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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20. We thank E. Godfrey and D. Rush for their contributions to this study. This work was supported by PHS grant FO2 HD 55299-01 to C.N.C. and NSF grant 6B 43526 and NIH grant NS 12151 to J.P.

3 August 1976; revised 9 November 1976

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.*

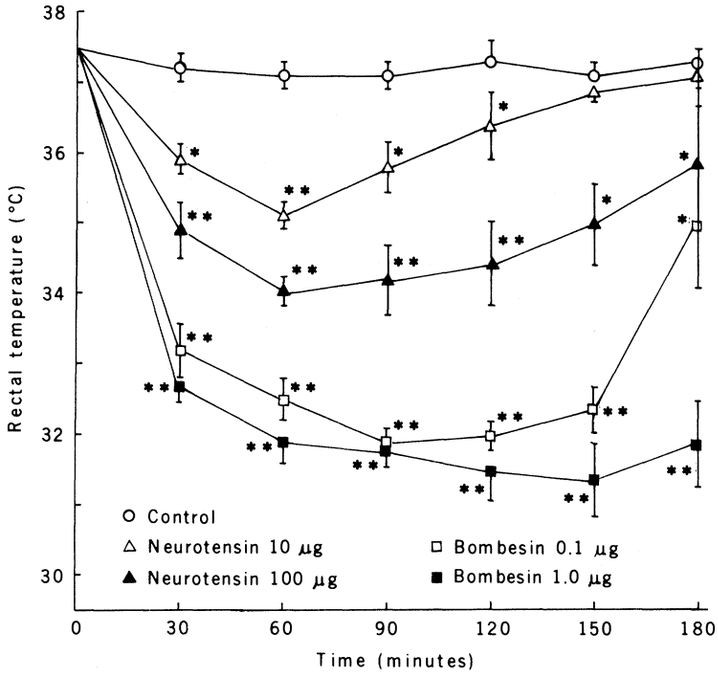
The tridecapeptide neurotensin, isolated and characterized from bovine hypothalamus (*1, 2*), has been reported to lower blood pressure, increase vascular permeability, induce gut contraction (*1*), extend the duration of barbiturate sedation (*3*), and increase plasma levels of glucagon (*4, 5*), glucose (*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.



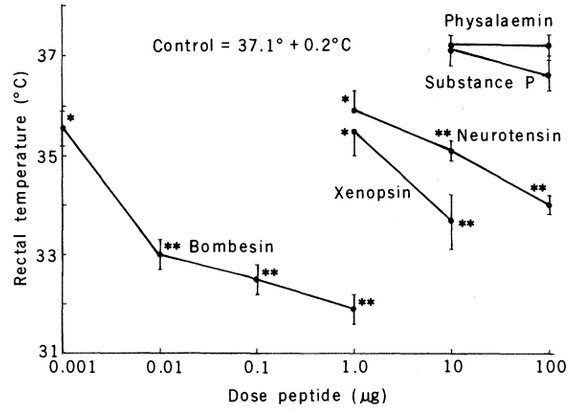


Fig. 1 (left). Temporal effects of various doses of neurotensin and bombesin on rectal temperature of rats exposed to cold (4°C). Each point represents eight animals; *, $P < .05$; **, $P < .01$. Fig. 2 (right). Effects of graded doses of bombesin, xenopsin, neurotensin, physalaemin, and substance P on rectal temperature of rats 60 minutes after exposure to cold (4°C). Each point represents eight animals; *, $P < .05$; **, $P < .01$.

Because of the similarities of the biologic activities of these peptides and their distribution in the CNS (*9, 11–14*), we have tested several naturally occurring peptides with partial sequence homologies to neurotensin and substance P. These peptides—bombesin, physalaemin, and xenopsin—were isolated from the skin of several anuran species (*15*), and like neurotensin and substance P have been found to alter blood pressure in the rat (*2, 16, 17*). This report describes the striking effects of these peptides on thermoregulation in the rat exposed to cold.

Male Sprague-Dawley-CD rats weighing 180 to 200 g were obtained from Charles River. They were housed in temperature- and humidity-controlled quarters, and Purina Rat Chow and tap water were freely available. After rats were given anesthesia, the peptides (10 μl) were injected into the cisterna magna. Immediately following these injections, animals were transferred to a cold room maintained at 4°C. Rectal temperatures were recorded with a thermoprobe (Yellow Springs Instrument) at 30-minute intervals after injections. All experiments were carried out in a randomized block design. Following analysis of variance, differences between treatments were determined by the multiple range tests of Dunnett and Duncan.

Peptides were synthesized and purified as previously described (*17*). Homogeneity of samples was assessed by the use of high-pressure liquid chromatography. Peptides were dissolved in a buffer, *p*H 7.2, composed of 126 m*M* NaCl, 6 m*M* KCl, 1 m*M* NAH_2PO_4, 0.88 m*M* $MgSO_4$, 1.45 m*M* $CaCl_2$, and 25 m*M* Hepes.

Figure 1 shows the temporal effects of neurotensin and bombesin on core temperature. The effects of graded doses of neurotensin, substance P, xenopsin, physalaemin, and bombesin on core temperature of rats 60 minutes after their administration are given in Fig. 2. These results show that bombesin is at least 10^4 times more potent than neurotensin in producing hypothermia in the rat exposed to cold. Other peptides with structural similarities to bombesin isolated from anuran skin—litorin, ranatensin, and alytesin—are approximately 1, 10, and 100 percent, respectively, as potent as bombesin in lowering body temperature of the cold exposed rat (*18*). Similar results are obtained in rats if bombesin is administered via the lateral ventricle (*19*). None of the peptides produced hypothermia when administered intravenously at ten times the dose used in these experiments.

At room temperature, neurotensin and xenopsin produced inconsistent lowering of the body temperature of rats, but bombesin did not lower the temperature of these animals. Bombesin produced a stereotyped scratching of the head with the forepaw and hindpaw. After termination of experiments and removal of animals from the cold room, all animals lived and exhibited normal behavior the following day.

It is not surprising that xenopsin, which is closely related structurally to neurotensin (Table 1), also produces hypothermia in the rat exposed to cold. Physalaemin, which is structurally related to substance P, is inactive in production of hypothermia. The lowering of body temperature by bombesin (minimal effective dose, 1 ng) is one of the most potent reported effects (in vivo) of a peptide on the CNS, suggesting the existence of high affinity receptors to bombesin or closely structurally related peptides in the CNS. Since bombesin bears little obvious resemblance to neurotensin, these data may imply that the actions of bombesin and neurotensin are mediated by different biological receptors. The specificity of the hypothermia-producing actions of these peptides is supported by the inactivity of a variety of peptides, including luteinizing hormone–releasing factor, somatostatin (*8*), bradykinin, and α-MSH (α-melanocyte stimulating hormone) (*20*).

Since anuran skin glands that produce these peptides are derived from neural crest tissue (*21*) and since immunoreactive bombesin-like activity has been reported to occur in tissue extracts of birds and mammals and in human serum after meals (*22*), it would not be surprising to find these or similar peptides in the mammalian CNS. Thus the anatomical distribution of bombesin would be similar to that of neurotensin, substance P, somatostatin, gastrin, and vasoactive intestinal polypeptide, all of which are present in gut and neural tissue (*14*) and are presumably also of neural crest origin embryologically (*21*).

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15 November 1976

Mutagenic Activity of Nitrite-Treated Foods: Human Stomach Cancer May Be Related to Dietary Factors

Abstract. *By the* Salmonella typhimurium *test, extracts of Japanese raw fish treated in the laboratory with nitrite showed mutagenic activity which is prevented by addition of ascorbate. Extracts from similarly treated beef and hot dogs were nonmutagenic. The data conform to a working concept that the high stomach cancer incidence in Japanese and certain other populations may be due to specific dietary factors of an alkylnitrosamide type.*

We have described a working concept on the mechanism whereby gastric cancer in man may arise (*1*). It involves the endogenous formation of an alkylnitrosamide type of gastric carcinogen (*2*). Such chemicals can be formed in the stomach from nitrite and a suitable substrate (*3*). The sources of nitrite in the human environment have been described (*1*, *4*). In a search for the as yet unknown substrate, we have subjected to nitrosation under simulated gastric conditions several foods typically eaten by populations in countries with a high risk for gastric cancer, such as Japan, and with a low risk, such as the United States (*5*).

To provide preliminary information on the potential carcinogenicity, mutagenicity in indicator strains of *Salmonella typhimurium* was utilized (*6*). In this system, nitrosation of a type of fish commonly eaten in Japan, followed by extraction under mild conditions, yielded evidence of high mutagenic potential of this extract (Table 1). There was no increase in mutagenicity when the extract was exposed to the indicator organism in the presence of a 9000*g* supernatant of rat liver homogenate, indicating that a direct-acting mutagen, rather than one which requires metabolic activation, is formed in fish treated with nitrite. Furthermore, ascorbic acid in amounts twice equimolar to the nitrite used completely prevented the formation of the mutagenic principle. Similar treatment of homogenates of beef or hot dogs failed to give rise to measurable mutagenic activities.

Of all the *Salmonella* mutants tested, those sensitive to agents which yield base-pair substitutions were most responsive to the mutagenic principle in fish treated with nitrite (Table 2). The activity in base-pair sensitive strains and the lack of activity in the other strains is a useful guide for the future identification of the mutagenic principles present. It suggests that materials such as alkylnitrosamides and related structures can be candidates, and rules out compounds such as polycyclic or heterocyclic chemicals.

These results have dual significance. First, the absence of mutagenic activity in

Table 1. Mutagenic effects of various food extracts on *Salmonella typhimurium* strain TA 1535. Five grams of a food homogenate, including 5000 parts of sodium chloride per million (*10*), were incubated at *p*H 3 for 1 hour at 25°C with and without 5000 parts of sodium nitrite per million. Nitrosation was stopped by the addition of 5000 parts of ammonium sulfamate per million. Thereafter, the incubation mixture was extracted with hexane (twice), mainly to remove lipids, and then with ether (four times). Mutagenic activity of 10 μl of the reduced ether extract (total volume from 5 g of food homogenate, about 100 μl) was determined by spot-testing following the procedure described by Ames *et al.* (*6*). The results represent means ± standard deviations from two experiments with duplicate plates per point for hot dog and beef, from four experiments with duplicate plates per point for fish. The fish samples tested were purchased over an interval of 6 months and were from three distinct shipments. The S9 fraction is the 9000*g* supernatant of a liver homogenate from Aroclor-induced male CDF rats (*6*), with 400 μg of protein per plate. The hot dogs tested contained beef as well as pork. Beef and hot dogs were obtained from local stores. The Japanese fish (sanma hiraki) was imported deep-frozen from Japan and purchased from Main Street Foodstore, Flushing, New York. Ascorbic acid from Sigma Chemical Company was added to the fish homogenate to give a level of 28,000 parts per million, equivalent to twice the molarity of 5000 parts of nitrite per million.

Food extracts from	His^+ revertant colonies per plate	
	Without S-9 fraction	With S-9 fraction
Control	8 ± 3.5	8 ± 3.5
Methylnitrosourea (10 μg per plate)	280 ± 2.4	249 ± 8.5
Japanese raw fish	3 ± 2.1	7 ± 2.8
Japanese raw fish + nitrite	297 ± 8.5	290 ± 25.0
Japanese raw fish + nitrite + ascorbic acid	7 ± 0.7	
Hot dog	4 ± 2.4	6 ± 1.4
Hot dog + nitrite	6 ± 0	3 ± 2.1
Beef	9 ± 0.7	9 ± 2.1
Beef + nitrite	15 ± 5.7	9 ± 2.1

Table 2. Mutagenic effects of fish treated with nitrite on various strains of *Salmonella typhimurium*. Samples (10 μl) of extracts of Japanese fish treated with 5000 parts of sodium nitrite per million under the conditions given in Table 1 were applied to plates bearing various strains of *Salmonella* without metabolic activation system, and the revertants were determined as described (*6*). The results represent the mean number of his^+ revertant colonies ± standard deviation from four experiments with duplicate plates per point. No mutagenic activity in any strain was seen with extracts of hot dogs treated with nitrite.

Salmonella typhimurium strain	His^+ revertant colonies (No.)	
	Extract	Spontaneous
TA 1535	252 ± 29.0	14 ± 2.8
TA 100	320 ± 42.0	80 ± 2.8
TA 1537	7 ± 5.7	4 ± 2.8
TA 1538	14 ± 4.2	8 ± 5.0
TA 98	30 ± 5.7	17 ± 3.5