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Synapse formation between clonal neuroblastoma × glioma hybrid cells and striated muscle cells

(cell recognition/acetylcholine receptor/transmitter storage and release/regulation of synapse formation)

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ABSTRACT **Clonal neuroblastoma × glioma hybrid cells were shown to form synapses with cultured, striated muscle cells. The properties of the synapses between hybrid and muscle cells were similar to those of the normal, neuromuscular synapse at an early stage of development. The number of synapses formed and the efficiency of transmission across synapses were found to be regulated, apparently independently, by components in the culture medium. Under appropriate conditions synapses were found with 20% of the hybrid-muscle cell pairs examined; thus, the hybrid cells form synapses with relatively high frequency.**

Little is known at the molecular level about the process of synapse formation and the basis for the specificity of synaptic connections between cells. Some of the difficulties in studying this problem stem from the many cell types that are present in the nervous system and the lack of adequate criteria that can be used to distinguish one type of neuron from another. Clonal lines of neuroblastoma cells (1, 2) and somatic cell hybrids (3–6) derived from neuroblastoma cells have proved experimentally advantageous for the study of some neural properties. Such cells continue to proliferate; thus populations of cells that appear to be fairly homogeneous can be obtained, yet cells retain the ability to express various properties of differentiated neurons. One of the primary objectives in generating and characterizing these cell lines has been to obtain cell lines that are able to form synapses *in vitro* with high frequency. In this report, we show that clonal neuroblastoma × glioma hybrid cells which are known to synthesize, store, and excrete acetylcholine* form synapses with striated muscle cells *in vitro*.

MATERIALS AND METHODS

Cell Cultures. NG108-15 hybrid cells were derived by fusion of mouse neuroblastoma clone N18TG-2 resistant to 6-thioguanine (4), with rat glioma clone C6BU-1, resistant to 5-bromodeoxyuridine (5). NG108-15 cells routinely were cultured at 37° in medium D (90% Dulbecco-Vogt modification of Eagle's minimal essential medium, GIBCO H-21; 10% fetal bovine serum; 1×10^{-4} M hypoxanthine; 1×10^{-6} M aminopterin; and 1.6×10^{-5} M thymidine) in a humidified atmosphere of 10% CO_2–90% air. NG108-15 cells were cultured for 1–3 weeks in the presence of 1 mM N^6, O^2-dibutyryl-adenosine-3′:5′-cyclic monophosphate (dibutyryl cAMP) to shift the hybrid cells to a more differentiated state*. All experiments were performed with NG108-15 cells that had been subcultured 14 to 20 times.

It was necessary to remove butyric acid and perhaps other contaminants from dibutyryl cAMP prior to use. The following method was used. Dibutyryl cAMP dissolved in H_2O (1 g/20 ml) was adjusted to pH 3.0 with 0.1 M HCl and then extracted vigorously (and sometimes repeatedly) with 10 volumes of diethylether. The aqueous layer was recovered, adjusted to pH 6.5 with 0.1 M NaOH, and lyophilized.

Cultures of striated muscle cells were prepared by dissociation of cells from the hindlimbs of 18 to 21-day-old C57BL/6N mouse embryos with 0.25% trypsin for 40 min at 37°. The concentration of fibroblasts was reduced by incubating cell suspensions for 20 min in Falcon plastic petri dishes resulting in the preferential attachment of fibroblasts. A suspension of 3.5×10^5 single cells that did not attach to the petri dish was plated in a collagen-coated 35 mm plastic culture dish containing either 1.5 ml of 80% DMEM, 10% fetal bovine serum, 10% horse serum; or 40% DMEM, 40% MEM (adjusted to 33 mM dextrose and 44 mM sodium bicarbonate), 10% fetal bovine serum, and 10% horse serum. Cells were grown at 36° in a humidified atmosphere of 10% CO_2–90% air. The medium was replaced 24 and 48 hr later; thereafter, cultures were fed twice weekly with 90% DMEM and 10% horse serum, except where noted. Usually on the ninth day of incubation of muscle cells 3×10^4 NG108-15 hybrid cells were added to each muscle culture. NG108-15 and muscle cells usually were cocultured for an additional 1–2 weeks in 90% DMEM, 10% horse serum; 1 mM dibutyryl cAMP; 0.1 mM hypoxanthine; and 0.016 mM thymidine before cells were assayed for synapses.

Electrophysiology. Intracellular recording and stimulation techniques were essentially as described (7). *d*-Tubocurarine chloride solutions (0.6 to 6×10^{-5} M) in pipettes with tips 5–20 μm in diameter were applied to muscle cells by allowing the solution to diffuse from the pipette tip. Usually *d*-tubocurarine chloride was dissolved in the same medium that the cells were exposed to; to avoid nonspecific effects, the pH was maintained at 7.0–7.5.

The medium bathing cells during the electrophysiologic experiments was either DMEM or modified MEM without serum usually adjusted as follows (except where stated): 3.8 mM $CaCl_2$; 20 μM eserine sulfate (freshly prepared); 1 mM dibutyryl cAMP, purified as described above; 0.1 mM hypoxanthine; and 0.016 mM thymidine. In some experiments cells were treated with 1 mM N^2,O^2-dibutyryl-guanosine-3′:5′-cyclic monophosphate (dibutyryl cGMP) for 1 day prior to the assay in place of dibutyryl cAMP. This substitution had no apparent effect on synapse formation.

Materials. The following materials were used: aminopter-

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

* B. Hamprecht, T. Amano, P. Simpson, and M. Nirenberg, unpublished observations.

in, choline chloride (Nutritional Biochem. Corp.); hypoxanthine (General Biochem, Inc.); *d*-tubocurarine chloride (Abbott or Sigma Chem. Co.); eserine sulfate, dibutyryl cGMP, sodium salt (Sigma Chem. Co.); thymidine (Worthington Biochem. Corp.); and dibutyryl cAMP, sodium salt (JEM Research).

RESULTS

Neuroblastoma × glioma NG108-15 hybrid cells usually were grown for 1–3 weeks in the presence of 1 mM dibutyryl cAMP, and then were dissociated and plated on a monolayer of mouse striated muscle cells formed by fusion of myoblasts *in vitro* during the previous 1–2 weeks. The electrical excitability of the cells was improved greatly when contaminants were removed from dibutyryl cAMP by extraction with diethylether prior to use as described in *Materials and Methods*. The hybrid cells attached readily to the muscle monolayer and extended long branched processes with varicosities. Processes with terminal swellings were found which were attached to muscle cells (Fig. 1A). Pairs of interacting hybrid and muscle cells that were well differentiated morphologically and were not obscured by other cells were selected for electrophysiological study to determine whether synapses were present. The muscle and hybrid cells were impaled with separate microelectrodes, the resting membrane potentials were determined; then the hybrid cell was stimulated electrically to elicit action potentials, and muscle responses were recorded. Nearly all adequately penetrated NG108-15 cells exhibited action potentials in response to electrical stimulation, and spontaneous repetitive action potentials were found with 5–10% of the hybrid cells tested.

As shown in Fig. 1C, about 50% of the action potentials elicited in the NG108-15 hybrid cell by repetitive electrical stimulation resulted in transient depolarizing shifts in the muscle membrane potential which varied in amplitude. The proportion of hybrid action potentials that evoked muscle responses varied with different cell pairs, but usually was <50%.

As shown in Fig. 1B, electrical stimulation of an NG108-15 cell (trace 6) elicited an action potential in the hybrid cell (trace 5) which was followed after a delay of several msec by a muscle response. Four examples of muscle responses evoked by hybrid action potentials are shown (traces 1–4). Some variation was observed in the delay and in the amplitude and duration of muscle responses to different hybrid action potentials. These muscle responses clearly were not due to direct electrical coupling between hybrid and muscle cells since hyperpolarizing currents passed across the hybrid or the muscle cell membrane did not change the membrane potential of the other cell. Electrical coupling between NG108-15 and muscle cells were observed infrequently (<1% of the cell pairs examined).

The action potential of a muscle cell (Fig. 1C) elicited by electrical stimulation of the muscle cell characteristically had a faster rate of rise than that of NG108-15 and a time course and amplitude that was easily distinguished from muscle responses to NG108-15 action potential.

In Fig. 2 the amplitudes of muscle responses are shown as a function of response frequency. The response amplitudes of the muscle cells varied from 0.4 mV, just above the noise level, to 14 mV. Most muscle responses involved relatively small shifts in membrane potential; the mean response amplitude was 4.3 mV. Analysis of other synapses revealed amplitude distribution similarly skewed towards the lower amplitudes, but with a wide range of response amplitudes.

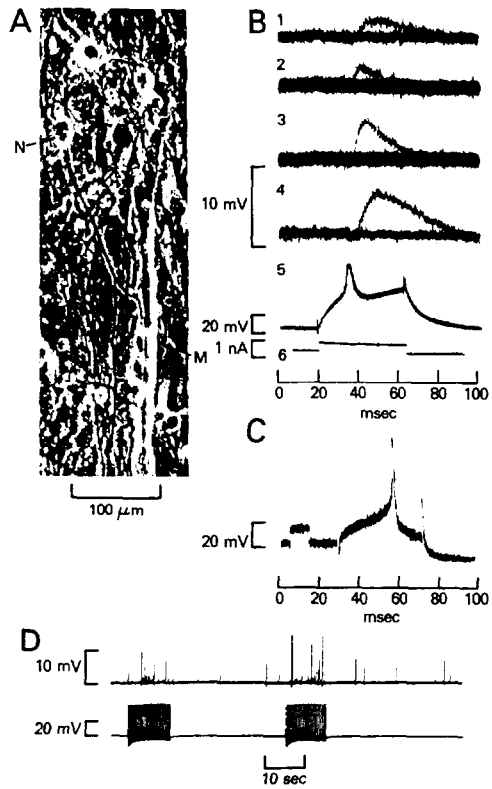

FIG. 1. (A) Phase contrast photomicrograph of a culture containing neuroblastoma × glioma NG108-15 hybrid cells and normal mouse muscle cells. NG108-15 cells, grown in the presence of 1 mM dibutyryl cAMP for 10 days, were dissociated and added to a culture of muscle cells that had been cultured for 20 days. The medium used to assay cells for synapses consisted of MEM minus sodium bicarbonate, 15 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), pH 7.1, 8.0 mM CaCl, 260 μM choline chloride, 1 mM dibutyryl cAMP, 0.1 mM hypoxanthine, 16 μM thymidine, and 33 mM dextrose in a humidified atmosphere of 5% CO_2–95% air. (B) Intracellularly recorded responses of the muscle cell labeled M shown in panel A to action potentials electrically elicited in the NG108-15 hybrid cell labeled N. The resting membrane potential of the muscle cells was −50 mV. *Traces 1–4:* Each high gain trace shown represents 5–10 superimposed oscilloscope sweeps showing 1 muscle response per set of sweeps. *Trace 5:* An example of an NG108-15 action potential that elicited a response such as those shown in traces 1–4. Traces 1–5 are aligned so that temporal relationships between the neuron action potential and muscle response are shown. *Trace 6:* Current pulse used to elicit an NG108-15 action potential. The hybrid cell was stimulated three times per sec. The resting membrane potential of the hybrid cell was −30 mV; the membrane potential was adjusted to −75 mV with constant current. (C) Action potential of a muscle cell elicited by electrical stimulation of the muscle cell (0.4 nA, 40 msec). Resting membrane potential was −50 mV. (D) Penwriter recordings from a muscle cell obtained with intracellular electrodes (upper record) and from an NG108-15 cell with an intracellular electrode (lower record) showing spontaneous muscle events and muscle responses to NG108-15 action potentials elicited by repetitive electrical stimulation of the hybrid cell. The largest muscle responses in the second set of evoked responses are action potentials attenuated by the penwriter frequency response.

d-Tubocurarine inhibits cell responses to acetylcholine that are mediated by nicotinic acetylcholine receptors and thus would be expected to inhibit muscle responses to acetylcholine released by NG108-15 cells. The effect of *d*-tubocurarine is shown in Fig. 3. When the tip of a pipette 10 μm in

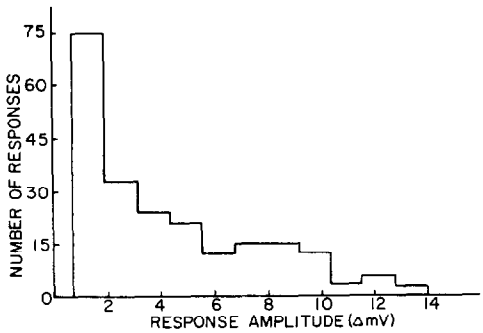

FIG. 2. Muscle response amplitude (Δ mV) to NG108-15 action potentials plotted as a function of the number of muscle responses observed. A total of 456 action potentials were evoked in NG108-15 by electrical stimulation; 215 muscle responses were observed. The mean response amplitude ($\bar{x}$) is 4.3 mV. The resting membrane potential of the muscle cell was −44 mV.

diameter that contained 6×10^{-5} M *d*-tubocurarine dissolved in DMEM was positioned near the site of the synapse, the frequency and amplitudes of muscle responses to NG108-15 action potentials were reduced greatly, whereas little or no change (<2 mV) was observed in the membrane potentials of muscle and hybrid cell or in NG108-15 action potentials. Muscle responses returned when the pipette was withdrawn; thus the inhibition was reversible. As shown in Fig. 3B, the muscle response rate was not affected when the tip of a control pipette that contained only DMEM was placed near the site of the synapse. In Fig. 3C a graded inhibition of muscle response was obtained by varying the distance between the tip of the *d*-tubocurarine pipette and the site of the synapse. Perfusion studies (10^{-6} M) also show that *d*-tubocurarine inhibits muscle responses evoked by NG108-15 action potentials as well as spontaneous muscle

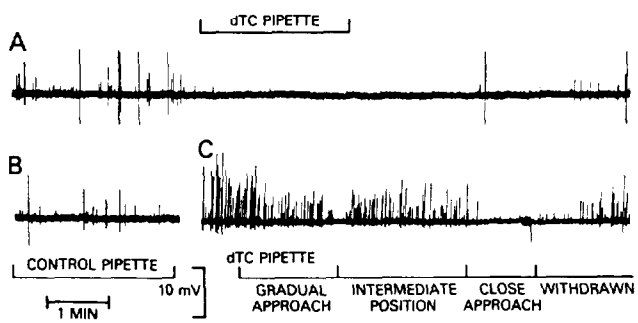

FIG. 3. Inhibition by *d*-tubocurarine of muscle responses evoked by NG108-15 action potentials. (A) The tip of the pipette containing 6×10^{-5} M *d*-tubocurarine chloride (dTC) dissolved in DMEM (the cells were also in DMEM) was placed near the site of the NG108-15 synapse with the muscle cell shown in Fig. 1A while the hybrid cell was stimulated three times/sec to evoke action potentials. The duration of the application of *d*-tubocurarine is indicated by the bar above the tracing. The muscle resting membrane potential was −50 mV. (B) Recording obtained from the same muscle cell as in panel A after recovery from exposure to *d*-tubocurarine was almost complete. A control pipette containing medium without *d*-tubocurarine was placed in the same position as the *d*-tubocurarine pipette of panel A. The amplitudes of the muscle events were essentially normal. (C) Experiment with a different NG108-15 muscle cell showing a graded reversible inhibition of muscle responses by *d*-tubocurarine chloride which depended on the proximity of the tip of the pipette containing the *d*-tubocurarine solution to the site of synapse between the hybrid and muscle cells. The position of the *d*-tubocurarine pipette tip is indicated below the tracing. The muscle resting membrane potential was −48 mV.

events, and that the inhibition by *d*-tubocurarine is reversible. The inhibition of spontaneous muscle events by *d*-tubocurarine indicates that spontaneous events were of synaptic origin.

To test the relation between the amplitude of muscle responses and the membrane potential of the muscle cell, constant current was passed across the muscle cell membrane through the recording microelectrode The amplitudes of muscle responses evoked by hybrid cell action potentials were increased by steady polarizing currents which increased the muscle membrane potential, and were decreased by steady depolarizing currents. The muscle responses changed in the directions expected for membrane polarization reactions mediated by nicotinic acetylcholine receptors; however, the nonlinearity of the muscle membrane resistance precluded a meaningful estimate of the reversal potential for the muscle response. Further experiments are needed to clarify this important point.

During the course of these studies, exploratory experiments were performed to determine the effect of culture and assay conditions upon the abundance of synapses (Table 1). Increasing the $CaCl_2$ concentration from 2.7 to 8 mM did not appreciably affect the frequency with which synapses were detected. Synapses were found with 20% of the cell pairs tested when the following conditions were used. NG108-15 cells were grown in the presence of 1 mM dibutyryl cAMP, purified as described in *Materials and Methods*, for 2 weeks, and muscle cells were grown for approximately 9 days as described in *Materials and Methods*. Hybrid and muscle cells were then cocultured for an additional 8–15 days in 90% DMEM, 10% horse serum, 1 mM purified dibutyryl cAMP, 0.1 mM hypoxanthine, 0.016 mM thymidine and were assayed for synapse formation in this medium without serum modified as follows: 3.8 mM $CaCl_2$, 270 μM choline chloride, and 20 μM eserine sulfate. Increasing the choline concentration to 270 μM (124 μM in more recent experiments) when assaying for synapses may be helpful since the intracellular choline concentration may limit the rate of acetylcholine synthesis, whereas growth of cells in the pres-

Table 1. Effect of growth and assay conditions on synapse frequency

Cell growth*	Synapse assay*		Synapses found	Cell pairs tested	Pairs with synapse (%)
Choline μM	Choline μM	$CaCl_2$ mM			
6†	6†	1.8	0	54	0
24	6†	1.8	1	12	8
24	6†	3.8	4	45	9
24	6‡	8.0	1	15	7
24	260‡	8.0	2	8	25
24	24	3.8	1	1	
24	270	3.8	21	104	20
270	24	3.8	0	15	0
270	270	3.8	1	35	3
		Total	31	289	11%

* The medium was DMEM, except where specified, plus the components described under *Materials and Methods*.

† MEM medium adjusted to 33 mM dextrose and 44 mM sodium bicarbonate plus the components described under *Materials and Methods*.

‡ MEM with 15 mM Hepes, pH 7.1, and 33 mM dextrose without sodium bicarbonate plus the components described under *Materials and Methods*.

ence of 270 μM choline may decrease the number of synapses that are present. These results suggest that both synapse formation and the efficiency of transmission across the synapse are regulated by choline or other media components and that synapse frequency and efficiency are regulated by different processes.

DISCUSSION

In previous studies, neuroblastoma × glioma NG108-15 hybrid cells were shown to synthesize, store, and excrete acetylcholine and to generate action potentials in response to electrical stimulation*, whereas striated muscle cells have abundant nicotinic acetylcholine receptors. Thus, the components that are known to be required for synaptic communication between the two cell types can be expressed *in vitro*. In this report, we show that clonal NG108-15 cells do, indeed, form chemical synapses with striated muscle cells. The evidence for synapse formation is based upon the following observations: (*a*) action potentials in NG108-15 hybrid cells elicit muscle responses after a variable delay; (*b*) *d*-tubocurarine, an inhibitor of the nicotinic acetylcholine receptor, inhibits muscle responses reversibly; and (*c*) muscle response amplitudes increased when the muscle membrane potential was adjusted with constant hyperpolarizing current, and decreased, when adjusted with constant depolarizing current, as expected for reactions that are mediated by the nicotinic acetylcholine receptor.

Muscle responses to NG108-15 action potentials of the type shown in Fig. 1 were shown not to be due to direct electrical coupling between muscle and hybrid cells. We have considered a more complex electrical circuit involving a second muscle cell electrically coupled to both the NG108-15 and the muscle cell recorded from but reject this interpretation because it is not in accord with the findings that the incidence of direct electrical coupling between hybrid and muscle cells is low (<1% of the cell pairs tested) compared to the higher incidence of synapse formation (11% of the 276 cell pairs tested and, in some cultures, 20–25% of the cell pairs examined). In addition, the inhibition of muscle responses by *d*-tubocurarine agrees well with receptor-mediated synaptic communication, but not with an electrical coupling mechanism of communication. Electrical connections between NG108-15 and muscle cells, although infrequent, represent a second mode of communication between cells and deserve further study.

According to the quantal hypothesis (8), a neuron action potential may induce the release of acetylcholine in packets of transmitter which may correspond to one or more storage vesicles and thereby simultaneously activate many muscle acetylcholine receptors. If the release mechanism obeys Poisson statistics, the mean number of vesicles that release their contents per action potential (the mean quantal response) can be calculated from either the number of neuronal action potentials that fail to elicit muscle responses or the coefficient of variation of muscle response amplitudes (9–11). There was excellent agreement between the values obtained by the two methods of calculation with the four NG108-15 synapses analyzed in this way, with the mean number of vesicles released per NG108-15 action potential ranging from approximately 0.1 to 0.5. The low quantum content indicates that most of the muscle responses observed were due to the release of acetylcholine from a single vesicle. The mean amplitude of the muscle responses ranged from 0.5 to 5.0 mV; in all cases studied the range of amplitudes was large. These observations may be due to the presence in NG108-15 cells of both clear vesicles 600 Å in diameter and dense core neurosecretory vesicles 1800 Å in diameter (6). Whether one or both types of vesicles contains acetylcholine and releases this compound following an NG108-15 action potential remains to be determined.

Early in the development of synapses between normal mammalian motor neurons and striated muscle cells, a single muscle cell is innervated by more than one neuron (12, 13), whereas at a later developmental stage, only one neuron synapses with a single muscle cell. The efficiency of synaptic communication during the early stage in synapse formation is low, and most muscle responses are below the threshold for activation of action potentials (12–14). At a later stage in the development of the synapse the efficiency of transsynaptic communication becomes 100% since every neuronal action potential elicits muscle cell contractions. The synapses formed between NG108-15 hybrid cells and striated muscle cells closely resemble those formed between normal motor neurons and striated muscle cells during the early stage of synapse development. The frequency of synapse formation between hybrid and muscle cells equals or exceeds the synapse frequency reported for normal dissociated spinal cord neurons and striated muscle cells *in vitro* (15). The efficiency of synaptic transmission might be affected by many factors. For example, high concentrations of nicotinic acetylcholine receptors have been found at points of contact between neuroblastoma and striated muscle cells (16); however, similar receptor "hot spots" are found on muscle cells in the absence of neuroblastoma cells (17, 18).

Establishment of synapses between nerve and muscle poses a paradox, for on the one hand, muscle movements are highly coordinated, which suggests that neuromuscular synapses and other synapses in the neural circuits are assembled with high precision, whereas the demonstrated ability of autonomic neurons of the vagus (19), sympathetic ganglion neurons (20), and clonal NG108-15 hybrid cells to synapse with striated muscle cells suggests that functional synapses can form that may not be dependent upon highly specific cell recognition molecules. Although most striated muscle cells in mammals are innervated by spinal motor neurons, it is interesting to note that the esophagus contains striated muscle cells that normally are innervated by autonomic neurons of the vagus.

The molecular nature of the cell interactions that lead to synapse formation is not known. It is clear that NG108-15 cells adhere firmly to muscle cells; however, the hybrid cells also adhere to one another, to fibroblasts, to other cell types, and to the polystyrene petri dish. Whether NG108-15 hybrid cells have specific cell recognition molecules that are required for synapse formation remains to be determined. Since NG108-15 hybrid cells are clonal cells and under appropriate culture conditions synapses formed between 20 and 25% of the hybrid-muscle cell pairs examined, the simplest hypothesis is that NG108-15 may constitute one class of cells with respect to synapse formation, and muscle cells, another class, and that any sufficiently well differentiated NG108-15 cell may be able to form synapses with any muscle cell in the culture. If specific cell recognition molecules are required to establish synapses between NG108-15 hybrid cells and mouse striated muscle cells, it seems likely that few kinds of recognition molecules may be required to establish synaptic connections during the early stage of development.

Synapses between NG108-15 and muscle cells resemble in various ways the early form of the normal neuromuscular

synapse. The results suggest that synapse formation and the efficiency of transmission are regulated *in vitro*, apparently by independent processes. It may be possible to find conditions for the conversion of the early form of synapse to the late, mature form. It also seems probable that NG108-15 cells may synapse with clonal muscle cells, since normal spinal cord neurons reportedly synapse with clonal muscle cells (21). Clearly, the ability of NG108-15 hybrid cells to form synapses affords many opportunities to correlate biochemical aspects of synaptogenesis with developmental and electrophysiologic aspects and to explore the molecular basis for the specificity of synaptic connections between cells.

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