DEVELOPMENT OF THE NEUROMUSCULAR JUNCTION

III. Degeneration of Motor End Plates After Denervation and Maintenance In Vitro by Nerve Explants

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ABSTRACT
To determine the effects of nerve explants on the integrity of motor end plates in vitro, cholinesterase activity and structure of end plates were compared in newt muscle denervated in vivo, cultured in the absence of nerve explants, and cultured in the presence of sensory ganglia. In neuromuscular junctions denervated in vivo or in vitro, the synaptic vesicles become clumped and fragmented. A few intact vesicles escape into the synaptic cleft. Axon terminals degenerate until they are left as residual bodies within the Schwann cell cytoplasm. Junctional folds on the muscle surface are reduced in height and are no longer evident once traces of axoplasm within the Schwann cell disappear. End plate cholinesterase activity is reduced as junctional folds are lost. When muscle is cultured in the presence of a sensory ganglion, the terminal axoplasm degenerates in the same manner but junctional folds persist on the muscle surface. Moderately intense cholinesterase activity remains in association with the junctional folds, so that normal motor end plates are maintained in the absence of innervation. These results show that degenerative changes in the structure of the motor end plate and loss of cholinesterase activity occurring in organ culture as a result of denervation can be retarded by nerve explants that do not directly innervate the muscle.

INTRODUCTION
Trophic effects of neurons have been defined as long-term interactions between nerve cells and other cells which initiate or control molecular modifications in the target cell (Guth, 1969). An example of trophic activity is the role of the nerve fiber in the maintenance of the structural, chemical, and functional integrity of the motor end plate of skeletal muscle. The motor nerve fiber is considered to exert a morphogenetic effect on the formation of the motor end plate because the structural specializations of the end plate arise in developing or regenerating muscle only where axon terminals become closely apposed to the muscle surface (Teräväinen, 1968; Kelly and Zacks, 1969; Lentz, 1969). Conversely, when the nerve supply to muscle is interrupted, the neuromuscular junction degenerates although the end plate itself may remain unaltered for relatively long periods (Tower, 1939; Birks et al., 1960; Gutmann and Zelená, 1962). Innervation also has an effect on acetylcholinesterase (AChE) activity at the motor end plate. End plate cholinesterase (ChE) activity appears in differentiating muscle in relation to innervation (Mumenthaler and Engel, 1961; Zelená, 1962; Guth and Brown, 1965; Eränkö and Teräväinen, 1967; Lentz, 1969), although the myoblast does synthesize ChE before innervation (Tennyson et al., 1971). When the
muscle is denervated, there is a loss of ChE activity at the motor end plate (Brzin and Majcen-Tkac, 1963; Guth et al., 1964; Filogamo and Gabella, 1966; Eränkö and Teräväinen, 1967). On these bases, the motor neuron seems to exert a regulatory effect on the structure and ChE activity of the myoneural junction (Guth, 1968).

The nature of the trophic effect of nerve and its mechanism of action remain unknown; possibilities include muscular activity (Lågno and Rosenthal, 1972), acetylcholine (Drachman, 1967), and unidentified trophic substances released by nerve cells (Singer, 1952; Gutmann and Hnik, 1962). In an effort to study the nature of the trophic process, a bioassay system has been devised in which the effects of nerve explants, nerve extracts, or chemical substances on ChE activity of muscle placed in organ culture are measured (Lentz, 1971). By means of this assay system, nerve explants and homogenates have been found to retard or prevent the loss of ChE activity occurring as a result of denervation. With light microscope histochemistry, end plates in muscle cultured with nerve explants retain greater ChE activity than end plates of untreated muscle.

The present study was undertaken to determine the effects of nerve explants on the fine structure of the neuromuscular junction and the cytochemical localization of end plate ChE activity in cultured muscle. Sensory ganglia were employed as a source of nerve tissue because, while they can sustain limb regeneration of the newt (Kamrin and Singer, 1959), they do not directly innervate the cultured muscle in the time course of these experiments. Thus, any effects on the motor end plate can be maintained in vitro in the absence of direct innervation.

**MATERIALS AND METHODS**

Adult newts, *Triturus viridescens*, were used in these experiments. The fine structure and cytochemical localization of ChE activity of neuromuscular junctions were studied in muscle denervated in vivo, in untreated cultured muscle, and in muscle cultured with sensory ganglia. Denervation of the forelimb in newts anesthetized in 0.2% chloretone was achieved by exposing the brachial plexus and removing a 1 mm segment of the brachial nerve cords. This procedure eliminates the motor and sensory supply (nerves 3, 4, and 5) to the limb and prevents reinnervation during the time course of these experiments. Newts were kept on moist paper towels in covered fingerbowls and fed two times a week. The triceps muscle was removed at 3, 7, 14, and 21 days after denervation for fixation for electron microscopy. Muscles from at least ten newts were examined at each time period. Innervated muscle in the opposite limb served as a control.

The procedures for organ culture of newt muscle have already been outlined (Lentz, 1971). Triceps muscle from one limb of a normal newt was removed under sterile conditions and placed in culture in an amphibian culture medium (Wolf and Quimby, 1964; Grand Island Biological Co., Grand Island, N. Y.). Cultures were maintained at room temperature in a moist atmosphere of 95% air and 5% CO₂. Culture medium was changed twice a week. Muscle

**FIGURE 1** Neuromuscular junction in a normally innervated limb (arrows) on the side opposite that on which nerve transection was performed. A thin layer of Schwann cell (SC) cytoplasm covers the axon. The axoplasm contains glycogen (Gly), mitochondria, and large numbers of synaptic vesicles (SV). The axon and muscle membranes are separated by the sarcotubule layer. Junctional folds (JF) project from the muscle surface. Dense material occurs beneath the sarcolemma at the tips of the junctional folds (arrows). X 38,000.

**FIGURE 2** Neuromuscular junction 7 days after nerve section. Segments of axoplasm are enveloped by Schwann cell (SC) cytoplasm. At A, synaptic vesicles are intact. At B, they are densely packed and mitochondria are degenerating. At C, the axonal remnant exists as whorls of concentric lamellae (myelin figure). Junctional folds are normal in appearance opposite C. At B, they are present only as low ridges (arrows). No folds are present in the region of A. X 25,000.
from the opposite limb was cultured in the same manner and treated experimentally by placing a sensory ganglion (C5 or C4) directly on top of the muscle. After 3, 7, 14, and 21 days in culture, muscles were fixed for microscopy. Ten or more pairs of muscle were examined at each time period. In cultures maintained longer than a week, ganglia were replaced at weekly intervals.

For electron microscopy, muscles were cut into small blocks and fixed for 1 hr in cold 3% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2). The blocks were rinsed in cold buffer and fixed for an additional hour in cold 1% osmium tetroxide in 0.05 M cacodylate buffer (pH 7.2). The tissues were dehydrated, embedded in Maraglas, and sectioned with glass or diamond knives on a Sorvall MT-2 microtome (Ivan Sorvall, Inc., Norwalk, Conn.). Thin sections were stained with lead citrate alone or uranyl acetate followed by lead citrate and examined with an RCA EMU 3F or a Hitachi HU11-ES electron microscope.

For the cytochemical localization of ChE, the thiolacetic acid-lead nitrate method (Crevier and Bélanger, 1955; Barnett, 1962) was employed as described previously (Lentz, 1969). Glutaraldehyde-fixed tissues were incubated in the reaction medium and incubation in the presence of physostigmine (eserine) (10^-4 M) or diisopropylfluorophosphate (DFP) (10^-4 M).

RESULTS

Motor End Plate After Denervation

Neuromuscular junctions in the limb on the side opposite that on which denervation was performed are normal in appearance (Fig. 1). A thin layer of Schwann cell cytoplasm covers the outer surface of the terminal axon. The axoplasm contains glycogen granules, mitochondria, and synaptic vesicles. Junctional folds arise from the muscle surface along the entire region of nerve-muscle contact. A thin band of dense material is found on the inner surface of the sarcolemma at the tips of the junctional folds. (See Lentz, 1969, for further details of the normal newt neuromuscular junction.)

The following descriptions represent the most common sequence of changes based on observation of several hundred motor end plates. It should be noted, however, that there is considerable variation in individual end plates at each time. Changes in the neuromuscular junction of muscle denervated in vivo or placed in culture are largely the same, the only difference being that they occur more rapidly in vitro. Within 3 days after denervation, the terminal axoplasm becomes more completely enveloped by Schwann cell cytoplasm (Figs. 2, 3). Tongues of Schwann cell cytoplasm intervene between the axonal and myofiber surfaces. Envelopment by the Schwann cell appears to divide the terminal axon into segments (Fig. 2).

Within the axoplasm, synaptic vesicles become clumped into tightly-packed clusters (Figs. 2-4). Glycogen granules are no longer evident and mitochondria are rounded and increased in density (Fig. 3). Vesicle and mitochondrial membranes become less distinct and fragmented. Breakdown of vesicles and mitochondria leaves membrane-enclosed masses of dense material or concentric lamellae in the Schwann cell (Figs. 2, 4, 6). Later, these residual bodies and myelin figures disappear.

FIGURE 3 Degenerating junction 3 days after nerve section. The Schwann cell encloses large numbers of synaptic vesicles. In most places, the axolemma has disappeared so that the vesicles actually lie free within spaces bounded by Schwann cell plasma membrane. Where there are gaps in the Schwann cell covering (between arrows), the synaptic vesicles are exposed to the synaptic cleft. X 45,000.

FIGURE 4 Degenerating junction 3 days after nerve section. Portions of the terminal axon are embedded in the Schwann cell. In the large axonal fragment, synaptic vesicles are tightly clustered. The other portions of axoplasm are in more advanced stages of degeneration. Note the presence of vesicles (V) in the synaptic cleft. The vesicles are on the axonal side of the band of dense material occupying the synaptic cleft. The muscle surface is devoid of junctional folds, but dense material (arrow) like that at the tips of normal folds can be seen beneath it. X 33,000.

FIGURE 5 Portion of a neuromuscular junction 3 days after denervation. Vesicles (V) are free in the intercellular cleft between the sarcolemma and a concavity in the Schwann cell. A single junctional fold is present on the muscle surface. X 49,000.

FIGURE 6 Neuromuscular junction 7 days after denervation. The axoplasm has degenerated and is left as a residual body (R) and myelinated figure (M) within the Schwann cell. Junctional folds (arrows) do not occur over a large extent of the muscle surface but persist opposite the remnants of axoplasm. X 21,000.
In some terminals, the membrane enclosing the axoplasm is no longer discernible so that the axonal synaptic vesicles are exposed to the intercellular cleft through gaps in the Schwann cell covering (Fig. 5). Small vesicles with the appearance of intact synaptic vesicles are often seen within the synaptic gap (Figs. 4, 5). Generally, the vesicles are situated on the axonal side of the band of dense material (basement lamina) lying in the synaptic cleft. Intercellular vesicles often lie within or near a concavity in the Schwann cell (Fig. 5).

While the axonal changes are occurring, the junctional area also undergoes alteration. After denervation, the junctional folds of the motor end plate gradually disappear. By 3 days, both in vivo and in vitro, some portions of the junctional region are devoid of folds and have a wide intercellular cleft between muscle and Schwann cell (Figs. 2, 4). In other regions, the junctional folds are reduced in height and appear as short hillocks on the muscle surface (Fig. 2). Dense material like that found beneath the membranes of normal folds persists in the hillocks or along the muscle surface after the folds have disappeared (Figs. 4, 7, 8). Relatively few junctional folds can be found 21 days after denervation in vivo or after 7 days in culture. They seem to persist longest opposite the parts of the Schwann cell that contain remnants of axoplasm (Fig. 6). However, once the axoplasm disappears completely, junctional folds are rarely evident and these are usually short and few in number (Figs. 7, 8).

Schwann cells contain the fragments of degenerating axoplasm, dense bodies (lysosomes), and residual bodies. At this time, the hyaloplasm of the Schwann cell is of low density and contains an increased number of microtubules (Fig. 7). Later, as the dense bodies disappear, short cisternae of rough-surfaced endoplasmic reticulum and clusters of free ribosomes appear in the cytoplasm (Fig. 8). These changes, which give the Schwann cell the appearance of a connective tissue cell, occur earlier and are more prominent in vitro. Cells with these characteristics become separated from the muscle surface by a large space (Fig. 11).

Cytologically, physostigmine- and DFP-sensitive ChE activity associated with the motor end plate is reduced in intensity concomitantly with the loss of morphological specialization in the junction. In general, loss of ChE activity parallels reduction of the junctional folds. In advanced stages, when the axoplasm is degenerating and the junctional folds are not prominent, very little enzymatic activity is detectable cytologically in the former synaptic cleft or associated with the sarcolemma (Fig. 9). Increased enzymatic activity was observed in some Schwann cells (Figs. 9, 12). Deposits of final reaction product occur scattered over the hyaloplasm not associated with any discernible structures or are present within dense granules (lysosomes). The latter activity is not sensitive to inhibitors and is most likely due to an enzyme other than ChE. Dense deposits observed over myofilaments and intercellular collagen commonly occur after incubation in lead and thus are nonenzymatic.

**Motor End Plate of Muscle Cultured in Presence of Nerve Explants**

When skeletal muscle is cultured in the presence of a sensory ganglion, envelopment and degeneration of the axon terminal occurs in the same way and the Schwann cell undergoes the same changes.

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**Figure 7** Advanced stage of end plate degeneration 7 days after nerve section. The Schwann cell (SC) is situated adjacent to the muscle surface. The cytoplasm of the Schwann cell is of low density and contains microtubules (Mt). The end plate is identified by the persistence of a single secondary synaptic cleft (C). Elsewhere, dense material (arrow) is still present beneath the sarcolemma. X 26,000.

**Figure 8** Neuromuscular junction of muscle after 1 wk in organ culture. The axon has completely disappeared from the Schwann cell (SC) which contains an increased number of ribosomes and short cisternae of endoplasmic reticulum. Junctional folds have regressed on the muscle surface. One low fold (F) remains identifying this structure as a former neuromuscular junction. X 26,000.

**Figure 9** Neuromuscular junction of muscle after 1 wk in culture reacted for ChE activity. Axoplasm has degenerated, leaving the Schwann cell adjacent to the muscle, and junctional folds are no longer evident. Enzyme activity has largely disappeared from the synaptic cleft. Compare with the intensity of reaction in a normal junction (Lents, T. L. 1969. J. Cell Biol. 42:440; Fig. 10). X 26,000.
as in muscle cultured in the absence of nerve explants (Fig. 10). Many motor end plates, however, are largely unchanged after 1 wk (Figs. 10, 11). As the axon degenerates, junctional folds remain of normal height and arise over a normal extent of muscle surface. The end plate region remains intact when the axoplasm has completely disappeared and the Schwann cell is situated adjacent to the muscle (Fig. 10). The number of end plates that can be found and their well-differentiated state is in marked contrast to the situation in untreated muscle, where there is only the occasional persistence of folds. Even after the Schwann cell begins to withdraw from the surface of the muscle fiber, normal end plates can be found on the surface of muscle cultured with nerve explants (Fig. 11).

Not all end plates in the cultured muscle are preserved. Some disappear by 1 wk as in muscle cultured alone. By 3 wk in the treated muscle, all junctions are in advanced stages of degeneration. Thus, the explants appear capable of maintaining normal end plates for 1 wk, but thereafter only retard the disappearance of the junctions.

The cytochemical procedure for ChE shows that moderate accumulations of final reaction product persist in the synaptic cleft and in association with the sarcolemma after 1 wk in culture (Fig. 12). Although the intensity of reaction is not so great as normal, it is more intense than in the remnants of end plates in untreated muscle in vitro or muscle denervated in vivo (Fig. 9).

**DISCUSSION**

In the newt, the structure of both the neural and muscle components of the neuromuscular junction as well as end plate ChE activity are rapidly and markedly affected by denervation. Axon terminals, both in vivo and in vitro, show disruption of internal structure after nerve section and apparently are lysed within the Schwann cell. Junctional folds of the myofiber show regression and gradually disappear after denervation. It was noted that the end plate or portions of it may be maintained as long as remnants of axoplasm remain in the Schwann cell. Presumably, the degenerating axonal fragments retain the ability to supply whatever factor is necessary for maintenance of the end plate.

ChE activity rapidly diminishes in intensity at the motor end plate after denervation, both in vivo and in vitro. Total muscle ChE as determined quantitatively, however, shows little change after 1 wk in culture, but diminishes thereafter (Lenz, 1971). While the quantitative procedure provides no information on changes in ChE activity at specific sites, the cytochemical method shows a close dependence of end plate ChE on innervation. Loss of activity generally parallels loss of junctional folds. The increased activity in the cytoplasm of Schwann cells corresponds to the increase in butyrylcholinesterase (nonspecific ChE) in Schwann cells after denervation (Eränkö and Teräväinen, 1967). In marked contrast to motor end plates in untreated muscle in vitro and denervated in vivo, end plates can be preserved in vitro by means of nerve explants. Although the axon endings degenerate in the usual way, junctional folds are maintained on the muscle surface. Cytochemically, ChE activity, although not so intense as normal, persists in the end plates of treated muscle. These

**FIGURE 10** Neuromuscular junction of muscle cultured 1 wk in the presence of a sensory ganglion. This junction is comparable to that in Fig. 8 in terms of the state of axoplasmic degeneration and Schwann cell (SC) configuration. The axoplasm has disappeared from the Schwann cell which now contains cisternae of rough-surfaced endoplasmic reticulum. The motor end plate is normal in appearance and junctional folds show no evidence of regression. × 32,000.

**FIGURE 11** End plate of muscle cultured in the presence of a sensory ganglion for 1 wk. The Schwann cell is no longer closely associated with the muscle surface and may have given rise to the cell located in the intercellular space. This cell contains ribosomes and endoplasmic reticulum and resembles a connective tissue element. The end plate is unchanged in appearance. Junctional folds are elongated and constricted at their base as in normally innervated junctions. × 15,000.

**FIGURE 12** Motor end plate in muscle cultured for 1 wk in the presence of a sensory ganglion. Thiolaetic acid-lead nitrate method for ChE activity. The axoplasm has degenerated but junctional folds have maintained their normal structure. Moderately intense enzyme activity is preserved in the synaptic cleft. Deposits of final reaction product occur over the cytoplasm of the Schwann cell (SC). Dense deposits in the intercellular space and over myofibrils are nonenzymatic. Compare with Fig. 9. × 36,000.
results show that changes in the structure of the motor end plate and loss of ChE activity occurring in culture as a result of denervation can be prevented by nerve explants which do not directly innervate the muscle fibers. Extracts of nerve homogenates have also been shown to maintain ChE activity of muscle in vitro (Lentz, 1971). It appears that some factor absent after denervation and necessary for the maintenance of the end plate is supplied by the addition of nerve explants and homogenates. If this maintenance is due to specific trophic interaction, these observations support the notion that the trophic effect is mediated by a diffusible chemical substance or nerve trophic factor. The possibility remains, however, that the nerve explants or homogenates supply other nonspecific hormonal or nutrient factors lacking in the culture medium. Biochemical studies will be necessary to determine the nature of the active substance in the nerve homogenates.

The changes in the structure of the neuromuscular junction and decrease in intensity of ChE occur shortly after nerve section in the newt, and are more rapid in vitro than in vivo. Equally rapid modulations in the neuromuscular junction occur in the course of muscle dedifferentiation after limb amputation (Lentz, 1970). Several studies, however, have shown a much longer persistence of motor end plates (Couteaux, 1955; Nickel and Waser, 1968) or ChE activity (Couteaux, 1955; Filogamo and Gabella, 1966; Eränkö and Teräväinen, 1967) after nerve section. Thus, there appear to be species differences in the stability of the end plate after denervation or in the degree to which the end plate is dependent on innervation for maintaining the formed structure.

Physiologically, nerve section is followed by a disappearance of junctional transmission and miniature end plate potentials (mepps) (Birks et al., 1960; Miledi, 1960). However, after a few days of electrical quiescence, subthreshold activity is again detectable at the end plate region. The mepps resuming in denervated muscle resemble those occurring in normal innervated end plates, but their frequency is much lower and their amplitude distribution is highly asymmetrical. It was suggested that the spontaneous potentials were the result of production and release of acetylcholine packets by the Schwann cell which continued to occupy the site of innervation after degeneration of the axon (Birks et al., 1960; Miledi, 1960). The possibility that remnants of the axonal stock were persisting was not ruled out.

The present observations indicate that the acetylcholine responsible for mepps after denervation may originate from synaptic vesicles escaping from axonal remnants. After nerve section, the bulk of the terminal axoplasm is enveloped by and eventually degenerates within the Schwann cell. However, a few synaptic vesicles appear to gain access to the synaptic cleft. Breakdown of the vesicle membrane would liberate acetylcholine, resulting in the appearance of mepps. Thus, intermittent release of small numbers of synaptic vesicles from the degenerating axon could account for the subthreshold electrical activity occurring in denervated end plates. Escape of vesicles into the synaptic cleft may not be restricted to degenerating terminals, as Grillo (1970) has described extracellular vesicles in the heart of the normal mouse.

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