Formation de novo and development of neuromuscular junctions in vitro

By GIORGIO VENERONI1 & MARGARET R. MURRAY2

From the Department of Surgery, College of Physicians and Surgeons, Columbia University, New York

The purpose of this study has been to examine, by means of isolation in vitro, the conditions under which neuromyal junctions develop. More than a hundred years ago Doyère (1840) observed in the water-bear Milnesium tardigradum that the nerve fibers terminate in characteristic eminences of muscle fibers. This observation, made on an arthropod, stimulated research into the connexion between muscle and nerve fiber, and resulted in abandonment of the older concept that motor nerves after having formed loops around the muscles return to the central nervous system as sensory pathways. Although investigations during the intervening century have identified and characterized morphologically the 'motor end plate' (as it was designated by Krause, 1863), and have revealed microscopic and fine-structural differences between types of motor endings, there is as yet no general agreement on the respective roles played by nerve, muscle and ambient influences in the development of the neuromuscular junction.

The comprehensive light-microscope description of the motor end plate by Couteaux (1941, 1947) has been confirmed and extended at the electron microscopic level by Reger (1955) and subsequently by others (reviewed by Couteaux, 1960). A correlation of the structural and functional characteristics of the sub-neural apparatus has been proposed in a monograph by Csillik (1965). Experimental approaches in vivo to the nerve–muscle relationship using denervation have been reviewed by Gutman & Zelená (1962) and by Zelená (1962).

In tissue culture studies it has been shown conclusively that skeletal muscle tissue can proceed to an advanced stage of morphological differentiation, coupled with function, in the absence of nervous tissue (cf. Murray, 1960, 1965). Crucial evidence to support these points has been accumulated from experiments utilizing explants of varying ages, primordial to adult, which commenced with the early work of the Lewises (1917) and has continued to the present day. Van Weel (1948) demonstrated that mesenchymal areas of the embryonic chick

1 Author's address: Istituto Camillo Golgi, Università di Pavia, Italia.
2 Author's address: Department of Surgery, College of Physicians and Surgeons, Columbia University, New York, New York 10032, U.S.A.
destined to become muscle were self-differentiating when isolated at a stage preceding their histological recognition as muscle rudiments. Poggeff & Murray (1946) explanted adult rat muscle from which some regenerating fibers in the outgrowth developed \textit{de novo} the definitive striation pattern and continued to contract for as long as a year. Others (e.g. Capers, 1960; Okazaki & Holtzer, 1966; Ezerman & Ishikawa, 1967; Shimada, Fischman & Moscona, 1967) have described patterns of muscle differentiation which varied with the age of the tissue explanted. Nevertheless, influence of nerve on muscle development \textit{in situ} has been invoked repeatedly (Hamburger, 1963), and Holtzer & Detwiler (1954) and Avery, Chow & Holtzer (1956) have reported that spinal cord (and not notochord) stimulates the growth of somitic muscle in amphibians and birds.

Observations of functional relationships developing between spinal cord and somitic muscle explanted together \textit{in vitro} have been recorded repeatedly. In mammals, Bornstein & Breitbart (1964) and Crain (1964), utilizing fetal mice of 12- to 14-day gestation, reported that both central and peripheral nervous tissues developed normally, and also that cross-striations appeared in the skeletal muscle. Fibrillary twitches as well as synchronous contractions of a number of fibers took place, and electrical stimulation of various elements of the cultures gave responses consistent with a high degree of functional organization \textit{developed and maintained \textit{in vitro}}. However, these last experiments, like earlier ones, had the intrinsic limitation of using preparations in which neuromuscular connexions were likely to have been already established and the muscle tissue had reached a degree of differentiation at explantation.

In order to conduct a more rigorous examination of junction development \textit{de novo}, and to explore possible circumstances under which this may take place, the following procedure has been followed: (a) explantation of nerve–muscle primordia (cord-somite) substantially before neuromyal junctions can have been established \textit{in vivo}; and (b) explantation of the two tissues, separated spatially, in combined cultures at several developmental stages.

**MATERIALS**

Chick embryos from 72 h to 11 days of incubation, and mouse embryos from 10 to 17 days gestation, were employed in four types of preparations:

1. (A) Ten-day mouse embryos were dissected to produce explants consisting of the entire posterior body wall containing the spinal cord and paraspinal masses in continuity (24 cultures). (B) From 72 to 80 h chick embryos the rostral somitic region was removed and the remaining axial region was cut transversely to produce small explants containing the neuromuscular primordia (42 cultures).

2. From fetal mice 14–17 days \textit{in utero} cervical and lumbar regions of the spinal cord with attached roots and ganglia were excised, and sliced transversely or divided dorsoventrally, to produce explants which were then confronted with fragments of metamerically related muscles from the same embryo, i.e. cervical
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3. From chicks 7—11 days in ovo cervical, thoracic and lumbar regions of the cord with attached ganglia were dissected out separately. In Exp. A these were combined at explantation with a suspension of autologous trypsinized muscle; in Exp. B the cord explants were cultured alone for 8—10 days, then seeded with dissociated muscle cells from 9- to 11-day chick embryos (100 cultures in all). The cells to be suspended were taken from pectoral and wing muscle or from thigh and leg, in accordance with the metameric level of the cord explant. 3 or 4 x 10^5 cells per culture were used.

4. Several cultures of muscle alone, dissociated or not, from both mouse and chick were carried for comparison.

METHODS

The Maximow double-overslip assembly, with reconstituted rat-tail collagen substrate (Peterson, Crain & Murray, 1965) was used. A thin overlay of clotting chicken plasma was sometimes added, especially on cultures of chick material. Three times a week cultures were washed in balanced salt solution (BSS) and fed with a medium consisting of equal parts human placental serum, bovine serum ultra-filtrate, Eagle’s (1955) minimal essential medium (MEM) and saline extract of 9-day chick embryos. This medium was supplemented with glucose, to a final concentration of 900 mg/100 ml. Some cultures of pure trypsinized muscle were also exposed during the first week of culture to the medium of Okazaki & Holtzer (1966) consisting of Eagle’s (1955) MEM 8 parts, horse serum 1 part, chick embryo extract 1 part. For muscle dissociation a mixture of 2 % trypsin + 0.08 % Versene in equal parts was diluted 20 times with Moscona (1952) Ca^2+ and Mg^2+-free saline.

Cultures were examined and photographed:

1. As living material.
2. After staining for cholinesterase activity by the method of Karnovsky & Roots (1964), with prior fixation in 10 % formol-calcium. Iso-OMPA (tetra-iso-propyl-pyro-phosphoramide; L. Light and Co. Ltd.) 2 x 10^{-4} M and 2 x 10^{-5} M was used to repress non-specific cholinesterase.
3. After silver impregnation according to the Holmes (1947) and Bodian (1937) methods.
4. After standard histological procedures such as haematoxylin and trichrome staining.
5. During supra-vital staining with methylene blue or Janus green.

RESULTS

1. (A) Explants dissected from 10-day mouse embryos (26—28 somites) develop quite well in vitro. At the time of explantation the only recognizable tissue is the
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spinal cord, with its large neurocoel, surrounded by undifferentiated mesenchyme. A few days later three rather dark areas appear, two of them lateral to the cord and one of them ventral. By the fourth day the explant contains all the primordial elements of the as yet undifferentiated posterior wall. The neurocoel is reduced in size and the surrounding areas of condensation contain embryonic cartilage; in the interspace between the spinal cord and the paraspinal masses the dorsal root ganglia have appeared and anterior to the vertebral body a few sympathetic cells, sometimes intermingled with the emerging efferent fibers, are quite easily recognized. When the explant has been well oriented—that is, in perfect cross-section—all these elements are distinctly evident and symmetrically arranged, although the outgrowth is denser in the ventral part of the section.

On the fifth to sixth day in vitro muscle tissue becomes clearly distinguishable, with its myotubes showing only longitudinal striation. At this time single twitches or slow, strong, synchronous contractions begin, and, as the result of this mechanical disturbance within the whole paraspinal mass, the explant partially loses its previous topography. It tends to open posteriorly, providing space for glial and sometimes sensory ganglion outgrowth. The cartilage masses

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Fig. 1. With the exception of (A) (× 900), all figures are equally magnified (× 1450)

(A) Neuromuscular junctions from a culture of a 10-day mouse embryo (en bloc preparation) after 17 days in vitro. Holmes stain (1947).

(B) Simple contact of an exploring fiber (arrow) from an 11-day chick embryo with recombined muscle from an embryo of the same age; 8 days in vitro. Karnovsky & Roots (1964) method for cholinesterase shows diffuse enzymic activity.

(C) Neuromuscular junction formed de novo in a culture from 7-day chick embryo spinal cord with recombined muscle from an embryo of 11 days; 26 days in vitro. Karnovsky & Roots (1964) method for cholinesterase and silver proteinate (Bodian, 1937).

(D) From the same embryo as in (C). Notice the fine branching of the nerve terminal (arrow) in an area containing many nuclei. Karnovsky & Roots (1964) method followed by silver proteinate (Bodian, 1937).

(E) Development de novo of a neuromuscular junction in a culture of spinal cord from a 7-day chick embryo with reassociated muscle from an embryo of the same age; 23 days in vitro. (a) Contact between a nerve fiber and a muscle fiber; (b) an area of highly localized cholinesterase activity on the muscle membrane, separate from the nerve contact; (c) a muscle nucleus in hypolemmal position, also cross-striation of the muscle fiber as expected in fully differentiated muscle; (d) a "bulging" nucleus from the muscle fiber. Karnovsky & Roots (1964) method for cholinesterase.

(F) Semi-schematic drawing combining several optical levels of the fibers photographed for (E).

(G) Fiber in another culture from a chick embryo of the same age as in (C), showing full development of the neuromuscular junction with the nuclei of the sole and the positive cholinesterase reaction (arrow) at the nerve terminal. Karnovsky & Roots (1964) method for cholinesterase followed by silver proteinate (Bodian, 1937).

(H) Reassociated muscle fiber from a 9-day chick embryo in a culture also containing an explant of spinal cord. No evidence of innervation, but dark areas of cholinesterase activity (arrows) are shown at sarcolemmal level in the newly recombined muscle. Karnovsky & Roots method for cholinesterase (1964).
are oriented so as to give the general appearance of a broadly open U. A few
days later (ninth day) cross-striation appears, and by the twelfth day in vitro
almost all the cultures show fully differentiated muscle fibers; these have a
smaller diameter than fibers in situ, even allowing for the tendency of cultured
fibers to flatten. The fixed and stained preparations allow more detailed observ-
ations than the living cultures; as shown in Fig. 1A, not only the muscle fibers
but such a delicate structure as a neuromuscular junction can be discerned with
the light microscope.

(B) Chick embryos of 72–80 h (30–36 somites), from which transverse sections
of the posterior axial region were explanted, gave analogous results. Morpho-
genetic movements in the chick explants were essentially similar to those in the
mouse, but the rate of growth and differentiation was greater in the chick. After
3 days, 74 h chick explants already showed strong muscle contractions although
only myocytes appeared to be present. At 8 days in vitro myotubes had developed
and at 13 days a pale, incomplete and sometimes unstable cross-striation had
already appeared as the intensity of the fibrillation was decreasing. In explants
from 80 h chick rhythmic muscular contraction appeared at 6 days in vitro and
by 20 days the fibers had developed stable and definitive cross-striation and their
nuclei had adopted the hypolemmal position. At 24 days (in two cultures) a
bundle of myelinated nerve fibers could be seen emerging from the spinal cord
and penetrating the paraspinal muscle tissue.

(2) The behaviour of cultures comprising separated explants of autologous
spinal cord and muscle from 14- to 17-day mouse fetuses was quite different
from the above. While the cord explants developed in the usual way (cf. Peterson
et al. 1965) after producing within 24 h an outgrowth of neurites associated with
meningeal and glial cells, the muscle explants did not advance pari passu but
soon entered a phase of dedifferentiation, so that by the third day in vitro most
of their cross-striation had been lost and the fibers had reverted to the myotube
stage with central nuclei and only longitudinal striae. At the periphery of the
muscle explants it was common to find typical regenerative forms such as large
pear-shaped plasmodia packed with nuclei. The outgrowing nerve fibers showed
very little tendency to penetrate the muscle areas; more often the neurites
courses around, over or under them, emerging free a few days later. No difference
in this respect was noted among the different types of spinal cord explants.

While differentiation of the nervous tissue gradually progressed, dedifferentia-
tion of the muscle tissue continued, so that by the fifteenth day in vitro the latter
appeared as an almost undifferentiated mass, with which some cells of fibrocytic
or histiocytic aspect were associated. A few myoblasts and myotubes were
present, however, and among these it was possible to see some twitching.
Whether these simple contractions had been present some time before or
whether they were commencing at the time of first observation, as an expression
of redifferentiation, is difficult to say. However, at 20–22 days some cross-striated
fibers could be seen in parallel formation with myelinated nerves which did not
show, in living preparations, any connexion with the sarcoblasts. Although the myelin sheaths were quite stable, the individual muscle fibers varied frequently in aspect, showing within a space of 24 h either cross or longitudinal striation. At 34 days, when our experiments were terminated, fixed and stained preparations indicated that the attributes of cross-striation and hypolemmal nuclear position had been lost. No evidences of junction between nerve and muscle were found.

Observation of the living muscle explant was impeded by the constant emergence, at the seventh to tenth day in vitro, of brown-fat cells whose primordia could not be eliminated from the explants. These subsequently increased rapidly in number and size, masking and eventually overwhelming the muscle explant; by the end of the month of culture these adipose cells together with the regressed muscle had become so numerous that the few differentiated muscle fibers were hardly visible. According to Sidman & Fawcett (1954) the dorsal brown-fat bodies of the mouse are richly innervated in situ; after nerve section they increase by both hypertrophy and hyperplasia.

(3) Spinal cord and dissociated muscle from 9- to 11-day chick embryos. (A) Autologous cord and muscle-suspension explanted together: within 3–5 days in vitro the dissociated myoblasts had fused to form myotubes. During this period some pulsation of myoblasts was observed, and a good deal of twitching by myotubes. By 8–9 days a fine pale cross-striation had appeared, starting from the surface of the myotube and proceeding toward the central part of the fiber. As the cross-striation became more pronounced the nuclei moved to an hypolemmal position. This picture was common to all of the cultures although not all of them showed differentiated muscle throughout. Some myotubes underwent a partial differentiation only, with an arrest of the cross-striation at its beginning, and without displacement of the nuclei. Sometimes many nuclei aligned themselves at one surface of the fiber only, the one opposite the striation. Sometimes they were gathered toward one end of the tube, thus forming a cluster similar to the pear-shaped bag of the regenerating fiber. In a very few cultures we also observed a fragmentation of the tubes already formed, with the appearance of rounded hyaline bodies in the center of which pyknotic nuclei were visible.

(B) Cultures of cord to which homologous dissociated muscle cells from 9–11 day chick embryos were added 7–14 days after explantation of the cord. (a) At 14 days after explantation of 11-day cord, trypsinized muscle from an embryo of 11 days was added. Eight days later a nerve fiber with its branching end touching a muscle fiber was observed (Fig. 1B). This was not regarded as a junction, but as a simple contact between an exploring fiber and a muscular element. (b) In other experiments, where younger cord explants (7 days) were used, and 11-day trypsinized muscle was added after a shorter interval (7 days), much more intimate contacts were observed (Fig. 1C, D, E, G). In these cases the muscle fiber developed a higher degree of maturity than in (a); the nerve terminal appeared to adhere to it closely and was also associated constantly
with nuclei whose features, however, cannot be classified as either teloblastic or sarcoblastic. Moreover, at 26 days in vitro there was a highly localized and strongly positive cholinesterase activity at the site of these primitive contacts, which seems consistent with specialized myoneural structures.

Cholinesterase staining shows in addition other sites of strong enzymic activity randomly scattered throughout these 24- to 26-day cultures. Such areas are situated at the level of the sarcoblast membrane and they may or may not be associated with nuclei; in profile they appear as elongated half-moons (hemiepiphyseal) close to the surface of the fiber, and in full view as ovoids or ellipsoids at the surface; in some cases there are several in one fiber (Fig. 1H). It is difficult in a black and white photograph to show these as other than dark spots; however, by microscopic examination with close focusing it is possible to discern a fine lamellar structure. These lamellae are parallel to the fiber axis, and the whole deeply staining area may appear to be bulging from it; the edge of the ovoid projects fine radiating lines at the fiber surface.

(4) Muscle from groups (2) and (3) was also cultured alone, i.e. in the absence of the nervous tissue. The behaviour of fragments of muscle was similar to that described in Exp. 2. However, in a muscle explant from a 14-day mouse embryo we observed rhythmical contractions for a few days before it underwent de-differentiation; the rhythm was of one strong synchronous contraction each 25 sec; from time to time another group of fibers functioned, with a rhythm of one contraction in 13 sec. Moreover in a limb-bud from an 84 h chick embryo we observed the pulsation of myocytes and the fibrillar twitching of the few myotubes which had formed before the explant began to regress morphologically. In cultures of dissociated muscle (as in Exp. 3) from both chick and mouse an initial fusion of myocytes occurred, but whilst the chick myotubes continued to differentiate as in (3A), mouse myotubes did not advance in parallel: as the infestation of brown-fat cells proceeded these newly reconstituted muscle fibers dedifferentiated or were masked as before.

DISCUSSION

Since the results from nerve-muscle preparations en bloc were essentially similar in mice and chicks, they are discussed together. A 10-day mouse embryo is approximately comparable to a 3-day chick embryo (Rugh, 1964, 1968); the chick of 96 h has become clearly recognizable as a bird, and the mouse of 11 days gestation presents the major mammalian organs. Our results confirm and extend backward the morphological observations of Bornstein & Breitbart (1964) on fetal mouse material of 12-14 days gestation, and show that neuro-myal junctions can be developed in vitro from much earlier primordia (10 days).

It is from explants of combined nervous and muscular tissues separated by gaps of space and time that one might hope to glean information regarding nervous influences on muscle differentiation. From these it is clear that cross-
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striation and the hypolemmal position of nuclei, as well as focal cholinesterase reaction, can be produced in sarcoblasts which have reformed from dissociated muscle before evidence of innervation appears (Exp. 3); also that dedifferentiation of regenerated muscle may take place in the presence of well-maintained spinal cord nervous elements (Exp. 2). It may be unreasonable to expect that complete adult-type cytodifferentiation could occur and be maintained in the absence of blood circulation, general hormonal influences and the mechanical support of skeletal and other connective tissues, circumstances which, aside from the nervous contacts, are essential to prolonged muscular function in vivo.

For neuromyal junction formation, Couteaux proposed (1941) the following schema: the nerve fibers after having reached the muscle tissue as exploring neurites make contact with the muscle fibers which already have their nuclei in hypolemmal position. At this point a muscle nucleus divides and forms the outer layer of the sole. Simultaneously other cells resembling Schwann cells (lemmoblasts) move along the fiber and form a syncytium near the antler, thus completing the junction. This interpretation, which provides for joint contributions to the sole apparatus from muscle tissue and nervous supporting cells, has been accepted in general terms, with later modifications. Kupfer & Koelle (1951), studying the genesis of cholinesterase in rat skeletal muscle, reported that the sole-cholinesterase is derived from muscle nuclei; and, since the sole-nuclei are present before a connexion is made between nervous and muscular fibers, they proposed that 'the cholinesterase of the sole-plate may serve to attract the motor nerve fiber and thus directly influence the peripheral distribution of motor nerves to skeletal muscle'. It has been shown by Mumenthaler & Engel (1961) that in chick myoneural junctions the enzyme does not appear in Schwannian elements but in surface areas of the muscle fibers as soon as the nerves approach them.

In mammals Savay and Csillik (Csillik, 1965, pp. 63–74) report that after nerve section reinnervation occurs at the site of the original end-plate if this structure has not degenerated, but in its absence the regenerating nerve fiber induces a new end-plate in a different area of the muscle fiber. Emergence of new sites of intense acetylcholinesterase activity in denervated muscles of young rats has been reported recently by Lubińska & Zelená (1966) in experiments which prevented reinnervation by removing parts of lumbar and sacral cord segments. These new foci, dark spots on the contour of the muscle, were sometimes flush with the fiber surface, but more often bulged from it. Their occurrence was related to the age of the animal, and they were not observed in adults. As a possible explanation the authors suggest that denervated muscle itself can produce an accumulation of cholinesterase; they do not exclude, however, the Schwannian origin favored by Csillik (1965) for mammals, since Schwann cells become migratory after axon degeneration.

Positive cholinesterase reaction has been described in chick muscle cultured in vitro by Engel (1961). This investigator found, in explants of unspecified
muscle from 13-day embryos, that the cholinesterase activity in cells from myoblast to wide, multinucleated strap (striated sarcoblast) could be visualized as small brown granules located diffusely in the cytoplasm; no enzymic activity was seen in the sarcolemmal region or in the nuclei.

To us it seems probable that the foci of Lubinska & Zelená (1966) are equivalent to the foci that we observed as rings or lines on the surface of our reintegrated chick sarcoblasts. Such a conclusion is morphologically credible, and it is not necessarily in disagreement with the negative findings of Engel in cultures of muscle alone, or with those of other authors who have reported a lack of cholinesterase concentration in embryonic muscle before innervation was believed to have taken place. Our spots of concentration were found in cultures which also contained spinal cord and its exploring nerve fibers, although these latter had not made permanent contacts with the sarcoblasts under observation (Fig. 1H).

It would appear that the circumstances leading to myoneural junction formation are operative in very early stages of development. According to Visintini & Levi-Montalcini (1939), exploring fibers from the posterior root are present in chick somites as early as the fourth day in ovo; the ventral fibers become evident on the fifth day. Mumenthaler & Engel (1961) found diffuse cholinesterase activity in chick muscle from 4–7 days in ovo. If we assume, as does not appear impossible, that the exploring nerve fibers discharge some impulse or convey some signal (chemical, electrical or other) we can perhaps reconcile the above data.

The uninnervated muscle fiber shows a diffuse cytoplasmic cholinesterase activity (Engel, 1961); during innervation foci appear, activity becomes very high in the end-plate region and regresses elsewhere. Since in the experiments of Lubinska & Zelená the muscle had already been exposed to exploring fibers during development, the areas of cholinesterase activity reappearing after denervation could represent the potential sites of anchorage originally offered to ingrowing nerve fibers, which had returned to latency (with diffusion of cholinesterase) once definitive innervation had been achieved. After nerve section and loss of the pristine end-plate, functional inhibition of their activity might be released and concentration of enzyme triggered anew in the muscle, by contact with the exploring regenerative fibers.

In Engel's cultures of muscle alone (1961) new-formed fibers grew out and differentiated morphologically without any nervous connexion. These explants came from a muscle (13 days in ovo) which had originally been in contact with exploring fibers and therefore should have possessed the potential for renewed enzyme concentration. But in his paper only newly grown muscle cells were considered, and very little was said of the original explant, except that '...none of the cultured muscle cells, including those of the original explant, had sites of thiocholinesterase in the pattern of an embryonic or adult motor end-plate, even after 37 days in vitro'.
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These data, as well as our own observation of an exploring fiber touching a young myotube which showed diffuse cholinesterase reaction (Fig. 1B) and our findings of more complex associations between nerve and muscle (Fig. 1C, D, E, G) where localized areas of high cholinesterase activity are present, lead us to suggest the following hypothesis, which represents a further modification of the interpretations of Couteaux (1947) and Kupfer & Koelle (1951):

The exploring nerve fibers trigger the sarcoblast to displace its nuclei into an hypolemmal position and to concentrate cholinesterase activity in several surface areas. These areas bulge somewhat and are capable of serving as anchorage points for a nerve fiber destined to establish permanent contact. Once the fiber is anchored, morphogenesis of the neuromyal junction proceeds. It is possible that the establishment of the junction itself modifies the whole surface of the muscle fiber electrostatically, or alters it in such a way as to prevent enzyme accumulation elsewhere, during its functional existence. Hence the superfluous foci must regress to a state of diffuse reactivity, though the fiber retains for a considerable period the potentiality for providing new junction sites by redifferentiation in response to an appropriate stimulus.

SUMMARY

Combined cultures of nerve and muscle tissue from the following stages of development in chick and mouse were maintained for several weeks on collagen-coated coverslips in Maximow slide assemblies.

1. *En bloc* preparations of cord-somite regions were explanted from 26-somite mouse embryos (10 days *in utero*) and from equivalent chick-embryos (72–80 h *in ovo*), before neuromuscular connexions have been established. In these, both nerve and muscle tissue continued to develop *in vitro* and presented morphological evidence that characteristic neuromyal junctions had been formed.

2. Spatially separated explants of autologous spinal cord and muscle from 14 to 17 day mouse embryos showed continued differentiation of nervous elements but limited development of muscle. No evidence was found of junction formation, or of simple reinnervation. The regenerating muscle appeared to be overgrown by adipose cells from brown-fat rudiments which could not be eliminated.

3. Segments of chick spinal cord of 7–11 days incubation were explanted with dissociated muscle tissue from the same embryo or an embryo of different age. A high degree of differentiation was achieved by both nervous and re-aggregated muscle tissue. Exploring nerve fibers from the cord explants made contact with the sarcoblasts, and areas of cholinesterase activity appeared in the sarcolemma of the newly integrated fibers. Simple neuromuscular junctions formed *de novo*.

These observations are discussed in relation to problems of nervous influence
upon muscle development. As a hypothetical schema for neuromyal junction formation, it is proposed that contact by an exploring nerve fiber exerts a triggering action on the myotube membrane, causing it to bulge locally and anchor the neuritic terminal, to form the primordial end-plate.

**RÉSUMÉ**

*Formation de novo et développement des jonctions neuromusculaires in vitro*

Des cultures combinées de tissus nerveux et musculaire prélevés aux stades de développement suivants chez le Poulet et la Souris, sont maintenues pendant plusieurs semaines sur des lamelles recouvertes de collagène dans un ensemble de Maximow:

(1) Des préparations 'en bloc' de région chordo-somitique sont prélevées sur des embryons de Souris de 26 somites (10 jours in utero) et des embryons de Poulet de stade équivalent (72–80 heures in ovo), avant que les connexions neuromusculaires se soient établies in situ. Les tissus nerveux et musculaire continuent à se développer in vitro. L'étude morphologique met en évidence les jonctions neuromusculaires caractéristiques.

(2) Des explants séparés de muscle et de tube nerveux autologues, pris sur des embryons de Souris de 14–17 jours montrent une différenciation continue des éléments nerveux mais un développement limité du muscle. On ne voit aucune connexion ni même une simple réinnervation. Le muscle en régénération apparaît recouvert de cellules adipeuses provenant de rudiments de graisse brune qui ne peuvent être éliminés.

(3) Des segments de tube nerveux de Poulet de 7 à 11 jours d'incubation sont explantés avec du tissu musculaire dissocié provenant du même embryon ou d'un embryon d'âge différent. On obtient dans ce cas un haut degré de différenciation du tissu nerveux et du tissu musculaire réagrégé. Les fibres nerveuses qui partent des explants de tube nerveux entrent en contact avec les sarcoblastes et des aires d'activité cholinestérasique apparaissent dans le sarcolemme des fibres nouvellement intégrées. Des jonctions neuromusculaires simples se forment de novo.

Ces observations sont discutées vis-à-vis du problème de l'influence nerveuse sur le développement musculaire. Comme schéma hypothétique de la formation des connexions neuromusculaires, on propose qu'au contact d'une fibre nerveuse en croissance, la membrane du myotube réagit en se bombant localement pour ancrer la terminaison neuritique et former la plaque terminale primordiale.

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REFERENCES


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