Synapse Formation Between Two Clonal Cell Lines

Abstract. *Clonal neuroblastoma × glioma hybrid cells frequently formed synapses with clonal mouse striated muscle cells. Clonal myotubes were similar to cultured mouse embryo myotubes with respect to acetylcholine sensitivity and other membrane properties examined. However, acetylcholine sensitivity measurements indicate that acetylcholine receptors of clonal myotubes are distributed more uniformly over the cell surface than the receptors of cultured mouse embryo myotubes.*

A primary reason for the use of cell tissue culture is the possibility of analyzing a relatively simple system which displays the relevant phenomenon to be investigated (*1*). Certain clonal cell lines provide a large and readily manipulated population of homogenous cells with neural or muscle characteristics and recently have been demonstrated to be synaptically competent. Kidokoro and Heinemann (*2*) found that striated myotubes formed from clonal myogenic lines can be innervated by normal neurons in explants of embryonic spinal cord. We have previously shown that clonal neuroblastoma × glioma hybrid NG108-15 cells form functional synapses with cultured striated myotubes from the embryonic mouse (*3*). In this report, we describe the establishment from mouse muscle cultures of a myogenic clonal line, G-8, and show that the clonal hybrid cells form synapses with clonal striated myotubes.

The clonal line, G-8, was obtained from M114, an uncloned myogenic cell line which arose spontaneously in a culture of cells dissociated from Swiss Webster mouse hindlimb muscle (*4*). Whereas the diploid number of chromosomes in the mouse is 40, the modal chromosome number of G-8 cells is 75. When confluent, most G-8 cells form parallel arrays of spindle-shaped mononucleated cells which fuse to form multinucleated myotubes up to 500 μm in length; multinucleated spherical cells also are found in some older cultures. The myotubes attach to the plastic surface of the petri dish and to neighboring cells. Well-differentiated G-8 myotubes possess striations and closely resemble normal mouse myotubes in morphology. Many G-8 myotubes contract spontaneously.

Neuroblastoma × glioma NG108-15 cells generate action potentials in response to electrical or chemical stimuli and synthesize, store, and release acetylcholine (*3*). When added to plates con-

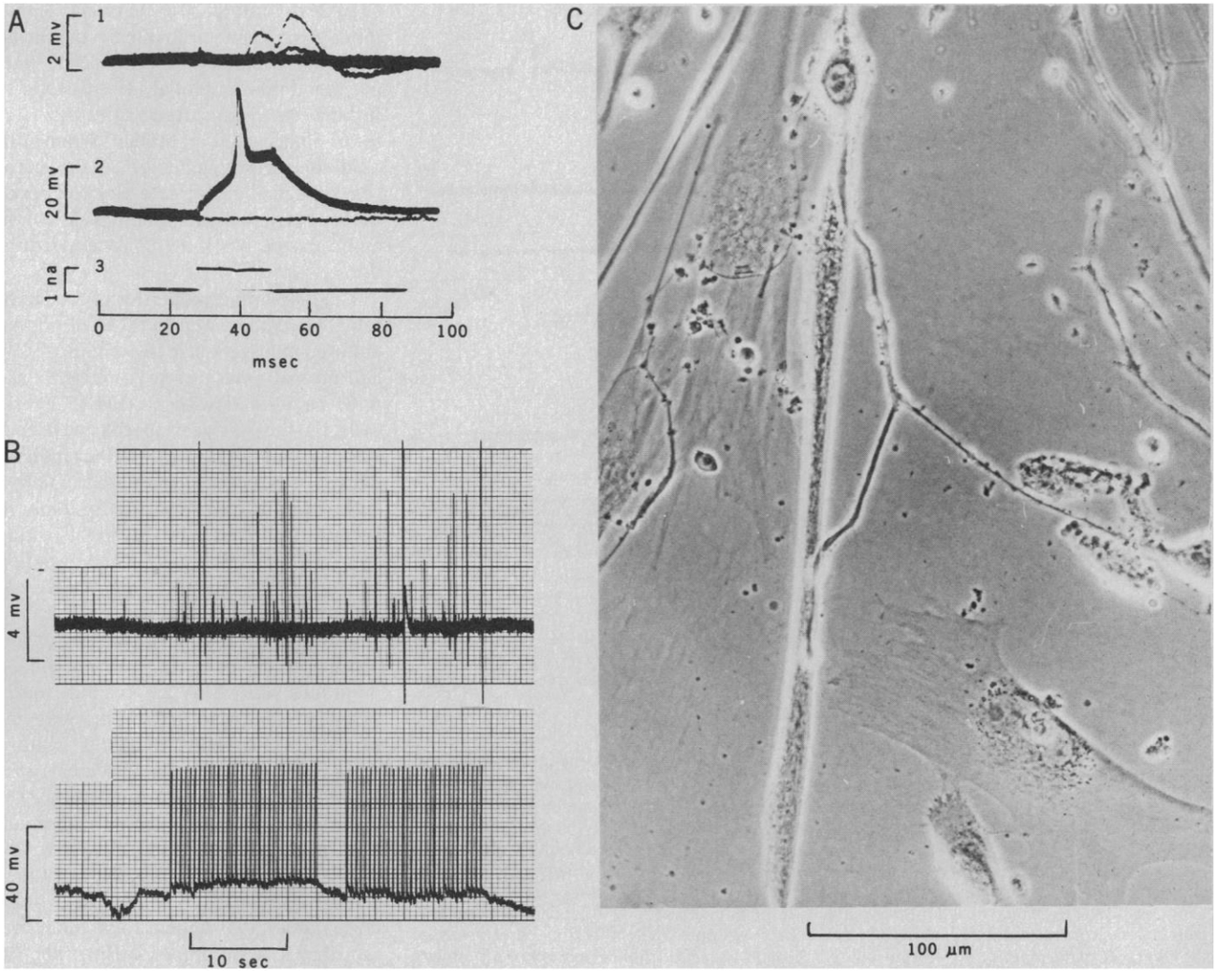

Fig. 1. Innervation of a G-8 myotube by a hybrid cell NG108-15. (A) Multiple oscilloscope sweeps demonstrating a functional connection between a hybrid and myotube. 1, Myotube recording; resting membrane potential is 45 mv. 2, Hybrid recording. 3, Current through hybrid recording electrode. (B) Chart record of same connection showing dependence of myotube responses (upper trace) on spikes elicited in hybrid (lower trace) by depolarizing currents passed through hybrid recording pipette. (C) Appearance of a coculture plated 31 days previously with myotubes and 18 days previously with hybrid cells.

taining G-8 myotubes (5), the hybrid cells attach readily to the plastic substratum and to neighboring hybrid or muscle cells and then extend long processes (Fig. 1C). The formation of synapses between NG108-15 hybrid cells and G-8 muscle cells was assessed after the cells had been cocultured for 3, 5, and 9 days. During the synapse assay the concentration of horse serum in the medium was reduced to 3 percent and the concentrations of choline chloride and calcium chloride were increased to 124 μM and 3.8 mM, respectively. The NG108-15 hybrid and G-8 muscle cells in physical contact were impaled with separate microelectrodes; the hybrid cell was stimulated electrically to elicit action potentials and the muscle responses were recorded. In 8 of 23 pairs tested, NG108-15 cell action potentials evoked depolarizing responses in myotubes. An example is shown in Fig. 1, A and B. The amplitude of the myotube responses to NG108-15 action potentials ranged from 0.3 mv, the electrical noise level, to 8 mv, and their latency varied from 1 to 40 msec. The efficiency of transynaptic communication varied from one synapse to another; but in most cases 10 to 50 percent of the NG108-15 hybrid action potentials evoked muscle responses.

As shown in Fig. 2B, myotube responses to NG108-15 cell action potentials were mimicked by the iontophoretic application of acetylcholine. Responses in G-8 myotubes produced by NG108-15 cell innervation were reversibly blocked by $3 \times 10^{-5} M$ *d*-tubocurarine released from a nearby blunt micropipette, as previously demonstrated (*3*, *6*) in normal mouse myotubes innervated by NG108-15 cells. The NG108-15 cells thus appear to form chemical synapses with either normal mouse or G-8 myotubes, utilizing acetylcholine as a neurotransmitter.

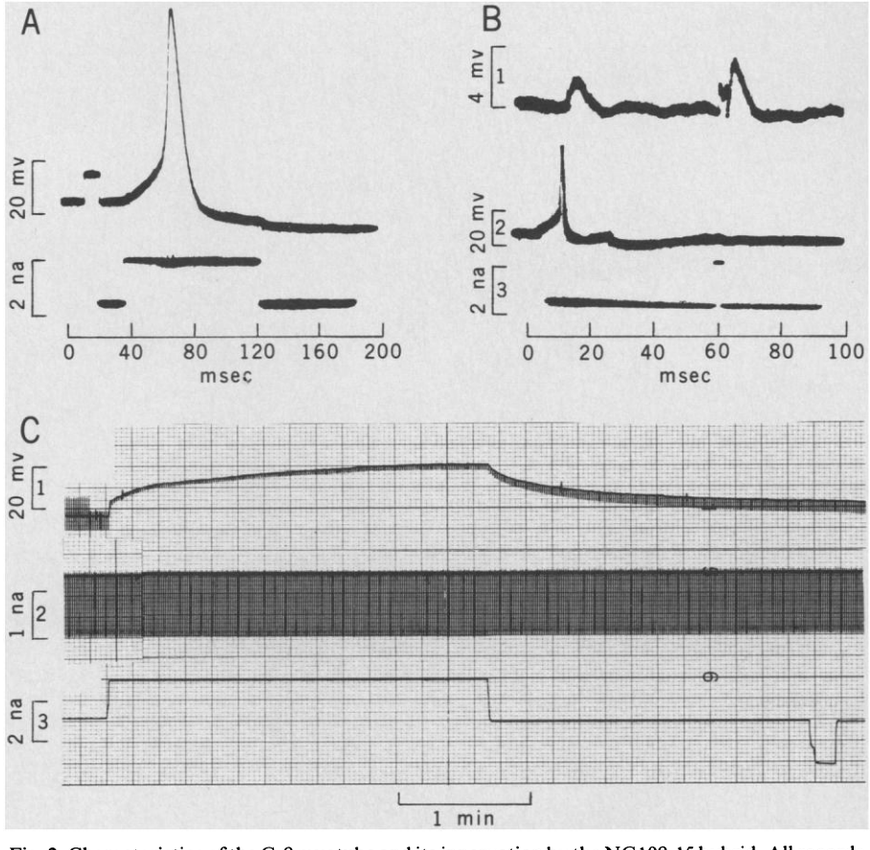

Fig. 2. Characteristics of the G-8 myotube and its innervation by the NG108-15 hybrid. All records are from tissue culture dishes of fused myotubes, cocultured for at least 3 days with hybrid cells. (A) An oscilloscope trace of an action potential (upper trace) in a myotube elicited by anodal break stimulation. A constant hyperpolarizing current (lower trace) was passed through a second electrode in the myotube, producing a membrane potential of 65 mv, then briefly terminated to produce an action potential. (B) An oscilloscope trace demonstrating the similarity of a myotube depolarization produced by an action potential in a nearby hybrid and by direct focal application of acetylcholine from an iontophoretic pipette. 1, Intracellular recording from a G-8 myotube. 2, Intracellular record of contiguous hybrid cell caused to spike by depolarizing current passed through recording pipette. 3, Current passed through an acetylcholine iontophoretic pipette placed close to the myotube membrane. (C) Chart record demonstrating a maintained myotube depolarization and conductance increase caused by continued application of acetylcholine. 1, Myotube membrane potential; resting potential 48 mv. 2, Depolarizing pulses (one per second) passed through a second intracellular pipette. 3, Current passed through an acetylcholine iontophoretic pipette. Baseline represents the −2 na holding current, which was turned off for approximately 3 minutes to release the drug.

In the present study, synapses were found with 34 percent of the NG108-15 hybrid cell and G-8 muscle cell pairs tested. Thus, these clonal cell lines form synapses with each other with high frequency. The hybrid cells form synapses with normal mouse embryo myotubes with the same high frequency (*3*). The formation of synaptic connections between clonal hybrid cells and clonal myotubes demonstrates that synapse formation is not dependent upon Schwann cells or other cell types.

To determine whether G-8 myotubes could be innervated by normal spinal cord neurons as well as by the hybrid cells, myotubes were examined 12 to 19 days after the addition of spinal cord cells. Spontaneously occurring postsynaptic potentials were recorded from 13 of 26 myotubes. Muscle response amplitudes were similar to those found in the biclonal system, as was the muscle resting membrane potential, 49 mv (± 2.9) for innervated compared to 47 mv (± 2.2) for noninnervated myotubes. When added to the recording medium, *d*-tubocurarine produced a complete block of spontaneous postsynaptic potentials at $10^{-8} M$ and a partial block at $10^{-9} M$ concentrations.

The membrane properties of G-8 myotubes were compared to those of normal cultured mouse muscle cells (*7*). Both G-8 and normal mouse embryo muscle cells were cocultured with NG108-15 hybrid cells, but the passive properties were analyzed without regard to whether individual myotubes were innervated. Table 1 shows that clonal and mouse embryo myotubes differed primarily in the resting membrane potential and in the variation in sensitivity to acetylcholine at different sites on the membrane surface.

The average measured resting membrane potential of cultured normal mouse myotubes was 11 mv greater than found in clonal G-8 myotubes. This may indicate that normal myotubes mature more quickly in vitro than do clonal myotubes. The measured resting membrane potential of mouse (*8*), rat (*9*), and chick (*10*) myotubes has been shown to increase with age as the cultured myotubes mature in vitro. At the time of testing, normal myotubes appear to be more mature than G-8 myotubes with respect to striations and hypolemmal nuclei.

Many of the myotubes in both types of cultures contracted spontaneously, an effect accentuated by the presence of dibutyryl 3′,5′-adenosine monophosphate

(dibutyryl cyclic AMP). In all G-8 myotubes tested, action potentials could be elicited by intracellular depolarizing pulses, although as with rat muscle clone L-6 cells (*11*), it was often first necessary to hyperpolarize the G-8 myotube membrane potential (Fig. 2A). Most G-8 myotubes contracted when adequately stimulated.

All G-8 myotubes tested responded to short pulses of iontophoretically applied acetylcholine with brief monophasic depolarizations (Fig 2B); longer applications of acetylcholine were accompanied by an approximately threefold decrease in membrane resistance (Fig. 2C). The polarity of the response reversed when the membrane potential was adjusted to a more positive value than −5 mv. These results suggest that the response of the G-8 cells to acetylcholine involves an increase in permeability to sodium and potassium ions, as reported for other cultured myotubes (*12*).

The G-8 muscle cells were highly sensitive to acetylcholine, although the average maximum sensitivities of G-8 myotubes were somewhat lower than those of primary mouse myotubes (875 as opposed to 1474 mv/ncoulomb). This may be due to the lower resting membrane potential and membrane resistance of the G-8 myotubes, rather than to differences in the maximum acetylcholine receptor concentrations on the two types of myotubes. Whereas the acetylcholine sensitivity of normal myotubes at sites other than the site of maximum sensitivity often was not detected with short iontophoretic acetylcholine pulses, the responsiveness of G-8 myotubes was uniformly high over the entire surface of the cell (at least 400 mv/ncoulomb in a number of myotubes). Such uniformly high sensitivity to acetylcholine was not observed with clonal L-6 rat muscle cells (*13*) or normal cultured myotubes (*14*).

To compare the efficiencies of the synapses formed by the hybrid cell on the clonal and normal cultured myotubes, we determined the peak postsynaptic conductance change caused by the presumed release of a single quantum of acetylcholine. Because synapses on both types of myotubes had high failure rates (> 0.5), and the wide variability of response latencies indicated that more than one quantum released on any trial would be detected as two separate events, each muscle response elicited by hybrid stimulation was taken to result from the release of the contents of a single vesicle. The average highest rate of voltage increase of synaptic responses in a number of myotubes was used to compute the average conductance change (*15*). As shown in Table 1, the conductance increase associated with the release of a single quantum from a hybrid cell was 1.3×10^{-8} mhos in primary mouse cells and 1.38×10^{-8} mhos in G-8 myotubes [10 to 25 percent of the conductance changes found at frog or snake neuromuscular junctions (*16, 17*)]. Spontaneous responses in a G-8 myotube cocultured with normal mouse spinal cord cells had an average conductance change of 1.2×10^{-8} mhos. These conductance changes would correspond to 5 to 10×10^{6} Na^{+} ions passing across the muscle membrane during a synaptic event.

Table 1. A comparison of passive membrane properties, acetylcholine sensitivity, and postsynaptic parameters of normal mouse and G-8 myotubes. Electrical membrane constants were determined in normal mouse myotubes 22 days in culture, and G-8 myotubes 23 days after plating. Both plates were cocultured with hybrid cells and x-irradiated. For each myotube, foci of maximal and minimal acetylcholine sensitivity were determined by iontophoretic application of the drug. Some normal mouse myotubes had areas of membrane which failed to yield detectable responses when up to 10 ncoulombs of current were passed through the iontophoretic pipette; they were assigned a response amplitude of 0.5 mv. Maximum rates of voltage increase of evoked responses were determined visually from records of G-8 myotube connections obtained in the present study, and from records of mouse myotube connections previously described (*3*). For each myotube, response current and conductance change were calculated from the average maximum rate of voltage increase of its synaptic responses (*15*) with the assumption of a reversal potential of −5 mv and a fixed capacitance given in this table. The data are expressed as means ± standard errors.

Parameter	Unit	Mouse	*N*	G-8	*N*
		Passive electrical properties			
Resting potential	Millivolt	59.9 ± 2.8	7	49.0 ± 2.2	6
Input resistance	Megaohm	10.2 ± 2.4	5	7.5 ± 1.7	6
Time constant	Millisecond	5.8 ± 1.3	5	8.7 ± 1.0	6
Specific resistance	Ohm-cm^2	1855 ± 531	5	2555 ± 467	6
Specific capacitance	Microfarad/cm^2	4.3 ± 1.4	5	4.1 ± 0.9	6
		Sensitivity to acetylcholine			
Maximum sensitivity	Millivolt/ncoulomb	1474 ± 295	6	875 ± 137	6
Minimum sensitivity	Millivolt/ncoulomb	< 61 ± 22	4	299 ± 47	4
Ratio		> 24		2.9	
		Quantal postsynaptic response parameters			
Maximum rate of rise	Volt/second	1.03 ± .09	7	0.50 ± .03	3
Maximum current	Nanoamp	0.58 ± .05	7	0.57 ± .03	3
Conductance change	Nanomhos	13.0 ± 1.8	7	13.8 ± 1.2	3

If one assumes that a similar number of acetylcholine molecules are released by the hybrid at synapses with both normal and G-8 myotubes, and that the response magnitude is a function of acetylcholine receptor concentration, then the similar magnitudes of the postsynaptic conductance changes imply that at regions of synaptic contact, normal and G-8 myotubes may have approximately the same acetylcholine receptor concentration and the same access to released transmitter.

This would suggest that synaptic sites on normal mouse myotubes are restricted to the relatively small (< 10 percent) portion of the normal myotube surface which has acetylcholine sensitivity comparable to at least the lower range of sensitivity of the G-8 myotubes (greater than 200 mv/ncoulomb). The results indicate that NG108-15 hybrid cells interact with normal myotubes so that the acetylcholine release sites of hybrid neurites are closely apposed to areas on the normal myotube surface membrane which have relatively high concentrations of nicotinic acetylcholine receptors. Evidence compatible with the interpretation is furnished by immunohistochemical data which show areas of normal mouse myotubes with high concentrations of nicotinic acetylcholine receptors in apposition to NG108-15 hybrid neurites known to innervate the myotube (*18*). Whether specific interactions between molecules on the hybrid and myotube membranes are needed to juxtapose acetylcholine release sites and acetylcholine receptors remains to be determined.

If we assume that the activation of one acetylcholine receptor results in a change in conductance of 50×10^{-12} mhos (*19*), then the conductance change produced by a hybrid cell quantum represents the action of at least 240 acetylcholine molecules. Although somewhat lower than the estimates of the minimal number of acetylcholine molecules found in a quantum at neuromuscular junctions (*17*), this number indicates that the formation and release of acetylcholine from the hybrid cells and the response of the myotubes to quanta are well developed at biclonal synapses. The most prominent functional difference between the NG108-15 hybrid synapse and the mature neuromuscular synapse is that the hybrid cell releases fewer quanta than a spinal cord motorneuron.

The synapses between the NG108-15 hybrid cell and myotubes resemble nor-

mal immature, neuromuscular synapses in this regard. The normal maturation of the neuromuscular junction involves a hundredfold increase in the number of quanta released by a nerve cell action potential. It is an attractive possibility that the number of quanta released by the hybrid cell may be regulated and that its regulatory mechanisms are the same as in the normal maturational process. Relatively little is known about these regulatory mechanisms at the molecular level. The demonstration that clonal cells form synapses provides a system that can be used to explore both presynaptic and postsynaptic regulatory events.

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4. Proliferating M114 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 percent horse serum (HS) and 10 percent fetal calf serum (FCS) in plastic flasks; in the third and subsequent passages flasks were coated with collagen. After approximately six generations, the M114 cells were dissociated and plated into collagen-coated cloning wells at an average of less than one cell per well. A myotube-forming clone, G-8, was subcultured by three serial passages in flasks and frozen [J. Peacock, in preparation]. The G-8 cells have been subcultured 15 times (an estimated 50 cell divisions) without loss of the ability to form myotubes. The G-8 cells were grown in DMEM, 10 percent fetal bovine serum, 10 percent horse serum, penicillin (50 unit/ml), sodium salt, and streptomycin sulfate (50 μg/ml; Microbiological Associates) in 250-ml Falcon plastic flasks, without collagen. When cultures became confluent, but before cell fusion occurred, they were dissociated with a solution of 0.125 percent crude trypsin (Microbiological Associates) in Puck's balanced salt solution D_1, adjusted with NaCl to 340 mosmole per kilogram of H_2O and subcultured at a 20-fold lower cell concentration. Myotube cultures were prepared by plating 10^6 dissociated G-8 cells in a 35-mm Falcon plastic culture dish, in growth medium supplemented with 50 μg/ml of acid soluble collagen (Calbiochem). Fusion was promoted by feeding the culture infrequently, that is, only as needed to maintain the *p*H at 7.0 to 7.4, thus allowing myoblasts to "condition" the medium [I. R. Konigsberg, *Dev. Biol.* **26**, 133 (1971)].
5. NG108-15 hybrid cells were grown as previously described, and "predifferentiated" for at least 1 week by the addition of 1 m*M* N^6,O^2-dibutyryl cyclic AMP to the growth medium. Seven days after the G-8 cells were plated, cocultures were established by the addition of 3×10^4 NG108-15 cells in 90 percent DMEM, 10 percent HS plus 1 m*M* dibutyryl cyclic AMP, 0.1 m*M* hypoxanthine, and 0.016 m*M* thymidine. To compare G-8 myotubes with normal myotubes, cultures were prepared from dissociated cells of the hindlimbs of 18- to 21-day-old C57B1/6N mouse embryos [E. L. Giller *et al.*, *Science* **182**, 588 (1973)] and plated with NG108-15 cells after 11 days. Occasionally, both types of muscle plates were x-irradiated (4000 rad at 274 rad/minute) which stopped the proliferation of both clonal and primary cells. The x-irradiation did not inhibit synapse formation between NG108-15 cells and normal mouse muscle cells (unpublished observations). To compare the synaptogenesis of G-8 and normal spinal cord neurons to the biclonal condition, cultures were prepared by the addition of dissociated cells from spinal cords of 12- to 14-day-old Swiss Webster mouse embryos to 7-day-old G-8 cultures. 5-Fluorodeoxyduridine and uridine were used to inhibit proliferation of nonneuronal cells derived from the spinal cord (E. L. Giller *et al.*, *J. Cell Biol.*, in press.)
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7. Each myotube was penetrated with two micropipettes filled with 3*M* potassium acetate, one electrode recording the cell's response to intracellular current passed through the second electrode. Input resistance was calculated from the amount of current producing a hyperpolarizing response of from 20 to 30 mv. The time constant of the membrane was taken to be the time necessary to reach 66 percent of the maximal hyperpolarizing response, and membrane area was approximated by two rectangles of the same dimension as the myotube. After determining membrane constants, each myotube was tested for sensitivity to acetylcholine by ionotophoresis of the drug from high-resistance pipettes. Pulses lasting less than 4 msec were used to eject acetylcholine onto the surface of the myotube, and various areas were assayed until its maximal sensitivity was determined. Back currents of less than 2 na were sufficient to prevent desensitization.
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Bombesin: Potent Effects on Thermoregulation in the Rat

Abstract. *Several vasoactive peptides administered intracisternally have been assessed for the effect of lowering the core temperature of rats exposed to cold. Peptides structurally related to neurotensin lower core temperature while those related to substance P do not. The tetradecapeptide bombesin, originally isolated from the extracts of the skin of the frog* Bombina bombina, *is 10^4 times more potent than neurotensin in lowering core temperature, with a minimal effective dose less than or equal to 1 nanogram per 200 grams of body weight. Thus bombesin is one of the most potent peptides reported to affect the central nervous system.*

The tridecapeptide neurotensin, isolated and characterized from bovine hypothalamus (*1, 2*), has been reported to lower blood pressure, increase vascular permeability, induce gut contraction (*1*), extend the duration of barbiturate sedation (*3*), and increase plasma levels of glucagon (*4, 5*), glucose (*4–6*), growth hormone, and prolactin in rats (*7*). Bissette *et al.* (*8*) have recently reported the hypothermic effects of neurotensin. In these studies, neurotensin given intracisternally, but not intravenously, produced a lowering of basal body temperature of mice at room temperature or of mice exposed to cold (4°C). These results, along with the recent demonstration of selected anatomic distribution of neurotensin in the central nervous system (CNS) (*9*) and CNS binding sites for neurotensin (*10*), strongly support a neurotropic role for this peptide.

The undecapeptide substance P shares a number of common actions with neurotensin, such as lowering of blood pressure, producing gut contraction (*11*), and elevating the plasma levels of glucagon, glucose (*4*), growth hormone, and prolactin (*7*). However, substance P has not been found to lower body temperature.

Table 1. Amino acid sequence of several peptides. Abbreviations: Ala, alanine; Arg, arginine; Asn, asparagine; Asp, aspartic acid; Gln, glutamine; Glu, glutamic acid; pGlu, pyroglutamic acid; Gly, glycine; His, histidine; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; Phe, phenylalanine; Pro, proline; Trp, tryptophan; Tyr, tyrosine; and Val, valine.

Peptides*	Amino acid sequence
Neurotensin	pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-OH
Xenopsin	pGlu-Gly-Lys-Arg-Pro-Trp-Ile-Leu-OH
Substance P	H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2
Physalaemin	pGlu-Ala-Asp-Pro-Asn-Lys-Phe-Tyr-Gly-Leu-Met-NH_2
Bombesin	pGlu-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2

*See text for references.