Epidermal Growth Factor Immunoreactive Material in the Central Nervous System: Location and Development

Abstract. *Epidermal growth factor (EGF) is a potent mitogen with hormonal activity in the gastrointestinal tract. Material cross-reacting with EGF was detected in the central nervous system of the developing and adult albino rat by the indirect immunofluorescence technique. High concentrations of EGF–cross-reacting material were identified in forebrain and midbrain structures of pallidal areas of the brain. These include the globus pallidus, ventral pallidum, entopeduncular nucleus, substantia nigra pars reticulata, and the islands of Calleja. Thus, EGF may represent another gut-brain peptide with potential neurotransmitter-neuromodulator functions in pallidal structures of the extrapyramidal motor systems of the brain.*

Epidermal growth factor (EGF) purified from male mouse submaxillary glands is a well-characterized polypeptide containing 53 amino acid residues and having a molecular weight of 6045 (*1*). EGF is potently mitogenic in vitro for diverse cells isolated from tissue of ectodermal and mesodermal origin (*2*). Evidence that EGF may have important physiological functions stems from studies in vivo, in which large quantities of EGF injected into perinatal mammals resulted in precocious opening of the eyelids and closing of the palate (*2*). Urogastrone, which presumably represents the human form of EGF, is found in urine; it is a gut peptide that inhibits gastric hydrochloric acid secretion (*3*). Because many of the other gut peptides are also distributed within the central nervous system (*4*), and because EGF is mitogenic for rat brain astrocytes (*5*), we undertook studies to see if EGF, or an EGF-like molecule, might be present in the rat brain. In similar studies, Byyny *et al.* (*6*), using a radioimmunoassay, were unable to detect significant quantities of EGF in whole brain extracts. However, specifically localized regions containing EGF, even in relatively high concentrations, might be missed by this approach since the amount of EGF relative to the total protein content of whole brain extracts would be extremely small under these circumstances. We used immunofluorescence and immunochemical techniques to look for EGF–cross-reactive material (EGF-CRM) in the late neonatal and adult albino rat brain. The results of these studies demonstrated the presence of densely stained regions of EGF-CRM in five discrete regions of the brain.

We used the indirect immunofluorescence method (*7*) with three different antiserums to EGF prepared in two different laboratories (*8*) to locate EGF-CRM in the rat brain. Identical results were obtained with each. Forty-seven albino rats were studied. Of these, 20 animals, 1 to 90 days old, that did not receive colchicine were used to determine the age at which EGF-CRM first appeared. One percent colchicine was injected into several brain structures of five adult animals to determine the location of immunoreactive cell bodies (*9*); and 22 other adult animals were used in antibody control experiments that included (i) the omission of primary or secondary antisera, (ii) the use of serum from unimmunized animals in place of immune serum, and (iii) the use of an immune serum that was depleted of EGF-specific immunoglobulin G (IgG) by being passed over an affinity column containing EGF covalently linked to Sepharose.

EGF was found in both forebrain and midbrain structures. These included the globus pallidus, entopeduncular nucleus, substantia nigra pars reticulata, ventral pallidum, and the islands of Calleja complex (Fig. 1). The EGF-CRM immunofluorescence staining patterns were almost identical with each of the three different antiserum preparations. EGF-CRM appeared to be located in long (> 1 mm, in some cases) fibers and small terminal-like puncta (Fig. 2). The immunofluorescent staining of EGF-CRM was most intense in the core and cap regions of the islands of Calleja complex and was moderately intense in the other structures described above. The medial and ventral bed nucleus of the stria terminalis also showed the presence of EGF-CRM, although the staining was much less intense (Fig. 1). In some cases, EGF-CRM was also seen in radial fibers in layers I to V of the somatosensory, cingulate,

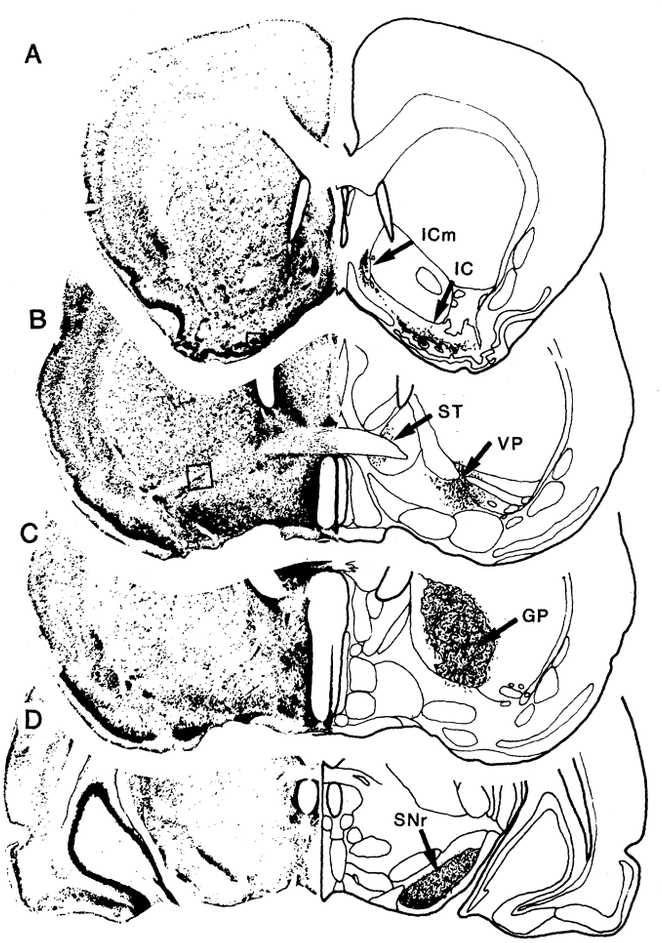

Fig. 1. Semischematic drawings of coronal sections of the adult albino rat showing the location of fibers containing EGF-CRM. The left side of each diagram is a Nissl-stained section of neuronal cell bodies, and the right side of each diagram is a drawing of the same brain structures. Sections run (A) rostral to (D) caudal in the brain. (A) EGF-CRM in the small islands of Calleja (*IC*) and large island of Calleja (*ICm*). The EGF-CRM–containing fibers are located in the core, cap, and "pallidal bridge" regions of the islands of Calleja, which have been shown by other methods to be pallidal (*13*). (B) EGF-CRM–containing fibers in the ventral pallidum (*VP*) and light to moderately sparse EGF-CRM in the medial sector of the bed nucleus of the stria terminalis (*ST*). (C) EGF-CRM in fibers in the globus pallidus (*GP*), which is the homolog of the external segment of the globus pallidus in primates. EGF-CRM fibers are also present in the entopeduncular nucleus (not shown), which is the homolog of the internal segment of the globus pallidus in primates. (D) EGF-CRM fibers in the pars reticulata of the substantia nigra (*SNr*), which has also been shown to be a pallidal structure (*11*). Boxed-in areas in the left side indicate the sites in the islands of Calleja (A) and ventral pallidum (B) from which the photomicrographs in Fig. 2, A and B, were taken.

and pyriform cortices (data not shown).

To determine whether neuronal cell bodies might represent the site of synthesis or storage of EGF-CRM, we injected colchicine directly into the brain to block axoplasmic transport. If EGF-CRM is produced or stored in the neuronal cell bodies, then this blockage of transport should result in a buildup of EGF-CRM in the cell body. Injections of colchicine into pallidal structures resulted in the loss of EGF-CRM in fibers; however, neither these injections nor injections of colchicine into the caudate putamen, neocortex, subthalamus, brainstem, or lateral ventricle led to the appearance of intense staining of EGF-CRM in cell bodies. Therefore, we were not able to determine the source or sources of EGF-CRM. In control studies in which either the primary or secondary antiserum was omitted, or serum from unimmunized rats was used, specific staining of EGF-CRM was completely absent. In control experiments in which antiserum was used that was depleted of IgG specifically directed against EGF, a 75 to 85 percent reduction in staining was observed. Taken together, these results strongly suggest that the immunofluorescent staining observed was due to the presence of the EGF-specific IgG in the immune serum and not to any incidental IgG's present in the antiserum preparations.

We also investigated the age dependence of the appearance of EGF-CRM in the rat brain. In preliminary experiments we observed the presence of EGF-CRM in brain samples from 15-day-old rats but not in samples from 7-day-old rats. To determine the age dependence of the appearance of EGF-CRM more precisely, we tested brain samples from animals aged 1, 7, 10, 13, 14, 15, and 21 days. The appearance of EGF-CRM proved to be sharply age-dependent; samples from animals 14 days and younger showed no staining, whereas samples from animals 15 days old and older showed a staining pattern that was conserved throughout adulthood (90 days).

What is the significance of EGF-CRM in the central nervous system? The two most likely explanations for its presence in the brain are (i) that it acts as a mitogen or developmental hormone or (ii) that it acts as a neurotransmitter or neuromodulator, or both. The possibility that EGF serves as a mitogen for specific classes of brain cells comes from studies in which EGF has a demonstrated mitogenic effect on cultured astrocytes (*5*). However, because the EGF-CRM does not appear until the animal is 15 days old, which is after the peak of astrocyte proliferation (*10*), it is not likely that EGF functions as a physiological mitogen for astrocytes in the brain. We cannot rule out the possibility that EGF functions as a brain mitogen before day 15, but is undetectable by the methods used. The more likely explanation for the presence of EGF-CRM in the brain may be related to its role as a neurotransmitter-neuromodulator. This interpretation gains support from the recent demonstration that a number of gut-related peptides, of which EGF is one, have been identified in the central nervous system (*4*). Our results would support the hypothesis that EGF serves as a neurotransmitter-neuromodulator on the basis of the complex relation between the discrete regions in which EGF-CRM was located. The globus pallidus and entopeduncular nucleus of the rat are homologous, respectively, to the external and internal segments of the primate globus pallidus, and the substantia nigra pars reticulata appears to be yet a third subdivision of the pallidum (*11*).

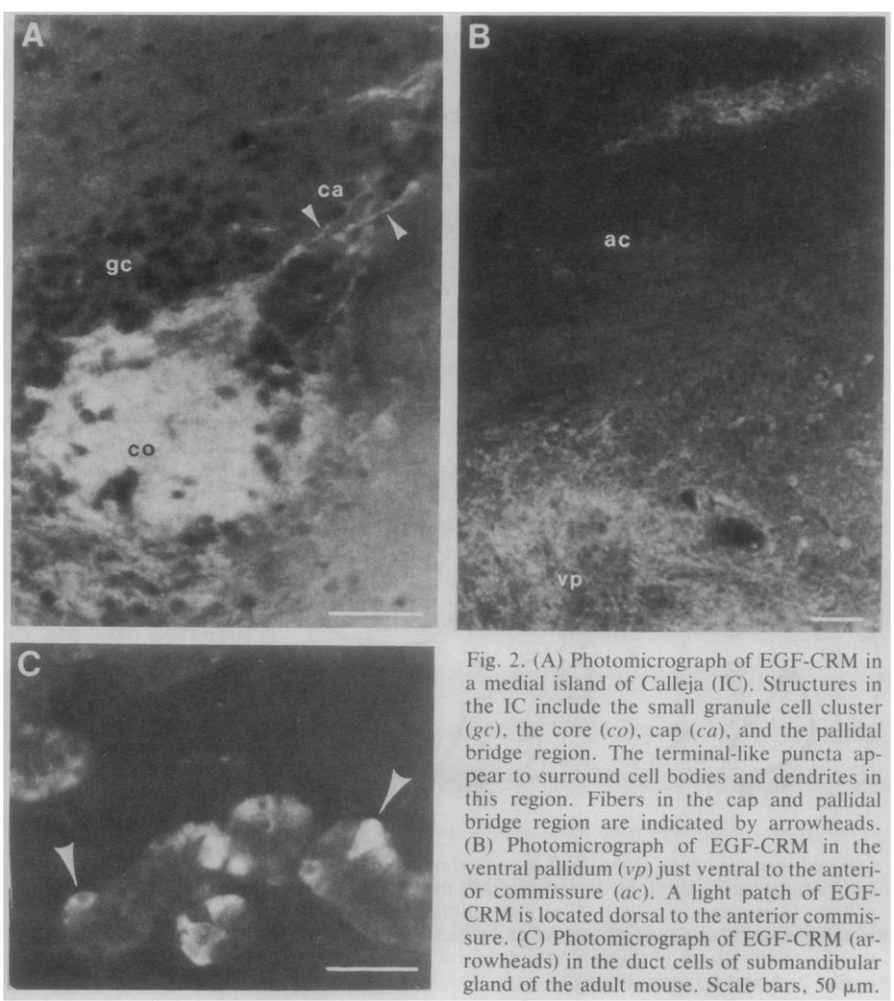

Fig. 2. (A) Photomicrograph of EGF-CRM in a medial island of Calleja (IC). Structures in the IC include the small granule cell cluster (*gc*), the core (*co*), cap (*ca*), and the pallidal bridge region. The terminal-like puncta appear to surround cell bodies and dendrites in this region. Fibers in the cap and pallidal bridge region are indicated by arrowheads. (B) Photomicrograph of EGF-CRM in the ventral pallidum (*vp*) just ventral to the anterior commissure (*ac*). A light patch of EGF-CRM is located dorsal to the anterior commissure. (C) Photomicrograph of EGF-CRM (arrowheads) in the duct cells of submandibular gland of the adult mouse. Scale bars, 50 μm.

What relation do these pallidal structures have to the two other regions (subcommissural region and islands of Calleja complex) that contain intense EGF-CRM? The subcommissural region containing EGF-CRM is the area that has, on the basis of connections and cell types, been redefined as ventral pallidum (*12*). The EGF-CRM zones in the core and cap regions of the islands of Calleja complex overlap the pallidal zones in this region (*13*). Because intense EGF-CRM staining is found only in these five pallidum-like structures, we conclude that EGF-CRM is the best pallidal marker known, although less intense EGF-CRM staining was seen in the ventral medial part of the bed nucleus of the stria terminalis. If EGF-CRM is a pallidal marker, this region may be an excellent new candidate for a pallidal component related to the amygdala.

Unfortunately, we were unable to determine the source of the EGF-CRM in these studies. Because of the morphology, location, and length of the fibers in which EGF-CRM was observed, however, we believe that EGF-CRM is located in neurons and not in glia. Electron microscopic investigations will clarify the issue as to the subcellular location of EGF-CRM. Neuronal or glial uptake of

EGF or EGF-like materials may be the source of EGF-CRM in the brain, but whether the blood-brain barrier is permeable to EGF has not been determined. The presence of EGF-CRM only in pallidal structures suggests a neurotransmitter-neuromodulator role for this gut-brain peptide in extrapyramidal motor function. The pallidum receives convergent information from the cortical mantle via the striatum and has divergent outputs to brainstem and to subthalamic, thalamic, and hypothalamic motor- and endocrine-related target areas (*13*). EGF or an EGF-like molecule could then influence extrapyramidal motor function at key nodal points in the pallidum. Further morphological and functional studies are needed to clarify the role of EGF in the central nervous system.

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8. Two of the primary rabbit antisera to EGF were provided by F. Fox. Secondary fluorescein isothiocyanate–coupled IgG directed against rabbit antiserum was purchased from Miles or Antibodies, Incorporated, and was diluted to 1:40 in phosphate-buffered saline with 0.2 percent Triton X-100. Incubation in secondary antiserum was done at room temperature for 1 hour.
9. One percent colchicine (0.5 to 2.0 μl) in saline was injected into the lateral ventricle or other brain structure with a 1- or 5-μl syringe (Unimetrics). The colchicine prevents axoplasmic flow of material (in this case EGF) from the neuron cell body through its axon to the terminals. Neuronal substances such as polypeptides are still produced in the cell body but are not transported. The substance is, therefore, more concentrated in the cell body and is easier to visualize with the immunohistochemical techniques.
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Saccharomyces cerevisiae Produces a Yeast Substance That Exhibits Estrogenic Activity in Mammalian Systems

Abstract. *Partially purified lipid extracts of* Saccharomyces cerevisiae *contain a substance that displaces tritiated estradiol from rat uterine cytosol estrogen receptors. The yeast product induces estrogenic bioresponses in mammalian systems as measured by induction of progesterone receptors in cultured MCF-7 human breast cancer cells and by a uterotrophic response and progesterone receptor induction after administration to ovariectomized mice. The findings raise the possibility that bakers' yeast may be a source of environmental estrogens.*

Saccharomyces cerevisiae contains an estrogen-binding protein (*1, 2*) and produces a lipid-extractable substance capable of displacing [^{3}H]estradiol from the yeast estrogen-binding protein (*1*). We have hypothesized that this substance represents the endogenous ligand for a yeast hormone receptor system. The yeast ligand also displaces [^{3}H]estradiol from rat uterine estrogen receptors (*1*). These findings suggested to us that yeast ligand might exhibit estrogenic activity in higher organisms, including mammals. To evaluate this hypothesis, we used partially purified preparations of ligand and examined them for estrogenic functional bioactivity. We report that yeast ligand exhibits estrogenic activity as measured by induction of progesterone receptors in cultured MCF-7 human breast cancer cells and by a uterotrophic response and uterine progesterone receptor induction after in vivo administration to ovariectomized mice. Estrogen receptor binding activity and reversible inhibition of bioactivity by the antiestrogen tamoxifen both support the hypothesis that yeast ligand acts through the estrogen receptor pathway in the manner of estradiol itself. The determination that *S. cerevisiae* ligand is estrogenic leads us to speculate that bakers' yeast may be a source of environmental estrogens.

Our methods to obtain partially purified yeast ligand from organic solvent extracts of *S. cerevisiae* by a four-step

Table 1. Scheme for partial purification of yeast ligand. Shown are the results of a typical purification experiment in which the decreasing mass required to achieve half-maximal binding or functional responses indicates increasing potency of the preparation. Yeast ligand was partially purified by extracting ~ 500 g of *S. cerevisiae* dried cells (Sigma) with ethanol. After concentration the residue was solubilized in ethanol, washed with water, and reextracted with ethyl acetate. The extract was passed over a 6-cm (outer diameter) column packed with 400 g of silica gel (Kieselgel 60, Merck) and eluted with a stepwise gradient of methylene chloride, ethyl acetate, and methanol (95:5:0, 75:25:0, 50:50:0, 50:45:5, and 50:40:10) (step 1). The active fractions were rechromatographed over a smaller (3.4-cm) silica gel column (160 g) eluted in a similar fashion (step 2). Active fractions were then dried under vacuum and resolubilized in acetonitrile and water (70:30) and rechromatographed on a reversed-phase HPLC semipreparative column (Whatman Partisil-10 C-8, 250 by 9.4 mm) eluted with acetonitrile and water 70:30 (step 3). Step 4 was a series of purifications on an analytic scale HPLC column (Alltech C8, 10 μm, 250 by 4.6 mm) eluted with acetonitrile and water (40:60).

Step	Procedure	Active fraction	
		Binding activity* (μg)	Functional activity† (ng)
1	Silica column	100	
2	Silica column	20	500
3	Semipreparative HPLC	2	50
4	Analytical HPLC	< 1	10

*Binding activity represents the amount of semipurified yeast ligand that displaced ~ 50 percent of [^{3}H]estradiol from MCF-7 cells. †Functional activity represents the amount of semipurified yeast ligand that induced ~ 50 percent of the maximum estradiol-stimulated increment in [^{3}H]R5020 binding sites in MCF-7 cells.