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5 December 1988; accepted 3 March 1989

Synaptic Connections in Vitro: Modulation of Number and Efficacy by Electrical Activity

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The functional architecture of synaptic circuits is determined to a crucial degree by the patterns of electrical activity that occur during development. Studies with an in vitro preparation of mammalian sensory neurons projecting to ventral spinal cord neurons show that electrical activity induces competitive processes that regulate synaptic efficacy so as to favor activated pathways over inactive convergent pathways. At the same time, electrical activity initiates noncompetitive processes that increase the number of axonal connections between these sensory and spinal cord neurons.

PATTERNS OF NEURONAL ELECTRICAL activity play an important role in determining the development of appropriate functional synapses, as has been shown in studies on the vertebrate visual cortex (*1*). Activity-dependent changes in synaptic organization occur in many areas of the nervous system in response to sensory deprivation (*2*) or experimental regulation of neural activity (*3*). Data suggest that multiple afferents converging on a common target interact competitively, resulting in the weakening or elimination of "inappropriate" connections and the maintenance of functionally effective afferents (*4*). "Appropriate" connections are those provided from sets of neurons that fire so close to synchronously that they produce a critical state of activation of the target neuronal population (*5*).

In the present studies, part of the circuitry from the developing spinal cord was reconstructed in vitro, which made it possible to manipulate activity from sensory neurons to spinal cord neurons and measure intracellularly the strength of synapses from competing inputs. Neurons dissociated from 13-day fetal mouse spinal cords were cultured in a three-compartment tissue culture chamber (*6*) (Fig. 1). After approximately 1 week in culture, axons from dorsal root ganglion (DRG) neurons plated in the two side compartments penetrated the thin fluid space under the barrier to form synapses on ventral horn (VH) spinal neurons in the central compartment. Horseradish peroxidase injection and electrophysiological investigation gave no evidence of DRG projection from one side compartment to the other in these experiments. Nine to 12 days after the DRG neurons were plated, a phasic pattern of electrical stimulation was applied to the axons extending from one of the side compartments (*7*). The stimulation did not produce any change in the *p*H of the culture medium and had no substantial effect on DRG survival. The electrode for the center compartment was more than 1 cm removed from the VH neurons in the center slot. For these reasons we conclude that the stimuli were not producing nonspecific physicochemical effects on the system. After 3 to 5 days of stimulation, intracellular recordings were obtained from VH neurons to determine the number of functional inputs and the amplitudes of excitatory postsynaptic potentials (EPSPs) generated by axons from each side (Fig. 2A) (*8*). Control preparations were treated similarly except that neither side chamber was stimulated. VH neurons were selected from random locations in the central compartment, and the experimenter was not aware of the stimulus condition at the time of recording.

The protocol results in three categories of afferents to VH neurons: (i) stimulated afferents, (ii) nonstimulated convergent afferents, and (iii) afferents from controls in which neither side compartment was stimulated. Long-term stimulation of the DRG afferents affected the EPSPs significantly. In unilaterally stimulated chambers, the median EPSP produced by the long-term stimulation of the DRG axon was 10.0 mV, whereas the median EPSP produced by the nonstimulated convergent DRG axons was 3.9 mV ($P < 0.03$, Mann-Whitney U test). In control chambers, this value was intermediate at 5.5 mV (Table 1). This data set shows that stimulation affects EPSP amplitudes with a significance level of $P < 0.005$ based on the Kruskal Wallace test (*9*).

The change in synaptic strength could be due to a change in the number of axons that establish functional connections with the target VH neuron. To investigate this ques-

Table 1. The strength of synapses that form during development is influenced by differences in the level of electrical activity between axons that converge on the same neuron. The strength of synapses was increased in afferents exposed to a phasic pattern of stimulation during development, but the amplitude of EPSPs from inactive afferents was reduced below that of controls in which neither side compartment was stimulated.

	Amplitude of EPSPs evoked in VH neurons by DRG axon stimulation (mV)				
	Median	Mean rank	Mean	SD	No. of sides
Controls (no stimulation)	5.49	128	11.7	13.11	108
Stimulated side	10.00*	149	15.8	14.55	79
Nonstimulated side	3.89	122	10.5	13.37	77

*$P < 0.03$, Mann-Whitney U test, versus nonstimulated side.

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tion, we determined the number of inputs contributing to the EPSP. This was accomplished by a graded increase in stimulation intensity, which sequentially recruits inputs from separate axons according to differences in their threshold excitability (Fig. 2B). Although the number of functional inputs increased from a mean of 1 per side in controls to 1.6 per side after long-term stimulation ($P < 0.001$, Mann-Whitney U test), this increase did not result in a competitive advantage for the stimulated pathway relative to the nonstimulated convergent pathway in terms of the number of synaptically connected axons (Table 2). Although converging inputs exhibited activity-dependent changes in EPSP amplitudes, the number of inputs was not significantly different between stimulated and nonstimulated sides (1.6 versus 1.4 inputs per side).

The strength and number of synaptic connections vary with the location of VH neurons within the central compartment. We randomized such spatial effects in the present study by pooling VH neuron recordings from all locations so that the stimulation effects were not dependent on spatial factors.

Synaptic efficacy (EPSP amplitude) is a function of the probability of release by individual synaptic boutons, the sum of all boutons from each afferent, and the total number of separate inputs (axons) contributing to the synaptic potential. Postsynaptic factors (receptor density, dendritic size) are also relevant. If the activity-dependent changes in EPSP amplitude involved trimming of synaptic boutons from the weaker inactive convergent input and if the mechanism for this process were localized on the dendrites in the vicinity of active inputs, then inactive inputs at dendritic branches remote from the active inputs might escape elimination. Thus, the anatomical complexity of the dendritic field might make complete elimination of less effective inputs

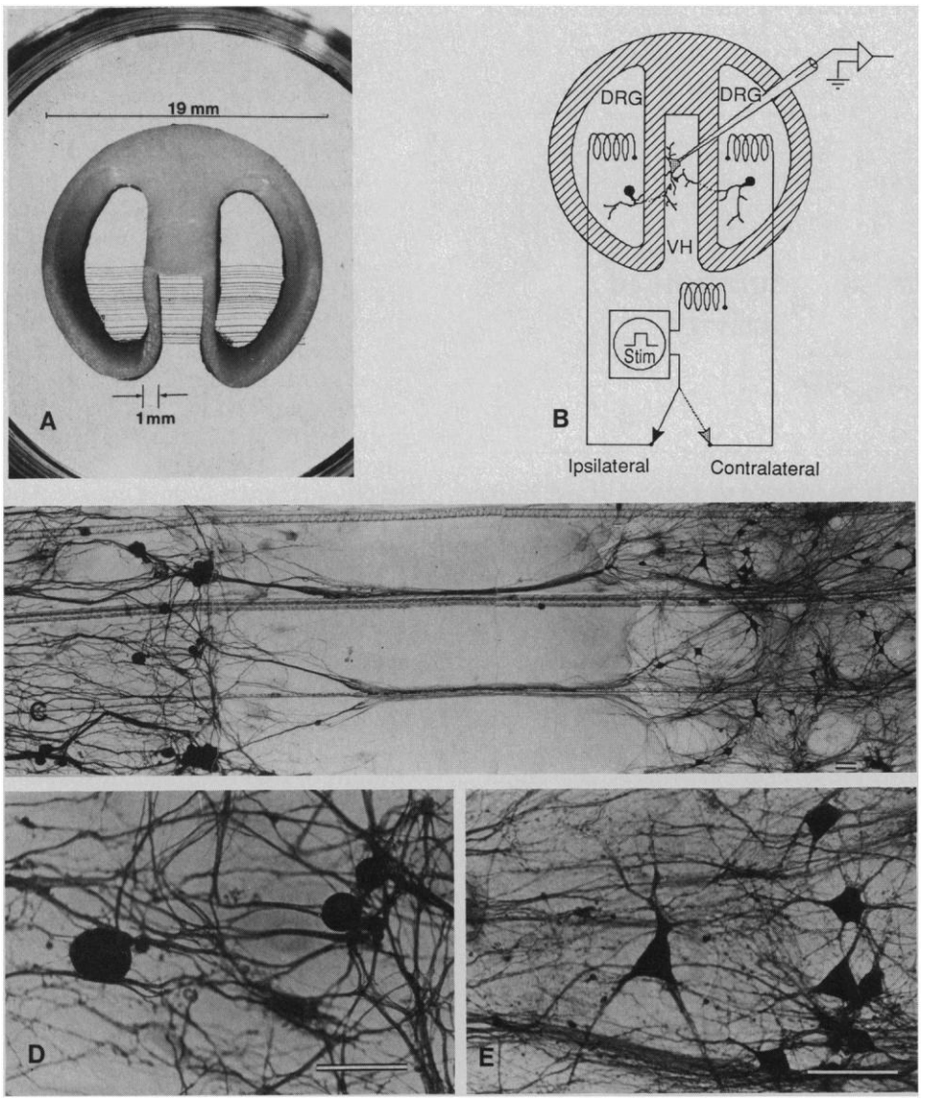

Fig. 1. Multicompartment chamber for establishing and selectively stimulating inputs from discrete populations of dissociated neurons in vitro. (**A**) Chambers were constructed of Teflon (*12*) and attached with silicone grease to collagen-coated 35-mm culture dishes. A series of shallow parallel scratches were made in the bottom of the dish, and a drop of 1% methylcellulose in Eagle's minimum essential medium was applied to this area before assembly. This procedure forms a fluid-tight seal under the barrier but provides sufficient space for the growth of neurites from DRG neurons in the side compartments to innervate VH neurons in the central compartment. VH neurons were dissociated from 13-day mouse fetuses (*13*) and plated in the central compartment onto a confluent monolayer of cortical glia. A week later, DRG neurons from 13-day mouse fetuses were plated in the side compartments. After an additional week, DRG neurites began extending under the barrier to form synapses with the VH neurons. (**B**) A phasic pattern of electrical sitmulation was delivered to axons crossing under the barrier of one side compartment; neurites from the opposite compartment were not stimulated. Stimulation commenced 9 to 12 days after the plating of the DRG neurons and was continued for 3 to 5 days, after which the number of functional inputs and the size of monosynaptic EPSPs were determined by intracellular recordings in the VH neurons. (**C**) After removal of the Teflon insert and staining (*14*), neurites from DRG neurons in the side compartment (left) can be seen traversing the barrier zone (center of panel) to form synapses with VH neurons in the central compartment (right) (*15*). (**D**) DRG neurons. (**E**) VH neurons. Bar, 50 μm.

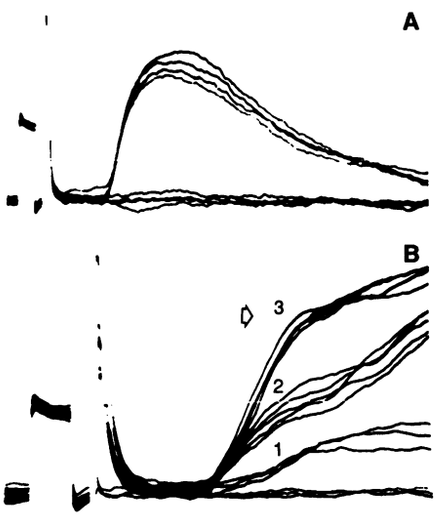

Fig. 2. Monosynaptic potentials recorded in VH neurons in response to stimulating axons of DRG neurons in one of the side compartments. (**A**) Several superimposed sweeps showing typical monosynaptic EPSPs elicited by stimulating DRG axons. In this example, gradually increasing the stimulus intensity elicits only a single all-or-none synaptic event, indicating that only a single DRG axon from the side chamber is connected to this VH neuron. (**B**) In another neuron, graded stimulation intensity from 0 to about 3 V resolves an EPSP into three discrete components representing inputs from separate axons with slight differences in threshold sensitivity (*16*). The peak amplitude of the monosynaptic component was recorded (arrow). The 2-ms calibration pulse = 10 mV.

Table 2. Electrical activity during development determines the number of synaptic inputs that form. The number of functional inputs to VH neurons was increased significantly in the activated pathway, compared to nonstimulated controls, but, unlike data on EPSP amplitudes (Table 1), there was no evidence for elimination of inputs from nonstimulated afferents converging on neurons with stimulated inputs.

	Number of inputs from DRG neuron chamber to VH neurons*			
	Mean	SD	Mean rank	No. of sides
Controls (no stimulation)	1.0	0.917	131	110
Stimulated side	1.6†	1.21	168	93
Nonstimulated side	1.4	1.22	150	93

*$P < 0.001$, Kruskal-Wallace nonparametric multiple comparisons test for differences among three stimulus treatments. †$P < 0.001$, Mann-Whitney U test, stimulated side versus no stimulation.

more unlikely than in morphologically simpler targets such as the neuromuscular junction. Alternatively, increased postsynaptic activity might enlarge the capacity of the postsynaptic target to sustain synapses, so that inactive connections would not be completely eliminated. The persistence of relatively ineffective, even subliminal synapses has been documented in the mammalian spinal cord (*10*).

The major consequence of long-term synapse activation is that the amplitude of EPSPs from the long-term activated afferents is increased relative to the nonactivated convergent afferents in the same preparation. The data do not establish whether the efficacy of stimulated afferents was increased or whether the efficacy of nonstimulated convergent afferents was decreased relative to that for controls but suggest that both processes occur (Table 1).

We interpret the results of our experiments as indicating that long-term stimulation of synaptic inputs to the target VH population has two effects, one that results in an increase in the number of inputs and is not restricted to the stimulated pathway and another that initiates a competitive process that diminishes the relative synaptic efficacy of the inactive convergent pathway. The net result is the development of the nervous system according to functional considerations: circuits with greater activity are favored over relatively inactive ones. The culture system described here makes these neuronal properties more amenable to detailed, mechanistically oriented studies (*11*).

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7. Pulses (3 V) were delivered from a constant-voltage stimulator through extracellular platinum electrodes fitted into culture dishes. Stimulation consisted of a 10-Hz burst of five biphasic (separation about 5 ms), pulse pairs per 2 s. Each pulse was 0.2 ms in duration. These bursts of stimuli were chosen because of results in other studies [see (*3*)] that showed this to be an effective way to produce synaptic competition.
8. EPSP amplitude represents the peak amplitude of the monosynaptic component of the synaptic potential. Monosynaptic responses were distinguished from polysynaptic components by their shorter and invariant latency (less than 10 ms) and their ability to respond to high-frequency stimulation. High divalent cation concentrations (2 m*M* for both Mg^{2+} and Ca^{2+}) and 5 n*M* tetrodotoxin were used in the recording medium to minimize spontaneous and polysynaptic activity by elevating spike threshold. If evoked monosynaptic activity produced action potentials, the postsynaptic cell was hyperpolarized to block the evoked spike.
9. The statistical significance of differences among these three groups was tested by the Kruskal-Wallace multiple comparisons test. This nonparametric test is appropriate, because the EPSP amplitudes were not normally distributed and the number of functional inputs is a discontinuous ordinal variable with a nonnormal distribution. Comparisons between two groups were made with the Mann-Whitney U nonparametric test. Similar conclusions were obtained when parametric tests were applied to these data (analysis of variance and *t* test). Means and standard deviations have been included in the data tables along with mean ranks to describe the groups more fully. See R. S. Sokal and F. J. Rohlf, *Biometry* (Freeman, San Francisco, 1981).
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15. Electrophysiological recording, microscopic examination, and neuronal tracing methods indicate that penetration of the barrier by VH neurites or cortical background cells was unusual; nevertheless, antidromic activation of VH axons was detected in 1 to 5% of the cases (as indicated by the occurrence of relatively short-latency action potentials that could be blocked in an all-or-none manner by hyperpolarizing current through the recording electrode). These would have represented a minor contribution to the synaptic activity evoked in the VH neurons by stimulation delivered across the chamber barrier. We have not examined the properties of such VH neuron–related PSPs in the absence of DRG neurons. The VH neurons that were antidromically activated were not included in the synaptic analysis because the antidromic response made adequate evaluation of the synaptic response impossible.
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4 November 1988; accepted 3 March 1989