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On the specificity of synapse formation

(neuroblastoma × glioma hybrid cells/nicotinic acetylcholine receptors/cell recognition)

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ABSTRACT **Clonal neuroblastoma × glioma hybrid cells form synapses with striated muscle cells from different muscles and from different organisms, such as chick, mouse, and rat. Under appropriate conditions 65–80% of the muscle cells exhibited synaptic responses. The results indicate that most striated muscle cells have the same specificity for synapse formation and suggest that synaptogenesis is not dependent on interactions between complementary molecules on neuron and muscle cells that code for different synaptic connections.**

The problem of how neurons form synaptic connections and assemble into neural circuits with precision remains one of the most challenging issues in neurobiology. In essence most of the hypotheses which relate to this problem can be divided into two categories: either that cells which form synapses possess different kinds of cell recognition molecules which must interact with specificity prior to synapse formation; i.e., a code for matching one cell with another which leads to the formation of different kinds of synaptic connections (1); or the alternative hypothesis which is not based on a cell recognition code, that modification of synaptic connections dependent upon transmission across the synapse determines the final synaptic organization (2, 3).

To understand the molecular nature of the factors that determine the specificity of synapse formation it would be advantageous to have homogenous populations of neurons that form synapses. Since normal neurons do not divide, clonal lines of neuroblastoma cells and somatic cell hybrids derived from neuroblastoma cells were generated and characterized with respect to receptors, neurotransmitters, action potential ionophores, and other properties which are required for synaptic communication (4, 5). Fusion of mouse neuroblastoma cells with rat glioma cells yielded a clonal hybrid cell line NG108-15, which synthesizes, stores, and excretes acetylcholine, properties which are not expressed by the parental cell lines (B. Hamprecht, M. Nirenberg, and T. Amano, submitted for publication), and which forms synapses with cultured embryonic and clonal mouse striated muscle cells as reported by Nelson *et al.* (6), and C. Christian, P. Nelson, and M. Nirenberg (submitted for publication).

This clonal cell line provides an opportunity to test the specificity of synapse formation. If the formation of synaptic connections between neurons and muscle cells were coded by specific cell recognition molecules, then discrete classes of muscle cells with different specificities for synapse formation might be expected. In this report we show that NG108-15 hybrid cells form synapses with cells from different muscles and from different species. Thus, most muscle cells constitute a single class of cells with respect to the formation of synapses.

Abbreviations: DMEM, the Dulbecco-Vogt modification of Eagle's minimal essential medium; MEM, Eagles's minimal essential medium; dibutyryl cAMP, $N^6,O^{2'}$-dibutyryl-adenosine 3′:5′-cyclic monophosphate.

METHODS AND MATERIALS

Cell Culture. Neuroblastoma × glioma NG108-15 hybrid cells were derived (B. Hamprecht, M. Nirenberg, and T. Amano, submitted for publication) by fusion of mouse neuroblastoma clone N18TG-2, resistant to 6-thioguanine (5), with rat glioma clone C6BU-1, resistant to 5-bromodeoxyuridine (7). NG108-15 cells were cultured at 36° in Dulbecco–Vogt modification of Eagle's minimal essential medium (DMEM; Gibco Catalog no. H-21), 10% fetal bovine serum, 100 μM hypoxanthine, 1 μM aminopterin, and 16 μM thymidine in a humidified atmosphere of 10% CO_2–90% air. The hybrid cells were shifted to a more differentiated state by culturing them for 1–3 weeks in the presence of 1 mM $N^6,O^{2'}$-dibutyryl-adenosine 3′:5′-cyclic monophosphate (dibutyryl cAMP) (B. Hamprecht, M. Nirenberg, and T. Amano, submitted for publication) purified as described previously (6). The NG108-15 cells which were used in this study had been subcultured 15 to 20 times.

Cultures of striated muscle cells were prepared by dissociation of cells from the hindlimbs of 18- to 21-day-old fetal C57BL/6N mice, 18- to 21-day-old fetal or newborn Wistar rats, or 10-day-old chick embryos (White Leghorn) as described previously (6). Each collagen-coated 35 mm plastic culture dish usually was inoculated with 0.3 to 1.0 × 10^6 cells. Explants of pectoral muscle from 10-day chick embryos were established in collagen-coated 35 mm plastic culture dishes.

Mouse muscle cells were incubated in 40% DMEM, 40% Eagle's minimal essential medium (MEM) (adjusted to 33 mM D-glucose and 44 mM sodium bicarbonate), 10% horse serum, and 10% fetal bovine serum for 48 hr and thereafter in 90% DMEM, 10% horse serum. Rat muscle cells were incubated for 48 hr in 90% DMEM, 10% horse serum, 10% fetal bovine serum, and thereafter in 90% DMEM, 10% fetal bovine serum. Chick muscle cells were grown either in 90% F_{14} (8) and 10% fetal bovine serum or in 85% MEM, 10% horse serum, 5% chick embryo extract, 50 units of penicillin, Na^+ salt, and 50 μg of streptomycin sulfate per ml of medium. At 48 hr the concentration of chick embryo extract was reduced to 2%. The medium was changed 24 and 48 hr after the cells were plated; thereafter, cultures were fed twice weekly. Cytosine arabinonucleoside (10 μM) or 40 μM 5-fluorodeoxyuridine and 200 μM uridine were added to some cultures for 24–48 hr as cells approached confluency. This treatment greatly reduced the number of dividing cells. Cells were grown at 36° in a humidified atmosphere of 10% CO_2–90% air or, when unmodified MEM was used, in 5% CO_2–95% air.

Chick muscle cells were cultured for 3–6 days and mouse and rat cells for 5–9 days; then 2 to 6 × 10^4 NG108-15 hybrid cells that had been grown in the presence of dibutyryl cAMP were added to each muscle culture. Hybrid cells were cocultured with mouse muscle cells in 90% DMEM and 10% horse serum with rat muscle in 90% DMEM and 10% fetal bovine serum and with chick muscle in either 90% F_{14} or 90% DMEM and 10%

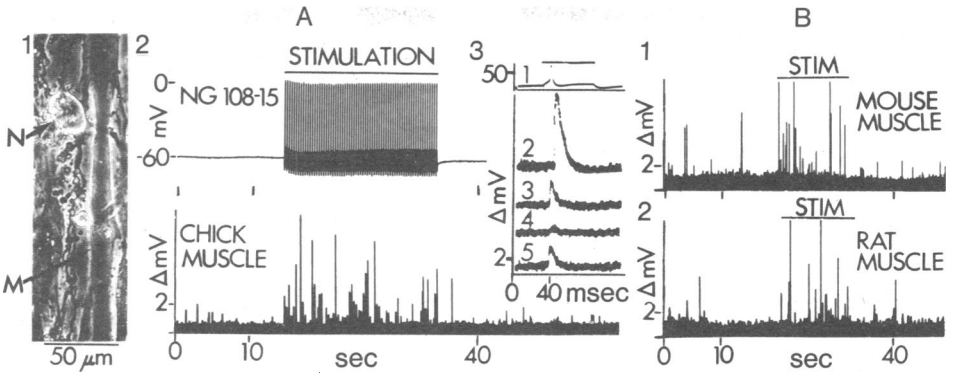

FIG. 1. Examples of NG108-15 synapses with chick, mouse, and rat muscle cells. (A1) Phase contrast photomicrograph of a neuroblastoma × glioma NG108-15 hybrid cell (labeled N) that formed a synapse with a chick muscle cell (labeled M). (A2) Penwriter recordings from intracellular electrodes in the same NG108-15 cell (upper record) and chick muscle cell (lower record) shown in panel A1. Repetitive electrical stimulation of the hybrid cell elicited action potentials in the hybrid cell which evoked an increase in the frequency and amplitude (ΔmV) of muscle responses. (A3) Oscilloscope traces of intracellular recordings from the same NG108-15 cell (trace 1) and muscle cell (traces 2–5) shown in A1. The traces are aligned so that temporal relationships between the NG108-15 action potential and the muscle responses (each evoked by a separate action potential) are shown. *Trace 1*: An example of an electrically elicited action potential in the NG108-15 cell which had a resting membrane potential of −60 mV and was stimulated two times per sec with 0.5 nA of depolarizing current (bar). *Traces 2–5*: These highly amplified traces show typical evoked depolarizing responses of the muscle cell. The muscle membrane potential was −70 mV.

(B1) Penwriter recordings from a mouse muscle cell obtained with an intracellular electrode showing muscle responses evoked by NG108-15 action potentials which were elicited by repetitive electrical stimulation of the hybrid cell (0.3 nA depolarizing pulse, 60 msec in duration, 3 pulses/sec). Both the muscle and hybrid cells had resting membrane potentials of −40 mV. The choline concentration of the assay media was adjusted to 34 μM. (B2) A synapse between NG108-15 hybrid cell and a rat muscle cell. The resting membrane potential of the muscle cell was −80 mV. The resting membrane potential of the hybrid cell was −40 mV; the membrane potential was adjusted to −80 mV with constant current. The hybrid cell was stimulated three times per sec with 0.5 nA of depolarizing current, 60 msec in duration. The choline concentration of the assay medium was adjusted to 124 μM.

fetal bovine serum. In addition, the coculture media always contained 1 mM purified dibutyryl cAMP, 100 μM hypoxanthine, and 16 μM thymidine. The medium was changed every 48 hr. After 3–21 days of incubation, pairs of hybrid and muscle cells were assayed for the presence of synapses.

Electrophysiology. Intracellular recording and stimulating techniques were similar to those described by Nelson *et al.* (9). Glass micropipette electrodes were filled with 3 M potassium acetate and had electrical resistances of 20–80 MΩ. The medium used for the assay of synapses consisted of DMEM without serum adjusted as follows (except where stated): 3.8 mM $CaCl_2$, 1 mM dibutyryl cAMP, 100 μM hypoxanthine, and 16 μM thymidine. The growth medium was replaced with the assay medium 1–2 hr before the experiment.

RESULTS

NG108-15 hybrid cells with cell bodies greater than 25 μm in diameter and processes that were close to well-differentiated myotubes were assayed for synapses. A hybrid cell and muscle cell were impaled with separate microelectrodes; the hybrid cell was stimulated electrically to elicit action potentials, and muscle responses were recorded.

We find that NG108-15 cells form synapses with chick, mouse, and rat muscle cells. Examples of the synapses are shown in Fig. 1. Repetitive electrical stimulation of the NG108-15 hybrid cell elicited action potentials in the hybrid cell (Fig. 1 A2, upper record) which often but not always evoked transient depolarizing responses in the chick muscle cell (Fig. 1 A2, lower record). The muscle responses varied markedly in amplitude. Spontaneous muscle responses also were observed which were less frequent and usually smaller in amplitude than the evoked responses. An oscilloscope trace of an NG108-15 action potential elicited by electrical stimulation is shown in Fig. 1 A3, trace 1. Traces 2 to 5 are muscle responses to NG108-15 action potentials. The amplitudes of the responses varied from approximately 0.5 to 5 mV; all were below the threshold for initiating action potentials. These traces also show that the intervals between NG108-15 action potentials and muscle responses vary. The muscle responses in traces 3 to 5 were initiated 1 msec after the onset of the NG108-15 action potential, while the muscle response shown in trace 2 occurred 5 msec after the NG108-15 action potential.

As shown in Figs. 1 B1 and 1 B2, NG108-15 hybrid cells also form synapses with mouse and rat muscle cells, respectively. Electrical stimulation of the hybrid cells (at 3/sec, shown by the horizontal bar) resulted in an increase in the frequency and amplitude of muscle responses. Oscilloscope tracings (not shown) revealed that muscle responses were evoked within a few msec of NG108-15 action potentials. The records of mouse and rat muscle responses also show that the amplitude of the muscle response varies, that the muscle cells respond to some but not all NG108-15 action potentials, and that spontaneous muscle responses occur. Although the efficiency of transmission across the synapses, the delay in the muscle response to the NG108-15 action potential, and the amplitude of the muscle response varied greatly from one synapse to another, no obvious differences in the properties of synapses of NG108-15 cells with chick, mouse, and rat muscle were observed.

As shown in Fig. 2A, repetitive stimulation of an NG108-15 cell (2/sec) could evoke repetitive action potentials in a rat muscle cell. In some cases, a single NG108-15 action potential resulted in a series of muscle action potentials. In addition, some NG108-15 action potentials either failed to evoke a muscle response or evoked responses which were below the threshold for the initiation of action potentials. The oscilloscope traces in Fig. 2A show an NG108-15 action potential (upper trace) followed within 10 msec by an action potential in the muscle cell (lower trace). In Fig. 2B intracellular recordings of both spontaneous and evoked activity of the same chick muscle cell cocultured with NG108-15 hybrid cells are shown. Most of the spontaneous muscle events were below the threshold for initiation of action potentials. Prolonged depolarizations of 45–50 mV in amplitude also can be seen which last for seconds (or for

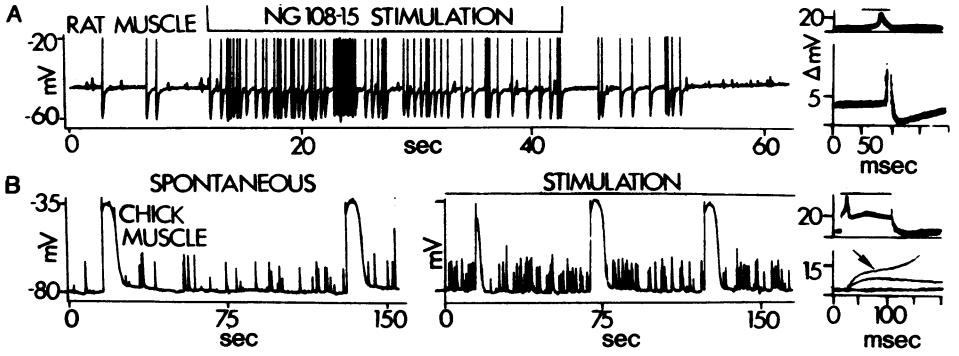

FIG. 2. (A) Penwriter recording from an intracellular electrode in a rat muscle cell. When an NG108-15 cell was stimulated repetitively (bar) at the rate of 2/sec, action potentials were evoked in the muscle cell. Subthreshold muscle responses also were evoked. The oscilloscope traces to the right of the penwriter record are from the NG108-15 cell (upper trace) and the muscle cell (lower trace). *Upper trace*: An example of an action potential elicited in the NG108-15 cell by electrical stimulation (0.4 nA, 45 msec in duration as shown by the bar). *Lower trace*: The evoked muscle response initiated an action potential. The peak of the action potential went off the oscilloscope screen. The resting membrane potentials of the hybrid and muscle cells were −80 and −40 mV, respectively.

(B) Penwriter recordings from an intracellular electrode in a chick muscle cell. Many spontaneous muscle events (left panel) were below the threshold for initiation of action potentials. Large depolarizations, approximately 10 sec in duration, probably are chloride spikes. As shown in the right panel (same scales as left panel) repetitive stimulation of an NG108-15 cell (3 pulses/sec) that elicited hybrid cell action potentials resulted in an increase in the number of subthreshold responses and action potentials in the muscle cell. The upper oscilloscope trace in B shows an NG108-15 action potential elicited by a 90 msec, 0.1 nA depolarizing pulse (bar). In the lower frame three successive oscilloscope sweeps from the muscle recording are superimposed. No response, a subthreshold response, and the onset of an action potential (arrow) are shown. For the oscilloscope records the vertical axis is mV, the horizontal, msec. The resting membrane potential of the muscle was −80 mV; that of the hybrid cell was −40 mV.

minutes with some muscle cells), which may be chloride spikes (10). NG108-15 action potentials elicited by repetitive electrical stimulation (3/sec) evoked an increase in the number of muscle responses. The upper oscilloscope trace in Fig. 2B shows an NG108-15 action potential elicited by electrical stimulation. In the lower frame three successive oscilloscope sweeps of muscle cell activity are superimposed. No response, a response below the threshold for initiation of an action potential, and the onset of an action potential (arrow) are shown. The action potential shown resembles a chloride spike; however, further work is needed to determine the kinds of ionophores of chick muscle which can be activated in response to NG108-15 action potentials. These results show that NG108-15 action potentials can evoke action potentials in rat, mouse, and chick muscle cells.

Rat and mouse muscle cells were found which received synaptic input from two NG108-15 cells. We also observed NG108-15 hybrid cells which formed synapses with two muscle cells from the chick, mouse, or rat in addition to forming synapses with the cells from the many muscles in the hindlimb, NG-108-15 cells also established synapses with explants of pectoral muscle from 10-day-old chick embryos which closely resembled those formed by NG108-15 cells and dissociated hindlimb muscle cells both in abundance and in the efficiency of transynaptic communication. Thus, the ability of NG108-15 cells to form synapses with striated muscle cells is not restricted to hindlimb muscles.

As a further documentation of the synaptic nature of the muscle responses evoked by stimulation of NG108-15 cells, the effect of *d*-tubocurarine, which blocks nicotinic acetylcholine receptors, on synaptic transmission between an NG108-15 cell and a chick muscle cell was studied. The local application of *d*-tubocurarine by diffusion from micropipettes (tip diameter 5–20 μm, filled with 10–30 μM *d*-tubocurarine) positioned near the synaptic sites resulted in a 70–80% decrease in the frequency of evoked muscle responses. These and other results (6) show that *d*-tubocurarine inhibits synaptic transmission between NG108-15 cells and muscle cells, and that the inhibition is reversible. Additional experiments with micropipettes filled with a *d*-tubocurarine solution and with 10 μM *d*-tubocurarine in the assay medium showed that certain spontaneous muscle activity is reversibly blocked by this drug. The spontaneous activity that is blocked is not rhythmic and varies in amplitude, usually from less than 0.5 mV to 5 mV. We consider such muscle activity to be spontaneous synaptic responses. This is in contrast to another type of spontaneous activity which is rhythmic and uniform in amplitude. In control cultures containing only muscle cells we found no activity resembling the kind that can be blocked by *d*-tubocurarine, although rhythmic activity was observed.

Nelson *et al.* (6) reported that increasing the concentration of the choline chloride in the synapse assay medium from 24 to 124 μM increases the efficiency of synaptic transmission

Table 1. Effect of assay conditions on NG108-15 synapses with rat muscle cells

Assay medium*		Muscle responses evoked by NG108-15 action potentials			Muscle cells with spontaneous synaptic responses		
mM $CaCl_2$	μM Choline chloride	Synapses found	Cell pairs tested	% With synapses	Cells with responses	Cells tested	% With synapses
1.8	24	1	14	7	5	17	29
3.8†	24	6	28	21	19	25	76
3.8†	124	8	17	47	10	15	67

* The assay medium was DMEM.
† The medium also contained 1 mM dibutyryl cAMP, 0.1 mM hypoxanthine, and 0.016 mM thymidine.

Table 2. Specificity of synapse formation between NG108-15 cells and striated muscle cells

Muscle source	Muscle responses evoked by NG108-15 action potentials			Muscle cells with spontaneous synaptic responses			
	Synapses found	Cell pairs tested	% With synapses	Cells with responses	Cells tested	% With synapses	Range* (%)
Chick	16	88	18	82	152	54	29–82
Mouse	7	42	17	12	33	36	27–67
Rat	15	59	25	36	57	63	30–76

* The range of the average values found for the cell growth or assay conditions used.

between NG108-15 cells and mouse muscle cells and the number of synapses detected. The effects of various choline chloride and calcium chloride concentrations on NG108-15 synapses with rat muscle are shown in Table 1. Increasing the calcium chloride concentration from 1.8 to 3.8 mM and adding 1 mM dibutyryl cAMP increased both the proportion of hybrid and muscle cell pairs that were found with synaptic connections and the proportion of muscle cells examined that exhibited spontaneous synaptic responses. Further adjustment by increasing the choline chloride concentration from 24 to 124 μM increased the proportion of cell pairs that were found with synaptic connections (47% of the pairs tested had synapses), but did not increase further the proportion of muscle cells that were found with spontaneous synaptic responses (67%).

The number of muscle cells with spontaneous synaptic responses may reflect more accurately the abundance of synapses than the number of muscle cells that can be demonstrated to respond to NG108-15 action potentials because small hybrid cells which probably form synaptic connections usually were not assayed. It seems unlikely that electrical coupling between muscle cells would result in a significant overestimate of the percent of muscle cells that were innervated, since diminution in amplitude between electrically coupled muscle cells would reduce most spontaneous synaptic responses to below the noise level. However, as opposed to spontaneous activity, muscle responses that are evoked by NG108-15 action potentials enable one to study many properties of synaptic transmission, and the documentation of a temporal relation between the NG108-15 action potential and the muscle cell response provides a more reliable synapse assay.

A summary of the results is shown in Table 2. Twelve to 25% of the NG108-15 hybrid cell and chick, mouse, or rat muscle cell pairs tested had functional synapses, and 27–82% of the muscle cells tested exhibited spontaneous synaptic responses. Synaptic responses were detected in up to 65–80% of the various species of muscle cells examined. These results show that NG108-15 cells form synapses with high frequency with chick, mouse, and rat striated muscle cells.

DISCUSSION

Our objective has been to study the specificity of synapse formation between clonal NG108-15 hybrid cells and striated muscle cells. The results show that NG108-15 hybrid cells form synapses with cells from different muscles and from different organisms such as the chick, mouse, and rat, and that most of the muscle cells tested were innervated by hybrid cells. We conclude that most striated muscle cells have the same specificity for synapse formation. No evidence was found for discrete classes of muscle cells with different specificities for synapse formation. In essence the results suggest that the formation of the neuromuscular synapse is not dependent upon a cell recognition code. The results also indicate that mechanisms of synapse formation are conserved during evolution and thus may be largely universal.

These results with clonal cells confirm and extend the findings of Crain *et al.*, which show that explanted mouse spinal cord neurons form synapses with mouse, rat, or human muscle cells (11, 12). In addition, Kidokoro and Heinemann have reported that neurons in explants of chick embryo spinal cord form synapses with clonal rat muscle cells (13). Under special conditions striated muscle cells have been shown to be innervated by functionally inappropriate motor neurons (14, 15), parasympathetic neurons (16), sympathetic ganglion neurons (17), cerebral cortex neurons (18), and neurons from the retina (D. Puro, F. DeMello, and M. Nirenberg, unpublished data). These results provide additional evidence there are few or no restrictions with respect to the type of cholinergic neuron that can synapse with a striated muscle cell.

Studies on the embryonic development of the mammalian neuromuscular synapse reveal that, at an early developmental stage when nicotinic acetylcholine receptors are distributed over the entire surface of the muscle cell (19), a single muscle cell usually is innervated by multiple neurons (20, 21). At this stage transmission across the synapse is relatively inefficient since some, but not all, neuron action potentials evoke muscle responses (20, 21). As the synapse matures the number of synaptic vesicles that release acetylcholine per motor neuron action potential increases 100- to 300-fold with a concomitant increase in the efficiency of transynaptic communication, and the concentration of nicotinic acetylcholine receptors decreases at all sites on the muscle cell other than the site of one synapse (19). Thus, the muscle cell is converted from a permissive state with respect to synapse formation to a nonpermissive state, and each muscle cell then is innervated by only one motor neuron.

The synaptic connections that form between NG108-15 hybrid cells and cultured striated muscle cells closely resemble the synapses that form between normal motor neurons and muscle cells at an early developmental stage. The demonstration that two hybrid cells can innervate a single muscle cell provides further evidence that these are immature synapses.

Lømo and Rosenthal (22) and Drachman and Witzke (23) have shown that the distribution of nicotinic acetylcholine receptors on muscle cells can be regulated by electrical stimulation of the muscle cell, and Stent (3) has suggested that regulation of acetylcholine receptor distribution may determine the assembly of certain neural circuits.

The evidence both on the development of the neuromuscular synapse and on the lack of specificity of synapse formation between clonal NG108-15 cells and striated muscle cells is compatible with the hypothesis that much of the specificity of the normal neuromuscular synapse is acquired after the synapses form by a process of selection that reduces the number of synapses and that is dependent upon effective transmission across the synapse, rather than by a process of matching com-

plementary molecules on neurons and muscle cells that code for different synaptic connections. Whether gradients of molecules provide positional information during synaptogenesis remains to be determined; however, it seems likely that both the regulation of the acetylcholine excreted from the neuron and the distribution of nicotinic acetylcholine receptors on the muscle cells are involved in the synapse selection process.

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1. Sperry, R. W. (1963) *Proc. Natl. Acad. Sci. USA* **50,** 703–710.
2. Hubel, D. H. & Wiesel, T. N. (1963) *J. Neurophysiol.* **26,** 994–1002.
3. Stent, G. S. (1973) *Proc. Natl. Acad. Sci. USA* **70,** 997–1001.
4. Minna, J., Nelson, P., Peacock, J., Glazer, D. & Nirenberg, M. (1971) *Proc. Natl. Acad. Sci. USA* **68,** 234–239.
5. Minna, J., Glazer, D. & Nirenberg, M. (1972) *Nature New Biol.* **235,** 225–231.
6. Nelson, P., Christian, C. & Nirenberg, M. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 123–127.
7. Amano, T., Hamprecht, B. & Kemper, W. (1974) *Exp. Cell Res.* **85,** 399–408.
8. Vogel, Z., Sytkowski, A. J. & Nirenberg, M. W. (1972) *Proc. Natl. Acad. Sci. USA* **69,** 3180–3184.
9. Nelson, P. G., Peacock, J. H., Amano, T. & Minna, J. (1971) *J. Cell. Physiol.* **77,** 337–352.
10. Fukuda, J. (1974) *Science* **185,** 76–77.
11. Crain, S. M., Alfei, L. & Peterson, E. R. (1970) *J. Neurobiol.* **1,** 471–489.
12. Peterson, E. R. & Crain, S. M. (1970) *Z. Zellforsch. Mikrosk. Anat.* **106,** 1–21.
13. Kidokoro, Y. & Heinemann, S. (1974) *Nature* **252,** 593–594.
14. Székely, G. & Szentagothai, J. (1962) *J. Embryol. Exp. Morphol.* **10,** 140–151.
15. Frank, E., Jansen, J. K. S., Lomø, T. & Westgaard, R. H. (1975) *J. Physiol. (London)* **247,** 725–743.
16. Landmesser, L. (1971) *J. Physiol. (London)* **213,** 707–725.
17. Nurse, C. A. & O'Lague, P. H. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 1955–1959.
18. Kodokoro, Y., Heinemann, S., Schubert, D., Brandt, B. L. & Klier, F. G. (1976) *Cold Spring Harbor Symp. Quant. Biol.* **40,** 373–388.
19. Diamond, J. & Miledi, R. (1962) *J. Physiol. (London)* **162,** 393–408.
20. Redfern, P. A. (1970) *J. Physiol. (London)* **209,** 701–709.
21. Bennett, M. R. & Pettigrew, A. G. (1974) *J. Physiol. (London)* **241,** 515–545.
22. Lømo, T. & Rosenthal, J. (1972) *J. Physiol. (London)* **221,** 493–513.
23. Drachman, D. B. & Witzke, F. (1972) *Science* **176,** 514–516.