THE DEVELOPMENT OF NERVE-MUSCLE JUNCTIONS IN MONOLAYER CULTURES OF EMBRYONIC SPINAL CORD AND SKELETAL MUSCLE CELLS

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Most of the previous work on the formation of nerve-muscle connections has been done either in whole embryos (1–3) or in organ cultures of tissue fragments (4, 5). As a step towards studying innervation mechanisms at the level of single cell interactions we have recently determined that neuromuscular junctions can develop in monolayer cultures of mixed spinal cord and skeletal muscle cells from chick embryos (6). The present report extends these observations to longer term cultures of these cells and presents evidence suggestive of the formation, in such cultures, of twitch-type ("en plaque") nerve-muscle contacts, in addition to the bulbous nerve endings ("en grappe") described in the previous report.

METHODS

Suspensions of myogenic cells were obtained from thigh muscles of 12 day chick embryos; nerve cell suspensions were prepared from the ventral half of the spinal cord of 6 day chick embryos. The tissues were dissociated with trypsin and dispersed into single cells according to the standard procedures of this laboratory (7). The myoblasts were dispersed in culture medium (Eagle's basal medium with glutamine, 10% horse serum, 10% embryo extract, and 1% penicillin-streptomycin [Microbiological Associates, Inc., Bethesda, Md.]) and distributed into 35 mm plastic culture dishes (Falcon Plastics, Los Angeles), each with 3 round glass-coverslips on the bottom, at a concentration of 5×10^4 cells per dish. After 2 days, 10^5 spinal cord cells suspended in the above culture medium were added to each culture dish. The coverslips were precoated with silicone, then covered with evaporated carbon (8) and overlaid with collagen (9). All cultures were maintained at 37°C in an atmosphere of 5% CO_2 in air, at saturation humidity. The culture medium was changed every 2 days. At the termination of culture, coverslips with the adhering cells were fixed in 10% formaldehyde (freshly prepared from paraformaldehyde) in 0.1 M phosphate buffer (pH 7.4), impregnated with silver according to a modified Richardson technique (10), and examined by light microscopy. To examine cross sections of nerve-muscle cultures, the cultures were processed by the same procedures as used previously for electron microscopy (8). Sections were cut at 0.5 μ with an LKB 4800 Ultrotome ultramicrotome and were stained with Toluidine blue.

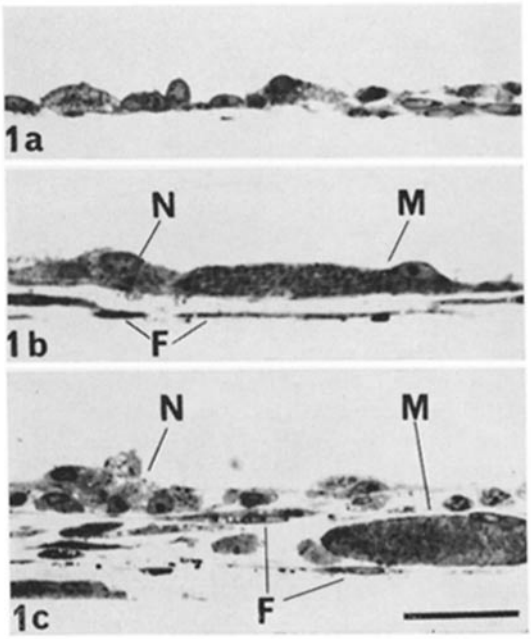

FIGURE 1 Three light micrographs of transverse sections through nerve-muscle cultures after varying times in vitro. Toluidine blue stained. × 580. Calibration bar = 100 μ. 1 *a*, 6 day culture. There is very little stratification of the cells at this time. 1 *b*, 8 day culture. A well delineated fibroblastic (F) layer underlies the culture. Nerve cells (*N*) are often closely applied to the muscle cells (*M*), yet the flattened muscle nuclei can be readily distinguished from the spherical nuclei within the neurons. 1 *c*, 11 day culture. Stratification of the culture is prominent at this time. Fibroblasts (*F*) separate clusters of neuronal cell bodies (*N*) from the muscle fibers (*M*).

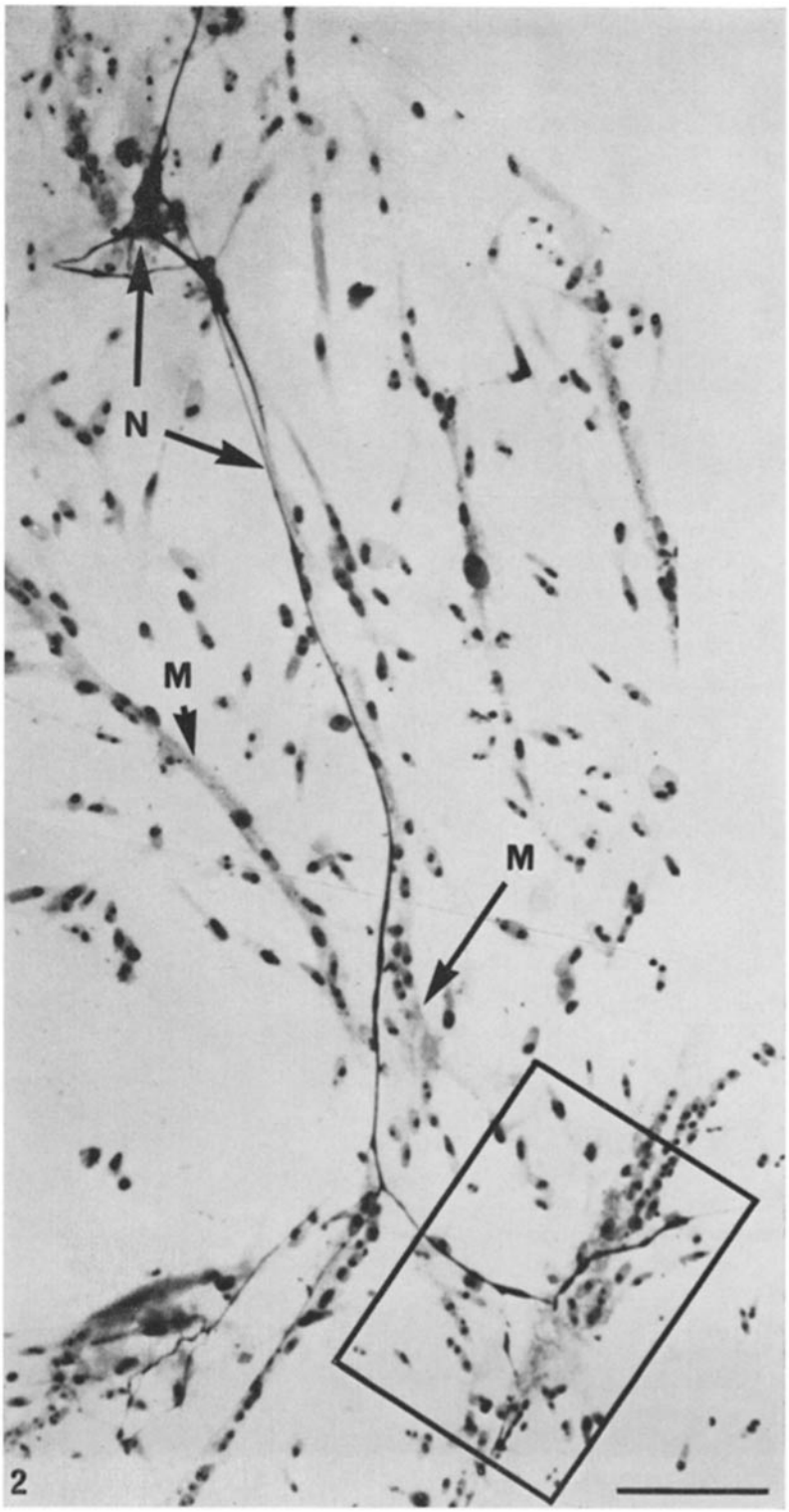

FIGURE 2 A silver stained whole-mount preparation of a nerve-muscle (*N-M*) culture 6 days in vitro. Nerve fibers (*N*) run along the upper surface of the myotubes (*M*), often branching near the intersection of two myotubes. Within the squared-off region multiple bulbous nerve endings are in contact with a myotube. × 390. Calibration bar = 200 μ.

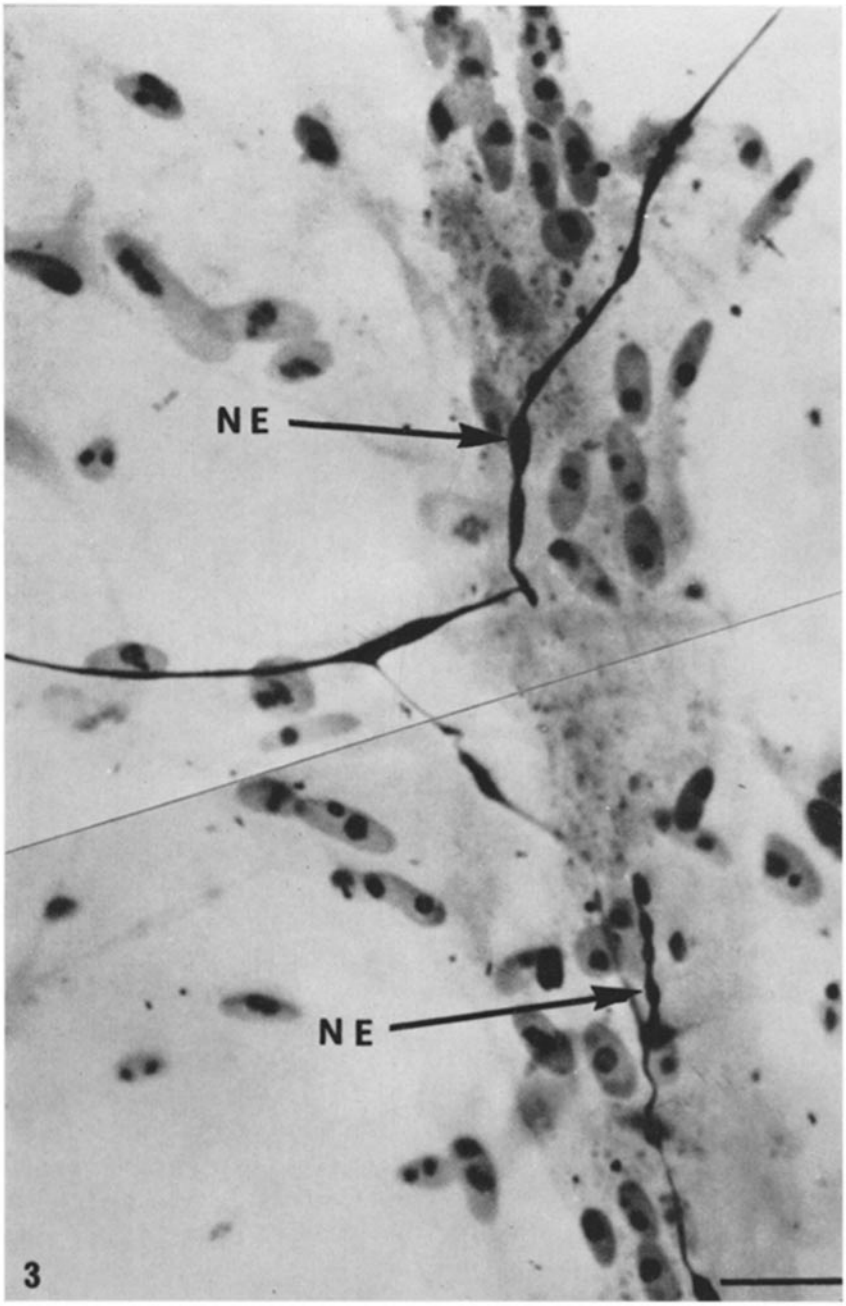

FIGURE 3 An enlarged montage of the squared-off region in Fig. 2. Note the bulbous swelling of the nerves (*NE*) on the myotube. × 1200. Calibration bar = 30 μ.

RESULTS

The freshly dissociated muscle cells settled rapidly out of suspension, attached to the substrate, and within hours assumed elongated shapes. During the first 2 days in culture the myoblasts exhibited prominent mitotic and migratory activity. Fusion of myoblasts and beginning of myotube formation were observed already after 48 hr in vitro as

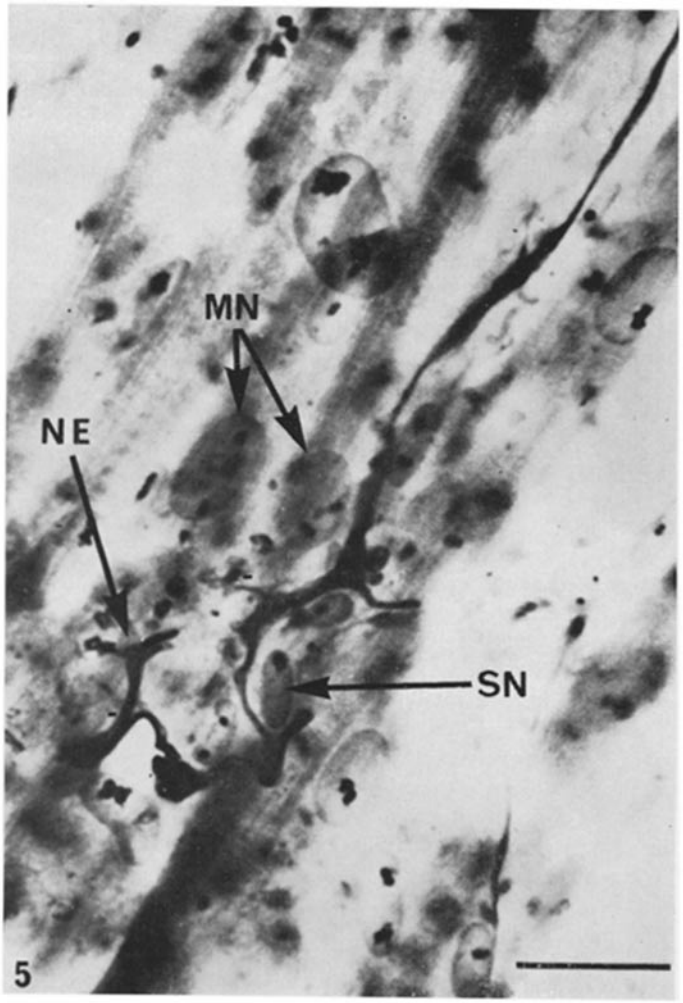

FIGURE 4 A silver stained whole-mount *N-M* preparation 20 days in vitro. As in the 6 day culture, nerves (*N*) send out branches which course along the muscle cells. Within the squared-off region a well-developed nerve ending (shown at higher magnification in Fig. 5) is seen. × 180. Calibration bar = 100 μ.

FIGURE 5 An enlargement of the rectangle in Fig. 4. Rather than the bulb like endings seen in the 6 day *N-M* cultures, the nerve endings (*NE*) after 20 days appear as complexly branched, finger-like projections directly applied to the muscle. Small oval nuclei (*SN*) which can be distinguished from the flattened muscle nuclei (*MN*) are often associated with the nerve terminal. These small nuclei are tentatively identified as Schwann cell nuclei. × 1200. Calibration bar = 25 μ.

described by Bischoff and Holtzer (11). At this time dissociated ventral spinal cord cells were added to the muscle culture. The settled neural cells sent out extended cytoplasmic processes which formed, within a week, an irregular network of branched argyrophilic fibers coursing over the myotubes. Frequently the nerve fibers were aligned parallel to myotubes or followed rows of fibroblastic cells, as if guided by them. It is of interest that although these cultures started out as monolayers of cells, they soon became several cell layers thick and thus did not exhibit "contact inhibition" of movement in the usual sense of that term (12). In general, the multilayered areas (Fig. 1) consisted of the following morphologically distinct cell layers (from the coverslip upward): 1) fibroblastic cells (*F*); 2) muscle (*M*); *3*) fibroblastic cells; 4) neural elements (*N*).

In silver impregnated preparations the nerve cells and their processes could be clearly discerned from other cellular elements. Along the length of the nerve fibers irregular thickenings were observed at points of contact with the myotubes or myofibers. Fig. 2 shows a 6 day nerve-muscle culture with an axon that extends along the myotubes, branches near the terminal, and has many bulblike swellings (squared-off area, Fig. 2) at points of contact with the muscle elements. In Fig. 3 the nerve endings in Fig. 2 are shown at a higher magnification. The nuclei in the immediate vicinity of the nerve endings were in the underlying muscle cells; no cells which could be identified as glial or Schwann cells were observed at the axonal terminals at this stage of development.

After 20 days (Fig. 4) the nerve-muscle cultures abounded in cross-striated muscle fibers and contained nerve fibers with many complex structures of axonal terminals in contact with the myofibers (squared-off area, Fig. 4). Fig. 5 shows such a nerve terminal at a higher magnification. Rather than the "bulb-type" nerve endings seen in the 6 day cultures, the axonal terminals after 20 days in vitro possess histological characteristics more typical of an "en plaque" ending (13, 14). In these later cultures small, oval nuclei are seen near the axon which are different in appearance from the flattened muscle nuclei and which may be Schwann cell nuclei.

Electron microscopic studies of these nerve-muscle contacts are in progress and initial results were presented elsewhere (6); these studies demonstrated that the intercellular contacts possess structural characteristics of a neuromuscular synapse.

Control experiments consisted of monolayer cultures which contained only spinal cord cells and no muscle cells. In these cultures, no bulblike endings or terminal plates were found of the kind present in cultures with muscle cells, supporting the possibility that such intercellular junctions are specific nerve-muscle contact differentiations.

Previous histological studies (15, 16) have shown that the morphology of motor nerve endings differs, depending upon the type of muscle (phasic or tonic) which is innervated. The nerve-muscle culture system described here lends itself to detailed studies along these lines; it should be possible to study the frequency and type of innervation in various combinations of nerve and muscle cells from different embryonic regions and thus examine problems of specificity and recognition during neuromuscular development.

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Note Added in Proof: While this manuscript was in press, two papers appeared (Nakai, J. 1969. *J. Exp. Zool.* **70**:85; Veneroni, G., and M. R. Murray. 1969. *J. Embryol. Exp. Morphol.* **21**:2, 369) in which structures similar to those described by us were demonstrated to develop in organ cultures of spinal cord and skeletal muscle fragments and identified as neuromuscular junctions by phase-contrast microscopy, silver impregnation, and acetylcholinesterase staining.