

PEPTIDES IN THE BRAIN. THE NEW ENDOCRINOLOGY OF THE NEURON

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by

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PART I

A. The Existence of Brain Peptides Controlling Adenohypophysial Functions. Isolation and Characterization of Their Primary Molecular Structures

In the early 1950s based on the anatomical observations and physiological experimentation from several groups in the USA and Europe, it became abundantly clear that the endocrine secretions of the anterior lobe of the hypophysis-well known by then to control all the functions of all the target endocrine glands, (thyroid, gonads, adrenal cortex) plus the overall somatic growth of the individual-were somehow entirely regulated by some integrative mechanism located in neuronal elements of the ventral hypothalamus (review Harris, 1955). Because of the peculiar anatomy of the junctional region between ventral hypothalamus (floor of the 3rd ventricle) and the parenchymal tissue of the anterior lobe of the pituitary (Fig. 1), the mechanisms involved in this hypothalamic control of adenohypophysial functions were best explained by proposing the existence of some secretory product(s) by some (uncharacterized) neuronal elements of the ventral hypothalamus, the products of which would somehow reach the adenohypophysis by the peculiar capillary vessels observed as if to join the floor of the hypothalamus to the pituitary gland.

That concept was definitely ascertained in simple experiments using combined tissue cultures of fragments of the pituitary gland and of the ventral hypothalamus (Guillemin and Rosenberg, 1955). The search for characterizing the hypothetical hypothalamic hypophysiotropic factors started then. Simple reasoning and early chemical confirmation led to the hypothesis that these unknown substances would be small peptides. After several years of pilot studies involving both biology and relatively simple chemistry in several laboratories in the USA, Europe and Japan, it became clear that characterizing these hypothalamic hypophysiotropic substances would be a challenge of (originally) unsuspected proportions. Entirely novel bioassays would have to be devised for routine testing of a large number of fractions generated by the chemical purification schemes; more sobering still was the realization in the early 1960s that enormous amounts of hypothalamic fragments (from slaughter house animals) would have to be obtained to have available a sufficient quantity of starting material to attempt a meaningful program of chemical isolation. The early pilot studies had indeed shown the hypothalamic sub-

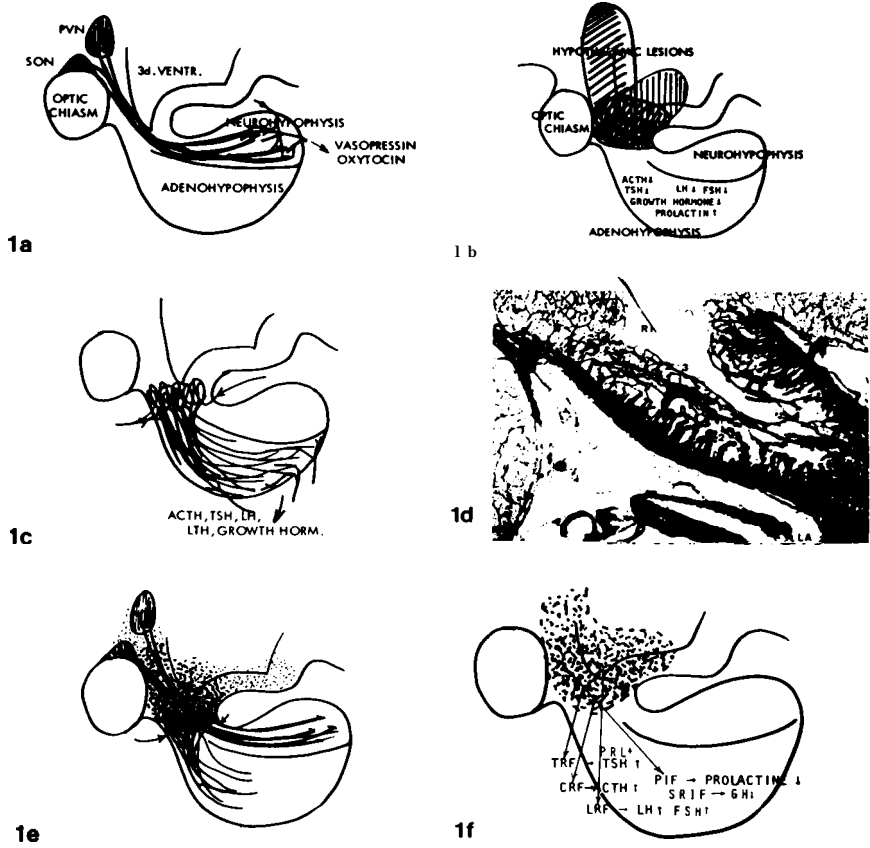


Fig. 1 : a) Diagrammatic representation of the pituitary gland and the innervation of the neurohypophysis by nerve fibers from the n.paraventricularis (PVN) and supra-opticus (SON). b) Localized lesions in the hypothalamus produce changes in the pituitary secretion of the various adenohypophysial hormones (increase ↑, or decrease ↓). c) Diagrammatic representation of the hypothalamo-hypophysial portal system. d) Photomicrograph of the hypothalamo-hypophysial portal system after injection with an opaque dye. e) Diagrammatic representation of the hypophysiotropic area. f) Changes in pituitary secretion of various adpnohypophysial hormones (increase ↑, or decrease ↓).

stances to be extremely potent and, on the basis of simple assumptions, to be present in each hypothalamic fragment only in a few nanogram quantities. Essentially one, then two groups of investigators approached the problem with enough constancy and resolution to stay with it for the ten years that it took to provide the first of its definitive solution, i.e. the primary structure of one of the hypothalamic hypophysiotropic factors: My own group, then at Baylor College of Medicine in Houston, Texas (with an episode at the Collège de France in Paris), organized the collection over several years of more than 5 million sheep brains, handling in the laboratory more than 50 tons of hypothalamic fragments. Schally and his collaborators, now in the Tulane

University School of Medicine in New Orleans, after he had left my laboratory at Baylor, collected also very large numbers of porcine hypothalamic fragments. Late in 1968, from 300,000 sheep hypothalami, Burgus and I isolated 1.0 mg of the first of these hypothalamic hypophysiotropic peptides, the thyrotropin releasing factor (TRF), the molecule by which the hypothalamus regulates through the pituitary the functions of the thyroid gland (Guillemin et al., 1962).

The following year, after more technical difficulties were overcome, we established the primary structure of ovine TRF by mass spectrometry as that of the deceptively simple tripeptide pGlu-His-Pro-NH₂. The material of porcine origin was shown by Schally and his collaborators to be identical. The synthetic replicate, rapidly available in unlimited quantities, was shown to be highly potent in all vertebrate species and particularly in man; it is now widely used throughout the world in a highly sensitive test of pituitary function and an early means of detection of pituitary tumors in man.

The isolation and characterization of TRF was the result of an enormous effort. It was also the turning point which separated doubt -- and often confusion, from unquestionable knowledge. It was of such heuristic significance, that I can say that neuroendocrinology became an established science on that event.

The characterization of the molecular structure of TRF was achieved in an unconventional manner, which will be briefly recounted here. I have with Burgus and Vale given an extensive technical review of the whole series of events that led to the characterization of ovine TRF (Guillemin et al., 1971); readers interested in the technical as well as historical aspects of these developments will find them in that review.

Publication, Isolation and Characterization of TRF

In January 1969, with the latest supply of highly purified ovine TRF available, - 1.0 mg obtained from 300,000 sheep hypothalamus fragments, amino acid analysis of 6 N HCl hydrolyzates of this preparation revealed only the amino acids, Glu, His, and Pro, in equimolar ratios and accounting in weight for 81% of the preparation (theoretical ponderal contribution of His, Pro, and Glu for a tripeptide monoacetate calculates to 86 %) (Burgus & Guillemin, 1970a). Furthermore the ultraviolet (UV), infrared (IR), and nuclear magnetic resonance (NMR) spectra obtained with that preparation of TRF were consistent with those of a polypeptide and upon close examination most of the characteristics of those spectra could be accounted for by the structural features of the amino acids found in the hydrolyzates of TRF. Moreover, the solubility properties and the lack of volatility observed in early attempts to obtain mass spectra or to perform gas chromatography, as well as other analytical data, were consistent with those of a polypeptide; also, the lack of effect of classical proteolytic enzymes, observed earlier, could be related to the particular amino acids observed. With the analyses of the more highly purified material unmistakably showing the amino acids to account for the total weight of the preparation, an earlier hypothesis that TRF could be a heteromeric poly-

peptide was therefore abandoned in favor of the possibility that it might be a cyclic or a protected peptide, a view compatible with failure to detect an N-terminus (Burgus et al. 1966b; Burgus and Guillemin, 1967, 1970a; Schally et al., 1966c, 1968, 1969) or a C-terminus (Schally et al., 1969; Burgus and Guillemin, 1970a) as well as the resistance of the biological activity to proteases.

The knowledge that the amino acids His, Pro, and Glu not only occurred in equimolar ratio in porcine and ovine TRF but indeed accounted for almost the theoretical total weight of the molecule in the case of ovine TRF, along with the previous knowledge of a lack of an N-terminal amino acid, led us to re-examine (this had been done by Schally in 1968) derivatives of synthetic polypeptides containing equimolar ratios of these amino acids to serve at least as possible models for the methodology to be used in the characterization of ovine TRF. We tested for TRF activity 6 tripeptide isomers containing L-His, L-Pro, and L-Glu synthesized upon our request by Gillessen et al. (1970) (containing only the peptides involving the α -carboxyl group of glutamic acid). The tripeptides proved to be devoid of TRF activity, confirming the earlier results of Schally et al. (1968, 1969). Our response to these negative results was, however, different from what had been that of Schally et al (see Schally et al., 1969).

I proposed treating each of the six tripeptides by acetic anhydride in an effort to protect the N-terminus as in natural TRF. The acetylation mixture from one, and only one of the peptides, namely H-Glu-His-Pro-OH, yielded biological activity qualitatively indistinguishable from that of natural TRF. It was active in *in vivo* and *in vitro* assays specific for TRF and its action *in vivo* was blocked by prior injection of the animals with thyroxine (Burgus et al., 1969a). The specific activity of the material obtained was lower (ca. 1×10^{-3}) than that of purified natural TRF. The nature of several possible reaction products was considered: mono- or diacetyl-derivatives, polymers of Glu-His-Pro, and cyclic peptide derivatives or derivatives containing pyroglutamic acid (pGlu) as the N-terminus. Subsequently we reported (Burgus et al., 1969a, b) that the major product by weight of this procedure was indeed pGlu-His-Pro-OH. The material was isolated from the reaction mixture and its structure was confirmed by mass spectrometry of the methyl ester and by its identity to authentic pGlu-His-Pro-OH (Gillessen et al., 1970) on TLC, the IR spectrum, as well as similarity of intrinsic biological activity *in vivo*.

This represented the first demonstration of a fully characterized synthetic molecule, based on the known composition of natural TRF, to reproduce the biological activity of a hypothalamic releasing factor.

Several other products present in the acetylation mixture, some possibly having higher specific activity than pGlu-His-Pro-OH, were not characterized. It is of interest that acetyl-Glu-His-Pro-OH obtained by total synthesis (Gillessen et al., 1970) was devoid of TRF activity in the *in vivo* assay at doses up to 250 μ g (Burgus et al., 196913).

Because of the differences between the specific biological activities of pGlu-His-Pro-OH and natural ovine TRF and the different behavior of these

two compounds in various chromatographic systems, it was evident that TRF was not pGlu-His-Pro-OH as such. It was the proposal of Burgus based on knowledge of the primary structures of other biologically active polypeptides (vasopressins, oxytocin, gastrins, etc.) that a likely candidate for the structure of the natural material would be pGlu-His-Pro-NH₂, and its synthesis was approached through the simple procedure of methanolysis of the methyl ester, pGlu-His-Pro-OMe (Burgus et al., 1969b, 1970 b, c). The ester, prepared by treatment of the pure synthetic pGlu-His-Pro-OH with methanolic HCl, was purified by partition chromatography and identified as pGlu-His-Pro-OMe, on the basis of its behavior on TLC, its IR spectrum, and by mass spectrometry (Burgus et al., 1970 b, c). It had biological activity *in vitro* and *in vivo* now approaching half of the specific activity of isolated ovine TRF. Ammonolysis of the methyl ester in methanol produced a material which upon partition chromatography gave a small yield of a substance presumably pGlu-His-Pro-NH₂, occurring in a Pauly positive zone separated from the starting material, which had a specific activity *in vivo* or *in vitro* statistically identical to that of ovine TRF. Among the derivatives tested, the properties of native ovine TRF most closely matched that of the amide, failing to separate from the synthetic compound in four different systems of TLC when run in mixtures. The IR spectra of several of the more highly purified preparations of the amide, including pGlu-His-Pro-NH₂, now prepared by total synthesis (Gillissen et al., 1970), were almost identical to that of ovine TRF, showing only minor differences in two regions of the spectra. These new observations, together with the demonstration that the specific activity of the pGlu-His-Pro-NH₂ was not statistically different from that of natural ovine TRF, led us to reconsider (Burgus et al., 1969c; 1970b) an earlier hypothesis (Burgus et al., 1969b) that ovine TRF may have a secondary or tertiary amide on the C-terminal proline, rather than correspond to the primary amide of the tripeptide pGlu-His-Pro.

Availability of large amounts of the synthetic tripeptides made possible a series of experiments with Desiderio, of Horning's group at Baylor, to modify the design of the direct probe of the then available low resolution mass spectrometer and simultaneously to obtain volatile derivatives of the peptides that would give clear mass spectra on only a few micrograms of the peptides. Once this was achieved, evidence was obtained (Burgus et al., 1969c) based on low and high resolution mass spectrometry, that the native ovine TRF preparation originally obtained in late 1968 had been all along essentially homogeneous and had unquestionably the structure pGlu-His-Pro-NH₂. Both synthetic pGlu-His-Pro-NH₂ (Burgus et al., 1969c; 1970b, c) and the highly purified ovine TRF (Burgus and Guillemin, 1970a) were introduced by direct probe into a low resolution mass spectrometer as the methyl or trifluoroacetyl (TFA) derivatives (Fig. 2); all preparations gave volatile materials in the temperature range of 150-200°C ($\leq 10^{-6}$ torr). Several mass spectra taken throughout the range of the thermal gradient (7 in the case of the isolated ovine TRF) showed fragmentation patterns corresponding to a single component. Although none of the spectra revealed a molecular ion, fragments

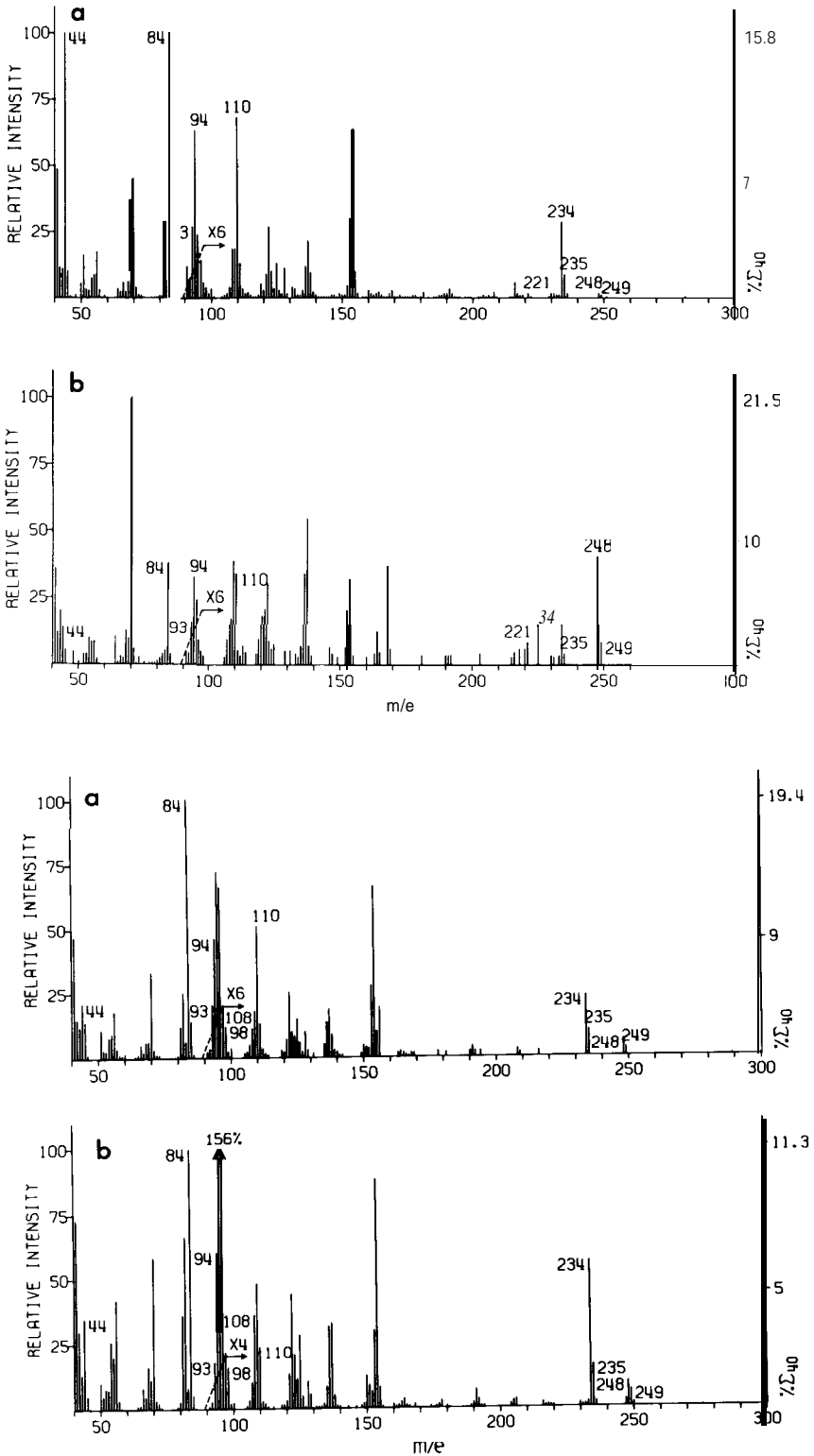


Fig. 2: Upper a, b: Low resolution mass spectra of trifluoroacetylated ovine TRF (a) and synthetic PCA-His-Pro-NH₂ (b).

Lower a, b: Low resolution mass spectra of methylated ovine TRF (a) and synthetic PCA-His-Pro-NH₂ (b) .

arising from the structures pGlu, methyl-pGlu, His, methyl-His, Pro, Pro-NH₂, CONH₂, pGlu-His, and His-Pro-NH₂ were observed. The low resolution mass spectra of the corresponding derivatives of synthetic pGlu-His-Pro-NH₂ and TRF were essentially identical. Fragments arising from unsubstituted pGlu or His were observed in the spectra of both types of derivatives.

The elemental composition of all the fragments, except *m/e* 221, the intensity of which was too weak for it to be observed on the photoplate used, were confirmed by high resolution mass spectroscopy of the methyl derivatives (Burgus et al., 1969c; 1970b).

Thus, the structure of ovine TRF as isolated from the hypothalamus was established as pGlu-His-Pro-NH₂ (Fig. 3). However, we did point out (Burgus et al., 1969c) that the possibility was not excluded that, as opposed to the isolated material, the native molecule of TRF may occur as Gln-His-Pro-NH₂, either free or conjugated to another structure such as a protein, which would not be necessary for biological activity *in vivo* or *in vitro*. At the time of this lecture we and others are still looking for a hypothetical prohormone of TRF.

The structure of *porcine* TRF was shown in a series of reports by Schally and his collaborators to be compatible with, and finally to be identical with, that of pGlu-His-Pro-NH₂; mass spectrometry was also the method of ultimate proof used by Nair et al. (1970).

STRUCTURE OF HYPOTHALAMIC TRF (OVINE)
WITH FRAGMENTATION POINTS IN MASS SPECTROMETRY

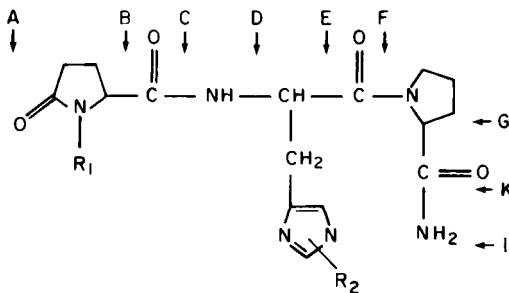


Fig. 3: The primary structure of TRF with indication of the fragmentation points in mass spectrometry. R₁, R₂, represent the methyl derivative prepared for mass spectrometry; in the native molecule, R₁ = R₂ = H.

It is most interesting that TRF from two widely different species of mammals should have the same structure and apparently the same specific (biological) activity in similar assays. It was rapidly shown that TRF shows no evidence of species specificity for its biological actions, pGlu-His-Pro-NH₂ being readily active in humans (see Fleischer et al., 1970; Fleischer and Guillemin, 1976).

Purification, Isolation and Characterization of LRF

In the early 1960s, several investigators reported experimental results that were best explained by proposing the existence in crude aqueous extracts of

hypothalamic tissues of substances that specifically stimulated the secretion of luteinizing hormone, and that were probably polypeptides (McCann et al., 1960; Campbell et al., 1961; Courrier et al., 1961). The active substance was named LH-releasing factor or LRF. Rapidly following these early observations, preparations of LRF, active at 1 μg per dose in animal bioassays, were obtained by gel filtration and ion-exchange chromatography on carboxymethylcellulose (Gullemin et al., 1963) an observation that was confirmed with similar methods by several investigators (Schally et al., 1968). In spite of the vagaries of the various bioassay methods available, several laboratories reported preparations of LRF of increased potency. Several of these early publications led, however, to contradictory statements regarding purification and separation of LH-releasing factor (LRF), from a follicle-stimulating hormone releasing factor (Schally et al., 1968; Dhariwal et al., 1967; Guillemin 1963).

Two laboratories independently reported in 1971 the isolation of porcine LRF (Schally et al., 1971a) and ovine LRF (Amoss et al., 1971), both groups concluding that LRF from either species was a *nonapeptide* containing, on the basis of acid hydrolysis, 1 His, 1 Arg, 1 Ser, 1 Glu, 1 Pro, 2 Gly, 1 Leu, 1 Tyr. Earlier results with the pyrrolidonecarboxylpeptidase prepared by Fellows and Mudge (1970) had led us to conclude (Amoss et al., 1970) that the N-terminal residue of LRF was Glu in its cyclized pyroglutamic (pGlu) form, as in the case of hypothalamic TRF, (pGlu-His-Pro-NH₂). The total amount of the highly purified ovine LRF that we had isolated from side fractions of the TRF program and that was available for amino acid sequencing was ca. 80 nmol (as measured by quantitative dansylation).

It is to the credit of Schally's group to have first recognized and reported (Matsuo et al., 1971a) that porcine LRF contained one residue of tryptophan (Trp), in addition to the other amino acids earlier observed by acid hydrolysis. On the basis of a series of experiments including enzymatic hydrolysis with chymotrypsin and thermolysin and analysis of the partial sequences of their decapeptide by Edman degradation-dansylation and selective tritiation of C-termini, Matsuo *et al.* (1971 b) proposed the sequence pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH₂, for porcine LRF as that best compatible with the partial sequence data. Their studies were carried out with cu. 200 nmol of peptide. They also stated that synthesis of that particular sequence had given a material with biological activity. A few weeks later, we reported the synthesis by solid-phase methods of the decapeptide pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH₂; after isolation from the reaction mixture it had quantitatively the full biological activity *in vivo* and *in vitro* of ovine LRF (Monahan et al., 1971).

Shortly thereafter, we reported (Burgus et al., 1971) the amino-acid sequence of ovine LRF obtained on 40 nmoles of peptide by analysis of hydrolysis products after digestion with chymotrypsin or pyrrolidonecarboxylpeptidase, using Edman-degradation followed by determination of N- and C-termini by a quantitative [¹⁴C]-dansylation technique. Confirmation of the positions of some of the amino-acid residues obtained by combined gas chromatographic-mass spectrometric analysis of phenylthiohydantoin (PTH) derivatives (Fales

et al., 1971; Hagenmaier et al., 1970) resulting from Edman degradations was described; we also reported results obtained by degradation of the synthetic decapeptide, since they confirmed and clarified some peculiarities observed upon enzymatic cleavage of the native peptide (Burgus et al., 1972).

The amino acid sequence of ovine LRF was established to be pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH₂. It is identical to that of the material of porcine origin.

Of considerable interest was the observation that the synthetic replicate of LRF, now available in large quantities, was shown to stimulate concomitant secretion of the two gonadotropins LH (luteinizing hormone) and FSH (follicle stimulating hormone) in all assay systems *in vivo* and *in vitro* in which it was tested. This confirmed the earlier results obtained with the minute quantities of the isolated ovine or porcine LRF (Amoss et al., 1971; Schally et al., 1971a). In other words, the stimulation of the release of FSH appeared to be inherent to the molecule of LRF - thus throwing considerable doubt on earlier reports (Schally et al., 1966; Schally et al., 1968; Igarashi et al., 1964) claiming to have obtained preparations of LRF free of FSH-releasing activity.

To the day of this lecture, no solid evidence has been produced which could be interpreted to indicate the existence of an FSH-releasing factor as a specific entity, discrete from the decapeptide LRF. Reports by Folkers *et al.* (1969) claiming purification of an FSH-releasing factor are difficult to appreciate in view of the paucity of data offered. Moreover, other evidence is against the existence of an FSH-releasing factor, separate from LRF: all synthetic analogs of LRF made so far, with no exception, can be shown to release LH and FSH with the same ratio of specific activity when related to the activity of LRF in the particular assay involved. Thus, none of these analogs has shown any evidence of dissociated activity for releasing FSH vs. LH. Also, there is increasing evidence that the two gonadotropins (LH and FSH) can be demonstrated (immunocytochemistry) mostly in the same pituitary cell (Moriarty, 1973). It is thus unlikely that one could be released without the other as they appear to be present in the same secretory granules.

Later on, both Schally's group (1971b) and our group (Ling et al., 1973) confirmed the primary structure of porcine and ovine LRF, respectively, using larger quantities of native material.

Purification, Isolation and Characterization of Somatostatin

It has been generally accepted that the control of the pituitary secretion of growth hormone would be exerted by a hypothalamic hypophysiotropic releasing factor, as is now proven to be the case for the secretion of thyrotropin and the gonadotropins. The nature of the postulated hypothalamic releasing factor for growth hormone, however, remains elusive to this day, mostly due to the difficulties and ambiguities of the various assay systems used so far in attempts at its characterization. For instance there is now agreement that the "growth hormone releasing hormone" (GH-RH), isolated on the basis of a bioassay and characterized by Schally *et al.* (1971c) as H-Val-His-Leu-Ser-Ala-Glu-Glu-Lys-Glu-Ala-OH, was actually a decapeptide fragment

of the N-terminal of the β -chain of porcine hemoglobin (Veber et al., 1971). The material has never been shown to be active in stimulating secretion of immunoreactive growth hormone. Similarly, biological activity of a tetrapeptide recently reported as a growth hormone releasing factor of porcine origin has not been confirmed by others, including our own laboratory (see Guillemin, 1973).

Searching to demonstrate the presence of this still hypothetical somatotropin releasing factor in the crude hypothalamic extracts used in the isolation of TRF (thyrotropin releasing factor) and LRF (luteinizing hormone releasing factor), we regularly observed that their addition in minute doses ($\leq .001$ of a hypothalamic fragment equivalent) to the incubation fluid of dispersed rat pituitary cells in monolayer cultures (Vale et al., 1972a) significantly decreased the resting secretion of immunoreactive growth hormone by the pituitary cells. This inhibition was related to the dose of hypothalamic extract added and appeared to be specific. It was not produced by similar extracts of cerebellum, and the crude hypothalamic extracts that inhibit secretion of growth hormone simultaneously stimulated secretion of LH and TSH. The inhibition of growth hormone secretion could not be duplicated by addition to the assay system of [Arg⁸]-vasopressin, oxytocin, histamine, various polyamines, serotonin, catecholamines, LRF, or TRF. We decided to attribute this inhibitory effect on the secretion of growth hormone to a "somatotropin-release inhibiting factor" which we later named *somatostatin*.

Inhibition of secretion of growth hormone by crude hypothalamic preparations had been reported by others (Krulich and McCann, 1969). The active factor possibly involved in these early studies based on various types of assays for growth hormone activity had not been characterized. The results on the inhibition by the hypothalamic extracts of the secretion of immunoreactive growth hormone by the monolayer pituitary cultures were so consistent and easily quantitated that we decided to attempt the isolation and characterization of the hypothalamic factor involved. We realized the possible interest of such a substance in inhibiting abnormally elevated secretion of growth hormone in juvenile diabetes; also, we considered that knowledge of the primary structure of a native inhibitor of the secretion of a pituitary hormone could be of significance in our efforts at designing synthetic inhibitors of the gonadotropin releasing factor LRF.

The starting material was the chloroform-methanol-glacial acetic acid extract of about 500,000 sheep hypothalamic fragments (Burgus et al., 1971; Burgus et al., 1972) used in the program of characterization of the releasing factors for the gonadotropins. The extract (2 kg) had been partitioned in two systems; the LRF concentrate was subjected to ion-exchange chromatography on carboxymethyl cellulose. At that stage, a fraction with growth hormone-release inhibiting-activity was observed well separated from the LRF zone; it was further purified by gel filtration (Sephadex G-25) and liquid partition chromatography (n-butanol, acetic acid, water, 4 : 1 : 5). Thin-layer chromatography and electrophoresis of the final product showed only traces of peptide impurities. The yield was 8.5 mg of a product containing 75 percent of amino

acids by weight; we will refer to this material by the name *somatostatin* which was actually given to it only after it had been fully characterized.

Analysis of amino acids obtained from somatostatin after acid hydrolysis in 6 N HCl-0.5 % thioglycollic acid gave the molar ratios Ala (0.9), Gly (1.1), Cys (0.2), Cys-SS-Cys (1.0), Lys (2.0), Asp (1.0), Phe (3.3), Trp (0.5), Thr (2.0), Ser (0.8), and NH, (1.1). Enzymic hydrolysis gave the ratios Ala (0.9), Gly (0.9), Lys (2.0), Phe (3.4) and Trp (0.9); Asn, Thr, and Ser were not well resolved, giving a total of about 3.6 mol/mol of peptide. Edman degradation of the carboxymethylated trypsin digests of somatostatin and mass spectrometry led finally to the final demonstration of the following primary structure for somatostatin: H-Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys-OH, in the oxidized form (Burgus et al., 1973).

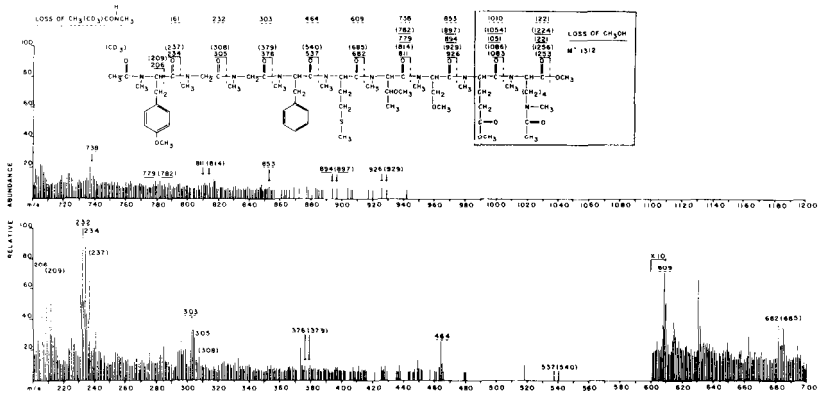
This peptide was reproduced by total synthesis using Merrifield method (see Rivier, 1974). It had the full biological activity of native somatostatin *in vivo* and *in vitro* (Brazeau et al., 1973, Vale et al., 1972b, Brazeau et al., 1974). Of interest was the unexpected observation that the peptide has the full biological activity either in the oxidized form (native) or reduced form.

B. Purification, Isolation and Characterization of the Endorphins, Opiate-Like Peptides of Brain or Pituitary Origin

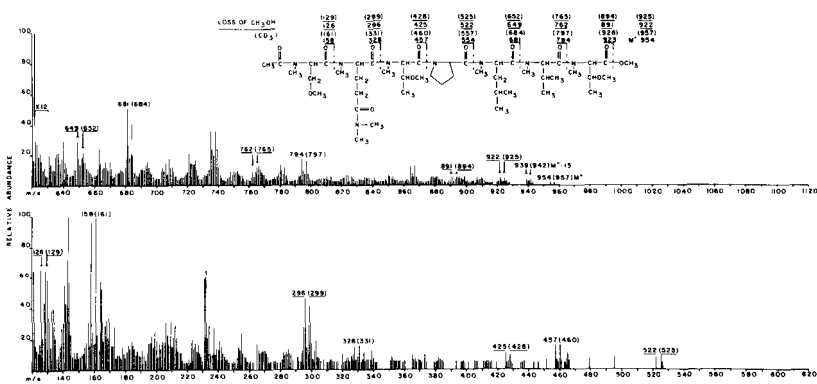
The concept and the demonstration, some years ago, of the existence in the brain of mammals of (synaptosomal) opiate-receptors (Pert and Snyder, 1973) led to the search of what has been termed the endogenous-ligand(s) of these opiate receptors. The generic name *endorphins* (from endogenous and morphine) was proposed for these (then hypothetical substances) by Eric Simon and will be used here. Sometime in the summer of 1975 I became interested in these early observations. Besides the challenge of characterizing an endogenous substance as the ligand of the brain opiate receptors, we could not ignore that, like morphine, the (then hypothetical) endorphins might stimulate the secretion of growth hormone; the nature of the growth hormone releasing factor was and still remains unknown. I thus decided to engage in the isolation and characterization of the endogenous ligand(s) for the opiate receptors. The isolation of these endogenous ligands of the opiate receptors turned out to be a relatively simple problem to which a solution was provided in less than a couple of months of effort.

Dilute acetic acid-methanol extracts of whole brain (ox, pig, rat) were confirmed to contain substances presumably peptidic in nature, with naloxone-reversible, morphine-like activity in the bioassay using the myenteric-plexus longitudinal muscle of the guinea pig ileum. Evidence of such biological activity in our laboratory was in agreement with earlier results of Hughes (1975), Terenius & Wahlstrom (1975), Teschemacher et al. (1975), and Pasternak et al. (1975). Searching for an enriched source of endorphins in available concentrates from our earlier efforts towards the isolation of CRF, TRF, LRF, somatostatin, I recognized that acetic acid-methanol extracts of porcine hypothalamus-neurohypophysis contained much greater concentrations of the morphine-like activity than extracts of whole brain. From such an extract

of approximately 250,000 fragments of pig hypothalamus-neurohypophysis we isolated several oligopeptides (*endorphins*) with opioid activity (Guillemin et al., 1976a; Lazarus et al., 1976; Ling et al., 1976). The isolation procedure involved successively gel filtration, ion exchange chromatography, liquid partition chromatography and high pressure liquid chromatography (Guillemin et al., 1976a; Lazarus et al., 1976; Ling et al., 1976). By that time, had appeared the evidence for the isolation and primary structure of Met⁵-enkephalin and Leu⁵-enkephalin (Hughes, et al., 1975). Hughes et al. (1975) had also made the remarkable observation of the identity of the amino acid sequence of Met⁵-enkephalin with that of the sequence Tyr⁶¹-Met⁶⁵ of β -lipotropin, a polypeptide of ill defined biological activity, isolated and characterized in 1964 by C. H. Li et al. (see Li & Chung, 1976). The primary structure of *α*-endorphin was established (Ling et al., 1976; Guillemin et al., 1976a) by mass spectrometry and classical Edman degradation of the enzymatically cleaved peptide and is H-Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-OH (Fig. 4 a, b). The primary structure of *γ*-endorphin



α-endorphin. NH² terminal fragment H-Tyr-Gly-Gly-Phe-Met-Thr-Ser



α-endorphin. COOH terminal fragment -Ser-Gln-Thr-Pro-Leu-Val-Thr-OH

Fig. 4: Mass spectra of *α*-endorphin after trypsin digestion, acetic and deuterioacetic anhydride acetylation and permethylation. The sequences are: (a) H-Tyr-Gly-Gly-Phe-Met-Thr-Ser-; (b) : H-Ser-Gln-Thr-Pro-Leu-Val-Thr-OH.

was similarly established by mass spectrometry and by Edman degradation: γ -endorphin has the same primary structure as α -endorphin with one additional Leu as the COOH-terminal residue in position 17.

Thus, it **was obvious that Met-enkephalin is the N-terminal pentapeptide** of α - and γ -endorphin, which have respectively the same amino acid sequence as β -lipotropin [61-76] and [61-77]. β -LPH-[61—91], a fragment of β -LPH isolated earlier on the basis of its chemical characteristics (Bradbury et al., 1975; Li and Chung, 1976) was shown also to have opiate-like activity (Bradbury et al., 1976; Lazarus et al., 1976; Cox et al. 1976) and has been named *β -endorphin* (Li and Chung, 1976). Recently we have isolated, from the same starting material of hypothalamus-neurohypophysis origin from which we originally isolated α - and γ -endorphin, two peptides characterized by amino acid composition as *β -endorphin* (β -LPH[61—91]) and *δ -endorphin* (β -LPH [61—87]). No effort was made to obtain the amino acid sequences of these two samples. The synthetic replicates of these two polypeptides have identical chromatographic behavior in several systems as the native materials.

PART II

Biological Activities of the Hypothalamic Peptides and Synthetic Analogs. Experimental and Clinical Studies

As soon as they were obtained in large quantity from total synthesis, TRF and LRF were extensively studied for their biological activities, both in the laboratory and in clinical medicine. Indeed, the observation was rapidly made that TRF, and later LRF, both characterized only from tissues of ovine and porcine origin, were biologically fully active in all species of vertebrates studied, including man (Fig. 5), the same was to apply for the synthetic replicate of somatostatin, as characterized from ovine brains (Fig. 6).

For early clinical studies with synthetic TRF see Fleischer et al., 1970 and more recently Fleischer and Guillemin, 1976; for early clinical studies with synthetic LRF see Yen et al., 1973; Rebar et al., 1973. Schally's group also published extensively on clinical investigations with either purified native or synthetic TRF and LRF (review in Schally et al., 1973).

Both in the case of TRF and of LRF chemists have prepared large numbers of synthetic analogs of the primary (native) structure for studies of correlation between molecular structure and biological activity. Also, biologists carefully screened these analogs, in the hope that some of them would prove to be antagonists of the native (agonist) releasing factor. This was of particular interest in the case of the gonadotropin releasing factor; a powerful antagonist of **LRF** would be of considerable interest as a chemical means of controlling or regulating fertility, thus introducing a totally new type of substances for contraception. In 1972, our laboratory reported the first partial agonist/antagonist analogs of LRF, (Vale et al., 1972c). They all had a deletion or a substitution of His² or Trp³ in the (otherwise identical) amino acid sequence of LRF. These were antagonists of low activity and of no possible practical value as clinically significant inhibitors of LRF. They showed, however, that analogs as competitive antagonists of the decapeptide LRF could be prepared.

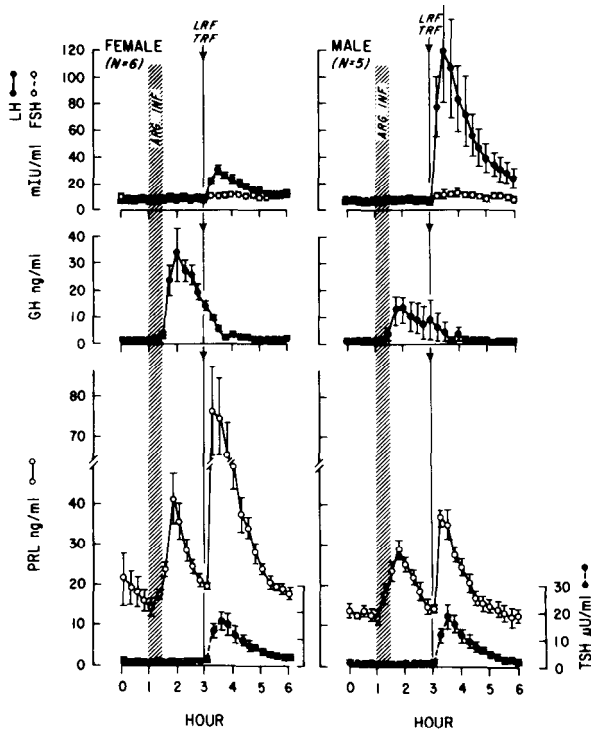


Fig. 5: Testing of the ability of the anterior pituitary to secrete GH, TSH, prolactin (PRL), LH and FSH in normal human subjects. Stimulation of the secretion of GH is achieved by i.v. administration of arginine; stimulation of the secretion of TSH and PRL, LH and FSH is produced by i.v. injection of a solution in saline of synthetic TRF (250 μg) and synthetic LRF (150 μg)-note that arginine infusion stimulates secretion of GH and PRL. All pituitary hormone plasma concentrations measured by radioimmunoassays (from Yen et al.).

To this day, the most potent antagonist-analogs of LRF still have the early deletion or substitution of His² or Trp³ of the amino acid sequence of LRF.

Also, analogs of TRF and LRF with increased potency (over that of the native compound) were expected, searched for, and obtained (see below).

a. Biological Activity of Thyrotropin Releasing Factor (TRF) and Luteinizing Hormone Releasing Factor (LRF)

The remarkable observation was originally made by Tashjian et al. (1971) that TRF stimulates the secretion of prolactin by the cloned line GH₃ of pituitary cells. This was confirmed by others and extended to show the observation to be valid also with normal pituitary tissues *in vitro* and *in vivo*, including the human pituitary. TRF can thus be considered as involved in the control of the secretion of thyrotropin and of prolactin. Of the many analogs of TRF which have been synthesized and studied biologically, only one has a significantly increased specific activity over that of the native compound. Described by our group and synthesized by Rivier (1971) a few years ago, it is the analog [3N-Methyl-His]-TRF. Its specific activity is approximately

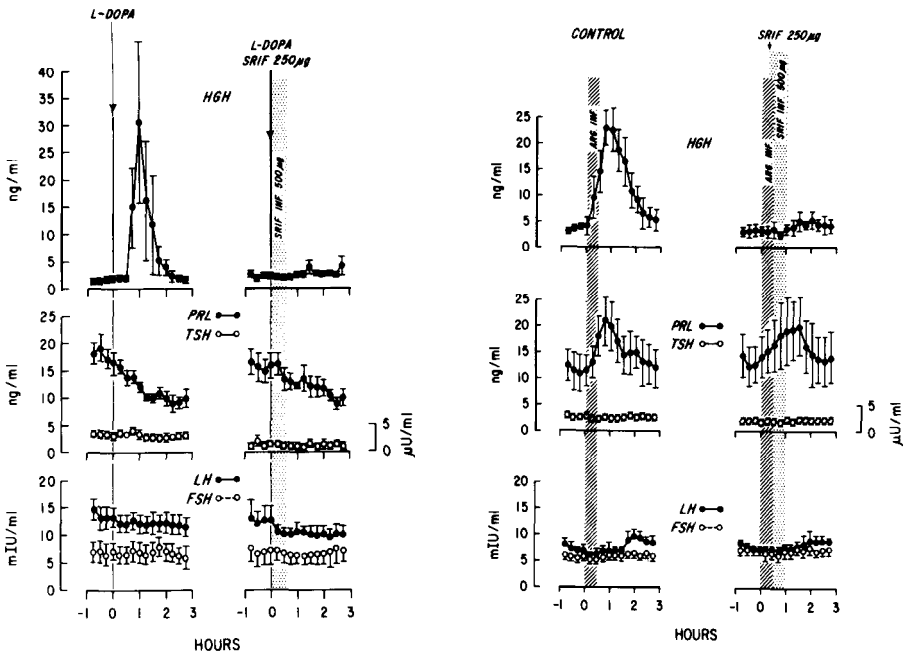


Fig. 6: Effects of the administration of synthetic somatostatin in normal human subjects. There is complete inhibition of the increase in GH secretion normally produced by infusion of arginine or oral administration of L-DOPA, where somatostatin is administered prior to or concurrently with the stimulating agent. Plasma concentrations of pituitary hormones were measured by radioimmunoassays (from Yen et al.).

10 times that of the native molecule, on the secretion of TSH as well as of prolactin. Of the several hundreds of TRF analogs synthesized, none has been found so far to be even a partial antagonist. They are all agonists with full intrinsic activity but variable specific activity; no true antagonist of TRF has been reported.

In contradistinction to the statement above regarding analogs of TRF, antagonist as well as extremely potent agonist analogs of LRF have been prepared by a number of laboratories. There are now available preparations of a series of what we may accurately call "super-LRFs", analogs which have as much as 150 times the specific activity of the native compound. In fact, in certain assays such as ovulation, they may have 1,000 times the specific activity of the native peptide. All the agonist-analogs or super-LRFs possess structural variations around two major modifications of the amino acid sequence of native LRF: They all have a modification of the C-terminal glycine, as originally reported by Fujino et al. (1974). The Fujino modification consists of deletion of Gly¹⁰-NH₂ and replacement by primary or secondary amide on the (now C-terminal) Pro⁹. In addition to the Fujino modification, they have an additional modification at the Gly⁶ position by substitution of one of several D-amino acids as originally discovered in our laboratories (Monahan et al., 1973). The most potent of the LRF-analog agonists prepared are [D-Trp⁶]-LRF; des-Gly¹⁰-[D-Trp⁶-Pro⁹-N-Et]-LRF, [D-Leu⁶, Pro⁹-N-Et]-LRF.

In an *in vitro* assay in which the peptides stimulate release of LH and FSH by surviving adeno-hypophysial cells in monolayer cultures, these analogs of LRF have a specific activity 50 to 100 times greater than that of the synthetic replicate of native LRF. There is no evidence of dissociation of the specific activity for the release of LH from that of FSH. All agonist analogs release LH and FSH in the same ratio (in that particular assay system) as does native LRF. Probably because of their much greater specific activity, when given in doses identical in weight to the reference doses of LRF, the super-LRFs are remarkably long acting. While the elevated secretion of LH (or FSH) induced by LRF is returned to normal in 60 minutes, identical amounts in weight, of [D-Trp⁶-des-Gly¹⁰]-N-Et-LRF lead to statistically elevated levels of LH up to 24 hours in several *in vivo* preparations, including man. These analogs are ideal agents to stimulate ovulation (Vilchez-Martinez et al., 1975). Marks and Stern (1975) have reported that these analogs are considerably more resistant than the native structures to degradation by tissue enzymes.

Injection into laboratory animals of large doses of the super-LRFs (i.e. doses of several micrograms/animal while minimal active doses or physiological range are in nanograms/animal) has been recently shown by several groups to have profound anti-gonadotropic effects, both in males and females; moreover, when such large doses of the super-LRFs are injected in the early days of pregnancy in rats, they consistently lead to resorption of the conceptus (see Rivier et al., 1977a); mechanisms involved in these observations have not been fully clarified as yet but are best explained by the current concepts of negative cooperativity between the peptidic ligands involved and their receptors at the several target-organ sites.

All of the antagonist LRF-analogs as originally found by our group (Vale et al. 1972c) or as later reported by others have deletion or a D-amino acid substitution of His². For reasons not clearly understood, addition of the Fujino modification on the C-terminal (Fujino et al., 1974) does not increase the specific activity (as antagonists) of the antagonist analogs. Administered simultaneously with LRF the antagonist analogs inhibit LRF in weight ratios ranging from 3 : 1 to 15 : 1. The most potent of these antagonists inhibit activity of LRF not only *in vitro*, but also in various tests *in vivo*. They inhibit the release of LH and FSH induced by an acute dose of LRF; they also inhibit endogenous release of LH-FSH and thus prevent ovulation in laboratory animals. The clinical testing of some of these LRF-antagonists prepared in our laboratory has recently started in collaboration with Yen at the University of California in San Diego.

b. *Biological Activity of Somatostatin*

It is now recognized that somatostatin has many biological effects other than the one on the basis of which we isolated it in extracts of the hypothalamus, i.e. as an inhibitor of the secretion of growth hormone (Brazeau et al., 1973). Somatostatin inhibits the secretion of thyrotropin, but not prolactin, normally stimulated by TRF (Vale et al., 1974); it also inhibits the secretion

of glucagon, insulin (Koerker et al., 1974), gastrin, secretin, by acting directly on the secretory elements of these peptides. I have recently shown (Guillemin, 1976) that somatostatin also inhibits the secretion of acetylcholine from the (electrically stimulated) myenteric plexus of the guinea pig ileum probably at a presynaptic locus - thus explaining at least in part the reportedly inhibitory effects of somatostatin on gut contraction, *in vivo* and *in vitro* (Fig. 7).

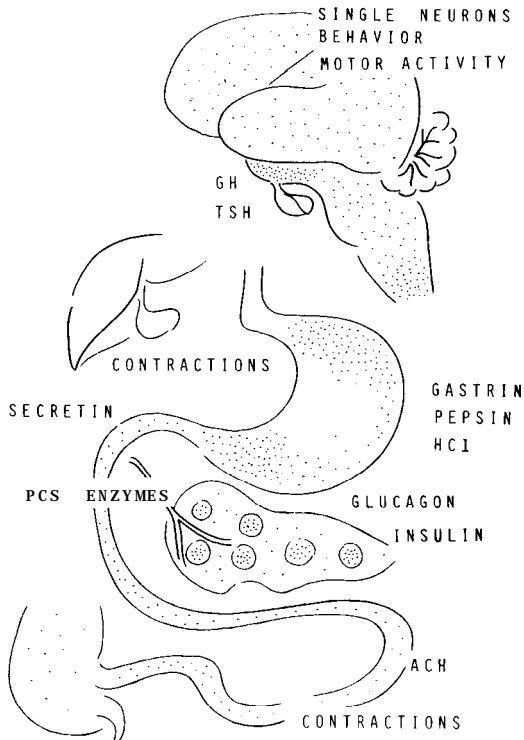


Fig. 7: Multiple locations of somatostatin and multiple effects of somatostatin.

It is also now well recognized that somatostatin is to be found in many locations other than the hypothalamus (Fig. 7), from which we originally isolated it. Somatostatin has been found in neuronal elements and axonal fibers in multiple locations in the central nervous system, including the spinal cord (see Hökfelt et al., 1976). It has been found also in discrete secretory cells of classical epithelial appearance in all the parts of the stomach, gut and pancreas (see Luft et al., 1974; Dubois, 1975) in which it had been first recognized to have an inhibitory effect.

Somatostatin does not inhibit indiscriminately the secretion of all polypeptides or proteins. For instance, as already stated, somatostatin does not inhibit the secretion of prolactin concomitant with that of thyrotropin when stimulated by a dose of TRF; this is true *in vivo* with normal animals or *in vitro* with normal pituitary tissue (see Vale et al., 1974). Somatostatin does not inhibit the secretion of either gonadotropin LH or FSH, the secretion of

calcitonin, the secretion of ACTH in normal animals or from normal pituitary tissues *in vitro*; it does not inhibit the secretion of steroids from adrenal cortex or gonads under any known circumstances (see Brazeau et al., 1973). Regarding the secretion of polypeptides or proteins from abnormal tissues of experimental or clinical sources, such as pituitary adenomas, gastrinomas, insulinomas, etc. somatostatin has been shown to be inhibitory according to its normal pattern of activity or being nondiscriminative. The latter must reflect one of the differences between normal and neoplastic tissue. This is in keeping with observation that TRF or LRF can stimulate release of growth hormone from the pituitaries of acromegalic patients though that does not happen with normal tissues.

Clinical studies have confirmed in man all observations obtained in the laboratory. The powerful inhibitory effects of somatostatin on the secretion not only of growth hormone but also of insulin and glucagon have led to extensive studies over the last three years of a possible role of somatostatin in the management or treatment of juvenile diabetes (Figs. 8-9). First of all, the ability of somatostatin to inhibit insulin and glucagon secretion has provided a useful tool for studying the physiological and pathological effects

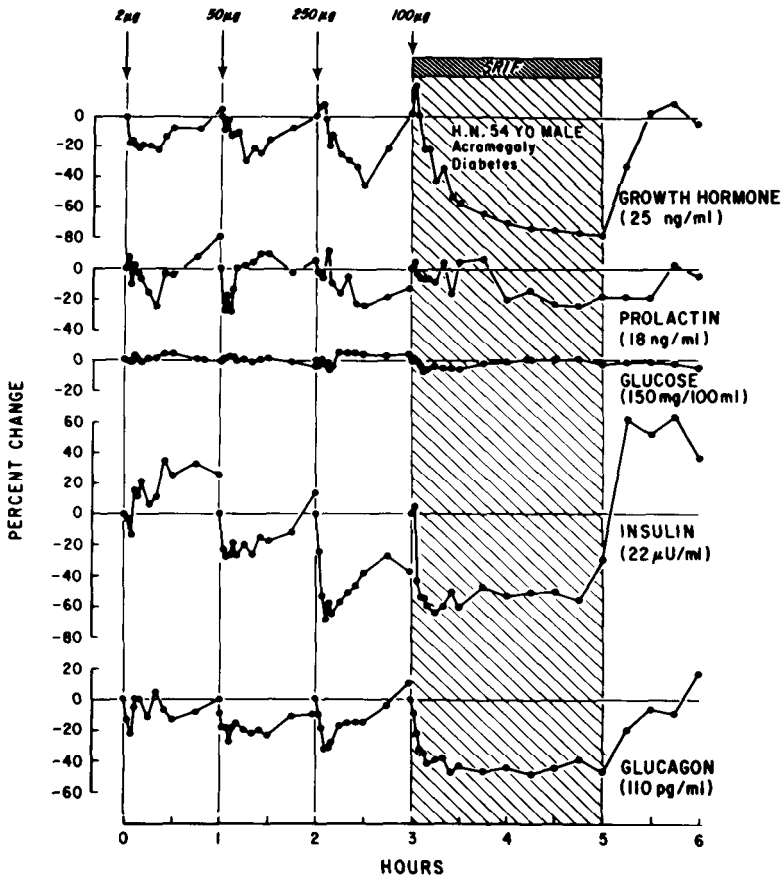


Fig. 8: Effect of multiple doses of somatostatin decreasing the plasma levels of growth hormone, insulin and glucagon in a patient with acromegaly and diabetes. (from Yen et al.).

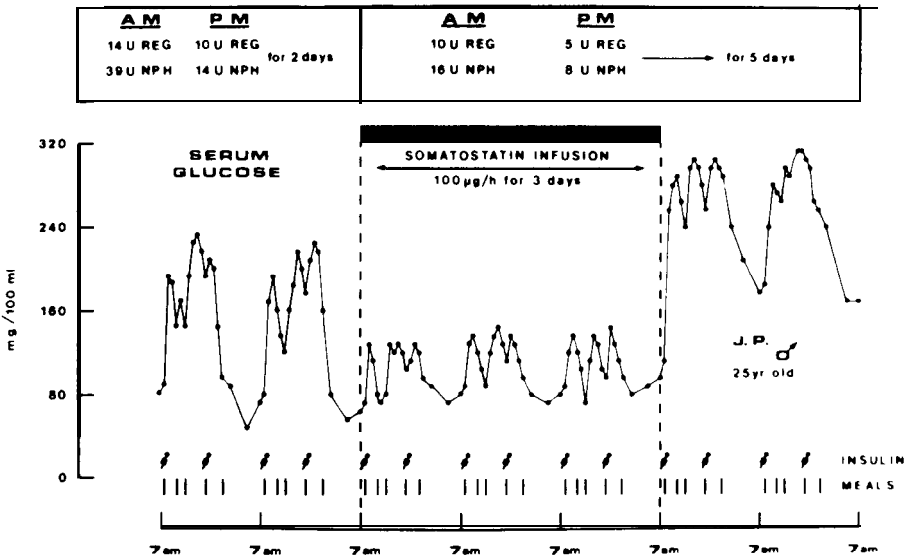


Fig. 9: Juvenile diabetic; improved control of glycemia during infusion of somatostatin and reduced amounts of insulin (from Gerich et al.).

of these hormones on human metabolism. Infusion of somatostatin lowers plasma glucose levels in normal man despite lowering of plasma insulin levels (Alford et al., 1974; Gerich et al., 1974; Mortimer et al., 1974). These observations provided the first clear-cut evidence that glucagon has an important physiological role in human carbohydrate homeostasis. Somatostatin itself has no direct effect on either hepatic glucose production or peripheral glucose utilization, since the fall in plasma glucose levels could be prevented by exogenous glucagon (Gerich et al., 1974).

In juvenile-type diabetics, somatostatin diminishes fasting hyperglycemia by as much as 50% in the complete absence of circulating insulin (Gerich et al., 1974). Although somatostatin impairs carbohydrate tolerance after oral or intravenous glucose challenges in normal man by inhibiting insulin secretion, carbohydrate tolerance after ingestion of balanced meals is improved in patients with insulin-dependent diabetes mellitus through the suppression of excessive glucagon responses (Gerich et al., 1974). The combination of somatostatin and a suboptimal amount of exogenous insulin (which by itself had prevented neither excessive hyperglycemia nor hyperglucagonemia in response to meals) completely prevents plasma glucose levels from rising after meal ingestion in insulin-dependent diabetics (Gerich et al., 1974). Through its suppression of glucagon and growth hormone secretion, somatostatin has also been shown to moderate or prevent completely the development of diabetic ketoacidosis after the acute withdrawal of insulin from patients with insulin-dependent diabetes mellitus (Gerich et al., 1975).

At the moment, clinical studies with somatostatin are proceeding in several clinical centers throughout the world.

From the foregoing description of the ability of somatostatin to inhibit

the secretion of various hormones, it would appear that it may be of therapeutic use in certain clinical conditions such as acromegaly, pancreatic islet cell tumors, and diabetes mellitus. With regard to endocrine tumors, it must be emphasized that while somatostatin will inhibit hormone secretion by these tissues, it would not be expected to diminish tumor growth (in view of its locus of action relating to that of c-AMP -- see Vale et al., 1972, 1973). Thus, in these conditions it is unlikely that somatostatin will find use other than as a symptomatic or temporizing measure.

In diabetes mellitus, however, somatostatin might be of considerable clinical value. First, it has already been demonstrated that it can acutely improve fasting as well as postprandial hyperglycemia in insulin-requiring diabetics, by inhibiting glucagon secretion. Second, since growth hormone has been implicated in the development of diabetic retinopathy, the inhibition of growth hormone secretion by somatostatin may lessen this complication of diabetes. Finally, through suppression of both growth hormone and glucagon secretion, somatostatin may prevent or diminish the severity of diabetic ketoacidosis and find application in "brittle diabetes." These optimistic expectations must be considered in light of the facts that the multiple effects of somatostatin on hormone secretions and its short duration of action make its clinical use impractical at the present time; moreover, its long-term effectiveness and safety have not been established as yet. Regarding the clinical use of somatostatin, see the recent review by Guillemin and Gerich (1976).

With the considerable interest in somatostatin as a part of the treatment of diabetics, "improved" analogs of somatostatin have been in the mind of clinicians and investigators. Analogs of somatostatin have been prepared in attempts to obtain substances of longer duration of activity than the native form of somatostatin; this has not been very successful so far. Other analogs have been sought that would have dissociated biological activity on one or more of the multiple recognized targets of somatostatin. Remarkable results have recently been obtained. The first such analog so recognized by the group of the Wyeth Research Laboratories was [des-Asn⁵]-somatostatin, an analog with approximately 4%, 10% and 1% the activity of somatostatin to inhibit respectively secretion of growth hormone, insulin and glucagon (Sarantakis et al., 1976). While such an analog is not of clinical interest, it showed that dissociation of the biological activities of the native somatostatin on three of its receptors could be achieved. Some of the most interesting analogs with dissociated activities reported so far were prepared and studied by J. Rivier, M. Brown and W. Vale in our laboratories; they are [D-Ser¹³]-somatostatin, [D-Cys¹⁴]-somatostatin and [D-Trp⁸, D-Cys¹⁴]-somatostatin. When compared to somatostatin, this latest compound has ratios of activity such as 300%, 10%, 100% to inhibit the secretions respectively of growth hormone, insulin and glucagon (Brown et al., 1976). These and other analogs are obviously of much clinical interest and are being so investigated at the moment in several laboratories and clinical centers. An international symposium was recently devoted entirely to the biology and chemistry of somatostatin and analogs (see Raptis, 1978).

c. *Biological Studies with the Endorphins*

1. *Relation of Endorphins to β -lipotropin*

So far, all morphinomimetic peptides isolated from natural sources on the basis of a bioassay or displacement assay for ^3H -opiates on synaptosomal preparations, and chemically characterized, have been related to a fragment of the C-terminus of the molecule of β -lipotropin, starting at Tyr⁶¹. In the case of Leu⁵-enkephalin, the relationship still holds for the sequence Tyr-Gly-Gly-Phe; no β -lipotropin with a Leu residue in position 65 has been observed.

β -LPH has no opioid activity in any of the tests above. Incubation of β -LPH at 37°C with the 10⁵ g supernatant of a neutral sucrose extract of rat-brain generates opioid activity suggesting the presence of peptidases in the rat brain that could cleave β -LPH to fragments with opioid activity. Thus, β -LPH may be a prohormone for the opiate-like peptides (Lazarus et al. 1976). This would imply that the biogenesis of endorphins may be similar to that of angiotensin with cleaving enzymes available in the central nervous system or in peripheral blood. There is also good evidence by immunocytochemistry (Bloom et al., 1977) and biosynthesis studies (Mains, Eipper and Ling, 1977;

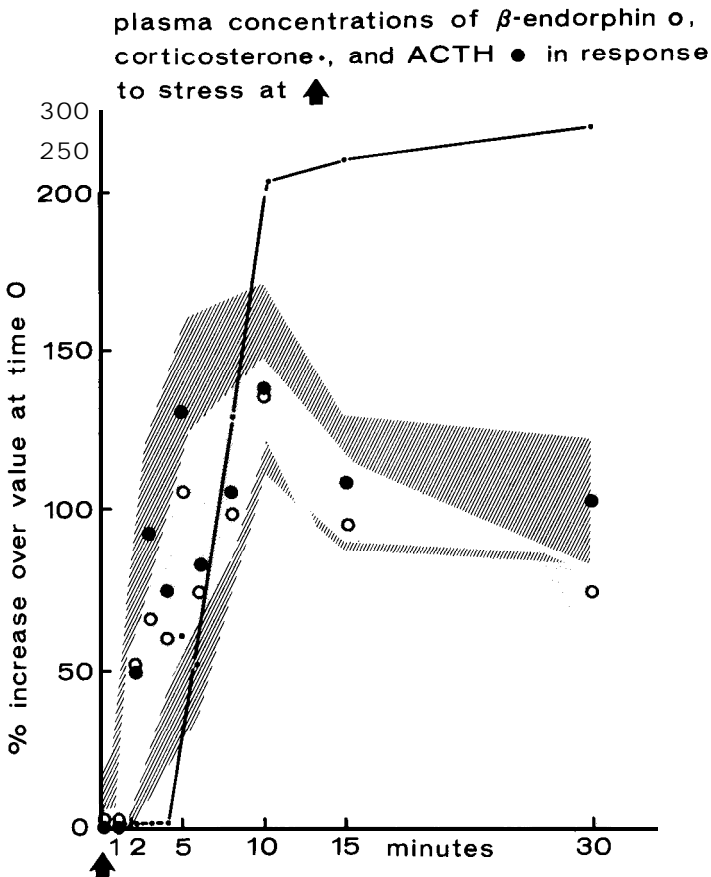


Fig. 10: Concomitant elevation of plasma levels of ACTH and β -endorphin upon acute exposure to stress applied at time 0. (Peptide measured by radioimmunoassays). Solid line shows plasma corticosterone levels measured by fluorometry.

Chrétien et al., 1977) that β -endorphin exists as such and as part of a larger precursor in discrete pituitary cells. We have indeed recently shown (Guillemin et al., 1977b) that β -endorphin and ACTH are secreted simultaneously *in vivo* or *in vitro* in all circumstances tested so far (Fig. 10).

β -LPH [61—63] has no opioid activity at 10^{-4} M; β -LPH[61—64], β -LPH[61—65]-NH₂, (Met (O)⁶⁵)- β -LPH[61—65], β -LPH[61—69], β -LPH[61—76], β -LPH[61—91] all have opioid activity. β -LPH[61—65]-NH₂, β -LPH[61—65]-NEt and all peptides larger than β -LPH[61—65] have longer duration of biological activity than met-enkephalin in the myenteric plexus bioassay. All these peptides were prepared by solid phase synthesis (see Ling and Guillemin, 1976). β -Endorphin is by far the longest-acting peptide when compared at equimolar ratios with all other fragments of the 61—91 COOH-fragment of β -LPH. In quantitative assays using the myenteric plexus, β -endorphin is approximately 5 times more potent than Met⁵-enkephalin; the two analogs of Met⁵-enkephalin amidated on the C-terminal residue have also 2—3 times greater specific activity than the free acid form of the peptide, with 95% fiducial limits of the assays overlapping those of β -endorphin. Several analogs of the enkephalins have recently been reported with greater specific activity than the native molecule; all involve substitution with D-amino acids (Pert et al., 1976; Ling et al., 1978).

A series of analogs of the endorphins was synthesized by N. Ling and further purified to high purity. All these peptides have parallel competition curves when studied at 5—6 dose levels in an opiate-displacement assay from rat brain synaptosomes (Lazarus et al., 1976) with the exception of β -LPH-[62—91] which is definitely divergent from the other curves. Comparing the values obtained in the bioassay (myenteric plexus-longitudinal muscle) and the synaptosomal displacement assay, it is obvious that the two assay systems do not give necessarily identical values.

Of considerable interest are some results observed with the analogs of α , β , γ , δ -endorphins in which a residue of leucine has been substituted for methionine in position 5 from the NH₂-terminus (Ling et al., 1978; Guillemin et al., 1977). [Leu⁵]- β -endorphin and [Leu⁵]- γ -endorphin are considerably more potent than their native congeners in the brain synaptosome assays, though not in the guinea pig ileum assay. It is tempting to speculate that the brain variety of endorphins might contain a residue of leucine in position 5. Proof of such a hypothesis would require isolation and characterization of such molecules. To this date, no [Leu⁶⁵]- β -lipotropin has been recognized and characterized. On the other hand, Hughes and collaborators (Hughes et al., 1975) and later Simantov and Snyder (1976) have isolated from brain extracts not only Met⁵-enkephalin but also Leu⁵-enkephalin. Leu⁵-enkephalin might come from an allele of β -lipotropin of brain origin. It is also possible that Leu⁵-enkephalin of brain origin is a sub-unit of a larger molecule with no relation to β -lipotropin (other than the common tetrapeptide Tyr-Gly-Gly-Phe). Recent studies in collaboration with Bloom (to be published) and Rossier (1977) have indeed shown remarkable dissociation in the distribution of neurons containing either β -endorphin or enkephalins.

2. Release of Pituitary Hormones by Endorphins

One of our original interests in engaging in the isolation and characterization of the endorphins was that the opiate-like peptides might be involved in the secretion of pituitary hormones, particularly growth hormone and prolactin, long known to be acutely released following injection of morphine.

We have shown (Rivier et al., 1977b) that β -endorphin is a potent releaser of immunoreactive growth hormone and prolactin when administered to rats by intracisternal injection. These effects were prevented by prior administration of naloxone. The endorphins are not active directly at the level of the pituitary cells; they show no effect, even in large doses, when added directly to monolayer cultures of (rat) pituitary cells. Thus, the hypophysiotropic effects of the endorphins, like those of the opiate alkaloids, are mediated by some structure in the central nervous system and are not directly at the level of the adenohypophysis. Similar results have been observed by several groups of investigators. We have recently shown that β -endorphin is a potent stimulator of the secretion of vasopressin, possibly acting at a hypothalamic level, since it is not active on the *in vitro* isolated neurohypophysis (Weitzman et al., 1977).

c. Neuronal Actions of Endorphins and Enkephalins Among Brain Regions

The existence of endogenous peptides with opiate-like actions suggests that these substances may function as neuromodulators or neurotransmitters in the CNS. Indeed, recent iontophoretic studies have shown that the enkephalins can modify the excitability of a variety of neurons in the CNS. Most neurons tested were inhibited by these peptides (Frederickson and Norris, 1976; Hill et al., 1976; Zieglgansberger et al., 1976), although Renshaw cells responded with an excitation (Davies and Dray, 1976). Studies have recently appeared exploring systematically the sensitivity of neurons to the endorphins or reporting a systematic regional survey of neurons responsive to the peptides (Nicoll et al., 1977).

A surprising finding in that study was the potent excitatory effects of the peptides and normorphine on hippocampal pyramidal cells (Fig. 11). The regional specificity of this excitatory action could be clearly demonstrated with the same electrode by recording from cells in the overlying cerebral cortex and the underlying thalamus during a single penetration. No tachyphylaxis was observed either to the excitatory or inhibitory action of the peptides in any of the regions examined, even though the peptides were often applied repeatedly to the same cell for periods in excess of one hour.

To determine whether the responses observed with the peptides were related to the activation of opiate receptors the specific opiate antagonist, *naloxone*, was administered both by iontophoresis from an adjacent barrel of the microelectrode and by subcutaneous injections. Administered by either route, naloxone antagonized both the excitations and the inhibitions.

All these effects of opiate-like peptides on neuronal activity, taken with biochemical and histochemical evidence for their existence in brain, are consistent with the hypothesis that these peptides are neurotransmitters in

NEURONAL EFFECTS OF OPIOID PEPTIDES

Region (Cell Type)	MET-Enkephalin		Beta-Endorphin		Normorphine	
	% Exc.	% Inh.	% Exc.	% Inh.	% Exc.	% Inh.
Cerebellum (Purkinje)	18 N=	21 34	23 N=13	23	20 N=	60 5
Cerebral Cortex (unidentified)	1 N=	79 58	25 N=44	49	26 N=27	52
Brain Stem (Lat. Ret. Nuc. +)	3 N=113	47	23 N=35	45	10 N=20	75
Caudate Nuc. (unidentified)	0 N=	83 18	10 N=35	86	9 N=20	73
Thalamus (unidentified)	0 N=	100 15	0 N=	100 5	0 N=	100 4
Hippocampus (Pyramidal)	90 N=	5 19	86 N=14	7	92 N=12	0

Fig. 11: Summary of neuronal effects of opioid peptides and morphine. In each category the total number of cells tested and the percentage of this total that were inhibited or excited is shown (from Nicol et al.).

the CNS. Moreover, I have recently observed in collaboration with Grumbath, Peternelli and Davis that purified synaptosomes of ventral hypothalamus origin (rat brain) release large amounts of immunoreactive β -endorphin when exposed to elevated [KCl] (to be published). When the cells of origin of these peptide-containing fibers have been determined, it may then be possible to proceed with studies into the effects on cellular activity and the secretion of the peptides in order to satisfy more completely the criteria for a neurotransmitter. Crucial points in such future analyses will be the questions of whether the endorphin and enkephalin containing fibers are mutually inclusive systems (recent studies indicate that they are not -- Rossier et al., 1977), whether the length of the peptide released by neuronal activity is subject to modulation, and whether intermediate length peptides (such as the α -, γ -, and δ -endorphins) may participate in such modulatory changes. Although the endorphins and β -LPH may be prohormones for Met⁵-enkephalin, there are at present no such candidates for Leu⁵-enkephalin. The results presented here indicate to us that the cellular roles of endorphin and enkephalin peptides cannot now be generalized across all brain regions where they are found, and that no simple cellular action of any peptide will yield an integrative picture of the way in which opiate alkaloids produce complex analgesic, euphoric, and addictive responses. Involvement of the endorphins in the control of adeno-hypophysial functions is still a subject for further study at the time of writing this review. So far, no direct hypophysiotropic activities of the endorphin peptides have been clearly demonstrated.

d. Behavioral effects of endorphins

The pharmacological properties of endorphins have so far been screened through application of tests *in vitro* or *in vivo* previously used to characterize opiate agonists and antagonists.

When injected into the cerebrospinal fluid, endorphins affect several behavioral and physiological measures, in addition to responses to noxious agents, and each of the peptides exhibits different dose-effect profiles on these measures: β -endorphin induces a marked catatonic state (Fig. 12) lasting for hours (Bloom et al., 1976) at molar doses 1/100 those at which Met⁵-enkephalin transiently inhibits responses to noxious agents (Belluzzi et al., 1976; Buscher et al., 1976; Loh et al., 1976). This potent behavioral effect of a naturally occurring substance suggests its regulation could have etiological significance in mental illness.

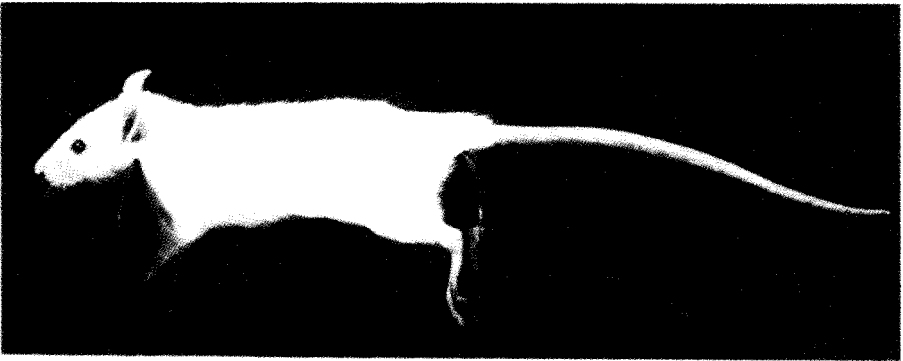


Fig. 12: Thirty minutes after the intracisternal injection of β -endorphin (15×10^9 mole) this rat exhibited sufficient rigid immobility to remain totally self-supporting when placed across metal bookends which are in contact only at the upper neck and base of the tail. Such postures were maintained for prolonged periods. Note the erect ears and tail, widely opened eyelids and extended lower limbs.

In terms of molar dose-effectiveness on the various parameters examined, β -endorphin is clearly the most potent substance tested.

Rats given seven daily intracisternal injections of 15×10^9 mole of β -endorphin continued to show the full set of responses and duration of action. The catatonic state induced by β -endorphin was not observed with the other endorphin peptides, even at considerably higher doses. At very high doses of α -endorphin, γ -endorphin or Met⁵-enkephalin, transient losses of corneal reflexes were observed, and α -endorphin seemed more potent in this regard than either γ -endorphin or Met⁵-enkephalin. No significant depressions of responsiveness to tail-pinch or pin-prick stimuli were observed with Met⁵-enkephalin, α -endorphin, or γ -endorphin, but such effects (Bradbury et al., 1976; Buscher et al., 1976; Ross et al., 1976) could have been missed by the 5-minute interval after injection and before testing began. In contrast to the syndrome induced by β -endorphin, rats given γ -endorphin

showed consistent elevations in rectal temperature (about 2.0°C at 30 minutes after 281×10^9 mole), and sometimes exhibited some degree of hyperresponsivity to sensory testing and handling, although there were individual variations in this response.

All of our observations suggest that normal variations-either qualitative or quantitative-in the homeostatic mechanisms regulating the postulated (Lazarus et al., 1976) conversion of β -LPH as a prohormone to its several endorphin cleavage products could constitute a system fundamentally involved in maintaining "normal" behavior; alterations of the mechanisms normally regulating β -lipotropin-endorphins homeostasis could lead to signs and symptoms of mental illness. Such a potential psychophysiological role of endorphins could logically be testable through the therapeutic administration of available opiate antagonists. This has already been attempted in several clinical centers throughout the world; results obtained have been interpreted differently and, in my own mind, are much too preliminary as yet to warrant any conclusion, positive or negative. The ultimate identification of endorphin-sensitive behavioral events and specific treatment of their dysfunctional states may require the development of more specific "anti-endorphins" than those now available; other naturally occurring brain peptides such as Substance P, have already been reported to be endorphin antagonists in some assay systems (Guillemin et al., 1976c). There is little doubt that the potential significance in any such studies is so great that major efforts in this area both in the laboratory and in clinical studies should be sponsored and pursued without fail.

PART III

Endocrine and Paracrine Secretions of the Brain. Hormones and Cybernins

It has been known for some time that TRF, LRF, and somatostatin originally isolated from extracts of the hypothalamus are actually to be found in non-negligible amounts throughout the central nervous system, including the spinal cord. This was demonstrated first by bioassays and has been amply confirmed by radioimmunoassays. Extrahypothalamic TRF, LRF, or somatostatin have not been isolated and chemically characterized as yet. There is thus no uncontrovertible evidence that the pertinent substances in extrahypothalamic brain-extracts are identical to those characterized in the hypothalamus. All the current circumstantial evidence is in favor, however, of the identity of the materials in their multiple locations (parallelism of dose-response curves in several bioassays, parallelism of displacement curves in several types of radioimmunoassays, etc. using purified tissue extracts and pure synthetic peptides as reference standards). Ubiquity of these peptides throughout the central nervous system does not imply that they are randomly distributed. Several groups have shown that each of these peptides has a unique distribution pattern as they have been identified by immunocytochemistry in axonal tracts and neuronal bodies in well characterized anatomical formations of the central nervous system (Hökfelt et al., 1977). It was this knowledge of the multiplicity of location of the releasing factors and

particularly of somatostatin in the central nervous system that lead a few years ago to the solution of one of the most puzzling dilemmas in this field, and that solution had far-reaching consequences: It was difficult to reconcile the short biological half-life of somatostatin (less than four minutes), when injected in the blood stream with its well established effects on the secretion of glucagon and insulin, with the hypothesis that *hypothalamic* somatostatin could be involved in the physiological control of the secretion of *pancreatic* glucagon and insulin. Luft in Stockholm and I independently wondered whether somatostatin could be delivered to the endocrine pancreas by means other than the general circulation, possibly by nerve fibers, known to innervate the islets of Langerhans. The remarkable observation was then made that in fact the endocrine pancreas of all vertebrates studied so far contain a discrete population of cellular elements containing somatostatin as shown by immunocytochemistry (Luft et al., 1974; Dubois, 1975). The somatostatin-containing cells belong to the D-cells of the endocrine pancreas, long known to the morphologists to be different from the u-cells containing glucagon and the β -cells containing insulin, but for which no specific secretory products had been recognized so far. Moreover, in these early reports a large number of secretory cells containing immunoreactive somatostatin were found throughout the gastrointestinal tract and it has now been shown that somatostatin can inhibit the secretion of gastrin, of secretin, of cholecystokinin, also the secretion of pepsin and HCl by acting directly at the level of the gastric mucosa (Nakaji, et al., 1975). TRF has recently been reported in extracts of the stomach and of the duodenum (Leppaluoto et al. in press). Neurotensin and substance P have also been located in the hypothalamus and throughout the gastrointestinal tract in specific cells and in crude extracts - as has been known in the case of substance P, since 1931 from the work of Gaddum and von Euler. There is now evidence that other peptides originally characterized from extracts of tissues of the gastrointestinal tract can be found and located in the brain; this is the case for gastrin/cholecystokinin, vaso-intestinal peptide (VIP), the gastric inhibitory peptide (GIP) (see review by Pearse and Takor, 1976); this is also true for the endorphins and enkephalins and for several of the small peptides such as bombesin, caerulein, physalmine, isolated years ago from extracts of the skin of several species of frogs. There are remarkable analogies and homologies between the amino acid sequences of several of these peptides of central nervous system origin and gastrointestinal origin, as well as those isolated from frog skin.

These peptides have been found by immunocytochemistry essentially in two types of cells: 1) They are seen in cell bodies and nerve fibers i.e. axonal and dendritic processes of neurons in brain, spinal cord, in spinal ganglia and in the myenteric plexus; 2) They are seen also in typical *endocrine cells*, for instance in the pancreatic islets of Langerhans, in the enterochromaffin cells of the gut and the adrenal medulla. Neuroblastomas have been reported to contain high levels of the vaso-intestinal peptide (VIP) (Said and Rosenberg, 1976).

There is already an interesting unifying concept to bring together these

rather startling observations. Much credit must go to A.G.E. Pearse for his visionary concept, formulated some ten years ago, of the APUD cells: Pearse observed that neurons and some endocrine cells producing polypeptide hormones shared a set of common cytochemical features and ultrastructural characteristics. APUD is an acronym referring to Amine content and/or Amine Precursor Uptake and Decarboxylation, as common qualities of these cells (Pearsé, 1968). The APUD concept postulated that these endocrine cells were derived from a common neuroectodermal ancestor, the transient neural crest. Pearse postulated further that a still larger number of endocrine cells would be eventually found sharing these common properties if one were to explore further in the adult, endocrine tissues derived from the neural crest. Recent observations with refined techniques, particularly the work of Le Douarin on topical chimeras with chromosomal markers, have led Pearse to modify the original APUD concept, and, as we will see, in a remarkable manner. The new evidence regarding the multiple sources of the several peptides mentioned above showed that tissues were involved that were not of neural crest origin; this is particularly true for the peptide-secreting cells of the gut. All these cells have been shown to arise from specialized neuroectoderm (Pearsé and Takor, 1976); that is, not only the neural crest but also the neural tube, the neural ridges and the placodes.

The expanded concept now postulates that all peptide hormone-producing cells are derived from the neural ectoderm, as are all neurons. With such ontogenic commonality, it is thus less surprising to recognize the presence of "gastro-intestinal peptides" in the brain, and of "brain peptides" in the gastro-intestinal tract.

ACTH, β -endorphin and growth hormone of pituitary origin, cholecystokinin, secretin, or gastrin of gastrointestinal origin are well recognized hormones which satisfy all the definitions of the word, particularly as it implies their distant action on target cells or organs far removed from the source of these peptides. In the case of the release of TRF, LRF, or somatostatin by hypothalamic neurons at the level of the hypothalamo-hypophysial portal vessels, these hypophysiotropic activities can also be considered as hormonal in nature. Let us note immediately that, while the means of conveyance are indeed blood vessels, the distance travelled by the hypophysiotropic peptides in these vessels is measured by a few mm. until they reach their target pituitary cells. This is very different from the long distance travelled by the classical hormones mentioned above. Moreover, even in the case of the hypothalamic peptides as involved in their hypophysiotropic functions, there is no generally accepted evidence that they enter the general circulation for any length of time and in a physiologically meaningful concentration.

When we consider these same peptides in parts of the brain other than the hypophysiotropic hypothalamus, the situation is altogether different: Both the optic and the electron microscope, combined with immunocytochemistry begin to show evidence of very punctual localizations which imply similarly punctual roles; i.e. to be played over distances measured in angstroms. Berta Scharrer has for some time described what she calls peptidergic synapses

(Scharrer, 1975). Moreover, recent studies with antibodies to somatostatin have yielded pictures which have been interpreted by Petrusz et al. (1977) as showing the localization of immunoreactive somatostatin in multiple dendritic endings. Some of these pictures are spectacular (Fig. 13). Their most heuristic interpretation is that each dark point is that of a dendritic contact either with another dendrite or abutting on a specialized locus of the axon or of the soma of recipient neurons. Clearly these recipient neurons do not seem to contain immunoreactive somatostatin. The cells of origin containing and sending the presumptive somatostatinergic terminals have not been characterized as yet. Similar pictures have already been observed in multiple locations in the brain and for several immunoreactive peptides. In this context these peptides which we called hormones earlier do not fit the definition of a hormone any more; they seem to be candidates for the definition of neurotransmitters. Recently Hökfelt (1977) has concluded that some neurons may contain both peptides and one of the catecholamines, a classical neurotransmitter (1977).

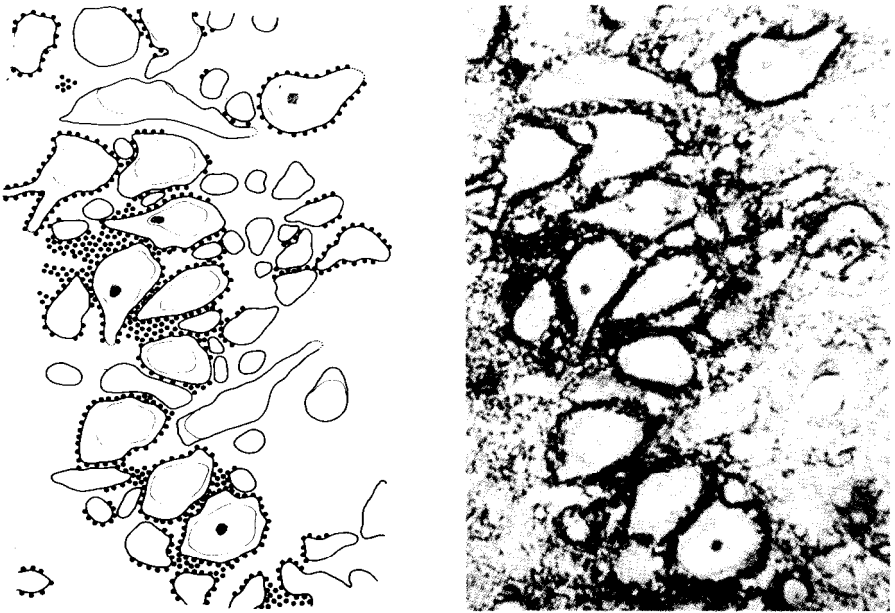


Fig. 13: Presumptive nerve terminals as somato-dendritic terminals around pyramidal cells of the hippocampus rat brain). Immunocytochemistry with antisera to somatostatin (from Petrusz et al.).

It will be obvious by now that we are only at the very beginning of the physiological significance of these peptides in the brain. The local punctual release that we have seen here with its multiple locations and short range of traffic would make them fit what Feyrter had called earlier the paracrine secretory system. Interestingly enough, Feyrter had evolved his concept of paracrine secretion while studying with very simple morphological tools and

a great deal of observational acumen the very cells of the gastrointestinal tract and of the pancreas that we know now to secrete the very peptides discussed in this lecture. I have proposed earlier the generic name *cybernin* for these substances; the etymology of the word implying local information. Obviously again, years of investigation are ahead to understand the mechanisms involved in the cell biology and the cell physiology of the neurons as they synthesize, release, respond to and metabolize the many peptides discussed here.

Of the many discoveries ahead of us will be those that will lead us to understand the role of each of these peptides in the brain, not only in their cellular physiology and biochemistry, but also in their significance in the higher functions of the central nervous systems. Though admittedly based on simple and enthusiastic teleology, it is difficult not to hypothesize that these peptides must indeed play some role in the functions of the brain. Once this simple proposal is made, if we recognize that not one word about the existence of these substances in the central nervous system is to be found in any of the classical texts of neuro-psychiatry, one can not but be optimistic that the early observations summarized in this lecture will lead to profound reappraisals of the mechanisms involved in the functions of the normal brain, but also of mental illness.

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