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Interaction with Membrane Remnants of Target Myotubes Maintains Transmitter Sensitivity of Cultured Neurons

Abstract. *Parasympathetic neurons, when cultured alone, lose sensitivity to acetylcholine, but if striated muscle is included in the culture, neuronal chemosensitivity is maintained. The membrane remnants of myotubes ruptured by osmotic shock also supported the responsiveness of the cultured neurons to transmitter, whereas muscle-conditioned medium or membrane remnants of nonmuscle embryonic skin cells did not support this responsiveness. The regulation of chemosensitivity by contact of neurons with the target cell membrane may be important in the formation and maintenance of neuronal circuitry.*

The maintenance of appropriate chemosensitivity by neurons is a prerequisite for adequate function of the nervous system. Although cell culture may allow the controlled study of the cellular processes that govern neuronal chemosensitivity, considerable variation in the sensitivity of cultured ganglionic neurons to transmitters and in the binding of labeled neurotoxins has been observed (*1, 2*). Thus, in one study (*3*), the formation of functional synapses between cultured ciliary ganglion neurons was not detected because of the loss of neuronal chemosensitivity in cultures lacking target myotubes, but in another study (*4*), synaptic activity was found in many neurons from similar cultures. When myotubes were included in the cultures along with the neurons, adequate neuronal chemosensitivity (*3*) and evidence of interneuronal transmission were more frequently found (*3, 4*). The present study indicates that specific interactions among the elements of a culture may determine neuronal responsiveness to transmitter [see also (*2*)]. Contact of the neurons even with the membrane remnants of myotubes sustains for at least 1 week the retention of active transmitter receptors.

Neurons of the chick ciliary ganglion were removed from embryos at 10 to 12 days of incubation, dissociated, and plated (*5*). The culture substrate was one of the following. (i) An air-dried layer of rat tail collagen. The neurons grow in the presence of ganglionic nonneural cells, but muscle tissue is absent. (ii) A monolayer of chick pectoral myotubes plated as myoblasts 1 week earlier and allowed to fuse. An immediately available target tissue is presented to the neurons; neuromuscular junctions are formed rapidly and are maintained over several weeks of culture (*3, 6*). (iii) "Muscle material"—a culture substrate composed of myotube membranes. Myotube cultures were lysed in sterile water for 1 to 2 hours before the addition of the suspension of embryonic neurons. Examination of this substrate under Hoffman-modulated optics or phase-contrast optics shows no live tissue, but the outlines of empty cell and myotube remnants are clearly visible (Fig. 1D). (iv) "Fibroblast material"—a culture of epithelial or fibroblast cells uncontaminated by myoblasts (*7*). Subsequent lysis with water leaves a nonmuscle membrane-material substrate.

Table 1. Sensitivity of neurons to iontophoretically applied ACh. Numbers in parentheses indicate numbers of neurons tested. Each neuron was tested for a response to a pulse (2 to 50 msec) of ACh at a minimum of three separate sites on the somal surface. For all culture conditions, except culture on myotubes and myotube material, 55 percent of the 54 neurons tested were sensitive to the transmitter at 2 to 4 days in vitro. However, the level of sensitivity was often low (5 to 50 mV per nanocoulomb).

Culture medium	Percentage of neurons responding at	
	6 to 8 days	> 12 days
Collagen	0 (32)	
Myotubes	100 (26)	100 (30)
Myotube material	88 (51)	0 (7)
Fibroblast material	0 (44)	
Collagen in muscle-conditioned medium*	0 (15)	
Collagen in fibroblast-conditioned medium*	0 (7)	

*Medium was conditioned for 24 hours (*3*).

Neuronal chemosensitivity was monitored by intracellular recording of the cultured neurons during iontophoresis of acetylcholine (ACh) with high-resistance (150 to 300 megohm) micropipettes (*3, 5*). Acetylcholine is the major transmitter for the ciliary ganglion, and chemical transmission through the ganglion begins before the stage of development attained by neurons used to establish the cultures in the present study (*8*). Therefore, in all cases, if recordings are taken 1 or 2 days after the cultures are seeded with neurons, the iontophoretic application of ACh results in a depolarization (*3*). This depolarization is accompanied by a drop in membrane input resistance and is completely blocked by the ACh antagonist *d*-tubocurarine (*3*). Eserine, an inhibitor of acetylcholinesterase, causes an increased amplitude and duration of the depolarization (Fig. 1C). The reversal potential for the response to ACh, estimated by extrapolation (Fig. 1B), in five cells was −10 to −22 mV, similar to published values for synaptic and iontophoretic ACh potentials (*9*). In addition, delivery of ACh pulses at frequencies higher than 2 Hz caused rapid desensitization, whereas the response was unchanged if several seconds elapsed between pulses. Therefore, the neuronal response to ACh is most likely mediated by specific receptors, activation of which results in an increase in membrane permeability to ions and thus a depolarization from the normal resting transmembrane potential.

No response to ACh was seen from neurons grown without muscle if the recordings were taken at six or more days after plating [Table 1 and (*3*)], and the percentage of responsive neurons progressively declined between 2 and 6 days in vitro. This confirms that the loss of active receptors during the first week of culture of neurons plated alone is responsible for the transmission failure of interneuronal synapses (*3*). In contrast, neurons plated onto myotubes retain active receptors for several weeks in vitro [Table 1 and (*3*)]. Acetylcholine sensitivity at the most responsive mem-

brane sites on neurons in the combined culture approximates that reported for parasympathetic neurons in vivo (*10*). However, even when the ACh delivery was at a membrane site less sensitive than the highest seen, a depolarization was recorded in response to the transmitter. Because the resolution of this method of ionotophoretic delivery has been found to be a circle of radius no less than 4 μm (*10, 11*), and the somal diameter of ciliary ganglion neurons in these cultures does not exceed 15 μm, only one or two sensitive areas on the somal membrane should ensure a response to ACh delivered at almost any site on the membrane. Thus, the lack of response reflects either a virtually complete loss of active transmitter receptors or the loss of all sites of sufficient receptor density.

The experiments with lysed membrane material as a substrate were undertaken to explain the reported formation of functional interneuronal synapses (implying retention of active receptors) by neurons plated onto fibroblast material rather than onto myotubes (*4*). The membrane remnants of myotubes, like the live myotubes, supported the retention of active receptors to ACh, throughout more than a week of culture (Table 1). However, after 10 or more days in culture on the myotube material, ciliary ganglion neurons progressively lost chemosensitivity. Thus, either an active interaction between live elements (nerve and muscle) is required for the long-term maintenance of active receptors, or the myotube material or its active components disappeared with time in culture. A progressive reduction in the amount of visible material over time was observed.

Fibroblast material prepared from cranial skin (*4*) by lysis of the monolayer 24 hours after plating also supported the chemosensitivity of a variable proportion of the neurons (not shown). This observation supports the report of functional interneuronal synapse formation in ganglion cell cultures on fibroblast material (*4*). However, if such cranial skin fibroblast cultures are allowed to grow for more than 24 hours, myotube formation occurs in a few days (*12*), suggesting that myoblast remnants are responsible for the retention of the capability for synaptic function. This possibility was confirmed by testing neurons grown on fibroblast material uncontaminated by myoblasts for the maintenance of ACh responsiveness. The course of these cultures was indistinguishable from that of neurons plated onto plain collagen (Table 1). At six or more days in culture, no neurons sensitive to ACh were found in a sample of 44 neurons from two separate culture sets. Neuronal chemosensitivity is supported by an interaction with some component of myotube membranes, and not by interaction with a nonspecific cellular component or membranes of another cell type. Tests for chemosensitivity of neurons cultured in medium "conditioned" by either myotubes or fibroblasts (Table 1) suggest that the active element responsible for sustaining neuronal sensitivity is not present in the medium, is extremely labile, or is not excreted in sufficiently high concentration to be active. Thus the regulation of neuronal transmitter sensitivity may be mediated by actual contact of the neuron with an appropriate target cell membrane.

The neurons used for this study were taken from embryos during the period of naturally occurring neuronal cell death (*13*). The period of cell death in this ganglion marks the first appearance of dependency on the target tissue for neuronal survival, normal development of transmitter metabolism, and ultrastructural development (*14*). Thus, in culture, ganglionic neurons at this developmental

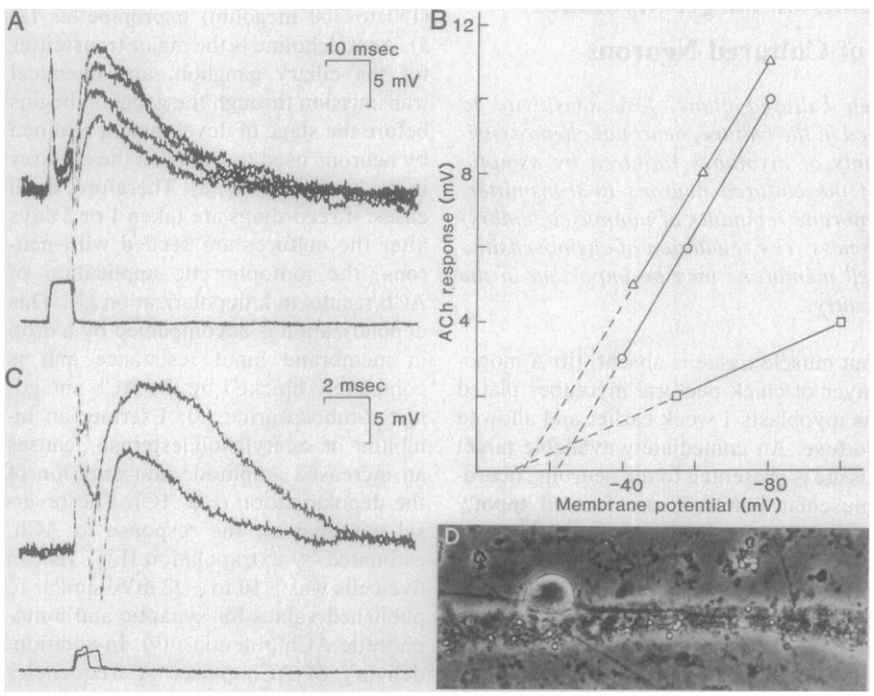

Fig. 1. (A–C) Responses of ciliary ganglion neurons in cell culture to iontophoretically applied ACh under several conditions and (D) a photomicrograph of a ganglion cell culture on a substrate of myotube membrane remnants. Culture methodology was as in (*3, 5*), except that ganglia from 10- to 12-day embryos were used, and the growth medium was supplemented (per milliliter) with 5 μg of insulin, 5 μg of transferrin, 6.3 ng of progesterone, 4 ng of selenious acid, and 8.8 ng of putrescine (*23*) and adjusted to 320 mos*M* with NaCl. All recordings shown were taken at 6 to 8 days of culture of the neurons. Recordings were essentially as in (*3*) but were made in a bath of 50 percent growth medium (base, minimum essential medium), 50 percent Tyrode's saline, with Ca^{2+} concentration raised to 6 m*M*. Iontophoretic pipettes were 150 to 300 megohms, filled with 3*M* ACh, and mounted in a constant-current "pump" equipped with reverse "holding" current capability (*20*). In each of the examples, the upper traces represent cell responses, and the lower trace is the iontophoretic monitor of pipette resistance during a pulse to ensure that bubble formation has not significantly altered ACh delivery during the pulse (*20*). Therefore, while the timing of the current is accurately reflected by the monitor trace, the amount of ACh delivered is proportional to the actual current passed. (A) Four superimposed traces of cell responses to a constant amplitude pulse of ACh, but with the membrane potential of the neuron adjusted to −80, −62, and −40 mV and resting potential (−55 mV) by direct-current passage through the recording pipette. These are used to extrapolate for an estimate of the reversal potential for the response to ACh. (B) Plots of three series of data, taken as in (A), and extrapolated to 0, an estimate of the "reversal potential." In no case did the response to ACh actually reverse to a hyperpolarization, despite adjustment of the membrane potential to + 10 mV. (C) Eserine (0.3 μg/ml), an inhibitor of the degradative enzyme for ACh, potentiated and prolonged the neuronal response to applied ACh. In the lower of the two superimposed cell traces, a longer pulse of ACh was delivered. In the upper trace, during superfusion with Eserine, a shorter pulse of the same current magnitude resulted in a larger depolarization of considerably longer duration. The shorter pulse of ACh was necessary to prevent the ACh response from passing threshold for an action potential. (D) Phase-contrast photomicrograph of a ciliary ganglion neuron plated onto myotube material at 5 days in vitro. Note the neurite extension along the surface of the myotube membrane remnant. The extension of neurites along live myotube surfaces is a prominent feature in combined cultures of nerve and muscle (*3*).

stage appear to require interaction with the target in order to remain responsive to ACh. If a similar requirement exists in vivo, either during development or throughout the neuronal life cycle, target interaction may be seen as a mechanism particularly suited to the formation and maintenance of appropriate neuronal circuitry. Activity through a synaptic pathway has been implicated as an important influence on the maintenance and relative efficacy of neuron-to-neuron and neuromuscular transmission (*15*). Neurons failing to compete successfully for target interaction might lose transmitter responsiveness and also lose a sustaining influence gained from tonic activity. This would be particularly true if the lack of contact with a target caused a loss of responsiveness to all transmitters, resulting in transmission failure at all incoming synapses. Because interaction with membrane remnants is as effective as interaction with live myotubes, at least in the short-term maintenance of neuronal ACh sensitivity, it is hard to envision a major role for tonic activity in this instance.

In cell culture, ciliary ganglion neurons can be released from the normal dependency on the target for survival by the addition of target-derived proteins (*16*). In addition, several other "trophic" proteins or activities support aspects of neuronal differentiation (*17*). These observations, along with studies of nerve growth factor (*18*), suggest an important role for readily soluble proteins in the development of embryonic neurons. However, for the ciliary ganglion, protein factors added to the culture medium are not capable of supporting neuronal development equivalent to that seen in vivo (*3, 19*). The failure of the soluble trophic factors to foster normal development in cell culture may be the result of other deficiencies in the culture environment, insufficient neuronal access to the factors, or as yet obscure factors that act in concert to promote neuronal differentiation. Nonetheless, the present results indicate that contact with the target membrane has a strong influence on neuronal development.

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Number of Receptor Sites from Scatchard and Klotz Graphs: A Constructive Critique

The Scatchard plot is now perhaps the most popular method for graphical analysis of ligand-receptor binding studies. With the increasing number of receptor studies in neurobiology, endocrinology, pharmacology, immunology, and numerous other biomedical fields, it is important that this method be used and interpreted correctly. Klotz (*1*) has called attention to some potential shortcomings of the Scatchard plot method. However, it should not be concluded that there is something inherently wrong with the Scatchard plot or that the graph preferred by Klotz, in which the concentration of bound ligand is plotted against the log of the free ligand ([*B*]/log [*F*]), is intrinsically better. Clearly, the statistical informational content of the data is not altered by presentation in one or the other coordinate system: a simple algebraic manipulation will convert one format to the other. Thus identical conclusions should be drawn from both plots, provided they are interpreted correctly.

A number of issues deserve comment.

1) When one uses a plot of bound versus free ligand or bound versus the log of the free concentration, it becomes apparent that the maximum binding capacity (B_{max}) is an extrapolated value and that one must extrapolate a very long distance (until the concentration of the free ligand equals "infinity"). Even if one reaches 80, 90, or 95 percent of the apparent upper plateau value, there is always the disturbing possibility that the curve might change its shape in the unobserved (and unobservable) region. Thus, one can never prove that one has determined the true value for B_{max}, utilizing any kind of plot or any kind of statistical analysis. This problem applies equally to the Scatchard and Lineweaver-Burk plots (*2*), although in these plots the graphical extrapolation appears to be only a short distance.

2) One can only make an estimate of B_{max} if one begins with a particular model (for example, homogeneous noninteracting sites or two, three, or more classes of independent sites, cooperativity based on a specified model) and then assumes that the model will continue to apply over the extrapolated range.

There are two distinct sources of uncertainty in the estimate of B_{max}. Random fluctuation in the observed data will result in corresponding uncertainty in the estimate of B_{max} even if we knew the exact model governing the biochemical reaction. The magnitude of this type of error will increase as the length of extrapolation increases, giving a warning when inadequate data are available. The error limits in the estimate of B_{max} may be calculated on the basis of assumptions about the error distribution for the data. More accurate limits are obtained if independent replicates of the experiment are available. Appropriate methods of calculation have been described elsewhere (*3*).

Uncertainty also results from incom-