Synaptic Potentials Recorded in Cell Cultures of Nerve and Muscle

Abstract. *Initially dissociated spinal cord and muscle cells derived from chick embryos differentiate sufficiently in tissue culture to form functional synaptic contacts. Spontaneous and evoked potentials recorded with intracellular microelectrodes resemble synaptic responses of adult spinal cord and neuromuscular junctions.*

Anatomic evidence has been presented for the formation in vitro of synaptic contacts between initially dissociated embryonic spinal cord neurons, between spinal cord neurons and limb muscle cells, and between dissociated retinal cells (*1*, *2*). Crain and Peterson (*3*) have demonstrated that newly formed synapses (*4*) between explanted slices of spinal cord and adjacent myotomes maintained as organ cultures are capable of transmitting impulses. The data presented here show that the contacts between the initially dissociated cells are also functional.

The methods of preparation for cell cultures of muscle and muscle plus spinal cord were similar to those reported in detail by Stockdale and Holtzer (*5*) and Shimada *et al.*, respectively (*2*, *6*). Myogenesis proceeded exactly as described by others who used the same culture system (*7*). Cross-striated, twitching fibers measuring 50 to 100 μm in diameter were abundant after 7 to 10 days and remained viable for as long as 8 weeks. Spinal cord cells, small and spherical when plated (Fig. 1a), developed, within 24 hours, processes that extended up to 100 μm. After 1 week in vitro, several morphological types of cells were evident. Relatively large, polyg-

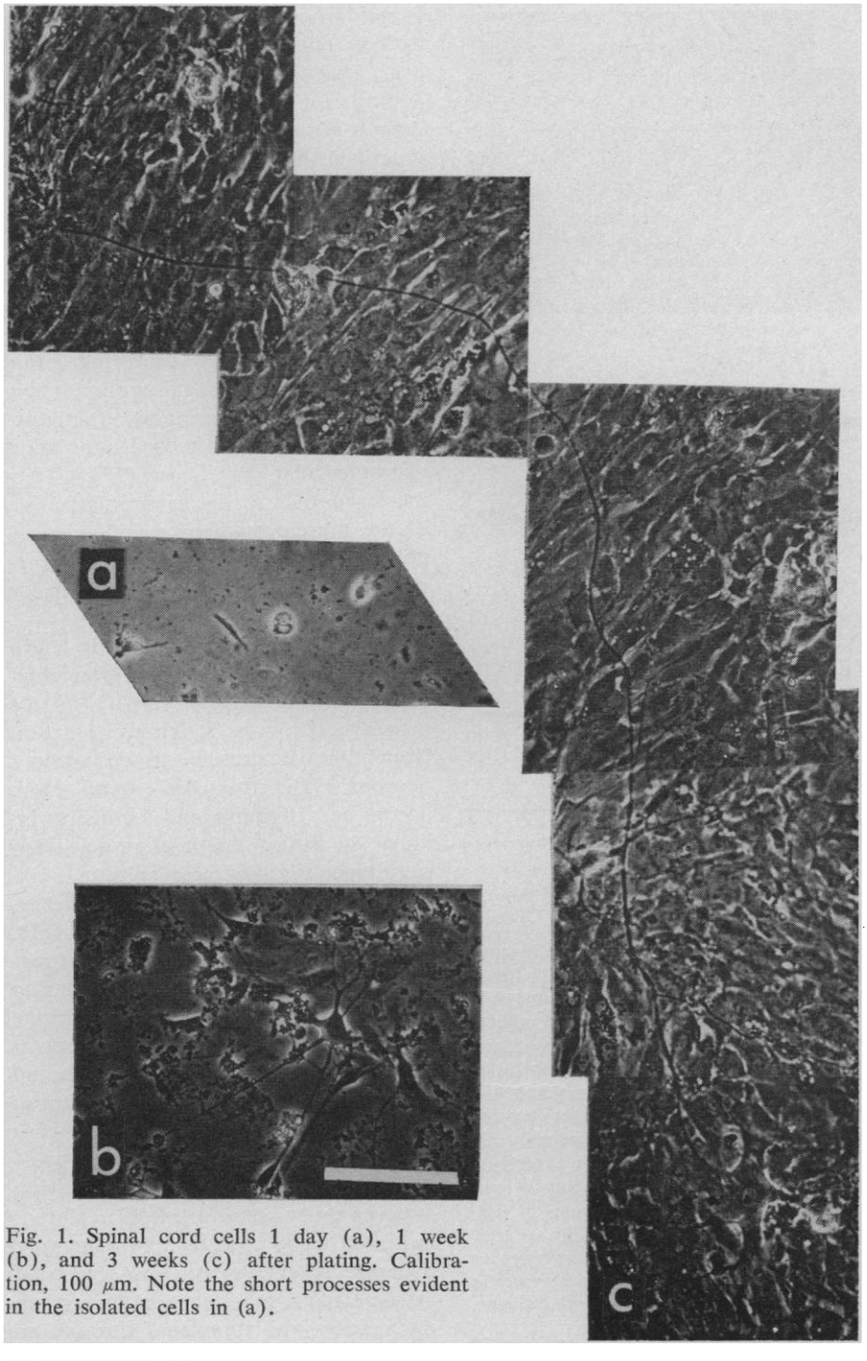

Fig. 1. Spinal cord cells 1 day (a), 1 week (b), and 3 weeks (c) after plating. Calibration, 100 μm. Note the short processes evident in the isolated cells in (a).

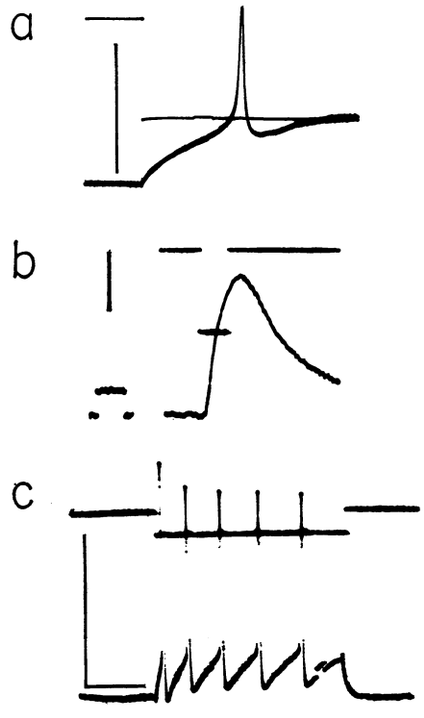

Fig. 2. (a) A muscle action potential (lower trace) is generated when the depolarization induced by a step of outward current (upper trace) reaches threshold. Vertical bar, 50 mv. (b) Muscle membrane response to iontophoretically applied acetylcholine (ACh). (Upper trace) Current pulse passed through the ACh pipette; vertical bar, 20 na; pulse duration, 50 msec. (Lower trace) Membrane potential; square pulse, 2 mv, 50 msec. (c) Repetitive action potentials recorded from a neuron, triggered by a prolonged pulse of outward current. Calibration bars, 50 mv and 20 msec. Note that the current traces do not represent the zero membrane potential level.

onal cells with at least one long process and a perinuclear ring of phase-dark material were particularly striking (Fig. 1b). They continued to increase in size over the next 1 to 2 weeks (Fig. 1c); eventually, after 4 to 8 weeks, they rounded up and detached from the culture surface.

The electrical properties of the cells were measured with conventional intracellular microelectrode techniques (*8*). The membrane parameters of developing muscle will be described in detail later (see *9*). The fibers studied in these experiments exhibited membrane potentials of 40 to 70 mv, were electrically excitable, and were sensitive to iontophoretically applied acetylcholine (Fig. 2, a and b). The membrane potentials of neurons, distinguished from other cells in the spinal cord cultures by their large size and distinctive processes and, ultimately, by their ability to generate action potentials, ranged from 20 to 60 mv. Many neurons responded to prolonged depolarizing pulses of current with repetitive action potentials (Fig. 2c).

In the spinal cord cultures, subthreshold, spontaneously occurring potentials, which resembled synaptic potentials recorded from adult spinal motoneurons (*10*), were recorded from neurons which appeared under phase-contrast microscopy to be in contact with other cells (Fig. 3, a and b). Similar potentials were never detected in cells growing in isolation. On several occasions, synaptic potentials could be evoked by passing depolarizing current pulses through a second microelectrode inserted in a nearby, "contacting" neuron (Fig. 3c). The evoked responses occurred after a delay of 1 to 2 msec. Direct electrotonic coupling between neurons has not been observed, but a more detailed search is required.

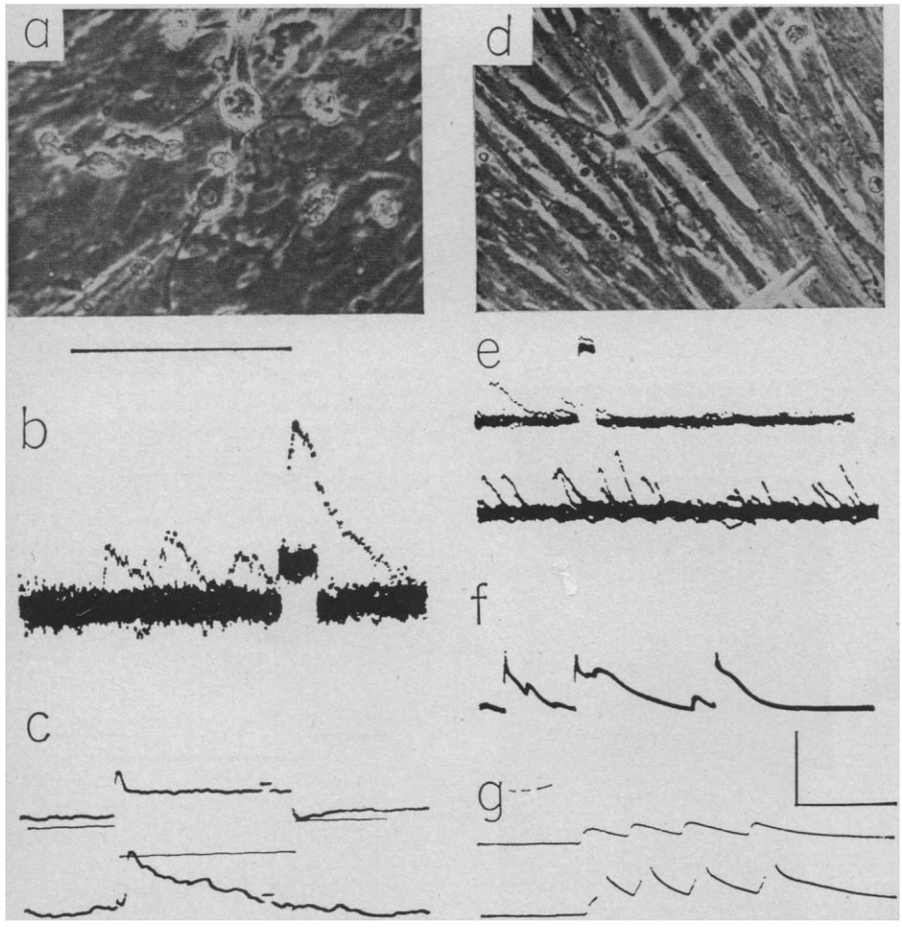

Fig. 3. (a) Adjacent neurons in a 25-day-old culture. (b) Superimposed oscilloscope traces showing four spontaneous, depolarizing potentials recorded from the lower neuron of the pair shown in (a). The calibration (2 mv, 5 msec) appears on each sweep. (c) An outward current pulse (middle trace) applied through a second microelectrode placed in the adjacent neuron that produced an active response in that cell (upper trace) also resulted, after a 1- to 2-msec delay, in a large synaptic potential in the lower neuron (lower trace). Calibration, 2 mv, 5 msec. Note that, although the active response recorded in the soma of the upper neuron was not an overshooting spike, the postsynaptic response was two to five times larger than the spontaneously occurring potentials recorded in the same cell (a). (d) An example of a neuron overlying a muscle fiber in a 10-day culture. (e) Superimposed traces showing spontaneous potentials at a similar nerve-muscle contact. Calibration, 2 mv, 5 msec. (f and g) Examples of larger spontaneously occurring potentials at nerve-muscle junction in another culture. Calibration of 50 mv, 50 msec applies to both (f) and (g). The two traces in (g) are segments of a continuous record from one muscle cell. The postsynaptic potentials occasionally triggered spikes evident as discontinuities in the potential traces. Note the regular pattern of synaptic potentials in (g).

Approximately 1 week after dissociated spinal cord cells were added to young myotubes, many cells could be found whose processes contacted or overlay muscle fibers (Fig. 3d). However, despite the 2- to 4-day delay in adding spinal cord cells to muscle, a definite tissue interaction similar to that described by Shimada (*11*) was observed; individual myotubes did not become as large or as obviously striated in areas where spinal cord cells were abundant. Nevertheless, synaptic potentials could be detected in these muscle fibers. Figure 3e shows examples of apparently randomly occurring rapid potential fluctuations of approximately 0.5 mv which resemble miniature end plate potentials recorded at adult neuromuscular junctions (*12*). They increased in size on hyperpolarization of the muscle membrane and reversed polarity when the membrane was depolarized to about +10 mv (inside positive).

In some combined cultures, in which the muscle development was more complete, the mechanical activity of the muscle cells was strikingly different from that of muscle grown without neurons. The twitches were more abrupt and rhythmic and seemed to involve the entire length of muscle fiber simultaneously. In these cultures, relatively large synaptic potentials were detected (Fig. 3f) which, in some cells, occurred in definite patterns and at regular intervals (Fig. 3g). On occasion they exceeded the muscle membrane threshold and generated action potentials (Fig. 3, f and g). The pattern and magnitude of these potentials suggest that they resulted from spontaneous discharges of the innervating neuron.

Functional synapses did not form between every nerve or muscle cell that appeared to be contacted by a neuron. Synaptic potentials were not recorded at some nerve-muscle pairs even when the neuron was stimulated through an

extracellular electrode with current strengths sufficient to activate the adjacent muscle fibers. Such "failures" might reflect a nonspecific tissue antagonism, immaturity, or inexcitability of the neurons. A more interesting alternative is that the synapses between neurons and those between neurons and muscle exhibit the same type of specificity demonstrated in the central nervous system (*13*).

In sum, isolated nerve and muscle cells, plated in cell cultures at relatively low densities, differentiate sufficiently to exhibt action potentials and to form chemically mediated synapses. The occurrence of synaptic potentials is consistent with the electron microscopic description of nerve-muscle contacts in a nearly identical culture system by Shimada *et al.* (*2*). The direct visualization of both pre- and postsynaptic elements in this culture system should permit a detailed study of the ontogeny and specificity of synaptic relations between cells.

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References and Notes

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6. Small fragments of pectoral muscles of 11-day chick embryos were exposed for 30 minutes to 0.25 percent trypsin in a balanced salt solution free of Ca^{2+} and Mg^{2+}, mechanically agitated in complete culture medium (see below), and filtered through a double layer of lens paper. The resulting mononucleated cells were plated in collagen-coated [M. Bornstein, *Lab. Invest.* **7**, 134 (1958)], 60-mm plastic petri plates at densities ranging from 100,000 to 500,000 cells per plate. Spinal cord cells prepared in an identical manner from minced fragments of whole cords of 7-day embryos were either plated in separate dishes or added to the muscle cultures 2 to 4 days after the muscle was plated, a time when young myotubes predominated. The cells were maintained at 37°C in a medium consisting of Eagle's minimum essential medium, horse serum, embryo extract, and a mixture of penicillin and streptomycin (in a proportion of 8 : 1 : 1 : 0.1) which was changed every 2 to 3 days. The medium was equilibrated with a mixture of 95 percent air and 5 percent CO_2.
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8. The cultures were mounted on the stage of an inverted phase-contrast microscope, and one or two KCl-filled microelectrodes which measured 20 to 50 megohm were introduced from above. Each microelectrode was connected in a bridge circuit such that it could be used simultaneously for transmembrane recording and stimulation. The stimulus was applied through a 10^9-ohm resistor in series with the microelectrode. During the recording periods, the medium was equilibrated with 5 percent CO_2 and maintained at 35° to 37°C.
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Turnover of the Brain Specific Protein, S-100

Abstract. *Rats were killed after intraventricular administration of [^{3}H]leucine, and the turnover rates of total soluble proteins and of the brain specific S-100 protein were determined. The half-life of S-100 was estimated to be 16 days. The S-100 protein, thus, has a turnover not unlike that of the average water-soluble protein of brain.*

The S-100 protein has been found in an immunologically cross-reacting form in the brains of all vertebrates and invertebrates examined (*1*). The fact that S-100 is present only in the nervous system but in no other organ (*2*), taken in conjunction with its apparent evolutionary stability, suggests that the protein may have an important, as yet undetermined, role in the function of the nervous system. Evidence obtained by several independent methods has also established that S-100 is localized predominantly, if not exclusively, in glial cells within the central nervous system (*3*). In an attempt to further characterize this glial protein, we have determined the turnover rate in vivo of S-100. Although it has been suggested that the S-100 protein turns over extremely rapidly in the brain (*4*), our work indicates a much slower turnover rate.

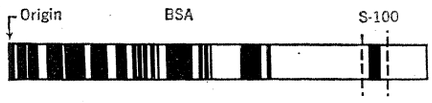

Fig. 1. Polyacrylamide-gel (7.5 percent) electrophoretic pattern of the DEAE-cellulose fraction containing the S-100 protein. The dotted line indicates the portion of the gel which was used to determine the specific activity of the S-100 protein.

Each of 14 male Holtzman rats, approximately 35 days of age, was stereotaxically implanted, under Nembutal anesthesia, with a lightweight cannula in the lateral ventricle according to standard stereotaxic procedures (*5*). Animals of this age were selected because the biosynthesis of the S-100 protein increases rapidly from birth to approximately 50 days in the developing rat brain (*1*), and we were thus assured of a relatively high labeling of the protein. At the end of a 5-day postoperative recovery period, the unanesthetized animals were infused with 200 μc of [4,5-^{3}H]leucine (New England Nuclear) in 100 μl of 0.15*M* NaCl over a 30-minute period by means of a chronic infusion system (*5*). Rats were killed by decapitation either at 5 hours, 24 hours, 48 hours, 7 days, 14 days, or 28 days after infusion. In all cases the brains were removed within 1 to 1½ minutes, quickly frozen on a block of dry ice, and stored at −80°C. Immediately before chemical analysis the brains were thawed, and the placement of each cannula in the lateral ventricle was grossly determined by sectioning the brain through the cannula track. The data reported here are based only on those animals in which the cannula appeared to rest in the lateral ventricle.

The brains were then weighed and homogenized in glass hand-operated homogenizers in ten volumes of the cold barbital-NaCl buffer which was used in subsequent complement-fixation assays (*6*); the homogenate was centrifuged at 25,000 rev/min for 90 minutes. The supernatants were saved, and total soluble proteins were determined by the method of Lowry *et al.* (*7*). Radioactive label in total soluble proteins was determined by precipitating the proteins in 0.3 ml of the supernatants with trichloroacetic acid (TCA) and then centrifuging at 2000 rev/min. The supernatants were discarded, and the precipitate was resuspended in 5 percent TCA and centrifuged again. Only one TCA washing was necessary since total radioactivity in the supernatant was negligible at this point. The resulting precipitate was then completely dissolved in 0.5 ml NCS (Nuclear-Chicago Solubilizer) reagent, 10 ml of toluene-base scintillation liquid was added, and the samples were counted in a Packard Tri-Carb scintillation counter.

The remainder of the original brain supernatants were chromatographed on a diethylaminoethyl (DEAE)-cellulose