Physiological Studies During Formation and Development of Rat Neuromuscular Junctions in Tissue Culture

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ABSTRACT Developing neuromuscular junctions in tissue cultures of rat embryo spinal cord and muscle were studied with intracellular recording. Excitatory junctional potentials (ejps) were found during nerve or spinal cord stimulation, and also arising spontaneously. The time-course of the potentials tended to be shorter in cultures older than 9 days in vitro than in more recently innervated cultures. Evidence of multiple innervation was found in many cells. In order to test the hypothesis that transmitter was released in integral multiples of a quantal amount and according to a Poisson distribution, mean quantum content was calculated from the coefficient of variation of ejp amplitudes, the percentage of zero responses, and the ratio of mean amplitude to mean of the smallest class of amplitudes. The three independent measures were in fair or good mutual agreement, implying that the mechanism of transmitter release in newly developed junctions is the same as in the adult. In newly formed junctions, ejps were subthreshold for action potential generation, but afterwards mean quantum content increased and action potentials were generated by single ejps. In fibers developing both with and without innervation, the entire muscle cell surface was as sensitive to acetylcholine as the adult end plate region. Innervation was related to cross-striation: every cross-striated fiber tested was found to be innervated, and denervation in vitro led to loss of distinct cross-striations.

INTRODUCTION

The functional development of a chemical synapse has not been traced from the onset of chemical transmission. In the case of the rat neuromuscular junction, studies with intracellular techniques begin at 17 days *in utero* (Diamond and Miledi, 1962). However, nerves are present in some muscles at 14 days (Kelly, 1966; Teravainen, 1968) and contraction in response to reflex or nerve stimulation is found at about 15 ½–16 days (Angulo y Gonzalez, 1932; Strauss and Weddell, 1940). Although there are technical difficulties in

dealing with small embryos, the use of tissue culture makes possible electrophysiologic investigations on neuromuscular junctions at any selected stage in early synaptogenesis. Peterson and Crain (1970) have shown, and we have confirmed, that coupled fragments of embryonic rodent spinal cord and muscle grown together in Maximow chambers can be observed under the microscope throughout the time that unmyelinated nerve fibers grow out from the spinal cord, make contact with developing myotubes, and finally produce functionally active cholinesterase-positive junctions between terminals of myelinated nerve fibers and cross-striated muscle. The present report concerns the physiological development of the neuromuscular junction in tissue culture during this formative period.

TECHNIQUE

I. *Cultures*

Fragments of limb muscle and spinal cord about 0.5 mm thick were taken from 11–19-day rat embryos, placed 0.5–2 mm apart on collagen-coated cover slips, and cultured at 37°C in Maximow double cover slip assemblies. In two instances, trypsin-dissociated cells from newborn muscle were added to cultures of 11 day embryonic spinal cord. Culture methods and feeding solutions are described elsewhere (Robbins and Yonezawa, 1971). Unless stated otherwise, all data presented below were obtained from rat material (embryos of 21 pregnant females).

II. *Electrophysiology*

At a selected stage in development (4–32 days in vitro) the cover slips were placed in a chamber fitted to an inverted microscope and slowly perfused with a mixture of 90% Gey's balanced salt solution and 10% horse serum at room temperature (24°–27°C). Conventional glass microelectrodes filled with 3 M KCl, with DC resistances of 15–30 MΩ and tip potentials less than 3 mv, were used for intracellular recording. Further details on recording and criteria for cell selection are given elsewhere (Robbins and Yonezawa, 1971).

Stimulating electrodes consisting of saline-filled glass pipettes with fire-polished orifices of 5–20 μm delivered current (10–30 μa, <0.1 msec) to the spinal cord or nerve fiber via an isolation unit. Acetylcholine was released iontophoretically from glass microelectrodes by the methods of Del Castillo and Katz (1955), and the current passed was measured. A steady "holding current" of 10^{-8} amp was used to prevent diffusion of acetylcholine from the pipette, although, where simultaneous intracellular recording was used, the current could be adjusted to a strength just sufficient to prevent depolarization.

RESULTS

I. *Résumé of Morphologic Observations*

The development of nerve-muscle contacts in vitro as seen through the light microscope was essentially as described by Peterson and Crain (1970). 1 or 2

days after explantation, myoblasts migrated from the muscle fragment, multiplied, and began to fuse into myotubes. Myotubes also grew as extensions of preexisting tubes in the muscle fragment. As early as 3 or 4 days in vitro, nerve fibers growing out from the spinal cord contacted myoblasts or myotubes. Some myotubes began to twitch at 4–5 days in vitro, and cross-striation was apparent about 6 days later. In many cultures, myelination of nerve fibers occurred after 2 or more wk in vitro. Histochemical stains for cholinesterase (Karnovsky and Roots, 1964) revealed a diffuse reaction in myotubes at 5–7 days in vitro. Later, with development of cross-striation, a progressively more dense and localized cholinesterase reaction appeared at the developing end plate regions, as demonstrated by combined cholinesterase and silver staining methods (Yonezawa and Robbins, in preparation).

A major problem in electrophysiologic studies was a thin layer of connective tissue covering the culture, which grew thicker with longer periods in vitro until it was no longer possible to dissect it away without damage to the underlying cells. A second problem in relation to developmental studies was the large variation between cultures and even within one culture in the rate of nerve, muscle, or junctional development, possibly due to differences in distance between spinal cord and muscle, or in local cell densities. Nevertheless, it was possible to draw conclusions about the course of development by noting the predominant characteristics of groups of cultures at different ages in vitro, and especially by choosing for study the most advanced cultures at any given age. Thus, on the average, three out of 30 cultures made from the embryos of each female were selected for electrophysiological study.

II. *Spontaneous Activity*

During intracellular recording in muscle, brief depolarizing potentials were observed in cells contacted by nerve endings in the absence of nerve stimulation (Fig. 1 A). In this type of cell, potentials elicited by stimulation of nerve or spinal cord were often similar in amplitude and time-course to some of the spontaneous potentials (Fig. 1 B). The briefest potentials, encountered in a striated fiber 11 days in vitro, had rise and ½ fall times of 1.9 and 2.3 msec, respectively. In cells up to about 9 days in vitro, the peak amplitudes of the potentials fluctuated considerably (Fig. 1 A and B). In some fibers, in the absence of curare, an occasional spontaneous potential reached the threshold for action potential generation (Fig. 1 C).

In older cultures, 1–25 μg/ml of *d*-tubocurarine or 10 mM magnesium was necessary to prevent twitching during intracellular recording (see following results). At a critical curarizing concentration, both spontaneous and evoked excitatory junctional potentials (sejps and ejps, respectively) were present but subthreshold; at higher concentrations, they were no longer detectable.

Action potentials were recognized by a relatively constant and large ampli-

tude and faster time-course compared to junctional potentials. Action potentials were followed by large afterhyperpolarizations in most but not all fibers. Positive afterpotentials occasionally followed large ejps as well.

Innervation was a necessary prerequisite for spontaneous activity since sejps were never found in muscle cells not contacted by nerve fibers. In most cultures studied, the neural connection between spinal cord and muscle was intact. Since spontaneous spinal cord electrical activity gives rise to muscle action potentials in this type of cord-muscle culture (Crain, 1970), some of the spontaneous activity must reflect neuronal firing. Yet, even immediately after division of the nerve bundle connecting spinal cord and muscle, twitching did not cease nor was the intracellular record silent (Fig. 1 C), although activity was diminished. In three cases cultured after this type of "denervation,"

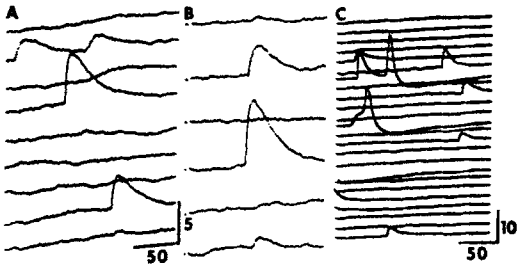

FIGURE 1. Intracellular recording of junctional potentials. (A) and (B): similar amplitudes and time-course of spontaneous (A) and evoked (B) junctional potentials recorded from a myotube 7 days in vitro. In (B), the spinal cord was stimulated at 1/sec. (C): spontaneous activity immediately after division of the nerve bundle connecting spinal cord and muscle. Note sejp giving rise to action potential with after-hyperpolarization. Calibrations in all figures: vertical millivolts, horizontal milliseconds.

twitching stopped only after 2–3 days, presumably when the nerve endings degenerated. Control nondenervated spinal cord–muscle pairs cultured on the same cover slip continued to show twitching, and the denervated muscle twitched when tested later with direct stimulation. Thus, sejp activity arises in nerve terminals, analogous to miniature end plate potentials, and/or in preterminal axons or possibly Schwann cells. Additional experiments are necessary to further localize the site of origin.

III. *Time-Course of ejps and sejps*

Junctional potentials with brief time-courses were more commonly found in older cultures. In 11 cells 4–8 days in vitro, only one had a rise time less than 3 msec and only two had ½-fall times of 5 msec or less. In nine cells 9–21 days in vitro, the corresponding numbers of cells were six and five. Moreover, there was no evidence of a change in membrane time constant with cell age in vitro. Measurements of chronaxie, which should be proportional to the time con-

stant if spike threshold stays the same through development (Katz, 1937), showed no decrease from 7–8 days in vitro (average 4.8 msec) to 10–18 days in vitro (average 6.8 msec).

IV. *Multiple Innervation*

Nerve fibers frequently branched profusely as they contacted developing muscle cells, and contacts were often seen at more than one region of any particular cell (see Fig. 1 A in Robbins and Yonezawa, 1971). Physiological evidence indicated that, in any given cell, more than one of these terminals was releasing transmitter:

(*a*) In three instances, absolute refractory periods of 3.2, 3.5, and 5.5 msec were found by recording nerve action potentials from electrically stimulated

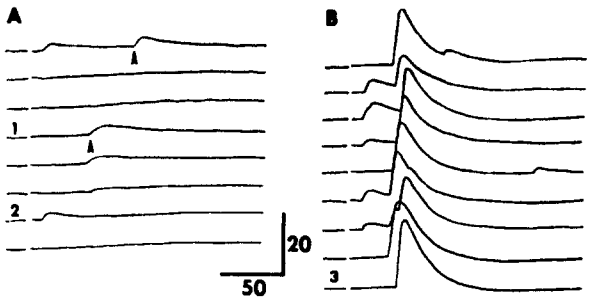

FIGURE 2. Intracellular evidence of multiple innervation. Ejps with different latency, amplitude, or time-course (numbered *1, 2, 3*) appearing during 1/sec nerve stimulation. Stimulus strength was just threshold in (A), higher in (B). Note ejp and sejp of same amplitude but different time-course (arrowheads). Newborn mouse muscle and spinal cord.

nerve trunks. On the other hand, ejps were sometimes double-peaked, the peaks separated by intervals of less than 3 msec, thus reflecting activity in more than one nerve fiber or terminal branch. Either multiple innervation or temporal dispersion of the quantal components of ejp (Liley, 1956) could have produced this result.

(*b*) In some cells, during threshold stimulation of nerve, ejps of similar amplitude at the recording electrode but differing rise and fall times were encountered (arrowheads, Fig. 2 A). Because of the cable properties of the cell, a larger ejp arising at a distance from the recording electrode would undergo attenuation and slowing of time-course (Fatt and Katz, 1951), so that a smaller ejp arising closer to the recording electrode might show an equal amplitude but faster time-course.

(*c*) Increasing the stimulus strength to the nerve led to the appearance of ejps with different latency or amplitude (Fig. 2 B) than was seen at lower stimulus strength.

If low-resistance coupling occurred between adjacent innervated myotubes in vitro, the intracellular record might be indistinguishable from that produced by multiple innervation of uncoupled single fibers. Although this possibility has not been excluded, evidence for multiple innervation as given above was also found in clearly isolated myotubes.

V. *Quantal Release*

Despite the presence of multiple innervation, it was occasionally possible to stimulate nerve or spinal cord at 1/sec, to record the resulting ejps, and to select for measurement those ejps with identical latency and time-course and without double peaks or composite wave form. The selected ejps were assumed to arise from a single junction, but the criteria of selection would not exclude data from two neighboring junctions with ejps of similar latency. The most favorable cells for the demonstration of quantal release showed subthreshold ejps and occasional failures of response in normal perfusing solution without *d*-tubocurarine.

If the release of transmitter were in integral (quantal) units, and if the number released per impulse followed a Poisson distribution with mean m, then m (mean quantum content) could be estimated either by the quantity $1/CV^2$, where CV is the coefficient of variation of ejp amplitudes, or by $\ln(N/n_o)$, where N is the total number of stimuli and n_o the number which produces no response ("failures") (Del Castillo and Katz, 1954; Martin, 1966). The best evidence for Poisson release was that these two independent estimates were in fair or good agreement in the five cases in which both quantities could be determined (Table I). The difference between the estimates in the two most discrepant cases may be partly accounted for by the large (10–20%) standard error (Martin, 1966). In addition, failures would be underestimated if ejps from another nerve terminal occurred during a failure of the nerve terminal of interest (see following discussions). On the other hand, undetectably composite ejps might increase the apparent CV. Although no correction was made for nonlinear summation of ejps (Martin, 1966), because neither the "true" resting membrane potential nor the reversal potential obtained with acetylcholine application were known, the estimates of m would not be materially changed; in most of the cells, the mean ejp amplitude was 5% or less of the measured membrane potential.

The data also fit the assumption of a binomial release process with $p = 0.14$ (Christensen and Martin, 1970). If m is calculated from $(1 - p)/CV^2$ and the number of quanta available, n, from m/p, the predicted number of failures, $N(1 - p)^n$, closely corresponds to the observed number in three cases, but not as well as the Poisson approximation in two others.

In any event, the agreement between estimates based on CV^2 and on N/n_o

(Table I) can hardly be fortuitous, and it supports the assumption that transmitter is released in integral (quantal) units.

Additional evidence for quantal release was obtained by dividing the mean ejp size ($\bar{v}$) by the mean size of the smallest class of ejps ($\bar{v}_1$) estimated from crude amplitude histograms based on small N. This quantity should supply a third estimate of m if the smallest class represents the unit response (Del Castillo and Katz, 1954). Although there was uncertainty in choosing limits for the smallest class, partly because the sample size was small, $\bar{v}_1$ was determined without knowledge of m estimated by $1/CV^2$ or $\ln(N/n_o)$. In most cases, there was again good agreement between these estimates and $\bar{v}/\bar{v}_1$ (Table I).

TABLE I

QUANTUM CONTENT OF NEUROMUSCULAR JUNCTIONS IN TISSUE CULTURE

See text for explanation.

Number of stimuli	m			$\bar{v}$	Membrane potential
	$1/CV^2$	$\ln(N/n_o)$	$\bar{v}/\nu_1$		
N				mv	mv
116	3.7	2.6	~3.5	3.2	—
223	1.1	1.9	—	1.5	44
	(1.0)*	(1.3)*	(~1.2)*		
20	2.4	—	~4	1.9	42
31‡	0.6	0.5	—	0.6	>30
82‡	1.4	1.4	~1.5	2.4	46
40§	1.5	1.4	~1.4	0.8	—

* Recomputed assuming small contribution of neighboring nerve terminal (see text).

‡ Muscle developed from dissociated cells, culture 10 days in vitro.

§ Culture 18 days in vitro, treated with *d*-tubocurarine from 5th day in vitro.

If transmitter is released in quantal amounts, then when m is small (and if nonlinear summation is negligible), the amplitude histogram should show peaks at integral multiples of the unit peak (Del Castillo and Katz, 1954). It was possible in only one case to hold a cell long enough during 1/sec stimulation to obtain sufficient data for a definitive amplitude histogram (Fig. 3). In this instance, integral multiples were discernible (arrows, Fig. 3), provided the unit peak was taken as 1.1 mv, i.e. excluding the small peak at 0.3 mv on the assumption that it represents the contribution of a nearby terminal. The presence of diffuse branching of nerve endings at immature junctions (e.g. Fig. 1 A in Robbins and Yonezawa, 1971) lends morphologic support to this assumption. Moreover, if the output of this small neighboring terminal were apparent only when the predominant terminal failed, so that values to the left

of the dotted line in Fig. 3 actually represented failures, the recomputed estimates of m (*Table I) were in better mutual agreement than when all data in the histogram were included.

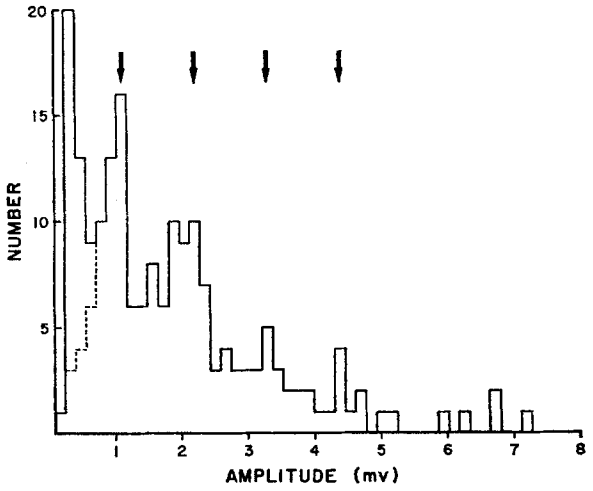

FIGURE 3. Histogram of ejp amplitudes from a myotube 5 days in vitro during 1/sec nerve fiber stimulation (223 stimuli). There were 33 failures of response (not plotted). Arrows point to presumed unit amplitude and its integral multiples, assuming values left of the dashed line arise from neighboring junction (see text).

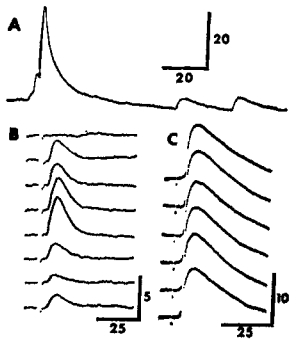

FIGURE 4. (A): Spontaneous activity in an innervated myotube 4 days in vitro. Summation of sejps reaches threshold for action potential (rising phase retouched) whereas single sejps are subthreshold. (B) and (C): comparison of ejps during 1/sec nerve fiber stimulation in cells from cultures 5 (B) and 19 (C) days in vitro. In (B), normal perfusing solution, and in (C), 7 μg/ml d-tubocurarine was added.

VI. *Increase of Transmitter Output with Development*

Qualitative observations suggested that transmitter output increased during synaptic development. Firstly, the incidence of both failures and widely fluctuating ejp amplitudes in successive responses during 1/sec stimulation decreased with duration of growth in vivo (compare Figs. 4 B and 4 C).

Secondly, in two cultures 4 and 5 days in vitro, when nerve-muscle contacts were first detectable, single ejps or sejps seldom or never gave rise to action potentials even in myotubes in which summated ejps produced threshold depolarization (Fig. 4 A). There were similar findings in 5 of 17 cultures tested at 6–8 days in vitro. In the other 12, single shocks to spinal cord or nerve produced a twitch response or, on intracellular recording, it was found that single ejps gave rise to action potentials. Even in this subgroup, there was considerable variability, some cells showing mainly subthreshold ejps.

Finally, in 28 cultures tested after 9–32 days in vitro, most innervated cells showed action potentials and twitches in response to single shock stimulation, and curarization was necessary for intracellular study of ejps. These ejps showed relatively smaller fluctuation in amplitude (Fig. 4 C). Throughout

TABLE II

QUANTUM CONTENT OF NEUROMUSCULAR JUNCTIONS DEVELOPING IN TISSUE CULTURE

See text for explanation.

Days in vitro	*d*-tubocurarine	Number of stimuli	*m*	$\bar{v}$	Membrane potential
	μg/ml	*N*	$1/CV^2$	*mv*	*mv*
4	0	116	3.7	3.2	—
5	0	223	1.1	1.5	44
7	0	20	2.4	1.9	42
8	5	22	34.2	2.3	—
11	5	60	8.7	0.7	24
12	10	100	55.8	2.3	48
18	4	50	13.6	1.5	32

development, spikes arose from junctional potential "humps" of 5–10 mv amplitude.

In a small number of favorable cases (Table II), direct quantitation showed that *m* was consistently higher in cultures 8–18 days in vitro than at 4–7 days in vitro, as expected from the qualitative observations preceding. In the older group the quantity of $1/CV^2$ (again, without correction for nonlinear summation) was the only means of estimating *m*, since failures were absent and the unit ejp was below the level of resolution because of curarization. However, transmitter release still appeared to be quantal, since 10 mM magnesium blocked the indirect twitch, and the intracellular record then showed ejps with large fluctuations in amplitude and frequent failures, as seen in the mature rat neuromuscular junction when quantum content is reduced by magnesium (Liley, 1956). Efforts to obtain more accurate estimates of *m* by recording extracellular ejps, as was done at the crayfish neuromuscular junction (Dudel and Kuffler, 1961), were thwarted by the dense connective tissue overlying the older cultures.

Other factors besides age in vitro influence the quantum content of developing junctions in tissue culture. For instance, two fibers 10 days in vitro (‡, Table I) with low quantum content were from a culture of dissociated newborn muscle and 11 day spinal cord in which myotube formation was delayed until 3–5 days in vitro. In another cell with low quantum content 18 days in vitro (§ Table I) both spinal cord and muscle had developed poorly. Although this culture was continuously exposed to 25 μg/ml curare from the 5th day in vitro, cultures of the same series grown in normal medium showed equally poor cord and muscle. Cells such as these, from specially treated cultures, are not included in Table II.

VII. *Acetylcholine Sensitivity*

Since nerves grow out of the spinal cord at 12 days *in utero* (Wehman and Plantholt, 1970) and are first described in muscle at 14 days (Kelly, 1966; Teravainen, 1968), limb muscle taken from an 11 day embryo has never been contacted by nerve. In order to determine if acetylcholine sensitivity of muscle depends on previous exposure to innervation, a culture of myotubes without spinal cord was grown from cells of an 11 day embryo and tested at 13 days in vitro for response to acetylcholine (ACh) released iontophoretically from a micropipette. In several fibers, regardless of where the pipette was placed along the fiber length, a twitch response was obtained when a brief (0.5–1.0 msec) pulse of positive current was passed through the pipette. The threshold value for twitch was 1×10^{-10} coul in one fiber, and 3×10^{-11} in two others. Release of the negative holding current, so that ACh passively diffused from the pipette, produced prolonged contracture. Although most of the cells tested were multinucleated, one cell with only three to four nuclei also gave a twitch response. Thus, it is clear that muscle cells can become diffusely sensitive to acetylcholine without prior innervation.

In two innervated cultures 9 days in vitro, the thresholds for twitch with iontophoretically delivered ACh ($0.5 - 4 \times 10^{-11}$ coul with pulses of 1 msec or less) were only slightly lower than in muscle grown without spinal cord. Again there was little variation in threshold when the ACh pipette was moved stepwise along the fiber over 500 μ.

Sensitivity of immature innervated muscle to ACh is about the same as that of the adult end plate. In two instances, intracellular recording of ACh responses of a few millivolts amplitude yielded calculated values of 68 and 128 mv depolarization/10^{-9} coul passed. Also, if the threshold depolarization for action potential generation is 5–10 mv, as found in several cells, then the data on ACh twitches can be expressed as sensitivities ranging from 12 to 200 mv/10^{-9} coul. These values are comparable to those found at adult rat end plates (Miledi, 1962; Albuquerque and McIsaac, 1969; Fischbach and Robbins, 1971). They are probably underestimates of the true ACh sensitivity

because no special effort was made to find regions of maximum sensitivity, and because connective tissue overlying the muscle cells impeded diffusion of ACh. It was not possible to determine a profile of ACh sensitivity in fibers of older, more mature cultures, because of the correspondingly thicker connective tissue.

VIII. *Trophic Effects of Innervation*

Developing rodent myotubes denervated in vivo (Zelena, 1962) or grown in tissue culture in the absence of spinal cord (e.g. Yaffe, 1968) develop cross-striations. However, in our particular culture medium which optimizes nerve growth and development, cross-striations in cultures of myotubes without nerve tissue appear only transiently (Peterson and Crain, 1970) or, in our hands, not at all. In contrast, well-maintained cross-striated muscle consistently develops in cord-muscle cultures in the same medium.

Physiological evidence suggested that innervation and not just mere presence of spinal cord was a significant factor in neural enhancement of muscle development and maintenance under these culture conditions. Over 30 cross-striated fibers were examined in 11 cord-muscle cultures derived from four pregnant females. Every fiber subjected to physiologic test was found to be innervated. The criteria of innervation included a twitch in response to indirect stimulation, a 4-14–fold increase in chronaxie with curarization, or the presence of ejps or sejps during intracellular recording.

Further evidence for a trophic effect of innervation was found in the course of in vitro denervation experiments mentioned before (section II). As the axon stump degenerated over 2–3 days, cross-striation became indistinct and muscle cell nuclei moved from a peripheral to a central position. The presence of spinal cord nearby or of functioning cord-muscle pairs in the same culture failed to prevent this reaction. This evidence and the constant association of cross-striation and innervation noted above imply that the influence of nerve requires intimate nerve-muscle contact.

DISCUSSION

With the use of tissue culture one can carry out physiological studies on partially or entirely visible neuromuscular junctions at any time of interest during synaptogenesis. There are distinct differences from in vivo development such as nutrient medium, input and output of spinal cord cells, lack of relatively fixed cartilagenous attachments of muscle, absence of normal microenvironmental cues in growth of nerve and muscle, distortion in timing of events by removal from the animal and redevelopment in vitro, and possibly nonspecific connections between neuron and muscle. Nonetheless, findings in many cultures 2 wk in vitro or longer closely resemble those reported in studies of pre-, neo-, or postnatal neuromuscular junctions. End plates with localized

cholinesterase activity are found in vitro (Peterson and Crain, 1970; Yonezawa and Robbins, in preparation), and, in somite cultures (Bornstein et al., 1968), the ultrastructure of the junction resembles the postnatal appearance in vivo. The shortest rise times of the ejp and sejp in vitro (1.9 msec) are essentially the same as those of miniature end plate potentials in pre- and neonatal junctions (2.1 msec, Diamond and Miledi, 1962), with both studies performed at room temperature. As in the adult junction, the in vitro synapse is cholinergic (the postsynaptic response is blocked by *d*-tubocurarine) and transmitter release is quantal following a Poisson (or perhaps binomial) distribution. Finally, many cells are multiply innervated, as reported for the neonatal rat neuromuscular junction (Redfern, 1970). Since the neuromuscular junction 8–10 days or more in tissue culture resembles its in vivo counterpart in the late prenatal and early neonatal period, we infer that in many respects the earlier course of development observed in tissue culture gives a representative picture of parallel events in vivo.

Both in vivo and in vitro, different stages of synaptogenesis may be present at the same time in different junctions of the same muscle tissue (Teravainen, 1968; Kelly and Zacks, 1969). Nonetheless, by relying on data from the more advanced junctions any time in vitro, the present results and those in a short report (Robbins and Yonezawa, 1971) can be combined to reconstruct events at an idealized developing neuromuscular junction.

Before Innervation Before the arrival of nerve endings, developing myotubes are sensitive to acetylcholine over their entire surface. There is probably no appreciable membrane response to neural acetylcholine release until contact at a distance of 1–2 μ or less is established between nerve ending and muscle; muscle fibers adjacent to innervated fibers do not respond when neighboring fibers are indirectly stimulated (Robbins and Yonezawa, 1971). Moreover, early neural release of acetylcholine is usually in quantal packets of 1–4 quanta so that the absolute amount diffusing to fibers at a distance from the junction would be minimal.

Early Contact Between Nerve and Muscle The earliest simple nerve-muscle contacts often give evidence of synaptic transmission, but at first the amount of acetylcholine released with each nerve impulse produces a response which is subthreshold for action potential generation. However, with the earliest contact or shortly thereafter, the quantal mechanism of transmitter release is the same as in the adult junction, but the mean quantum content is initially far lower. Many nerve impulses release no acetylcholine at all, either because of conduction block (see "Method" in Redfern, 1970) or because of the low quantum content, the expected number of failures being given by N/e^{-m}.

Kelly and Zacks (1969) have found close contacts between nerve terminals and developing muscle. If these represented low-resistance pathways, they would not have been detected by the methods employed in the present study.

Increase in Quantum Content As development proceeds, mean quantum content increases. The maximum value found during 1/sec stimulation up to 18 days in vitro is less than that of the adult rat neuromuscular junction stimulated once every 30 sec (Robbins and Fischbach, 1971). Since quantum content is the product of mean probability of release and number of available quanta, change in either or both factors could account for the 10–200-fold developmental increase. An increase in probability of this magnitude seems unlikely. The more plausible explanation is that the number of available quanta increases, perhaps related to the greater concentration of vesicles per terminal, larger terminal size, or larger number of terminal sprouts, observed during in vivo neuromuscular development (Teravainen, 1968; A. M. Kelly, personal communication). Similarly, in developing rat cortex, synaptosomal concentrations of vesicles and of acetylcholine increase during the perinatal period (Abdel-Latif et al., 1970; Jones and Revell, 1970). Correlated ultrastructural and physiological study of nerve-muscle junctions developing in vitro would be informative. In any event the development of quantum content in rat embryonic junctions differs from nerve regeneration in the frog (Miledi, 1960) where the first functional contacts release acetylcholine in amounts sufficient to generate action potentials.

Time-Course of Junctional Potentials Initially, histochemical cholinesterase is diffusely present in nerve and muscle with no junctional concentration (Yonezawa, in preparation). Presumably as a result, the duration of transmitter action depends mainly on simple diffusional escape from the junction to its surrounds. Later, junctional cholinesterase appears and the time-course shortens. Other factors, such as change in synaptic geometry (Eccles and Jaeger, 1958), rate of liberation from terminals, or postsynaptic receptor mechanisms could also affect the time-course.

Multiple Innervation Multiple innervation of myotubes is present early in synaptogenesis. It is not clear whether this results in part from fusion of already innervated myoblasts or, as seems likely, from profuse branching of growing nerve terminals, many establishing functional nerve endings. Multiple innervation may be a phylogenetic vestige of the type of innervation commonly found in adult lower vertebrates, or it may be a precondition for selection of proper nerve-muscle connections in development (see Redfern, 1970).

Trophic Effect The work of Peterson and Crain (1970) and the present study both demonstrate that innervation fosters the development and maintenance of muscle under a particular set of culture conditions. Since this trophic influence begins at the time of first nerve-muscle contacts (Peterson and Crain, 1970), and since it has been shown that transmitter is released at many of these contacts (Robbins and Yonezawa, 1971), it is possible that either acetylcholine is a trophic substance or that trophic substances are

released along with acetylcholine during early synaptogenesis. The present work further shows that not only development but also maintenance of cross-striation (under our culture conditions) depends on intimate or on functional nerve muscle association rather than on a chemical factor diffusely released into the medium by neural tissue.

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