibits the usual synaptic ultrastructure (Kullberg *et al.* 1977). The synapsing nerve fibre is covered by a thin Schwann cell process on its non-synaptic surface and contains clusters of clear vesicles and many mitochondria. There is basal lamina in the synaptic cleft and the postsynaptic membrane exhibits an increased electron density as well as underlying filamentous material.

TEMPORAL RELATIONSHIPS DURING THE DEVELOPMENT OF THE MYOTOMAL NEUROMUSCULAR JUNCTION *IN VIVO*

The early differentiation of myotomes and spinal cord

Myotomes begin to form at stage 17 (~ 19 h old) when the neural folds of the embryo are closely apposed (Nieuwkoop & Faber, 1967). Two hours later, at stage 19, 4–6 myotomes can be recognized. At this stage the neural folds are touching along much of their length thereby creating the neural tube. Some neurons can already be recognized in the spinal cord portion of the neural tube (Spitzer, 1979) but they do not yet exhibit synaptic contacts and few have any processes (Hayes & Roberts, 1973). Myotomal formation continues in a rostro-caudal direction (Nieuwkoop & Faber, 1967; Hamilton, 1969). By stage 20 (~ 22 h) there are 6–7 myotomes. At this stage some spinal cord neurons are electrically excitable (Spitzer, 1979), neural processes are more frequent, and some synaptic contacts are seen within the spinal cord (Hayes & Roberts, 1973). By stage 22 (24 h) there are 9–10 myotomes and some are now contractile (Muntz, 1975). The developing muscle cells are also electrically coupled to each other at these early stages, both within the same myotome and between neighbouring myotomes (Blackshaw & Warner, 1976*a*). About 1 day later, at stage 36 (50 h), the animal has about 36 myotomes and hatches to become a free-swimming tadpole (Nieuwkoop & Faber, 1967). In order to minimize variability arising from rostro-caudal differences in the state of differentiation, the developmental studies reviewed below were carried out on the first ten or so myotomes.

Arrival of nerve fibres in the myotomes

Nerve fibres were stained with nitroblue tetrazolium (Letinsky & DeCino, 1980) in order to reveal their arrival in the myotomes and their distribution at different stages of development (Chow & Cohen, in preparation). For these experiments sets of myotomes were isolated from each side of the spinal cord and were examined as whole mounts. At stage 19 (~ 21 h) only 7% of the myotomal preparations exhibited nerve fibre staining. This percentage increased progressively over the next few hours of development to more than 95% at stage 24 (~ 26 h). Throughout development the nerve fibres were localized mainly at the ends of the myotomes (Fig. 1 A, B).

Development of synaptic function

Synaptic potentials have been recorded from myotomal muscle cells in embryos as young as stages 20–21 (Blackshaw & Warner, 1976*b*; Kullberg *et al.* 1977). Since nerve fibres begin to invade the myotomes as early as stage 19 it follows that the delay between their arrival and the onset of synaptic function can be as little as 1–2 h. Studies on foetal rat diaphragm likewise suggest very little delay between the arrival of the nerve fibres and the onset of synaptic activity (Bennett & Pettigrew, 1974).

By stage 24 (~ 26 h) virtually all embryos exhibit synaptic function and reflex motor behaviour (Nieuwkoop & Faber, 1967; Kullberg *et al.* 1977). Focal external recordings indicate that many of the functional synaptic sites at these early stages are located at the ends of the myotomes, as is the case at later stages of development (Kullberg *et al.* 1977). One striking difference, however, concerns the time course of

Table 1. *Some early events in the normal development of the myotomal neuromuscular junction in* Xenopus *embryos*

	Onset	
	Stage*	Age* (h)
Myotomes acquire ACh receptors	19	20·75
Nerve fibres invade myotomes	19	20·75
Synaptic activity	20–21	21·75–22·5
Localization of ACh receptors at presumptive synaptic sites	21–22	22·5–24
Synaptic ultrastructure	25	27·5

* Stages and corresponding ages were determined according to the criteria of Nieuwkoop & Faber (1967).

transmitter action. The synaptic currents are some eight times longer-lasting at stages 24–26 than in the 2-week-old tadpole. Much of this developmental decline in synaptic current duration occurs very shortly after the onset of synaptic function. For example, by stage 34 (~ 45 h), less than 1 day after the beginning of synaptic activity, the synaptic currents are only about twice as long as at maturity. Cholinesterase inhibitors prolong the synaptic currents at stages 32–34 and at later stages, but not at stages 24–26, thereby indicating that the development of synaptic cholinesterase contributes to the developmental shortening of transmitter action (Kullberg *et al.* 1980). Other factors such as a developmental decrease in the characteristic open times of individual postsynaptic channels may also play a role, as has been found to be the case in newborn rat muscle (Sakmann & Brenner, 1978).

Localization of ACh receptors at presumptive synaptic sites

Acquisition of ACh receptors begins as early as stage 19 (Blackshaw & Warner, 1976*b*; Kullberg *et al.* 1977; Chow & Cohen, 1978). Until stage 21 (22·5 h) the receptors appear to be distributed randomly along the myotomes (Chow & Cohen, 1978). At this stage staining with fluorescent α-bungarotoxin revealed sites of high receptor density at the cell ends in only 3% of the myotomal preparations (Chow & Cohen, in preparation). This value increased progressively over the next few hours to about 60% at stage 24 and to more than 95% at stage 26 (~ 30 h). Throughout development the sites of high receptor density were localized mainly at the ends of the myotomal muscle cells.

The above findings suggest that synaptic transmission begins shortly before there is any pronounced localization of ACh receptors (Table 1). Evidence that this can occur has also been obtained in studies on cultured nerve and muscle (Cohen, 1976; Anderson, Kidokoro & Gruener, 1979). The data also suggest that the development of high receptor densities at presumptive sites of synaptic contact occurs after the arrival of the nerve fibres, albeit with a delay which can be less than 2 h (Table 1). A close temporal relationship between these two events has also been suggested during synaptogenesis in foetal rat muscle (Bevan & Steinbach, 1977) and is consistent with the notion that receptor localization during normal development is nerve-induced. Direct evidence for nerve-induced receptor localization has been obtained in culture (see below).

Development of synaptic ultrastructure

The early development of synaptic ultrastructure in *Xenopus* myotomes appears to follow the same course as that in muscles of rat (Teräväinen, 1968; Kelly & Zacks, 1969) and chick (Hirano, 1967; Atsumi, 1977; Jacob & Lentz, 1979). Prominent specializations are already apparent in the myotomes of *Xenopus* embryos by stage 34 (Kullberg *et al.* 1977). These include presynaptic clusters of vesicles, basal lamina-like material in the cleft, an increased electron density of the apposed muscle cell surface membrane, and the presence of filamentous material on its inner surface. Schwann cells do not arrive until later. The sites of postsynaptic ultrastructural specialization are presumably also sites of high receptor density as has been shown directly in developing chick muscle (Jacob & Lentz, 1979). Less pronounced forms of these synaptic specializations have been observed in embryos as young as stage 25. At earlier stages developing synaptic ultrastructure was not apparent although nerve processes were seen closely apposed to the muscle cell surface (Kullberg *et al.* 1977).

Detection of the onset of a particular developmental event naturally depends on the extensiveness of the sampling procedure. The sampling procedure for our electron microscope observations was necessarily much less complete than for the other observations listed in Table 1. For example, the onset of synaptic function was assessed by intracellular recording. Because of the tight electrical coupling between myotomal muscle cells in *Xenopus* embryos the electrode actually records from many if not all of the muscle cells within a myotome as well as from those in neighbouring myotomes (Blackshaw & Warner, 1976*a*). Likewise in assessing the presence of sites of high receptor density by fluorescent staining the myotomes are examined as whole mounts so that the sampling is essentially complete. By contrast, such extensive sampling is impractical with the electron microscope. The earliest synaptic ultrastructural specializations may also be relatively labile and difficult to preserve. In view of these considerations it seems likely that the delay between receptor localization and development of synaptic ultrastructure is shorter than that shown in Table 1 and that these events, together with the onset of synaptic function, occur in very close temporal relationship to each other during normal development.

DEVELOPMENT IN CULTURE

Cell cultures of myotomal muscle, with or without spinal cord, can be readily prepared from *Xenopus* embryos at stages 22–24 (Anderson *et al.* 1977). Nerve and muscle cultures can also be prepared from younger embryos, even from embryos which are still in the neural plate stage (Cohen, 1972; Spitzer & Lamborghini, 1976). The muscle and nerve cells in these latter cultures appear to develop and interact in the same way as in cultures derived from stage 22–24 embryos. However, the older embryos are more convenient for making cultures because the developing myotomes and spinal cord can be completely isolated from each other and from adjacent tissues. The findings on *Xenopus* cultures referred to below were made on cultures derived from stage 22–24 embryos.

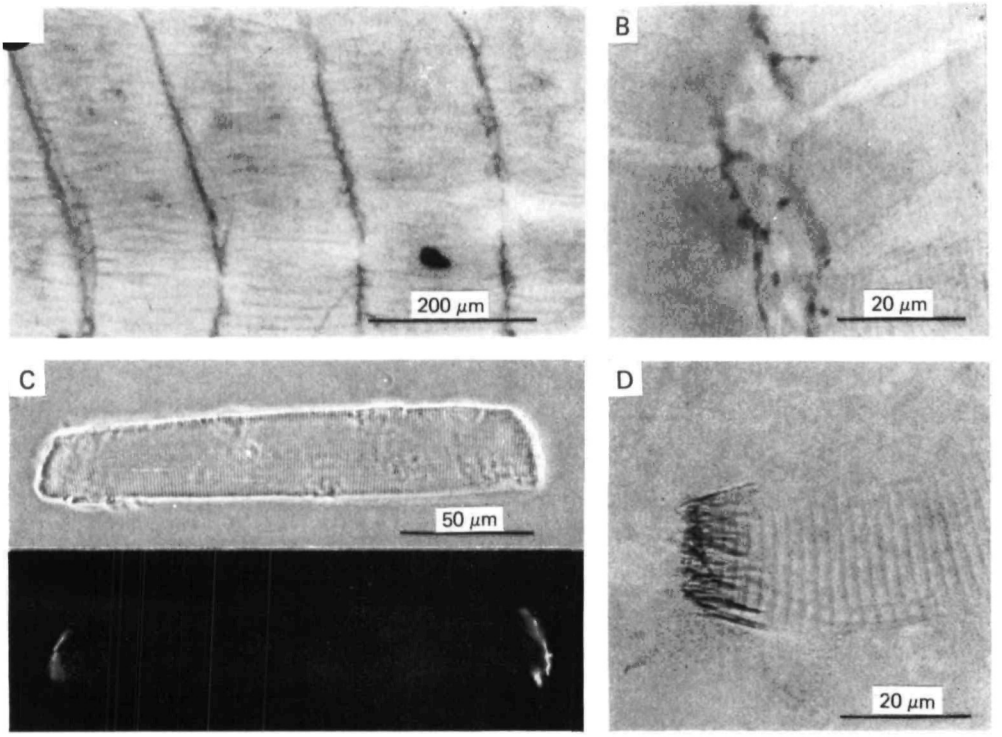

Fig. 1. Distribution of nerve fibres, ACh receptors and cholinesterase in *Xenopus* myotomal muscle.

(A) Myotomes stained for nerve fibres according to the method of Letinsky & DeCino (1980). Stained nerve fibres are seen mainly at the ends of the myotomes. Individual muscle cells can also be resolved. They are a little less than 200 μm long and about 15 μm in diameter. Stage 41.

(B) Higher magnification of stained nerve fibres. The nerve fibres exhibit intermittent varicosities and form simple patterns as they course across the ends of the myotomal muscle cells. Stage 48.

(C) Phase-contrast and corresponding fluorescence views of a single myotomal muscle cell stained with fluorescent α-bungarotoxin. The receptor stain is localized in narrow bands at both ends of the muscle cell. Stage 48.

(D) The end of a single myotomal muscle cell stained for cholinesterase by the method of Karnovsky & Roots (1964). The staining pattern is typical for cholinesterase at the myotendinous junction and obscures cholinesterase which is associated with the neuromuscular junction. Stage 49.

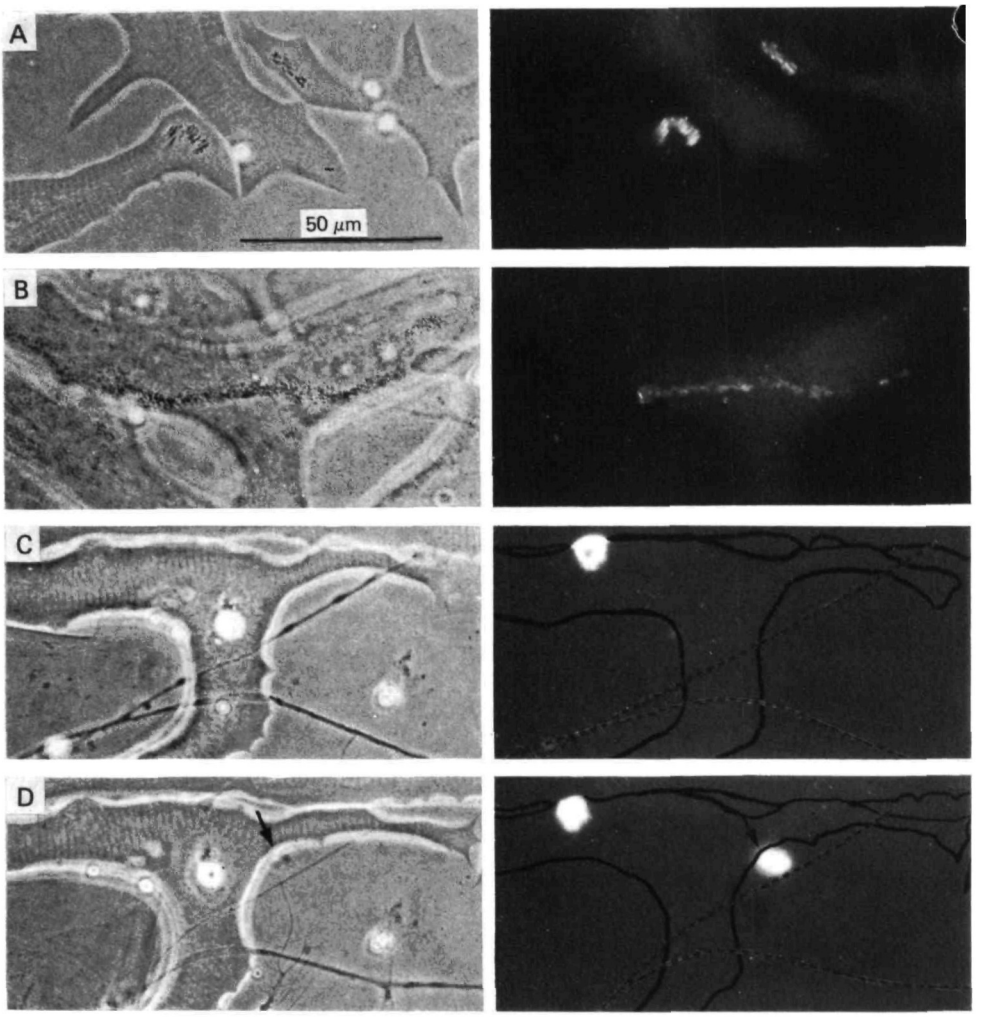

Fig. 2. Localization of cholinesterase and ACh receptors on cultured myotomal muscle cells. Magnification bar in A applies to all micrographs.

(A) Muscle cells not contacted by nerve. Cholinesterase (left) and ACh receptors (right) are localized together in characteristic patches. Four-day-old culture.

(B) Muscle cell contacted by SC neurite. Cholinesterase and ACh receptors are localized in a narrow band along the path of nerve–muscle contact. Widespread autofluorescence associated with muscle cells in A and B is due to fixation. Five-day-old culture.

(C) Three-day-old cultured muscle cell contacted by DRG neurite. Note the characteristic receptor patch similar to that seen on non-contacted muscle cells as well as the absence of receptor localization at nerve–muscle contacts. Dashed lines on fluorescence micrograph indicate neurites and solid line outlines muscle cells. The DRG explants in this culture were plated three days before the muscle cells.

(D) Same field as C, one day later. The pattern of receptor localization is unchanged. Arrow points to floating fluorescent debris.

Synaptic specializations on muscle cells not contacted by nerve

Sites of high receptor density have been observed on cultured non-innervated muscle cells derived from chick (Vogel, Sytkowski & Nirenberg, 1972; Fischbach & Cohen, 1973), rat (Axelrod *et al.* 1976; Land *et al.* 1977), mouse (Powell & Friedman, 1977), and *Xenopus* (Anderson *et al.* 1977). In *Xenopus* cultures these ACh receptor patches are seen on virtually all myotomal muscle cells by two days and occupy about 3% of the cell surface. They can occur anywhere on the cell but are most often located on the bottom surface (facing the floor of the culture dish) near the periphery of the cell or on the top surface in more central regions. Individual receptor patches vary considerably in size; they are usually less than 20 μm in their greatest dimension and never more than 40 μm. The density of receptors at these sites is some 20–30 times greater than over the rest of the cell surface (Anderson *et al.* 1977) and is probably almost as great as that at the adult neuromuscular junction (Peng & Nakajima, 1978).

Most of the receptor patches also exhibit cholinesterase activity (Fig. 2A) and those on the bottom surface of the muscle cells appear to be located at sites of increased adhesion to the culture dish (Moody-Corbett & Cohen, 1979). In addition, electron microscopy has revealed that the cultured myotomal muscle cells develop surface specializations which are ultrastructurally similar in appearance to those at the neuromuscular junction (Weldon & Cohen, 1979). These specializations include an increased electron density of the surface membrane together with basal lamina-like material on the outer surface and filamentous material on the inner surface. Similar ultrastructural specializations have also been observed in developing chick muscle and have been found to be sites of high receptor density (Jacob & Lentz, 1979). Presumably in *Xenopus* cultures too these ultrastructural specializations are the sites of high receptor density and cholinesterase activity. Since non-innervated myotomal muscle cells do not twitch spontaneously in culture (Anderson *et al.* 1977; see also Harrison, 1910), it is clear that all of these specializations can develop in the absence of muscle impulse or contractile activity.

Synaptic specializations at nerve–muscle contacts

Many of the myotomal muscle cells which are contacted by spinal cord neurites become functionally innervated and this innervation is apparent in less than a day (Anderson *et al.* 1977, 1979; see also Harrison, 1910; Cohen, 1972; Spitzer & Lamborghini, 1976; Peng *et al.* 1979). Most of the functionally innervated cells exhibit sites of high receptor density along the path of nerve–muscle contact (Anderson *et al.* 1977, 1979). Conversely muscle cells with a high receptor density along the path of contact invariably exhibit synaptic activity (Anderson *et al.* 1979). The sites of high receptor density can develop in less than 6 h after nerve-muscle contact is established (Cohen *et al.* 1979). Nerve–muscle contacts which have a high receptor density often exhibit cholinesterase activity as well (Fig. 2*B*).

Electron microscopy has also revealed pre- and postsynaptic specializations at nerve-muscle contacts in these cultures (Weldon & Cohen, 1979; Peng *et al.* 1979). The specializations are apparent even in 1-day-old cultures and include presynaptic clusters of vesicles, basal lamina-like material in the cleft, an increased electron

density of the muscle surface membrane and underlying filamentous material. As *in vivo* these sites of synaptic ultrastructure develop in the absence of Schwann cells and become more prominent and extensive with time. Their location correlates with sites of high receptor density (Anderson & Klier, 1979). On the basis of these and other findings (see subsection on neural specificity) it seems likely that most of the sites of high receptor density along nerve–muscle contacts in these cultures are synaptic sites. It is also apparent that the formation and early development of the myotomal neuromuscular junction in culture proceeds in much the same way as it does *in vivo*.

Receptor localization at nerve–muscle contacts in nerve-induced

The sites of high receptor density along paths of nerve–muscle contacts in *Xenopus* cultures tend to occur as narrow bands (Anderson *et al.* 1977), as *in vivo*. These narrow bands of high receptor density vary considerably in length but of most significance is the fact that some extend for more than 40 μm (Fig. 2 B), a distance which is greater than the largest receptor patch seen on non-contacted muscle cells. Such examples therefore cannot be explained simply in terms of the neurite having contacted a pre-existing receptor patch. They indicate instead that the spinal cord neurites can induce the development of a high receptor density at sites of contact with muscle (Anderson *et al.* 1977). This conclusion has been confirmed by experiments in which the development of these narrow bands of high receptor density has been followed on individual nerve-contacted muscle cells (Anderson & Cohen, 1977; Cohen *et al.* 1979). Evidence for nerve-induced receptor localization has also been obtained in cultures of chick nerve and muscle (Frank & Fischbach, 1979). In *Xenopus* cultures the narrow bands of high receptor density disappear within a day after spontaneous withdrawal of the neurite (Cohen *et al.* 1979) or after section of the neurite (Anderson & Klier, 1979), thereby indicating that their maintenance is dependent on the continued presence of a competent neurite. In addition nerve-contacted muscle cells which have a high receptor density along the path of contact often have no receptor patches elsewhere (Anderson *et al.* 1977). Apparently the muscle cells have a limited capacity to develop sites of high receptor density and the nerve-muscle interaction predominates in determining where these sites develop.

NEURAL DETERMINANTS IN RECEPTOR LOCALIZATION

Axons versus Schwann cells

As pointed out above, Schwann cells are not present at the synaptic contacts which form in *Xenopus* cultures. In fact, Schwann cells do not accompany the growing neurites (Weldon & Cohen, 1979), and this is in agreement with the early growth of axons in *Xenopus* myotomes *in vivo* (Kullberg *et al.* 1977). Synaptic contacts in cultures of chick nerve and muscle also appear to form in the absence of Schwann cells (Frank & Fischbach, 1979). Clearly Schwann cells are not involved in nerve-induced receptor localization or in the initial development of the synapse.

Neural specificity

In cultures of *Xenopus* spinal cord (SC) and muscle about 70% of the nerve-contacted muscle cells exhibit sites of high receptor density along the path of contact

(Anderson *et al.* 1979; Cohen *et al.* 1979). A simple explanation of this finding is that the spinal cord contains different types of nerve cells and only some of these are competent to form synapses with muscle and to induce receptor localization. An alternative possibility is that all neurites are competent to induce receptor localization but not all of the muscle cells are responsive. In order to distinguish between these possibilities experiments were undertaken in which *Xenopus* myotomal muscle cells were cultured with two other sources of nerve, sympathetic ganglia (SG) and dorsal root ganglia (DRG). In contrast to muscle cells contacted by SC neurites (Fig. 2B), most of the muscle cells contacted by SG or DRG neurites did not develop sites of high receptor density along the path of nerve-muscle contact (Fig. 2C, D). In the few cases where receptor localization was observed along the path of contact the sites of high receptor density did not take the form of narrow bands but rather appeared like the receptor patches on non-contacted muscle cells. Altogether 73% of SC-contacted muscle cells exhibited receptor localization along the path of contact whereas the corresponding values were 9% for DRG-contacted muscle cells and 5% for SG-contacted muscle cells (Cohen *et al.* 1979). These latter two values probably reflect chance contact between neurites and receptor patches. Similar differences between SC- and SG-contacted muscle cells were obtained when SG and SC explants were present together in the same culture dish, thereby excluding the possibility that the differences were due to some alteration of the culture medium by the nerve explants (Cohen *et al.* 1979). Electron microscopy has further revealed that the ganglionic neurites enter into close apposition with the muscle cell surface but do not form synaptic contacts (Cohen & Weldon, 1979). Taken together these findings indicate that the SC neurites possess a specific property which induces receptor localization at sites of contact with muscle and that this property is lacking in DRG and SG neurites. Presumably the same property is also important for the development of synaptic ultrastructure at these sites.

Activation of ACh receptors and muscle activity

Abolition of muscle impulse and contractile activity does not prevent the formation and development of the neuromuscular junction, as judged by motor behaviour (Harrison, 1904; Matthews & Detwiler, 1926), synaptic activity (Crain & Peterson, 1971; Cohen, 1972; Jansen & Van Essen, 1975; Kidokoro *et al.* 1975; Obata, 1977), ACh receptor localization (Steinbach, 1974; Anderson *et al.* 1977; Anderson & Cohen, 1977) and synaptic ultrastructure (Weldon & Cohen, 1979). Nerve-induced localization of ACh receptors also occurs in *Xenopus* cultures even when the cultures are maintained in concentrations of curare which completely obliterate end-plate potentials or in concentrations of α-bungarotoxin which saturate ACh receptors within minutes (Anderson *et al.* 1977; Anderson & Cohen, 1977). From these observations it follows that activation of ACh receptors by transmitter (and all subsequent events, such as the resulting ionic currents and changes in membrane potential) cannot be essential for nerve-induced receptor localization. Recently it has been found that applied electric fields can affect receptor distribution on cultured myotomal muscle cells (Orido & Poo, 1978). Clearly the electric fields generated by synaptic potentials or muscle action potentials are not required for receptor localization.

Neural factors

The neural factor which induces receptor localization could either be released from the axons or associated with their surface membrane. Attempts are now being made to isolate active neural factors. For these experiments muscle cells are cultured without nerve and the activity of the factor is assessed by the number of receptor patches that the muscle cells develop. Initial studies have indicated that extracts of brain or spinal cord do increase the number of receptor patches and the total number of receptors on cultured muscle cells (Podleski *et al.* 1978; Jessel, Siegel & Fischbach, 1979). Medium 'conditioned' by cloned neuroblastoma cells also appears to increase the number of receptor patches on cultured muscle cells but in this case without increasing the total number of receptors (Christian *et al.* 1978). These promising studies will doubtlessly be extended to isolate the active substances and to assess whether they participate at nerve–muscle contacts. In addition a recent elegant study by Burden, Sargent & McMahan (1979) has implicated the synaptic basal lamina in receptor localization in regenerating, adult frog muscle. It may be that the neural factor becomes incorporated into the synaptic basal lamina during development.

MUSCLE DETERMINANTS IN RECEPTOR LOCALIZATION

Protection against receptor degradation

The number of receptors on the surface of a muscle cell depends on the rate at which newly synthesized receptors are inserted into the surface membrane and the rate at which receptors are degraded or removed from the surface (Fambrough, 1979). In principle then the development of sites of high receptor density could arise if receptors at these sites were protected against degradation. In mature rat and chick muscle ACh receptors at the neuromuscular junction survive with a half-life of at least 5 days (Berg & Hall, 1974, 1975; Burden, 1977*b*; Chang & Huang, 1975; Linden & Fambrough, 1979) whereas the half-life of extrajunctional receptors in the surface membrane is only about 1 day (for a comprehensive review see Fambrough, 1979). The relative stability of junctional receptors is even apparent in muscles from newborn rats (Berg & Hall, 1975; Steinbach *et al.* 1979) and in 3-week-old chicks (Burden, 1977*b*). However, junctional receptors in embryonic chick muscle have the same short life-span as extrajunctional receptors even though their density at these early stages is comparable to that at the adult neuromuscular junction (Burden, 1977*a*). A similar short life-span has also been found for the ACh receptors in receptor patches and at sites of nerve–muscle contact in chick cultures (Schuetze, Frank & Fischbach, 1978). These findings therefore indicate that preferential protection against receptor degradation is not required for the maintenance of a localized high receptor density.

Local insertion of newly synthesized receptors

For a site of high receptor density to be maintained the rate of receptor loss from that site must be balanced by the rate of receptor replenishment. Replenishment could occur by direct insertion of newly synthesized receptors into the surface membrane at that site or by the movement of neighbouring surface receptors into the site. Neither of these supply routes is mutually exclusive and both could opera

together. Experiments to test whether the former route makes a significant contribution to the formation or maintenance of sites of high receptor density have not yet been reported.

Participation of mobile surface receptors

To study whether mobile surface receptors contribute to the development of sites of high receptor density receptors on cultured *Xenopus* muscle cells were stained with fluorescent α-bungarotoxin and the cultures were then maintained in a high concentration of unlabelled α-bungarotoxin. New sites of fluorescence developed along paths of nerve–muscle contact, thereby indicating that stained receptors which were originally located elsewhere on the cell surface had accumulated at these sites (Anderson & Cohen, 1977). Similar experiments have indicated that this process of receptor redistribution also contributes to the development of receptor patches on non-contacted *Xenopus* muscle cells (Anderson & Cohen, 1977). The disruption and reformation of receptor patches on cultured rat myotubes likewise appears to involve corresponding movements of surface receptors (Bloch, 1979). Mobile surface receptors have also been implicated in the formation of new receptor patches in response to 'conditioned medium' (Christian *et al.* 1978) and in the accumulation of receptors which occurs in response to an applied electric field (Orido & Poo, 1978).

Reduced mobility of receptors at sites of high receptor density

Evidence that surface ACh receptors are mobile and that their mobility varies according to their location was first obtained by Axelrod *et al.* (1976) in a study on cultured rat myotubes. This study indicated that many of the receptors which are present at lower density outside receptor patches are in fact mobile and have an effective diffusion coefficient in the same range as that of other surface macromolecules. On the other hand, receptors within receptor patches were found to be much less mobile and there appeared to be only a slight exchange of receptors between patches and their surrounding areas. ACh receptors at neuromuscular junctions in mouse (Axelrod *et al.* 1976) and rat (Fambrough & Pagano, 1977) have also been reported to be immobile. Such findings suggest that the maintenance of sites of high receptor density may be intimately dependent upon immobilization of the receptors at those sites. The progression of changes which occur during receptor patch disruption and reformation lends further support to this notion (Bloch, 1979).

The mechanism of receptor immobilization has thus become a major consideration in the quest to understand the development and maintenance of sites of high receptor density. Much attention is now being focused on the basal lamina and filamentous material which have been found to be associated with the outside and inside of the surface membrane at sites of high receptor density in adult muscle (Albuquerque *et al.* 1974; Fertuck & Salpeter, 1974, 1976; Porter & Barnard, 1975; Matthews-Billinger & Salpeter, 1978) and in developing muscle (Jacob & Lentz, 1979). High-resolution electron microscopy has revealed numerous attachments between the basal lamina and the postsynaptic membrane as well as between cytoplasmic microfilaments and the postsynaptic membrane in *Torpedo* electric organ (Heuser & Salpeter, 1979. Such attachments may restrict receptor mobility. Indeed, as pointed out above, the synaptic basal lamina has been directly implicated in the local development of a

high receptor density in regenerating frog muscle cells (Burden *et al.* 1979). When considered together with the finding that SC neurites can induce the development of a high receptor density at sites of contact with muscle perhaps the simplest hypothesis for the action of the inducing factor is that it becomes incorporated into the extracellular material at the contact site and immobilizes the receptors by directly interacting with them.

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