metastases obtained at autopsy from patients dying of SCCL ($N = 5$), bombesin concentrations were nine times higher than those of extracts of livers from patients dying of other forms of cancer ($N = 4$), (0.18 ± 0.04 compared to 0.02 ± 0.005 pmole per milligram of protein). Thus it is likely that biologically active bombesin is produced by SCCL tumor cells in vivo as well as in vitro. Multiple physiologic effects likely to be produced by bombesin hypersecretion include anorexia, hypothermia, and hyperglycemia.

Fetal lung contains a high concentration of bombesin-like immunoreactivity, and immunocytochemical evidence reveals that bombesin-like immunoreactivity is limited to a subset of endocrine cells of the respiratory epithelium of fetal and neonatal human lung, whose numbers are greatly reduced or even absent in the adult human lung (*11*). The finding of high bombesin-like reactivity in all 17 SCCL cultures examined suggests that SCCL is derived from "bombesinergic" precursor cells, which are plentiful during early development. The fact that we failed to identify a single SCCL cell line lacking bombesin-like immunoreactivity suggests that the presence of bombesin may be an essential property for the continued growth of these cancer cells ["autocrine" factor (*18*)] as well as a clue to the nature of the precursor cell. While many other hormones and neuropeptides can occur in SCCL tumors or cultures (*4*), thus far only bombesin is always present in SCCL and lacking in non-SCCL lines. Finally, it is possible that the presence of bombesin peptides in the blood of people at risk for lung cancer (such as heavy smokers) would allow the early detection of SCCL when treatment with chemo- and radiotherapy is most likely to yield a long-term cure (*19*). Already, we have data indicating that elevated blood levels of bombesin in SCCL patients are correlated with the extent of tumor burden (*20*).

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Novel Peptide Neuronal System in Rat Brain and Pituitary

Abstract. *Immunohistofluorescence studies of the rat central nervous system with antibodies to Phe-Met-Arg-Phe-NH_2 (molluskan cardioexcitatory peptide) revealed a widespread neuronal system in the brain, spinal cord, and posterior pituitary. Immunoreactive axons and cell bodies were mainly located in cortical, limbic, and hypothalamic areas. Immunostaining of serial sections of the brain and pituitary showed that the Phe-Met-Arg-Phe-NH_2 immunoreactive neurons were different from neurons labeled by antibodies to either Met-enkephalin or the putative Met-enkephalin precursor Tyr-Gly-Gly-Phe-Met-Arg-Phe, which is structurally related to Phe-Met-Arg-Phe-NH_2. Control staining by antiserum absorption and radioimmunoassay indicated that the antibodies that caused the specific immunofluorescence recognized peptides with an amidated Arg-Phe sequence at the carboxyl terminus.*

The endogenous opiate receptor ligand Met-enkephalin (Tyr-Gly-Gly-Phe-Met, YGGFM) (*1*) is widely distributed throughout the central and peripheral nervous system of vertebrate and invertebrate species (*2*). A number of large Met-enkephalin–containing peptides and proteins, because of their primary structure and anatomical distribution, may serve as precursors for the opioid pentapeptide (*3*). One of these putative Met-enkephalin precursors has the structure Tyr-Gly-Gly-Phe-Met-Arg-Phe (YGGFMRF) (*4*). This peptide is present in rat striata in amounts comparable to those of Leu-enkephalin (*4*, *5*), and, when measured with a specific radioimmunoassay (RIA), its regional distribution in rat brain follows closely that of Met-enkephalin (*6*). The carboxyterminal tetrapeptide fragment of this heptapeptide is strikingly similar to the molluskan cardioexcitatory peptide Phe-Met-Arg-Phe-NH_2 (FMRF-NH_2), which was isolated from clam ganglia by Price and Greenberg (*7*). Because brain contains peptidases capable of cleaving enkephalins at the Gly-Phe bond (*8*), we hypothesized that YGGFMRF might not only serve as a precursor to Met-enkephalin but also to FMRF-NH_2. To test this hypothesis we used antibodies to FMRF-NH_2 for the immunofluorescent staining of rat brain and pituitary sections. These antibodies did indeed detect a widespread neuronal system; however, this neuronal system was unrelated to the Met-enkephalin–YGGFMRF neuronal system.

The antibodies to FMRF-NH_2 were prepared by injecting five rabbits with a carbodiimide reacted peptide-thyroglobulin mixture (*9*) emulsified in Freund's adjuvant. All the rabbits produced serum with a high titer of antibodies. The antiserum from one rabbit (R3-1) was used for immunohistochemical mapping of FMRF-NH_2 immunoreactive neurons in brain, spinal cord, and pituitary. The antiserum from the other four rabbits also specifically labeled the same neurons. The immunofluorescent staining was performed on serial cryostat sections of paraformaldehyde-fixed brains

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and pituitaries from eight normal and colchicine-treated rats (*10*).

The most striking characteristic of the FMRF-NH_2 immunoreactive neuronal system was the high fiber density in cortical, limbic, and hypothalamic structures (Table 1). In the pituitary the immunoreactivity was associated with many neurosecretory endings in the neural lobe (Fig. 1d). No immunoreactivity in the intermediate or anterior pituitary was observed. The morphology of the FMRF-NH_2 immunoreactive neurons was very characteristic. In the cortical and limbic areas, the fibers were thin and long with a classical beaded morphology (Fig. 1a). In hypothalamus and brainstem, nerve endings were the predominant immunoreactive structures (Fig. 1c). To our knowledge, the distribution of this neuronal system is different from all peptide neuronal systems previously described. To establish the relation between the FMRF-NH_2 immunoreactive neurons and the neurons containing Met-enkephalin or YGGFMRF, we used the antiserums to FMRF-NH_2 and to Met-enkephalin or YGGFMRF to stain adjacent serial sections throughout the brain, cervical spinal cord, and pituitary (*11*). Both Met-enkephalin and YGGFMRF immunoreactivity were found in essentially all areas reported previously to contain Met- or Leu-enkephalin (Table 1) (*2*). The different neuronal morphology of the two systems strongly suggested that they were distinct. Whereas FMRF-NH_2 immunoreactive material was located in very thin, long beaded axons, the YGGFMRF-Met-enkephalin immunoreactive material occurred in thick, bundle-like structures that often formed a reticulum-like pattern. In a few areas, especially in the spinal cord and brainstem, the two systems seemed to overlap.

The specificity of the FMRF-NH_2 immunostaining was tested by antibody absorption with synthetic fragments and derivatives of FMRF-NH_2. All immunostaining reported above could be blocked by incubating the sections with FMRF-NH_2 antiserum in the presence of 5 μM FMRF-NH_2, YMRF-NH_2, MRF-NH_2, and YGGFMRF-NH_2. Arg-Phe-NH_2 could also block the immunostaining; however, a 100 μM concentration of this dipeptide was needed to block the immunostaining completely. In contrast, non-amidated YGGFMRF as well as FMRFY did not block the immunostaining (*12*). Thus, an amide group at a carboxyl terminal Arg-Phe configuration seemed to be an absolute requirement for the antibodies. Recently, a melanotropin-like peptide (γ_1-MSH) has been described in the intermediate lobe of the bovine pituitary (*13*). This peptide has an amidated Arg-Phe sequence at the carboxyl terminus. Large amounts (> 100 μM) of γ_1-MSH (*14*) also blocked the FMRF-NH_2-like immunostaining. Evidence suggests, however, that γ_1-MSH is mainly restricted to the bovine intermediate pituitary and that the neuronal system described here does not contain γ_1-MSH: (i) The FMRF-NH_2 antiserum does not detect immunoreactive material in rat intermediate pituitary (Fig. 1d). (ii) The FMRF-NH_2 immunoreactive neuronal system is different from and more widely distributed than the β-endorphin system which contains MSH-related peptides (γ_3-MSH) (*15*). (iii) By a specific radioimmunoassay to γ_1-MSH, no immunoreactive material can be detected in rat pituitary and brain (*16*) and of all bovine brain regions, only the hypothalamus contains very little γ_1-MSH–like immunoreactivity (*17*).

The carboxyl terminal tetrapeptide fragment of gastrin and of cholecystoki-

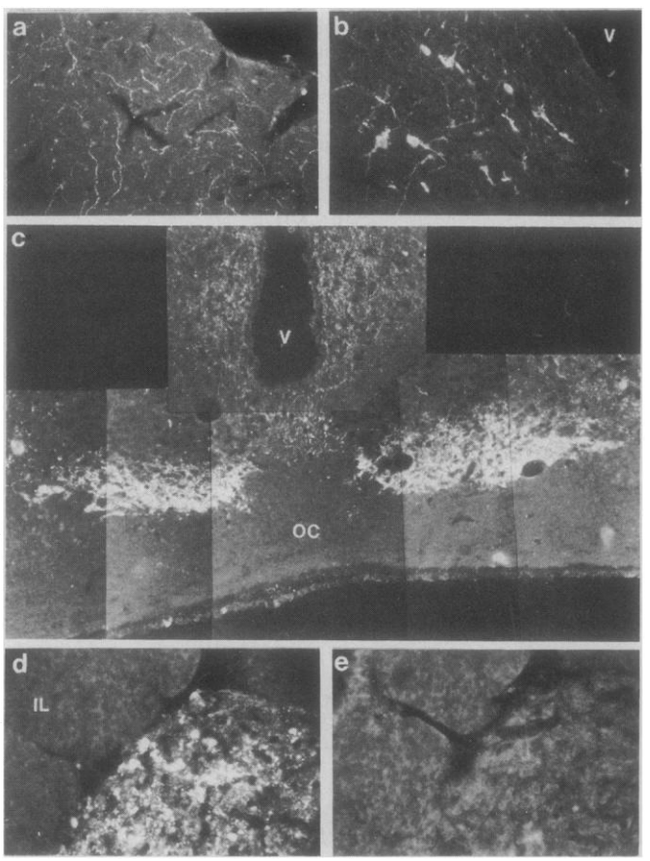

Fig. 1. Cell bodies, axons, and nerve endings showing immunofluorescence staining with antibodies to Phe-Met-Arg-Phe-NH_2. (a) Long, beaded axons in the outermost layer of the pyriform cortex (×270). (b) Cell bodies and axons in the dorsomedial nucleus of the hypothalamus of a colchicine-treated rat. These cell bodies were not visible without colchicine treatment, which blocks axonal transport of substances synthesized in the cell bodies. (c) Sections showing very dense immunostained nerve endings in the suprachiasmatic nucleus of a normal rat brain. Only the ventral aspect of the nucleus is stained. Nerve endings are also visible in the periventricular nucleus along the third ventricle (*V*) (*OC*, optic chiasm) (×170). (d) Positively stained neurosecretory endings in the posterior pituitary of a normal rat. No immunoreactivity is seen in the intermediate lobe (*IL*) (×270). (e) Specificity control to (d). An adjacent section was incubated with antiserum in the presence of 10 μM Phe-Met-Arg-Phe-NH_2. All immunofluorescence is blocked (×270).

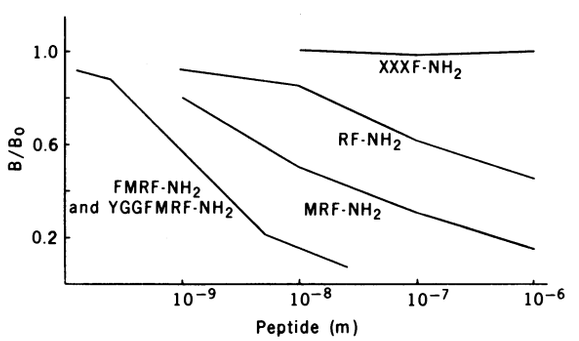

Fig. 2. Radioimmunoassay (RIA) specificity of the antibody to FMRF-NH_2 used for detecting immunoreactive nerve cells and axons in rat brain. The antiserum was diluted 12,000 times in RIA buffer, and trace amounts of ^{125}I-labeled Tyr-Met-Arg-Phe-NH_2 were added. The displacement of this trace from the antibodies by FMRF-NH_2 and by various fragments and analogs of it is shown. Arg-Phe-NH_2 is the smallest carboxyl terminal fragment which is recognized by the antibodies. Its cross-reactivity is 0.5 percent. *XXXF-NH_2* stands for gastrin or CCK-related peptides which have an amidated phenylalanine at the carboxyl terminus.

nin (CCK), both of which occur in brain (*18*), also has an amidated phenylalanine at the carboxyl terminus; the preceding amino acid, however, is an aspartic acid rather than an arginine. To test whether the antibodies cross-reacted with CCK-like peptides, we conducted blocking experiments with gastrin I, CCK(26–33) (sulfated form), CCK(27–33), and CCK(30–33). None of these peptides was capable of blocking the immunostaining at a 100-μM concentration. The neuropeptides Met-enkephalin, arginine-vasopressin, oxytocin, substance P, α-melanotropin, and somatostatin also did not cross-react. The FMRF-NH_2 antibodies were further characterized by RIA. Since FMRF-NH_2 does not contain an amino acid residue that can be radioiodinated, a tyrosine-containing analog (YMRF-NH_2) was synthesized (*12*). When radioiodinated YMRF-NH_2 was used as a trace (*19*) the specificity of the antibodies in RIA closely paralleled the specificity results obtained by immunocytochemical blocking controls (Fig. 2); YMRF-NH_2, FMRF-NH_2, and YGGFMRF-NH_2 were equally reactive; MRF-NH_2 and RF-NH_2 showed a cross-reactivity of 12 percent and 0.5 percent, respectively. Non-amidated YGGFMRF or FMRFY did not cross-react nor did the gastrin-CCK-related peptides. Of the other substances, only arginine-vasopressin and oxytocin showed a negligible cross-reactivity of 0.003 percent. Thus the antibodies seemed to be specific for the amidated carboxyl terminus of FMRF-NH_2, with RF-NH_2 being the smallest fragment recognized.

Table 1. Immunohistochemical distribution of FMRF-NH_2 immunoreactivity compared to Met-enkephalin/YGGFMRF immunoreactivity in rat brain and pituitary. All areas showing FMRF-NH_2 immunoreactivity are listed. Not all areas with Met-enkephalin/YGGFMRF–like immunoreactivity are listed. The Met-enkephalin/YGGFMRF distribution found is in agreement with reported maps for Leu-enkephalin and Met-enkephalin (*2*). Symbols: fiber and nerve endings, ++++ very dense, +++ dense, ++ moderate, + weak, and (+) occasional fibers; strong cell body staining, ●; weak cell body staining, ○.

Area	FMRF-NH_2–like immunoreactivity	Met-enkephalin/YGGFMRF–like immunoreactivity
Pituitary (Pars nervosa)	+++	+
Hypothalamus		
Nervus preopticus suprachias maticus	++	
Nervus preopticus paraventricularis	+	
Nervus preopticus medialis	+	+
Nervus preopticus lateralis	+	
Nervus periventricularis	++	
Nervus suprachiasmaticus	++++	
Nervus supraopticus	(+)	
Nervus anterior	(+)	●
Nervus paraventricularis	++ ○	●
Nervus arcuatus (rostral)	++	
Nervus arcuatus (caudal)	+++ ○	(+)
Nervus ventromedialis anterior		+ ●
Nervus ventromedialis		++
Nervus lateralis	++	
Nervus dorsomedialis	++ ●	
Nervus praemamillaris	++	●
Ventral border	++	
Stria terminalis	+	+
Cortical and limbic areas		
Frontal cortex	++ ○	
Olfactory tubercle	++ ●	+++
Pyriform cortex	+++ ●	
Corpus callosum	(+)	
Comissura anterior	(+)	
Entorhinal cortex	+++ ●	
Amygdala	+	
Amygdala (central nucleus)		+++ ●
Thalamus		
Nervus periventricularis rotundocellularis	+	+
Telencephalon		
Globus pallidus		++++
Nervus caudatus		+
Nervus accumbens		+++
Nervus septi lateralis	+	+++ ●
Mesencephalon		
Substantia grisea centralis	++	+
Formatio reticularis	+	+
Nervus interpeduncularis		+++ ●
Medulla		
Nervus solitarius	++	++
Nervus tractus spinalis nervi V	++	++
Nervus nervi X	++ ●	++
Spinal cord		
Lamina I	++	+++
Lamina II		+++

These results demonstrate a novel peptide neuronal system in brain and pituitary. Numerous peptides that were first isolated from tissues other than brain are present in the central nervous system (*20*). FMRF-NH_2 was first isolated from clam ganglia. However, we do not know whether the neuronal system described here contains the entire FMRF-NH_2 sequence. The antiserum-blocking controls and the RIA data taken together indicate that the specificity of the antibodies was such that RF-NH_2 itself or peptides with just the RF-NH_2 structure at the carboxyl terminus might produce a positive immunofluorescence. Although the RF-NH_2 fragment and RF-NH_2–containing peptides have a 200 times lower affinity for the antibodies than FMRF-NH_2, a cross-reactivity in this order can still cause a positive immunofluorescence (*21*). It is therefore possible that the neuronal system we detected in rat brain contains peptides which have the MRF-NH_2 or RF-NH_2 but not necessarily the entire FMRF-NH_2 sequence at their carboxyl terminus (*22*).

Note added in proof: After submission of this report, Dockray *et al.* (*23*) reported an FMRF-NH_2–like substance in various vertebrate tissues.

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10. Five normal and three colchicine-treated (50 μg of colchicine in 25 μl of 0.9 percent NaCl injected intraventricularly 48 hours before death) male Sprague-Dawley rats (200 to 250 g body weight) were anesthetized with pentobarbital and each animal was perfused via the ascending aorta with 250 ml of ice cold 4 percent freshly depolymerized paraformaldehyde in a 0.1*M* sodium phosphate buffer at *p*H 7.4. Brains, pituitaries, and cervical spinal cords were removed and further fixed for 2 hours. They were then placed in a 5 percent solution of sucrose in phosphate-buffered saline (PBS) overnight at 4°C. The tissue was frozen in liquid dichlorodifluoromethane and 14-μm-thick sections were cut in a cryostat. Sections were mounted on gelatin-coated slides and incubated at 4°C overnight with primary antiserum diluted 1:400 with PBS containing 0.3 percent Triton X-100 and 2.5 percent bovine serum albumin. The sections were then washed and incubated for 2 hours at room temperature with fluorescein-conjugated goat or sheep antiserum to rabbit immunoglobulin G (Cappel Laboratories) diluted from 1:10 to 1:40 in Triton X-100 buffer without albumin. After being rinsed the sections were covered with a 1:1 mixture of glycerol and PBS and examined with a Leitz Orthoplan light-microscope equipped with an epifluorescence attachment. Anatomical distribution of immunoreactive fibers and cell bodies was correlated with coronal sections from the stereotaxic atlases of J. F. R. Konig and R. A. Klippel [*The Rat Brain: A Stereotaxic Atlas of the Forebrain and Lower Parts of the Brain Stem* (Williams & Wilkins, Baltimore, 1963)] and of J. L. Pellegrino, A. S. Pellegrino, A. J. Cushman [*A Stereotaxic Atlas of the Rat Brain* (Plenum, New York, 1979)].
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12. The commercially available synthetic peptides were obtained from Peninsula Laboratories or from Bachem. The YMRF-NH_2, FMRFY, and MRF-NH_2 were synthesized by J. K. Chang at Peninsula Laboratories. The YGGFMRF-NH_2 and RF-NH_2 were a gift from D. Price, Tallahassee, Fla.
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19. Two micrograms of YMRF-NH_2 were labeled with 1 mCi of ^{125}I by the chloramine-T method of W. M. Hunter and F. C. Greenwood [*Nature (London)* **194**, 495 (1962)]. Radioiodinated peptide was purified on a Sephadex G-10 column in 50 percent acetic acid, and radioimmunoassays with antiserum R3-1 at a final dilution of 1:12,000 were performed according to a procedure described by E. Weber *et al.* (*21*). The 50 percent inhibition concentration of the radioimmunoassay was 500 p*M*. Interassay variability was < 11 percent.
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22. After completion of this work, we became aware of a study in which the identification of FMRF-NH_2–like immunoreactivity in the parabrachial nucleus of the mouse brainstem was described [H. H. Boer, L. P. C. Schot, J. A. Veenstra, D. Reichelt, *Cell Tissue Res.* **213**, 21 (1980)].
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24. Address correspondence and reprint requests to E.W. We thank D. Price, Tallahassee, for the YGGFMRF-NH_2 and RF-NH_2, J. K. Chang, Peninsula Laboratories, for synthesizing fragments and analogs of FMRF-NH_2, and N. Ling, La Jolla, for providing synthetic γ_1-MSH. We also thank I. Inman for technical assistance and S. Poage for preparing the manuscript. Supported by NIMH grant MH 23861, NIDA grant DA 01207, and ONR award SRO 001.

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Autoimmune Encephalomyelitis: Simultaneous Identification of T and B Cells in the Target Organ

Abstract. *Monoclonal antibodies to guinea pig T cells and antibodies to guinea pig immunoglobulin G were used in immunofluorescence studies to identify T and B cells in central nervous system tissue from guinea pigs with acute autoimmune encephalomyelitis. T cells appeared before B cells and were distributed within the white matter parenchyma, while B cells remained in perivascular spaces.*

Although it is recognized that experimental allergic encephalomyelitis (EAE) is a T cell–mediated autoimmune demyelinating condition (*1*), T cells have never been specifically identified in the target organ, the central nervous system (CNS). In the present study, fluorescein labeling of monoclonal antibodies to guinea pig T cells and rhodamine-labeled immunoglobulin G (IgG) were used to identify T and B cells in the CNS of guinea pigs with EAE. By means of this technique we found that T and B cells display different distribution patterns in the CNS. We also observed that, with increasing severity of the disease, there are correlated variations in the distribution of T and B cells, IgG, and the third component of complement (C3).

Acute EAE was induced in adult strain 13 guinea pigs by inoculation with an emulsion containing bovine white matter or isogeneic spinal cord tissue in complete Freund's adjuvant (*2*). A severe form of acute EAE was also induced in juvenile strain-13 guinea pigs similarly inoculated (*3*). Control animals consisted of uninoculated age-matched guinea pigs. Animals sensitized for EAE developed neurologic signs (paraparesis and incontinence) 12 to 24 days after inoculation. The animals were anesthetized and perfused with phosphate-buffered saline (PBS) and their CNS (brain and spinal cord) was removed. Slices were embedded in optimal cooling temperature medium and frozen in a bath of acetone and dry ice for cryostat sectioning. The sections were cut 10 μm thick and fixed for 20 minutes in ethanol at 4°C. Staining for T cells was achieved with mouse monoclonal antibodies to guinea pig T cells (*4*). This antibody (5CC2) was raised against peripheral lymphocytes from guinea pigs and gave immunofluorescent staining of circulating T cells and mature thymocytes (it also gave weak staining of B cells when used at a dilution of $1:10^4$ or lower). Tissue was exposed to 5CC2 at a dilution of $1:10^6$ in PBS and then incubated with fluorescein isothiocyanate–conjugated $F(ab')_2$ fragments of rabbit antibodies to mouse immunoglobulin at a dilution of 1:50 in PBS. For simultaneous demonstration of B cells, other IgG-containing cells (macrophages), and IgG deposits, we used rhodamine-conjugated $F(ab')_2$ fragments of rabbit antibodies to guinea pig IgG (specific for heavy and light chains) at a dilution of

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