Table 1. Effects of corticosterone and ACTH on firing rates of single units in the dorsal hippocampus of female rats.

Rats	Experiments (No.)	Effect on firing rate		
		Increase	Decrease	No change
Corticosterone				
Anesthetized	14	1	11	2
Telemetered, freely moving	9	1	7	1
ACTH				
Anesthetized	9	6	1	2
Telemetered, freely moving	8	4	0	4

Table 2. Effects of corticosterone and ACTH on cortical EEG of anesthetized female rats.

Hormone injected	Experiments (No.)	Hormone effect		
		Synchronization	Desynchronization	No change
Corticosterone	16	8	1	7
ACTH	8	1	1	6

(Table 1). Since both kinds of preparations gave similar results, it is unlikely that the effects recorded in the freely moving rats came from movement or other artifacts to which chronic recording is usually susceptible, and it is also unlikely that the effects of the hormones on hippocampal neurons are secondary to effects on behavioral activity. Histological examination showed that, in both acute and telemetered recording experiments, effects of corticosterone and ACTH were recorded from sites in the pyramidal layer and the dentate gyrus of the dorsal hippocampus.

These results indicate that ACTH and corticosterone have independent effects on hippocampal activity. Since the corticosterone effect was observed in hypophysectomized ($N = 12$) as well as intact animals ($N = 6$), it evidently was not mediated by inhibition of pituitary ACTH release. Similarly, the ACTH effect was not mediated by secretion of corticosterone because the ACTH effect was opposite to that of corticosterone, and because animals such as we used, hypophysectomized long before the experiments, secrete little steroid after a single injection of ACTH (*5*). Although steroids other than corticosterone were not used in these experiments, the pattern of uptake of radioactive hormones suggests the possibility of partial specificity: the hippocampus shows higher uptake of corticosterone than any other brain region (*1*), whereas the uptake of sex steroids is higher in the preoptic area and hypothalamus (*6*).

Our electrophysiological data fit well with the recent suggestion that ACTH has excitatory effects in the central nervous system and that corticosterone exerts the opposite influence, to counteract this excitation (*5*). We do not yet know the degree of specificity of these effects within the brain, but we have begun exploration of other anatomical locations in anesthetized animals. In nine experiments on the cerebral cortex, thalamus, and midbrain reticular formation only one corticosterone effect on unit activity has been recorded (decreased activity of a midbrain unit). However, injections of corticosterone were often followed by long periods of synchronized cortical EEG, sometimes accompanying the effect on hippocampal unit activity (Table 2).

The sensitivity of the hippocampus to hormonal influence is in keeping with other indications that this structure is affected by, and also regulates, pituitary-adrenal function. Radioactive corticosterone is concentrated more highly in the hippocampus than in other brain regions and shows nuclear uptake in hippocampal cells (*1*). Moreover, electrical stimulation of dorsal hippocampus can decrease basal concentrations of adrenal corticosteroids in the blood (*7*), whereas implantation of corticosteroids or lesions in the hippocampus are followed by increased concentrations (*8*). Although the relations between hippocampal neuronal firing and hormone secretion may not be simple or direct, these effects of stimulation, implantation, and lesions are consistent with the notion that corticosterone reduces dorsal hippocampal activity, which is in agreement with our results.

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Developing Neuromuscular Junctions: First Signs of Chemical Transmission during Formation in Tissue Culture

Abstract. *Electrophysiological study of rat neuromuscular junctions in the early stage of formation in tissue culture showed that chemical transmission begins with discrete, localized release of transmitter about the time when nerve-muscle contacts are first visible with light microscopy. Noncontractile myotubes with as few as three nuclei showed evidence of junctional transmission.*

The earliest form of chemical transmission in developing chemical synapses is unknown. In a classic case, the rat neuromuscular junction, nerves are present in developing muscle by 14 days in utero (*1, 2*), and muscle contraction in response to reflex or nerve stimulation begins at 15½ to 16 days (*3*). Studies with intracellular recording have shown that there is discrete synaptic release of transmitter at 17 days and thereafter (*4*), but there are no comparable studies before 17 days. It is unknown whether the earliest nerve-muscle contacts are functional synapses (*2, 5*) or whether the first form of neurochemical excitation of muscle occurs by long-range diffusion of transmitter (*6*). Moreover, it

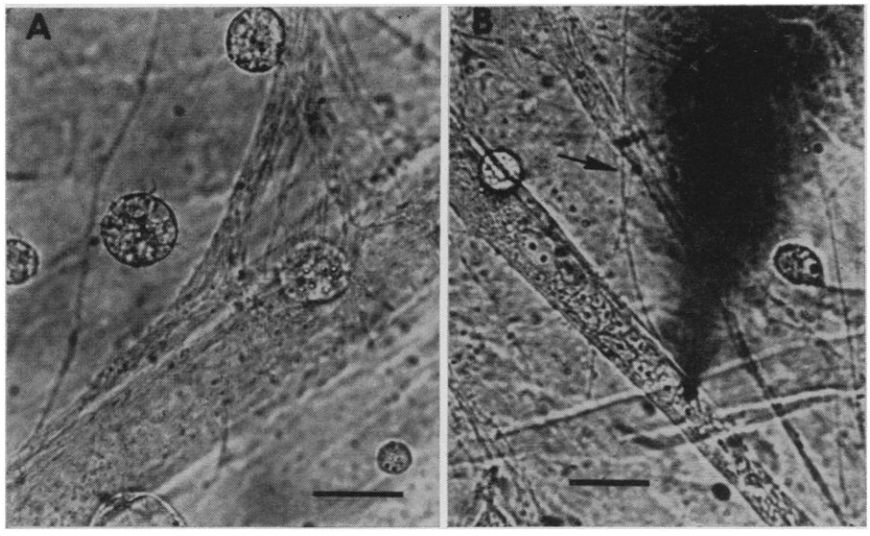

Fig. 1. Photomicrographs of early neuromuscular contacts formed in tissue culture. (A) Six days in vitro, formed from 14-day fetal spinal cord and trypsinized muscle from 2-day neonate. Marker, 25 μm. (B) Seven days in vitro, formed from initially separate explants of spinal cord and muscle from 12- to 13-day rat embryo. Marker, 25 μm. Arrow indicates nerve fiber going to multinucleated myotube impaled by a microelectrode (dark shadow coming to a point). The granular change due to muscle cell injury serves to highlight the nuclei.

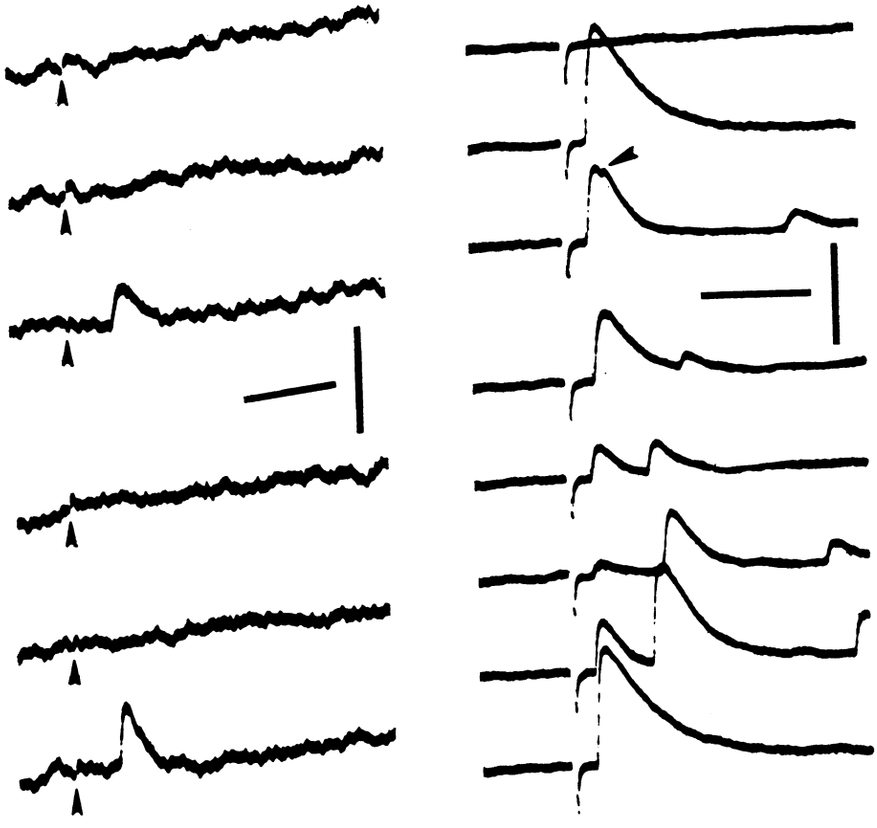

Fig. 2. (Left) Samples of intracellular record from a three-nuclei myotube in same culture as in Fig. 1B, during one-per-second stimulation of spinal cord (stimulus artefacts marked with arrows). Upper and lower three traces are sequences selected from a long series to show infrequent positive responses. Markers, 50 msec and 3 mv. (Right) Intracellular record from myotube with 10 to 11 nuclei. Upper three and lower four traces are sequences at different times during nerve fiber stimulation at one per second (downgoing stimulus artefact). At arrow, double-peaked excitatory junctional potentials, probably a response to activity in two adjacent nerve endings. Markers, 25 msec, 5 mv.

is unclear at what stage the developing muscle is capable of responding to transmitter.

Tissue culture presents a favorable situation in which to investigate these problems. Nerve-muscle junctions have been visualized during initial formation and development in culture (*7*), but functional neuromuscular synapses have been demonstrated only at later stages of in vitro development (*8*). In this study we have used tissue culture and intracellular recording technique to elucidate *early* events in synaptogenesis, about the time when growing nerve endings first contacted developing muscle. We found that chemical transmission from its onset was discrete and short-lived, qualitatively similar to the adult, and was in some cases established in immature noncontractile myotubes.

Fragments of muscle and spinal cord were taken from 11- to 19-day rat embryos, placed 0.5 to 2 mm apart on collagen-coated cover slips, and cultured at 37°C in Maximow assemblies (*9*). In two instances, dissociated newborn muscle was added to cultures of 11-day embryonic spinal cord. The cultures were frequently examined under the microscope, and used for physiologic experiments within hours or 1 to 2 days of the time when nerve fibers growing from the spinal cord had first made contact with muscle cells. At this time (4 to 10 days in vitro) the cover slips were removed, clamped in a 1.5-cm^3 chamber fitted to an inverted microscope, and slowly perfused with a mixture of 90 percent Gey's balanced salt solution and 10 percent horse serum, at room temperature (24° to 27°C). Only those areas of the culture containing clearly visible nerve endings and muscle cells were studied.

Stimulation of spinal cord fragment or nerve fiber and intracellular recording from muscle was carried out with conventional macro- and microelectrode techniques (*10*). Rat muscle cells in culture were small (usually 4 to 10 μm across) and flat, so that microelectrode penetrations were often traumatic. Nonetheless, excitatory junctional potentials (EJP's) (*11*) could be recorded when the resting membrane potential was 12 mv or more. Cells with lower membrane potential were rejected. The cells reported below had membrane potentials ranging from 12 to 46 mv, most 16 mv or more. Within any class of acceptable muscle cells, there was no

obvious correlation between presence or absence of EJP's and membrane potential. Furthermore, since the mean resting potential of rat muscle at birth is only 23 mv (*12*), the low values found in newly developed muscle in tissue culture could be representative. Cells were considered functionally innervated if stimulation of nerve or spinal cord produced contraction, or if EJP's (spontaneous or evoked by nerve stimulation) were found during intracellular recording; EJP's were never found in cells clearly devoid of nerve contacts.

The development of nerve-muscle contacts in vitro as seen through the light microscope (resolution about 0.5 μm) was essentially as described by Peterson and Crain (*5*). One or two 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 (these were not studied further). As early as 3 to 4 days in vitro, nerve fibers growing out from the spinal cord contacted myoblasts or myotubes. Before making contact, the nerves divided into either delicate weblike projections (Fig. 1A) or thick branches (Fig. 1B). This report concerns only young cultures chosen at a stage when contacts were rudimentary and when neither myelination nor teloglial cells were present. These cultures were clearly destined to develop further, since companion cultures from the same embryo at later times in vitro showed more mature nerve, muscle, and junctions, as described by Peterson and Crain (*5*).

Muscle cells were studied electrophysiologically in 19 cultures made from embryos taken from seven pregnant rats. Spontaneous or stimulus-evoked EJP's were found in only three out of 13 muscle cells with one to five nuclei and with one or more neural contacts. Two of the responsive cells had four or five nuclei, but the third (see Fig. 2, left) was a myotube with just three nuclei. On the other hand, EJP's were found in more than half of about 50 nerve-contacted myotubes with eight or more nuclei (Fig. 2, right). The EJP's fluctuated greatly in amplitude during one-per-second stimulation of spinal cord or nerve, with intermittent failures of response (top trace in Fig. 2, right). In adult nerve-muscle junctions, similar fluctuations and failures appear when mean quantum content of transmitter is reduced (*13*). Evidence will be presented elsewhere (*14*) showing that transmitter release at developing junctions is quantal and that quantum content increases with development in vitro, although the presence of multiple innervation (arrow in Fig. 2, right) complicates the analysis.

The transmitter in early junctions was probably acetylcholine, since in a few cases tested, *d*-tubocurarine completely eliminated EJP's. In all nerve-muscle contacts studied, chemical transmission appeared only in the form of EJP's whose shortest rise and half-fall times were 2.3 and 3.5 msec, respectively. There was no preceding stage in which stimulation of nerve produced long-lasting postsynaptic depolarization. Furthermore, the response to transmitter release was confined to innervated cells. No responses were detected in immediately adjacent noninnervated cells, even though in separate experiments noninnervated myotubes were found to be sensitive to iontophoretically applied acetylcholine (*14*). Finally, in several cases where visible early nerve-muscle contacts were present without EJP's, signal averaging (over 30 repetitions) synchronized with spinal cord or nerve stimulation did not reveal any postsynaptic response, presumably because appreciable transmitter release was absent or because nerve conduction failed even with strong stimulation (currents of 100 μa, 1 msec in duration). A likely interpretation is that these nonresponsive contacts were prefunctional and that cells with EJP's represent a later stage in synaptic development.

Contractility and functional transmission developed independently. Noncontacted myotubes occasionally contracted spontaneously or on direct stimulation. Other innervated myotubes contracted weakly when EJP amplitudes reached threshold for action potential generation. Still other innervated myotubes showed spontaneous or evoked EJP's before they were capable of contracting on either direct stimulation or with indirect stimulation giving rise to muscle action potentials.

This, to our knowledge, is the first description of function of a developing chemical synapse about the time it initially becomes active. The earliest chemical responses to nerve stimulation were brief "excitatory junctional potentials" restricted to the innervated cell, implying close apposition of nerve and muscle. Correlated electron microscopic studies will be necessary to establish the minimum structural requirements for junctional function, especially since nonfunctional contacts were also observed. The large fluctuation of EJP amplitudes and the failures of response suggest quantal release of transmitter with low mean quantum content by a mechanism similar to the mature neuromuscular junction.

Our results show that in tissue culture young noncontractile myotubes with as few as three nuclei can participate in chemical synaptic transmission. Although the timing of events may differ, morphologic development of neuromuscular junctions appears to be similar in vitro and in vivo (*15*). Thus it is likely that functional junctions of the kind described above form in vivo shortly after growing nerve fibers meet developing muscle and before the appearance of the indirect twitch. Our results do not entirely exclude the possibility that still younger muscle cell types receive functional innervation, nor do they rule out other possible forms of early nerve-muscle interaction, such as transfer of informational molecules or electrical transmission at tight junctions. However, since bare nerve endings are functionally active at a stage when Peterson and Crain, using similar cultures, first noticed neural enhancement of muscle development (*5*), the release of transmitter or some concomitant event could be responsible for this enhancement.

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balanced salt solution. The Maximow assembly is a slide with a sealed cylindrical compartment especially designed for repeated microscopic examination of developing cultures. The successive layers of the compartment are glass wall, air, and the culture growing on a thin cover slip held against an outermost cover slip by a film of fluid.

10. Stimulating electrodes were glass pipettes with fire-polished orifices of 5 to 20 μm. Currents of 10 to 30 μa and less than 0.1 msec in duration were supplied from an isolation unit. Conventional glass microelectrodes filled with 3*M* KCl, with d-c resistances of 15 to 30 megohms and tip potentials of less than 2 to 3 mv, were used for intracellular recording. Potentials were led via a chlorided wire to a high-input impedance negative capacitance amplifier and were further amplified and displayed on a Nihon-Kohden oscilloscope. An agar salt bridge contacting a chlorided silver wire connected the chamber to ground. The stimulating electrode, held by a micromanipulator, was gently applied to the surface of either spinal cord or nerve fiber. With another micromanipulator, the recording electrode was guided into a muscle cell in the vicinity of a visible nerve-muscle contact.
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Behavioral Sensitivity to Microwave Irradiation

Abstract. *Rats assayed by the technique of conditional suppression were able to detect the presence of 12.25-centimeter microwaves at doses of power approximating 0.5 to 6.4 milliwatts per gram. The assay, which controlled for sensitization, for pseudo and temporal conditioning, and for several possible sources of artifactual cueing, revealed that irradiation by microwaves, although lacking the saliency of an auditory stimulus, can function as a highly reliable cue. Efficiency of detection was strongly and positively related to the amount of microwave energy to which the rats were exposed.*

Nearly a decade ago, Frey (*1*) reported that human beings can detect pulse-modulated electromagnetic energy at wavelengths of 10 to 70 cm and at average power densities of 0.4 to 2.1 mw/cm^2. The sensations reported were usually auditory in character and were often described as "hissing, buzzing, and clicking sounds." Although no confirmatory studies have been published since Frey's reports, three instances of verification have been communicated to him (*2*), and he has referred to successful use of microwave energy as a signaling stimulus in cats (*3*). Two directly related studies yielded negative results. Jones (*4*) reported that none of 20 college students could discriminate presence from absence of 30- or 60-cm microwaves. Justesen and King (*5*) intermittently presented 12.25-cm microwave energy to each of six rats as a cue for obtaining sugar water, but none of the rats discriminated the cue. Since unmodulated energy was used in Jones's study, its negative findings comport with Frey's belief (*2*) that modulation is necessary for perception of microwaves. Modulated energy was used by Justesen and King, but the assay for perception was based upon appetitive rather than aversive motivation and may have lacked sensitivity. Much indirect evidence of relevance to detection of microwaves has been published, particularly in the Soviet literature (*6*); altered thresholds to physiologically adequate stimulation have been reported as sequelae of microwave irradiation in olfactory (*7*), auditory (*8*), visual (*9*), and cutaneous (*10*) modalities. Other aftereffects include cardiovascular changes (*11–13*), irritability and irascibility (*11*), neurasthenia (*13*), and headache and disturbance of sleep (*11*). Acute responses observed during irradiation include changes of blood pressure (*14*), heart rate (*15*), and cortical and subcortical electrophysiological activity (*3, 16*).

Although the mechanisms responsible for chronic and acute changes are unresolved and much debated (*3, 17*), the evidence suggests that microwaves at densities below the safety limit of 10 mw/cm^2 observed in the United States (*18*) can affect nervous activity and could, therefore, possess stimulus properties. We report here attempts to assess in rats the efficacy and the reliability of modulated 12.25-cm microwaves as a warning stimulus for impending electrical shock.

Six male albino rats of common age were obtained from the Simonsen Company of Minnesota. Three randomly selected rats (R-1, R-2, and R-5) served as subjects for irradiation; control rats (R-3, R-4, and R-6) were never irradiated, but were maintained in their home cages with unrestricted access to Purina Lab Chow and water. Although irradiated rats had the same access to water, they were partially deprived of food until their individual weights fell to 75 percent of that before experimentation; a diet that led to 75 percent of normal gains in weight was thereafter instituted on the basis of data on weight gained by the control animals. Weights of R-1, R-2, and R-5 at the commencement of experimentation were, respectively, 409, 455, and 427 g.

A highly sensitive measure of conventional sensory stimulation, the technique of conditional suppression (*19*), was used. With this technique, a subject is reinforced after making an operant response; then reinforcement is scheduled intermittently until the response occurs frequently and consistently. Finally, a Pavlovian conditioning regime is superimposed in which a warning signal is presented from time to time, always terminating in a brief, but aversive, unconditional stimulus (US). After repeated presentations of the warning signal and the US, a subject will respond stably except when the warning signal is being presented; that is, operant behavior is conditionally suppressed. The operant response required of our rats was the tongue lick, which was detected photoelectrically and reinforced by discrete volumes (30 μl) of sugar water (dextrose, 16 g/100 ml). A radiolucent ensemble by which licks were detected and reinforced is described elsewhere (*20*). An aperiodic schedule of reinforcement was used during all experiments; the passage of each 2-second interval after reinforcement led to availability of another reinforcer with a probability (*P*) of .25, .125, or .0625. The *P* value was not changed during a given experiment, but was varied across experiments to maintain stable responding. The US was unavoidable electrical shock to the feet presented by a radiopaque floor-grid of aluminum rods (*21*). A conventional warning stimulus, with which microwave irradiation was compared for cueing efficacy, was a 525-hz tone,