Target Recognition and Synapse Formation by Ciliary-Ganglion Neurons in Tissue Culture

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Cell-to-cell recognition must play an important role in the nervous system during the formation and maintenance of specific connections between neurons and their target cells. This process of recognition in the nervous system might be based on (1) complementarity of structural components in the presynaptic and postsynaptic membranes of appropriate pairs of cells, (2) the release of a chemical compound by the target cell, which acts as chemotactic 'lure' for the correct exploring nerve ending, (3) a profuse aspecific formation of cell-to-cell contacts followed by selective survival and maintenance of those connections that appear to be meaningful for the organism (in this case electrophysiological activity of the target cell or a biochemical process associated with this activity is supposed to be the crucial factor in recognition) and (4) a time-position mechanism in which differences in timing of maturation is a major factor in the control of the formation of the pattern of connections. For references see Gaze & Hope (1976) and Holliday et al. (1977). The relative contribution of any of these mechanisms is unknown.

The formation of junctions between neurons, and between neurons and muscle cells can be studied in tissue culture of embryonic neurons and in mixed cultures of embryonic neurons and skeletal-muscle cells respectively. In many studies the neuronal cells are obtained from embryonic spinal cord and the muscle cells from embryonic leg or breast muscle. Although such mixed cultures are likely to contain the genuine motor neurons they are bound to contain a host of other neurons, although no methods are presently available to identify a motor neuron in culture with any degree of certainty. The preparation is therefore not very attractive for the study of the recognition process involved in the formation of the neuromuscular junction.

A less complicated source of neurons suitable for this type of studies is the parasympathetic ciliary ganglion. In the pigeon and in the chick this ganglion is known to contain only two classes of neurons, which both are cholinceptive and cholinergic and that innervate the muscle fibres of the choroid plexus of the eye, the sphincter iridis and the ciliary body (Marwitt et al., 1971). The iris and the ciliary muscle in birds are both striated (Ovio, 1927), whereas in the mammal they are smooth. Neurons of the ciliary ganglion of the chick embryo can be cultivated in tissue culture (Hooisma et al., 1975) either in explants of whole ganglia or after dissociation, as individual cells. Branching neurites grow radially from the ganglia. A corona of neurites with a diameter of 500–1000 μm surrounding the ganglia is formed during 2 days in culture.

Mixed cultures of ciliary neurons and skeletal-muscle cells have been used for the study of the process of neuron-to-target recognition. The present paper deals with the following questions: (1) do the autonomic neurons of the chick ciliary ganglion recognize chick skeletal-muscle cells as appropriate target cells for functional innervation? (2) do neurons, which exclusively form neuromuscular connections with smooth-muscle cells in vivo, recognize striated muscle fibres as target cells in vitro? (3) does recognition of chick skeletal-muscle fibres by chick ciliary-ganglion neurons lead to neurotrophic support of these muscle fibres, analogous to the neurotrophic influence of the motor neuron on its target cells?

Muscle cells obtained by trypsin dissociation of leg muscles of the 11-day chick embryo were cultivated by the method of Slaaf (1977), on collagen-coated object glasses in Eagle's minimum essential medium and Earle's balanced-salt solution supplemented with 15% horse serum and 5% embryo extract at 35°C and with a final osmolarity of 320 mosmol, in a humid atmosphere of CO₂/air (1:19). d-Arabinofuranosyl-
cytosine was present in the medium on the third and fourth days of cultivation. Under these conditions fusion of myoblasts started within 24h. At day 5 long multinucleated myotubes had formed with pronounced cross striations. Ciliary ganglia were dissected from 6–7-day embryos and left to settle on the muscle cells in a thin layer of medium. The formation of functional neuromuscular junctions in mixed cultures of ciliary-ganglion neurons and skeletal-muscle fibres has been studied by intracellular electrophysiology. Already 24h after explanation of a ganglion on muscle cells of 2 days spontaneous end-plate potentials could be recorded from the muscle cells. These end-plate potentials were never recorded in cultures without ganglia or in muscle fibres far away from a ganglion. Morphological observations gave the impression that with most muscle fibres in the vicinity of the ganglion more than one neurite had made contact. Electrophysiological data showed that such myotubes were multiply innervated. End-plate potentials recorded from one myotube were usually quite different in amplitude (2–20mV) and had clearly different time courses. This indicates that end-plate potentials were generated at several sites in the same muscle fibre, i.e. at synapses located at various distances from the recording electrode. The formation of the newly formed neuromuscular junctions was also demonstrated by extracellular nerve stimulation. End-plate potentials could be evoked in a muscle fibre by electrical stimulation of an axon that seemed to end on that fibre. Moreover, local iontophoretic application of acetylcholine on neurons in the ciliary ganglion often resulted in the generation of end-plate potentials in a muscle fibre in the vicinity of the ganglion.

The nature of the receptors in these newly formed neuromuscular junctions was investigated. In seven experiments the sensitivity of neurotransmission to (+)-tubocurarine and atropine was tested. Addition of the former drug in a concentration of 1µg/ml almost completely blocked the generation of end-plate potentials, whereas as much as 100µg of atropine/ml was required to decrease the amplitude of the largest end-plate potentials to noise level. Apparently, nicotinic cholinergic neuromuscular junctions are formed in mixed cultures of the parasympathetic neurons and skeletal-muscle cells. It can be concluded that in tissue culture chick ciliary-ganglion neurons are able to recognize skeletal-muscle fibres as target cells.

The question of the specificity of recognition has been studied in mixed cultures of the mammalian ciliary-ganglion and skeletal-muscle cells. Neurons of this ganglion normally make exclusively cholinergic muscarinic connections with smooth-muscle cells. Ciliary ganglia taken from newborn rabbits appeared to form neurites in tissue culture. The neural outgrowth was slower than that of the chick ciliary ganglion with the consequence that, between the always present proliferating fibroblasts, only occasionally a point of contact between the nerve process and the muscle cells was observed. Nevertheless, in the myotubes in the vicinity of the ganglion, end-plate potentials could be recorded. The relative sensitivity of these end-plate potentials to (+)-tubocurarine and atropine was similar to that found in the muscle fibres innervated by the chick ciliary ganglion. This shows that neurons that exclusively form cholinergic muscarinic junctions in vivo, are able to form cholinergic nicotinic junctions in tissue culture. Apparently, the recognition of determinants of the muscle-cell membrane is of major importance in this process since, at least under the conditions studied, the character of the muscle and not that of the neuron determines the pharmacological nature of the neuromuscular junctions. The neuromuscular contact between skeletal-muscle fibres and spinal-cord neurons in vivo not only is a prerequisite for the transmission of nerve impulses to the postsynaptic cell, but is also required for the (neurotrophic) support of the structure and function of the muscle fibre (Gutman, 1976). In mixed cultures of spinal-cord neurons and muscle cells neurotransmission as well as neurotrophic support has been demonstrated. The innervated fibres develop more and better visible cross- striation, become thicker and survive longer. The ability of a neuron to exert a trophic action might be associated with or be the result of neurotransmission. It would therefore be interesting to compare the ability of neurons to recognize muscle cells as target cells and their ability to provide neurotrophic support. The ability to innervate skeletal-muscle cells can be expressed as the amount of functionally innervated fibres calculated as percentage
of the number of impaled fibres. The ability to provide trophic support can be assayed by a semiquantitative method for the evaluation of four morphological properties: cross striation, thickness, number of muscle fibres and absence of vacuoles (Hooisma, 1977). For each of these properties the condition of muscle fibres in the vicinity of the explant was rated on a five-point scale from 0 to 4 with 4 for the best condition.

The innervation ratio for muscle-cell cultures together with either spinal cord or ciliary ganglia was 80–90%. But the trophic influence of these two types of explants appeared to be quite different. In Fig. 1, on the abscissa the mean score is plotted of the morphological properties, corrected (by subtraction) for the morphological properties of corresponding muscle-cell cultures without neural explants. The ordinate gives the time of cultivation. The neuronal explants were always added after 2 days. The spinal-cord cells had a considerable trophic effect, but the innervation by the ciliary ganglion did not provide the support to maintain cross-striations. The results suggest that recognition of a cell as an appropriate target, followed by functional innervation, does not automatically lead to adequate neurotrophic support of this cell. Although for the gross morphology of the muscle fibres the innervation by ciliary neurons had little or no consequences, the innervation by such neurons had definite long-term consequences for their membranes on the molecular level. It is well known that innervation of muscle fibres in vivo by motor neurons leads to an increased acetylcholine-sensitivity at the site of innervation and to a restriction of the acetylcholine-sensitivity to this site. The increased local hypersensitivity has also been observed in mixed tissue cultures of spinal-cord material and skeletal-muscle cells, but the restriction of the sensitive area under such circumstances has not been reported. In our experiments, measurement of the sensitivity
to acetylcholine along the cell membranes of ciliary (innervated) myotubes, by recording the depolarizations brought about by local iontophoretic application of acetylcholine, demonstrated the existence of extremely localized areas of which the sensitivity was 4–36 times higher than the average sensitivity of the membrane of the same myotube. Such highly sensitive areas were not detected in non-innervated muscle fibres. So-called 'hot spots' i.e. localized clusters of acetylcholine receptors as were first described by Sytkowski et al. (1973) in non-innervated fibres, were not encountered in our cultures of muscle cells. These data have been confirmed by radioautography with the aid of 125I-labelled α-bungarotoxin, which binds irreversibly to the acetylcholine receptor. The distribution of acetylcholine receptors in mixed cultures either with spinal cord explants or with ciliary ganglion have been compared. In conclusion, the mixed culture of the ciliary ganglion and skeletal muscle is a useful preparation in the study of cell recognition of the target cell during innervation.


Membrane Glycoproteins in Recognition
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Carbohydrate is found exposed at the surfaces of cells of higher organisms, and it is clearly established that much of it is attached to proteins that insert into the lipid bilayer (for reviews see Hughes, 1975, 1976). In fact, it is possible that virtually all cell-surface proteins of mammalian cells are glycoproteins or proteins associated with glycoproteins. Viral and mitochondrial membranes also contain glycoproteins, but this is not a feature of all membranes since glycoproteins are rarely found in the membrane of bacterial cells (Mescher & Strominger, 1976). In evolution it may be that membrane glycoproteins are a feature of organisms (or their parasites) that form tissues.

There are few glycoproteins of plasma membranes that are well characterized, and fewer whose functions are known, but it is highly probable that a large proportion of these molecules are enzymes, or transport proteins, or receptors (e.g. for hormones or antigens). For example, in kidney brush-border membranes hydrolytic enzymes predominate and these are glycoproteins (Kenny & Booth, 1976). The anion-transport molecule of human erythrocytes (band 3) is a glycoprotein (Marchesi et al., 1976), as is the immunoglobulin antigen receptor of B-lymphocytes (Vitetta & Uhr, 1975). In these functions there is no obvious role for the carbohydrate structures.

A further point in considering cell-surface glycoproteins as proteins with diverse functions is that membranes of different cell types are highly dissimilar and probably differentiated for their unique functional roles. The brush border of kidney cells appears very different to the membrane of erythrocytes (Glossman & Neville, 1971), and many antigens of lymphocytes (this usually means protein antigenic determinants) are found on few if any other cell types (Williams, 1977). Thus one could say that cell-surface

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