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A STUDY OF THE EFFECT OF AGE AND HORMONES ON BRAIN AND PITUITARY ENDORPHINS

by

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ABSTRACT

Optimal methods were established to obtain reproducible estimates of endorphin concentrations in rat pituitary and brain extracts. Subsequently the influence of age, sex, endocrine changes and pharmacological treatments on regional brain endorphin concentrations was determined. The highest concentrations of β-endorphin were found in the pituitary, followed by the hypothalamus, hindbrain and midbrain. The principal immunoreactive species in brain extracts was β-endorphin. After hypophysectomy, a major reduction of β-endorphin concentration in the brain was observed. After adrenalectomy, the concentration of β-endorphin was increased significantly in the pituitary gland (359 µg/g wet wt. vs 540 µg/g), hindbrain (0.7 µg/g vs 1.5 µg/g), hypothalamus (3.9 µg/g vs 8.2 µg/g), and midbrain (0.53 µg/g vs 0.86 µg/g). After thyroidectomy, the concentration of β-endorphin was significantly increased in the hypothalamus (5 µg/g vs 11 µg/g). In rats treated with thyroxine (T_4), significant increases in β-endorphin were found in the midbrain (0.35 µg/g vs 0.60 µg/g), hypothalamus (4.8 µg/g vs 7.6 µg/g), and pituitary (187 µg/g vs 290 µg/g). In orchiectomized rats, a significant decrease of β-endorphin was found in rat pituitary (359 µg/g vs 189 µg/g). No differences in

β-endorphin in rat brain regions occurred after ovariectomy. Naloxone administration to rats increased β-endorphin in hypothalamus (4.7 μg/g vs 10.5 μg/g), while pentobarbital injection caused an increase in the pituitary (390 μg/g vs 607 μg/g). No differences in β-endorphin in rat brain were found between day 8 and day 24 old rats, but very significant increases were found between day 24 and day 60 old rats in most brain regions. The midbrain was the only brain region in which a significant sex difference in β-endorphin was found.

TABLE OF CONTENTS

NOMENCLATURE

ACTH	adrenocorticotropic hormone
BSA	bovine serum albumin
CRF	corticotropin releasing factor
CSF	cerebrospinal fluid
GH	growth hormone
LH	luteinizing hormone
LPH	lipotropic hormone
MLF	morphine-like factor
MSH	melanotropin stimulating hormone
RIA	radioimmunoassay
RRA	radioreceptorassay
PRL	prolactin
T_4	thyroxine
TRH	thyrotropin releasing hormone
TSH	thyrotropin stimulating hormone
V_o	void volume

LIST OF FIGURES

LIST OF TABLES

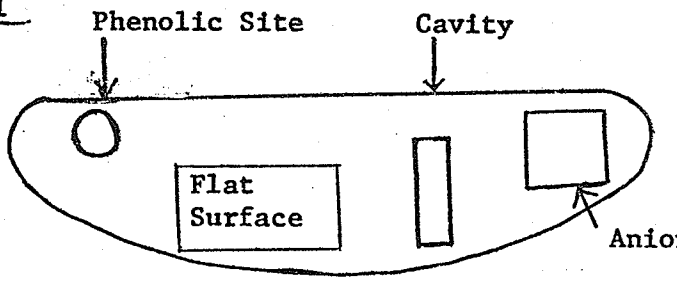
INTRODUCTION AND LITERATURE REVIEW

Opium, a word which means "poppy juice" has been used as a drug at least since the classical Greek period. It is an agent which kills pain and gives rise to euphoria. The drug is present in the milky exudate obtained by incising the unripe seedpod of the poppy "papaver somniferum". Derosine in 1803 and Serturner in 1805 both isolated crystalline material from crude opium, which Serturner called morphine and demonstrated that this material has opium like effects in dogs. The toxicity and addictiveness of morphine were recognized after the clinical utility of opium was established as a pain killer, anti-diarrheal agent and hypnotic. The search for nonaddictive synthetic opiates led to the discovery of numerous opiate agonists and antagonists. A landmark in this field occurred with the recent discovery of opiate receptors in the central nervous system of animals and man which shortly thereafter culminated in the identification and isolation of endogenous opiate-like ligands.

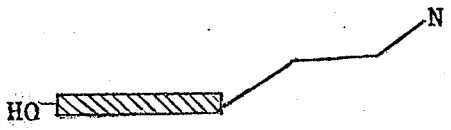
EVIDENCE FOR OPIATE RECEPTORS

Over the past 25 years (Beckett et al. 1954; Portoghese 1965; Jacobson 1972), much evidence supported the view that opiates act via specific receptors. Firstly, there was a general structural similarity among opiate agonists as shown in Fig. 1. The minimal structural requirements for opiate activity are the presence of an aromatic ring structure and a nitrogen atom, usually as a tertiary amine, that is located at a distance of 2 saturated carbon atoms from the aromatic ring (Eddy, et al. 1973). Secondly, only the naturally occurring levorotatory isomer is active, while the dextrorotatory isomer has little or no analgesic or addiction-producing activity. Thirdly, morphine cogeners such as etorphine have been synthesized that are 500-1000 times as potent as morphine, and differ in structure in only minor ways. Fourthly, Pohl (1975) found that morphine derivatives with small changes in structure may act as antagonists to opiates, for example : substitution of the N-methyl group by a large alkyl group such as allyl or cyclopropylmethyl, converts a potent analgesic to a drug that antagonizes morphine and related narcotic analgesics. Because of the steric and structural specificity of the opiates, pharmacologists assumed that specific opiate receptors must exist in brain and possibly in other tissues.

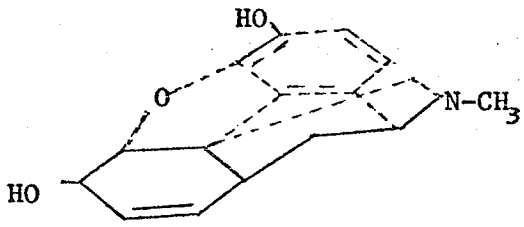
Fig. 1



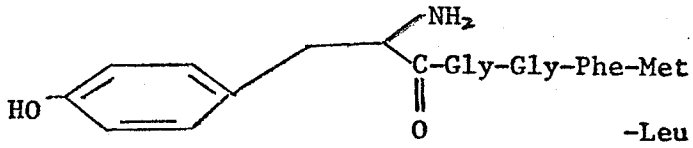
Postulated Opiate Receptor



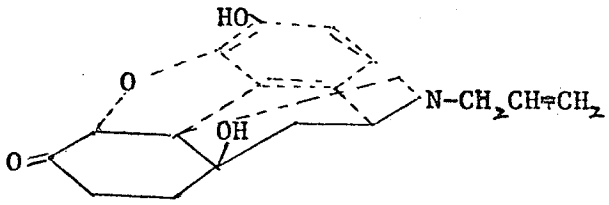
General Structural Similarity



Morphine



-Leu Enkephalins



Naloxone

Reference : Beckett et al. 1954; Hughes et al. 1975a, 1975b, 1975c.

DEVELOPMENT OF OPIATE RECEPTOR ASSAY

The search for putative opiate receptors was complicated by the difficulty of distinguishing nonspecific binding to various tissue components from specific binding to receptors and by the unavailability of isotopically labeled opiates with sufficiently high specific activity. Several early attempts (Simon et al. 1966; Ingoglia et al. 1970) were unsuccessful. In 1971, Goldstein et al. first established a set of criteria for stereospecificity of binding of opiates to their receptors. Mouse brain homogenates were incubated with ^{3}H-levorphanol in the presence of a large excess of unlabeled levorphanol and its inactive enantiomer dextrorphan. Stereospecific binding was defined as that portion of the binding of the labeled drug which is prevented by levorphanol but not by dextrorphan. Because he used a preparation of ^{3}H-levorphanol of very low specific activity (0.8 μCi/nM), he found only about 2% of the total binding to be stereospecific. Also he reported this binding to be localized in the nuclear portion of subcellular fractions and fairly evenly distributed throughout the brain. This result was discouraging, since it implied that it would be very difficult to distinguish the small amount of specific binding to the postulated receptor from the very large amount

of nonspecific binding. Other laboratories could not reproduce Goldstein's result. However, three laboratories (Pert & Snyder 1973a,1973b; Terenius 1973a,1973b; Simon et al. 1973a) using modifications of Goldstein's procedure, independently and almost simultaneously reported stereospecific opiate binding in rat brain homogenates. The modification involved the use of a very low concentration of labeled ligand of high specific activity ^{3}H-Naloxone (6.1 μCi/nM, Snyder), ^{3}H-Etorphine (Simon), and the washing of homogenate after incubation with cold buffer to remove contaminating unbound and loosely bound radioactivity. Their results differed in several respects from Goldstein's. They found that the binding was mainly in the synaptosomal fraction of brain homogenates and that there were marked regional variations throughout the brain. However stereospecific binding does not ensure that one is dealing with the opiate receptor, since various brain lipids e.g. cerebrosides (Loh et al. 1974; Abood et al. 1975) and even certain filters (Snyder et al. 1975) display "stereospecific binding" of opiates. When identifying receptors, it is desirable to examine (Cuatrecasas, 1976):(1) the binding of analogs and antagonists; (2) the capacity and affinity of binding sites; (3) the reversibility of binding; (4) the tissue distribution of specific binding sites; and (5) the simultaneous correlation of the binding data with

the biological dose-response curves in identical tissue preparations. The pharmacological relevance of the opiate receptor has been demonstrated by comparing binding and pharmacological activities in the same tissue - the guinea-pig ileum. (Creese & Snyder, 1975a; Kosterlitz & Waterfield 1975a). Their studies showed that the ability of the opiates to inhibit electrically induced contractions of the guinea-pig ileum parallels their analgesic potencies. Affinities of numerous opiate agonists and antagonists for binding sites in the guinea-pig ileum correlates remarkably well with their effects on electrically induced contractions in the same tissue, suggesting that this stereospecific binding sites was indeed a real receptor. Stahl et al.(1977) found very good correlations between the binding affinities of the drugs to sites in the brain and the ability of these opiates to inhibit contractions of the intestine, providing evidence for the similarity of opiate binding sites in the brain and the myenteric plexus of the intestine.

DISTRIBUTION OF OPIATE RECEPTORS

The stereospecific binding sites are found in the central

nervous system and in the myenteric plexus of the guinea-pig ileum. Apart from Abood's report (Abood et al. 1976) of stereospecific opiate binding in human erythrocyte membrane, opiate receptors have not been observed in non-nervous tissue. Receptor binding was detected in the brain of all vertebrates (Pert et al. 1974), but not in invertebrates. In the most primitive vertebrates such as the hagfish and dogfish shark brain, opiate receptors were as numerous as in monkey and human brain. Different species of mammals differ in their behavioral response to opiates. In many species, including man, monkey and dog, a depressive response is observed after opiate administration, while in others such as the cat, cow, sheep, horse and pig, excitatory features dominate (Jaffe, 1970). A survey of stereospecific binding of ^{3}H-Etorphine in selected area of the brains of several species - cow, cat, sheep, dog, monkey, and man (Kuhar et al. 1973; Hiller et al. 1973) revealed that except for the amygdala and frontal cortex, there was reasonably good reproducibility of binding sites in comparable anatomical regions in all six species. For species that exhibit depression after opiates at least a two fold greater ratio of opiate receptor levels in the amygdala and frontal cortex to that in the caudate nucleus was observed than for species that show an excitatory response to opiates. Opiate receptors are found throughout the brain but are concentrated in areas associated with the limbic system and the periaque-

ductal gray area is one of the few regions where microinjection of morphine elicits analgesia and where direct electrical stimulation causes analgesia that is blocked by naloxone.

Autoradiographic techniques (Jacquet et al. 1974; Pert et al. 1976a) have permitted discrete microscopic localization of receptors. Within the spinal cord, opiate receptors are localized in the substantia gelatinosa, which is the first region in the central nervous system for the intergration of sensory information. Within the brain stem, opiate receptors are particularly prominent in the solitary nuclei, which receive visceral fibers from the vagus and glossopharyngeal cranial nerves. Opiate receptors in the solitary nuclei may explain how opiates depress the cough reflex, elicit orthostatic hypotension and reduce gastric juice secretion. Also within the brain stem opiate receptors are concentrated in the area postrema, which contains the chemoreceptor trigger zone, the site at which opiates apparently induce nausea and vomiting. The greatest abundance of opiate receptors in the brain occurs in the amygdala. It is possible that receptors here are associated with influences of opiates on emotional behavior.
Duller, more chronic and less localized pain is quite effectively relieved by opiates and appears to be conveyed by a pathway that evolved earlier, called the paleospinothalamic system. This pathway ascends along the midline of the brain, its way

stations include the central gray matter of the brain stem and the central part of the thalamus. The map of the distribution of the opiate receptors strikingly parallels the paleospinothalamic pain pathway. In the subcellular studies, opiate receptors are highly associated with membrane fractions of tissue homogenates and have been reported to be most concentrated in the synaptosomal cell fraction of brain and guinea-pig ileum homogenates (Hitzeman et al. 1974; Pert et al. 1974b; Terenius et al. 1973c), suggesting a location in the vicinity of synapses. Whether the receptors are situated pre-or post-synaptically has not yet been established. Lamotte et al. (1976) found that after dorsal root section in the monkey, opiate receptor binding declined by 50% in the dorsal horn of the monkey spinal cord. Zieglgansberger et al.(1976a) found that opiates diminish spontaneous firing and glutamate induced firing of cells in the cerebral cortex and corpus striatum through a postsynaptic actions. A cell line in culture derived as somatic hybrids of a neuroblastoma clonal cell line and glioma cell line, NG108-15, is rich in opiate receptors (Klee et al. 1976). Both parent lines contain few if any opiate receptor binding sites.

FACTORS AFFECTING OPIATE RECEPTOR BINDING (Pert & Snyder, 1973b,

1974c; Soloman et al. 1973a, 1973b; Simon et al. 1973b, 1975a; Pasternak et al. 1973)

(1) Temperature: Stereospecific binding is temperature dependent with maximal binding at 35°C. At 4°C binding was reduced to 25% of values at 35°C. Heating for 10 minutes at temperatures higher than 50°C decreases specific naloxone binding by 90% or more.

(2) pH: Binding of both agonists and antagonists has a broad pH optimum between 6.5 and 8.

(3) Ions: Low physiologic concentrations of manganese and magnesium selectively increase the binding of opiate agonists by reducing receptor sensitivity to sodium. Calcium fails to enhance opiate agonist binding. Treating the brain membrane with EDTA decreases the binding of opiate agonist, while EGTA, which chelates calcium but not manganese and magnesium, has no influence on receptor binding. The most important feature is the role of sodium on binding of opiates. Inhibition of agonist binding by Na^+ was observed by Simon et al.(1973b) whereas binding of antagonists was not affected according to Pert and Snyder (1973b). Subsequent studies (Pert and Snyder, 1974c; Simon et al. 1975a) showed that Na^+ (and to some extent Li^+) specifically increases the binding affinity of antagonists and decreases that of opiate agonists. The enhan-

cement by sodium was remarkable requiring only 1 mM sodium. Anions e.g. F^-, Cl^-, Br^-, I^-, $SO_4^=$, SCN^- also favor binding of antagonists.

(4) Protein-modifying agents: The opiate receptor is highly sensitive to inactivation by various sulfhydryl regents such as N-ethylmaleimide (NEM), iodoacetamide, p-hydroxymercuribenzoate, Ellamn's regent etc.

(5) Enzymes and Detergents: Opiate receptor is extremely sensitive to digestion by proteolytic enzymes e.g. trypsin, chymotrypsin and detergents such as Triton X-100, sodium dodecylsulfate and deoxycholate. Low concentration of proteolytic enzymes selectively reduce opiate agonist binding with negligible effects on antagonist binding. Phospholipase A from Russell's Viper or bee venom block the binding while the enzyme obtained from rattle snake venom is ineffective. Phospholipase C is slightly inhibitory, while RNAase, DNAase and neuraminidase are without effect.

(6) Others: Implantation of morphine pellets or administration of opiate agonists or antagonists in vivo increases receptor binding of both agonists and antagonists to brain homogenates.

CONFORMATION OF THE OPIATE RECEPTOR

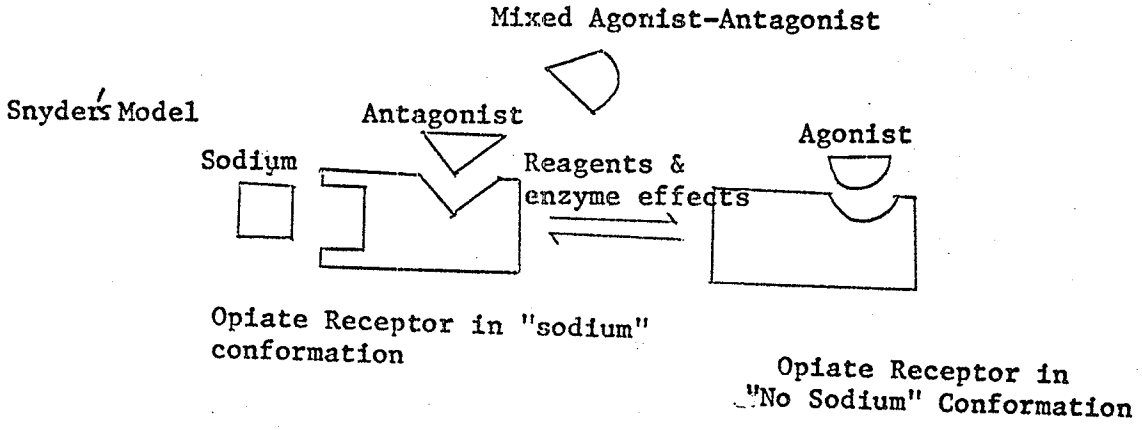
Due to the selective action of Na^+ in enhancing the opiate antagonist binding, two laboratories (Pert & Snyder, 1974c, 1975; Simon 1974, 1975c) have independently proposed similar allosteric models of the receptor. They hypothesize that the opiate receptor can exist in two states as shown in Fig. 2.

The essence of the models is that the sodium ion acts as an allosteric effector, the binding of which to an allosteric site on the receptor molecule results in a conformational change in the opiate binding site. The new conformer (Na^+- dependent) exhibits a higher affinity for antagonists and a lower affinity for agonists than the conformer that exists in Na^+- free media. Simon also found that conformational change in the receptor was produced by sodium at 37°C but that the change was amplified at 0°C. Similar results were obtained by Creese et al. (1975). The physiological significance of this conformational change in the receptor in the presence of sodium is still not clear.

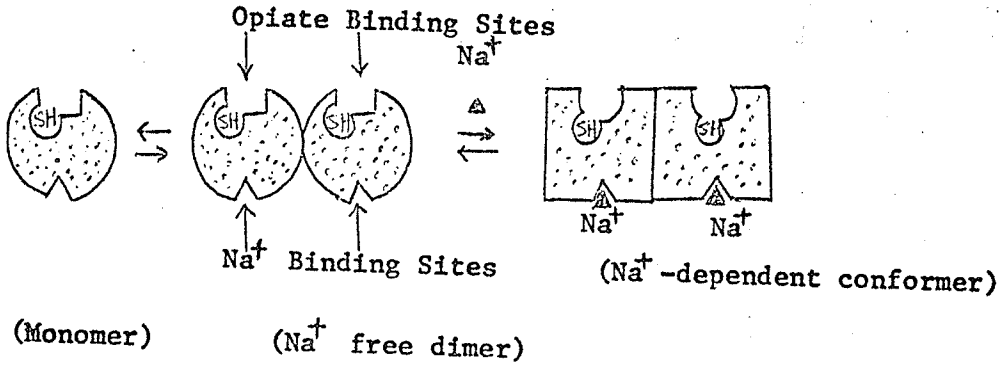
FURTHER CHARACTERIZATION OF OPIATE RECEPTOR

The solubilization and purification of the opiate receptor has proved to be difficult. The reason is that

Fig. 2



Simon's Model



Reference: Pert & Snyder 1974c, 1975; Simon 1974, 1975c.

these receptors appear to be unusually sensitive to detergents, even of the non-ionic variety. Also the concentrations of opiate receptors in even the richest brain regions is very low. Only two reports of successful solubilization of opiate-receptor complex have appeared. The detergent Brij36T was used, the receptor was preincubated with etorphine (Simon et al. 1975b) or enkephalin (Zukin, 1978). According to both studies, the molecular weight of the solubilized complex was about 400,000. Since opiate receptors are extremely sensitive to proteolytic enzymes, heat, sulfhydryl reagents, and phospholipase, it has been suggested that the receptor is composed of proteins and phospholipids.

MULTIPLE RECEPTOR THEORY

In 1965, Portoghese discussed the possibility that opiates might have more than one binding mode. In 1967, Martin introduced the concept of receptor dualism. The receptor dualism hypothesis proposed that nalorphine interacts as an antagonist with morphine receptors, but as an agonist with a receptor which is different from the morphine receptor. The basis of this hypothesis was the observation by Houde and Wallenstein(1956), that, in man, the interaction between morphine and nalorphine

is biphasic, low dose of nalorphine antagonize the analgesic action of morphine, whereas high doses increase analgesia. Martin and his colleagues examined this concept by studying the effects of various opioid drugs on the behavior and reflex activity of the chronic spinal dog. They distinguished between three types of receptors: morphine is the prototype agonist for the μ receptor, ketocyclazocine is the prototype agonist for the κ receptor, and N-allylnorcyclazocine is the prototype agonist for the ∂ receptor. Jacquet's(1977) observation of different actions of (+)morphine and (-)morphine also suggests that there are at least two classes of receptors, one stereospecific which is blocked by naloxone and the other only weakly stereospecific and not blocked by naloxone. Kosterlitz (Kosterlitz et al. 1968, 1975; Lord et al. 1977) concluded from the differences in binding observed with ^{3}H-Naloxone and ^{3}H-leucine-enkephalin in brain homogenates that opioid peptides act on populations of receptor that are different in the mouse vas deferens from those in the guinea-pig ileum. In the latter tissue, the peptides seem to interact mainly with μ-receptor, whereas in the mouse vas deferens they probably act on ∂-receptors(lord et al. 1976). These observations have been recently confirmed(Cuatrecasas, 1979) for the binding of leu-enkephalin and β-endorphin to brain membranes.

MEASUREMENT OF OPIOID ACTIVITY

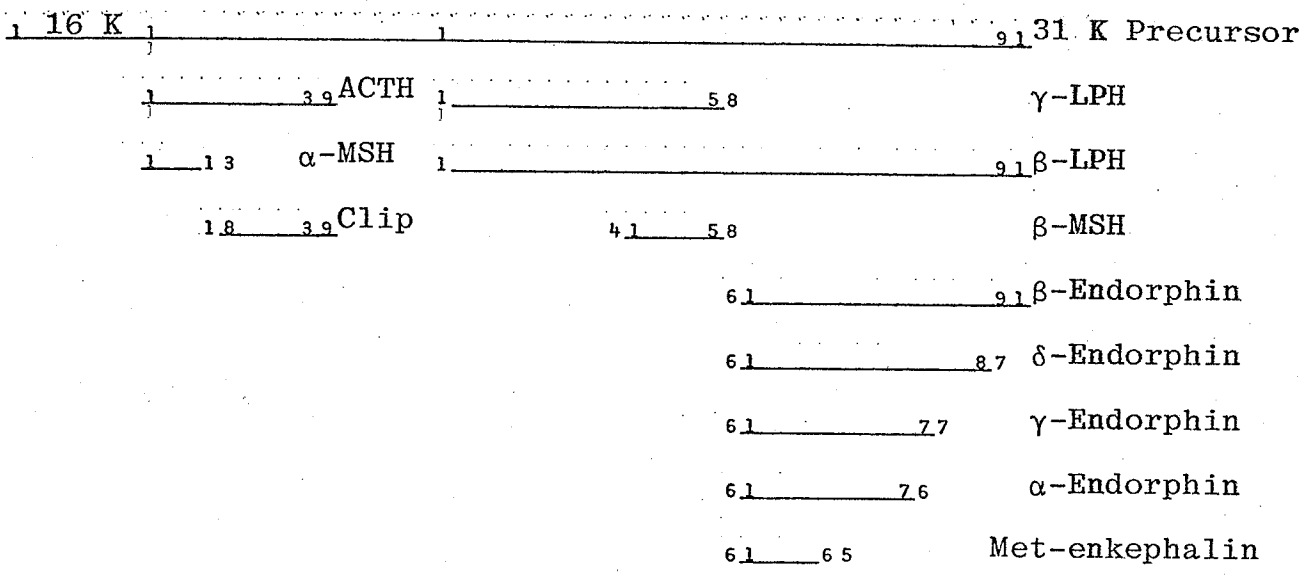
Opioid activity can be assayed in several ways. In vitro, the potency of opiate agonists is measured by their effectiveness in decreasing the amplitude of the contraction of the electrically paced guinea-pig ileum (Kosterlitz et al. 1970) or mouse vas deferens (Pert et al. 1976c). This effect is naloxone reversible. The inhibition of adenylate cyclase and modification of cAMP content of a neuroblastoma glioma hybrid has also been used as an index of opioid activity (Sharma et al.1975; Henderson et al. 1972). In vivo, most tests are based on the analgesic effects of opiates. The two most common tests are the "Hot-Plate Test" and the "Tail-Flick Test". In the first, a rat or mouse pretreated with the compound under investigation is placed on a plate heated to 55-70 ^{0}C, the latency of the period before the animal begins to lick its front paws and the latency before the animal jump is recorded.

In the second test, the tail is heated with a hot beam and again the time period until the animal moves the tail out of the beam is recorded (Woolfe et al. 1944; D'Amour et al. 1941). The increase in the latency is proportional to the analgesic effect of the agent.

DISCOVERY OF ENDOGENOUS OPIATES

In 1972, at the International Congress of Pharmacology in San Francisco, Collier first reported on the likelihood that endogenous ligands for opiate receptors exist. Liebeskind and his collaborators (Liebeskind et al. 1974; Mayer et al. 1974) had accumulated evidence for the presence of a central pain suppressive system. They were able to produce analgesia by electrical stimulation of the mesencephalic central gray and periventricular gray matter. This analgesia was found to be reversed by naloxone and showed cross tolerance with morphine induced analgesia (Mayer et al. 1975). These results suggested the existence of an endogenous pain suppressive system that uses as modulator a substance with morphine-like properties. In 1975, Hughes et al. (1975a,1975b,1975c,1975d) first isolated and identified two pentapeptides, methionine enkephalin (H-Tyr-Gly-Gly-Phe-Met-OH) and leucine enkephalin (H-Tyr-Gly-Gly-Phe-Leu-OH) in pig brain as opiate peptides. Simantov and Snyder (1976a) confirmed the structures of the same two peptides in bovine brain. They found the sequence of met-enkephalin to be identical with sequence 61-65 of ovine β-lipotropin (Li et al. 1965). This sequence also is present in β-lipotropin of porcine (Graf et al. 1971), bovine (Panker et al. 1972), camel (Li et al. 1976a), human (Li et al. 1976b) and rat (Rubinstein, 1977a) origin. The two pentapeptides are present in the CNS and interact with opiate receptors. Pig brain contains about

four times more met-enkephalin than leu-enkephalin, whereas the ratio is reversed in bovine brain. C.H.Li et al.(1976a) isolated another endogenous peptide from camel pituitary gland. He called it endorphin or C fragment of β-lipotropin with a sequence identical to residues 61-91 in ovine β-lipotropin. Subsequently β-endorphin was also purified from human (Li et al. 1976c), sheep (Chretien et al. 1976a), porcine (Bradbury et al. 1975), bovine (Goldstein et al. 1975), rat (Rubinstein et al. 1977b; Seidah et al. 1978) pituitary glands. The amino acid sequence of rat, ovine, bovine and camel β-endorphin are identical. Human β-endorphin sequence is different from that of ovine in position 87 with Tyr instead of His and in position 91 with Glu instead of Gln in ovine β-endorphin sequence. Porcine β-endorphin sequence differs from ovine in position 83 where Val was substituted for Ile. Other endogenous peptides such as α-endorphin (61-76), γ-endorphin (61-77), δ-endorphin (61-87) were obtained after acid extraction of tissue of hypothalamic and pituitary origin (Guillemin et al. 1976a). β-Lipotropin was subsequently shown to be part of a larger peptide called 31K precursor (Pro-opiocortin) which was also found in the rat pituitary gland (Mains et al. 1977; Roberts et al. 1977; Rubinstein et al. 1978; Yoshimi et al. 1978). A 37K precursor was found in the human pituitary extracts (Yoshimi et al. 1978). These relationships can be summarized as follows:



A comparison of the activities of the different endorphins is presented in Table 1.

ONTOGENY OF ENDOGENOUS OPIATES AND OPIATE RECEPTORS

There are three reports that have appeared on the ontogeny of opiate receptors (Clendeninn et al. 1976; Coyle et al. 1976; Francoise et al. 1976). There is only one preliminary report on the ontogeny of enkephalins and endorphins in which measurement of these substances was made by radioimmunoassay(Bayon,1978). The absolute amounts of both endorphin and enkephalin increase with age in all the regions studied (see table 2). When the concentrations were expressed on a protein basis interesting differences were revealed. Between day 20 of the fetus (ED 20) and postnatal

Table 1 : Comparison of Opioid Activities of Different Endorphins in Several Assays

Assay	Met-enkephalin (61-65)	α-Endorphin (61-76)	γ-Endorphin (61-77)	δ-Endorphin (61-87)	β-Endorphin (61-91)	Reference
(1)Analgesia (central admin.)						
Cat	0.01				100	a
Rat	0	0			50	b
Rat	0	0	0		4-10	c
Mouse&Rat	0.02	0.2			10-33	d
Rat	0.05				50	e
Rat	0.01-0.05					f
(2)Guinea-pig ileum						
	0.7				0.9	e
	1 (assume=1)		0.23		4.5	g
	1				1.4	h
	0.28				0.42	i
(3)Mouse vas deferens						
	38				7	g
(4)Neuroblastoma adenylate cyclase						
	100	10	10		10	j
(5)Receptor binding						
^{3}H-Naloxone						
	0.5	0.3			0.5	k
	0.3			0.2	9	l
^{3}H-Leu-enkephalin						
	340				470	g

Relative activity = morphine = normorphine = 1

Reference : (a):Smith et al. 1976. (b):Jacquet et al. 1976. (c):Bloom et al.1976. (d):Loh et al. 1976. (e):Gráf et al. 1976. (f):Belluzzi et al. 1976. (g):Lord et al. 1976.(h):Ling et al. 1976. (i):Cox et al. 1976. (j):Klee et al. 1976.(k):Bradbury 1975. (l):Pert et al.1976d.

Table 2 : Total Brain Content of Enkephalin and Endorphin by RIA

Age	Enkephalin Units*	Endorphin Units+	Brain Protein(mg)
ED-20	1.3±0.2	6.4±0.8	8.1±0.5
PN 6	7.4±0.5	11.2±1.3	48.5±1.8
PN 20	40.7±2.5	76.7±2.4	263.1±16.5

* : Enkephalin immunoreactivity is expressed as the ng of Leu-enkephalin that would give an equivalent trace displacement in the enkephalin RIA.

+ : Endorphin immunoreactivity is express as the ng of β-endorphin that would give an equivalent trace displacement in the endorphin RIA.

(ED : Embryonic Day PN : Postnatal Day)

Reference : Bayon et al. 1978.

day 6 (PN 6), the concentration of β-endorphin decreased in all regions studied. The greatest decrease (about 50%) occurs in the corpus striatum, and continues to decrease to a very low level in the adult rat. However, the concentration of enkephalin did not change significantly from ED 20 to PN 6 and remained almost constant after birth except for a marked increase (about 3 fold) in the preoptic area and septum. The corpus striatum shows the highest β-endorphin concentration in the brain before birth, whereas the hypothalamus is the richest in the adult. However the corpus striatum contains the highest concentration of enkephalin in both the embryonic and adult rat. In contrast to the brain, the pituitary concentrations of both β-endorphin and enkephalin remain constant from ED 20 to PN 6. Both peptides subsequently increase several fold by adulthood. Silman et al. (1978) found that the small molecular weight peptides α-MSH, CLIP, β-MSH and β-endorphin are characteristic of the fetal pituitary of the Rhesus monkey, whereas in the adult pituitary larger peptides ACTH, γ-LPH and β-LPH are the predominant forms. The striking increase of β-endorphin in the pituitary of the newborn monkey may serve to protect the body against the necessary but otherwise painful event of parturition.

The stereospecific binding of opiates was first detected in the total rat brain at 15 days of gestation. The rate of increase of opiate binding is greatest between the midfetal stage

and three weeks postpartum (three to four fold). (see Fig.3). Scatchard analysis has shown that the increase in binding is due to an increase in number of receptors rather than to enhanced affinity. However, studies of opiate binding to guinea-pig brain homogenates has demonstrated no significant difference in either receptor number or binding affinity between late fetal life and adulthood in this species. Binding in brain homogenates from a midterm fetus is about one half that observed late in pregnancy and in the adult guinea-pig. The fact that guinea-pigs are born with a full complement of receptors is perhaps expected considering the fact that the guinea-pig is born with almost full brain development whereas the rat is an animal in which a significant portion of brain development continues for at least three weeks after birth (Eayrs et al. 1959; Jones et al. 1974). In rat brain, the percentage increase of opiate receptors, from newborn to adult in various regions of the brain differs widely (see table 3).

REGIONAL DISTRIBUTION AND CHARACTERIZATION OF ENDOGENOUS OPIATES

As soon as radioimmunoassays for the enkephalins and the endorphins became available, numerous studies on the distribution of endogenous peptides in the central nervous system (CNS) have

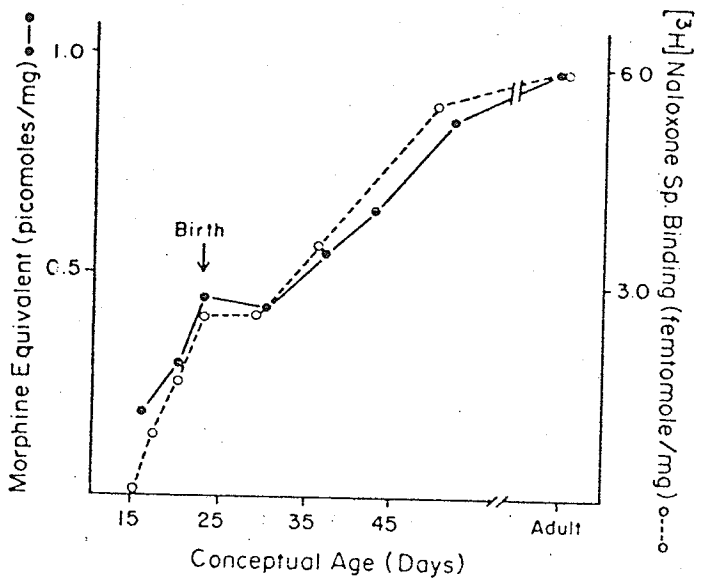


Fig. 3 : Development of ^{3}H-Naloxone specific binding site and endogenous MLF in rat brain. Each point is the mean of at least five separate preparations assayed in triplicate derived from at least two litters of rats. Results are presented in terms of the amount of ^{3}H-naloxone stereospecifically bound or the morphine equivalent of MLF per mg of whole brain.

(MLF : Morphine-like factor)

Reference : Francoise et al. 1976.

Table 3 : Regional Distribution of Opiate Receptor in Newborn and Adult Brain by ^{3}H-Naloxone Assay

Region	Newborn fmole/mg wet wt.	Adult	Increase Adult/Newborn
Parietal Cortex	1.04±0.03	7.12±0.50	6.85
Hippocampus	1.30±0.17	10.73±0.85	8.25
Striatum	7.43±1.12	22.41±0.92	3.01
Midbrain-Thalamus	3.65±0.51	23.32±1.59	6.38
Hypothalamus	5.38±0.30	20.71±0.73	3.85
Medulla-Pons	3.92±0.60	10.48±0.27	2.67

Reference : Francoise et al. 1976.

been performed. Some of this data is listed in the following tables (Table 4-7). Despite considerable variation in values reported from different laboratories, the immunoreactive β-endorphin-like substance is found in highest concentrations in the hypothalamus, midbrain and hindbrain. The concentration of endorphins are much higher in the pituitary than the brain and the reverse may be true for enkephalins. By gel filtration, at least two molecular weight forms of endorphins are found in brain extracts (Rossier et al. 1977a). One species representing about 60-70% of the material, closely coincides with the molecular weight of β-endorphin, while the other is a higher molecular weight substance in the range of β-LPH and the 31 K precursor. Rossier et al. (1977a) in other brain regions, such as the striatum, cerebellum, hippocampus and cerebral cortex found only the high molecular weight species. In hypothalamus, septum, pons, medulla, and mesencephalon, the fraction of total β-endorphin-like substances attributable to the larger molecular weight species is considerably lower. However, Krieger et al. (1977a) using an antiserum against porcine β-endorphin could detect only one peak in hypothalamic and striatal extracts in the bovine brain. This peak coincides with ^{125}I-β-endorphin upon gel filtration. In Dowling's(1978) experiment using human hypothalamic extracts and an antiserum against human β-endorphin, three immunoreactive peaks were detected upon gel

Table 4 : β-Endorphin-like Immunoreactivity in Different Regions of Brain (By RIA)

Species	Human	Human	Rat	Rat	Rat	Bovine
Reference	Liotta et al. (1978)	Dowling et al. (1978)	Rossier et al. (1977a)	Liotta (1978)	Höllt et al. (1978)	Krieger et al. (1977a)
Source & Tissue Obtained Method	Autopsy	Autopsy	Decapitation + Boiling	Decapitation + Boiling	Decapitation + Boiling	Exsanguination
Extraction Method	0.2 N HCl	0.1 N HAc	1 N HAc	0.2 N HCl	0.1 N HCl	0.2 N HCl
Units	ng/mg protein	ng/g tissue	ng/g tissue	ng/mg protein	ng/mg protein	ng/g tissue
Regions:						
Pituitary						
Whole	24.5	3500	2.7± 20x10^5			6100
Adenohypophysis			1.3± 9x10^5	1.5		
Neurohypophysis & Pars Intermedia		0	1.5±600x10^6			
Pineal			4.8± 1			
Whole Brain			108.0± 8			
			129.0± 18			
Hypothalamus		38.7	490.0± 30		113±30	19.9±5
			217.0± 32			
			600.0± 57			
Septum			234.0± 34			2.8±0.6
Olfactory Bulb						15.0
Midbrain		6.1	207.0± 15			
Medulla & Pons		4.7	179.0± 5			
Striatum			None			9.3
Thalamus		3.0	329.0± 19			
Cerebellum		1.0	None			
Globus Pallidus		1.7				
Hippocampus		0.0	None			
Caudate Nucleus		1.3				
Cortex		1.6	None			
Amygdada						7.0
Putamen		0.0				

Table 5 : β-LPH-like and ACTH-like Immunoreactivity in Different Regions of Brain (By RIA)

Species	Human	Rat	Bovine	Human	Rat	Bovine
RIA	β-LPH	β-LPH	β-LPH	ACTH	ACTH	ACTH
Reference	Liotta 1978	Liotta 1978	Krieger 1977a	Liotta 1978	Liotta 1978	Krieger 1977a
Units	ng/mg protein	"	"	"	"	"
Regions						
Pituitary						
Whole	585		17000	635		14000
Adenohypophysis		86.5			90.5	
Hypothalamus			5.17±0.81			17.00±4.80
Septum						0.11±0.03
Olfactory Bulb			1.38±0.94			0.05
Midbrain			2.58±0.15			0.24±0.06
Spinal Cord			2.00±1.40			0.06±0.01
Medulla			1.23±0.28			0.09±0.01
Pons			2.43±0.43			0.06±0.01
Striatum			1.63±0.41			0.17±0.02
Amygdala			0.49			1.20
Hippocampus			2.18±0.60			2.10±0.40
Cortex			1.24±0.07			0.24±0.06
Cerebellum			1.01±0.21			0.07±0.01
Thalamus			0.99±0.22			0.12±0.03

Table 6 : Met-enkephalin Immunoreactivity in Different Regions of Brain (By RIA)

Species	Bovine	Rat	Rat	Rat	Rat
Reference	Simantov 1977	Simantov 1976f	Catherine 1977	Yang 1977	Miller 1978
Units	ng/g tissue	ng/g tissue	ng/g tissue	ng/mg protein	ng/mg protein
Regions					
Whole pituitary					0.02±0.005
Whole brain		5±1		3.00±0.76(Microwave) 0.79±0.15(Decapitated) 0.81±0.13(M.+D.)	
Hypothalamus	55	20±3	235	4.80±1.30	135
Olfactory Bulb					3.05±0.45
Midbrain		13±2		1.10±0.17	
Medulla & Pons		9±2	100		
Medulla				2.60±0.34	
Striatum		23±4	615	9.20±1.70	3.00±0.35
Globus Pallidus					3.40±0.40
Caudate Nucleus	45				
Amygdala	150				
Hippocampus		8±2	25	0.71±0.14	0.45±0.005
Cortex	50	5±2	135	0.96±0.19	0.31±0.02
Cerebellum		2	25	0.36±0.02	0.01
Thalamus					o.52±0.04

Table 7 : Leu-enkephalin Immunoreactivity in Different Regions of Brain (By RIA)

Species	Bovine	Rat	Rat	Rat	Rat	Rat
Reference	Simantov 1977	Simantov 1976f	Rossier 1977a	Yang 1977	Miller 1977	Catherine 1977
Units	ng/g tissue	ng/g tissue	U/g tissue	ng/mg protein	ng/mg protein	ng/g tissue
Regions						
Pituitary						
Whole			74± 4			
Adenohypophysis			4± 1			
Neurohypophysis & Pars Intermedia			740± 5			
Pars Intermedia only	1					
Whole Brain		0.4±0.2	25± 2	*M 0.22±0.05	0.10±0.03	
				D 0.14±0.02		
				M+D 0.10±0.01		
Hypothalamus	135	1.5±0.3	120± 7	0.69±0.20	0.20±0.04	57
Septum			85± 7			
Olfactory Bulb					0.33±0.04	
Midbrain		1.1±0.3	32± 1	0.13±0.04	0.10±0.01	
Medulla & Pons		0.6±0.3	30± 4			6
Medulla				0.26±0.08		
Striatum		2.5±0.4	112±11	1.30±0.26	0.31±0.02	87
Globus Pallidus					0.38±0.004	
Caudate Nucleus	225					
Amygdala	50					
Hippocampus		0.3	13± 1	0.06±0.01	0.03±0.005	3
Cortex		0.4±0.2	15± 2	0.17±0.03	0.22±0.01	22
Cerebellum		0.3	5± 1	0.13±0.03	0.01	3
Thalamus			36± 5		0.17±0.005	

* M - Microwave

D - Decapitation

filtration. The major peak corresponded to β-LPH.

The regional distribution in mammalian brain of receptors and enkephalins parallel each other with the highest concentrations of both occurring in the striatum, anterior hypothalamus, mesencephalic central gray and amygdala and intermediate levels occurring in thalamic, cortical and brain stem areas and low levels of both in white matter, cerebellum and spinal cord, The medial thalamic region in monkey is unusual in having relatively low levels of enkephalin but high levels of receptors (Simantov et al. 1976b). The ratio of Met-enkephalin to Leu-enkephalin differs considerably from region to region. The significance of this observation is difficult to assess. It will be interesting to ascertain whether both Leu- & Met-enkephalins are localized in the same nerve fibers. Immunofluorescent histochemical analysis suggest both pentapeptides may be present in the same cell (Simantov et al. 1977; Catherine et al. 1977). Other explanations are possible for the change in ratio, such as a differential distribution of specific degradative enzymes. The variation between RIA and RRA estimates of enkephalins indicates that brain regions examined possessed a substantial amount of material which competes for receptor binding but does not react in the enkephalin RIA, One likely factor for this difference might be endorphin. The ratio of Met-enkephalin to Leu-enkephalin also differs in different species - guinea-pig, pig, bovine. Enkephalins were

not only found in the brain, but also in nerve plexi of the gastrointestinal tract and recently in exocrine cells of the stomach, intestine and pancreas (Polak et al. 1977).

Table 6 and 7 show that the concentrations of Met- and Leu-enkephalin obtained without taking any precaution in order to avoid their enzymatic degradation (Simantov et al. 1977; Miller et al. 1978; Simantov et al. 1976f) are significantly lower than those obtained when peptidases were heat-inactivated (Catherine et al. 1977; Rossier et al. 1977a; Yang et al. 1977). The early recognition of variation and the availability of completely specific antisera for the two enkephalins (cross reactivity between Met- and Leu-enkephalin of 0.3-10%) made their quantitation in discrete regions of the CNS a challenging task, but yielded comparable results in different laboratories. In the case of endorphin great variation in concentrations of β-endorphin in various brain regions have been reported (Table 4). There are two likely causes for these discrepancies. The extremely rapid degradation of β-endorphin in the nerve tissue "postmorten" and the widely different assay systems employed.

Table 8 shows that an almost instantaneous inactivation of brain enzymes is necessary in order to prevent the postmorten degradation of β-endorphin. At present, this can be obtained only with the use of microwave irradiation (Ogawa

Table 8 : Immunoreactive β-Endorphin in the Whole Brain of Male Rats

	Method of Killing	Enzyme Inactivation	ng*
I	Decapitation	None	34±5
II	Decapitation	Boil	46±4
III	Decapitation	Microwave	36±2
IV	Microwave	Microwave	304±23

* : Mean±S.E.M.

Reference : Ogawa et al. 1979.

et al.1977), since the time lapse between decapitation and microwave irradiation is sufficient to allow a 90% degradation of β-endorphin. It is worth noting that method II and IV displayed in Table 8 produced similar estimates of enkephalin (Yang et al. 1977). As shown in Table 9, a combinations of different antisera, tracer and standards have been used to assay human or rat β-endorphin concentrations. Moreover, only one of these assay systems (Rossier et al. 1977a) has been fully characterized for its use for rat samples. This is a crucial point, since human and porcine β-endorphin differ from rat β--endorphin as reported previously (Li et al. 1976c; Bradbury et al. 1975; Seidah et al. 1978). In a radioimmunoassay homologous for human β-endorphin, developed in our laboratory (unpolished results), camel and presumably rat β-endorphin cross react only 1%. In order to avoid any problem of specificity, in the rat studies to be presented, a radioimmunoassay homologous for camel (hence rat) β-endorphin was employed.
Study of brain and pituitary concentrations of β-endorphin is further complicated by the fact that all the antisera known at present show a 50-100% cross reactivity between β-endorphin and β-LPH or the 31 K precursor. As a consequence, further methods of separation have to be used to try to identify the cross reacting species. In the following study, gel chromatography has been employed to separate the 3 species, 31 K, LPH

Table 9 : Assay System Employed in Different Studies on β-Endorphin

Reference	Antiserum	Tracer	Standard
Rossier et al. 1977a	p*-β-endorphin	p*-β-endorphin	p*-β-endorphin
Liotta et al. 1978	h*-β-LPH	h*-β-endorphin	h*-β-endorphin
Yoshimi et al. 1978	p*-ACTH	h*-β-endorphin	h*-β-endorphin
Höllt et al. 1978	h*-β-endorphin	h*-β-endorphin	h*-β-endorphin

* : p : porcine

h : human

and β-endorphin. While there is great discordance in the quantitative estimates of β-endorphin concentrations, the data obtained by all laboratories are almost identical from a qualitative point of view.

In the pituitary, immunocytochemical and RIA studies indicate that α-and β-endorphin are found in every cell of the intermediate lobe and in discrete cells - corresponding to those reactive to antiserum against ACTH in the adenohypophysis. Very low levels of β-endorphin are present in the neurohypophysis (Rossier et al. 1979a). With the radioimmunoassay for enkephalin, immunoreactive species are primarily found in the intermediate lobe-neurohypophysis and almost absent in the adenohypophysis. β-Lipotropin is the predominant opioid peptide of the human pituitary and rat pars distalis. About 98% of the immunoreactivity corresponds to β-lipotropin in both regions by molecular sieve chromatography studies (Höllt et al. 1978, Liotta 1978). Based on the observation that ACTH, β-LPH and β-endorphin are found in the same granules in corticotrophs in the pituitary (Pelletier et al. 1977), Watson et al. (1977, 1978a) used immunocytochemical techniques to demonstrate that these three substances also coexist in the same cells and fibers of the brain. Using radioimmunoassay, anatomical lesions, and immunocytochemical techniques, they found that there are two separate systems; one is the β-endorphin system, the other

is the enkephalin system in rat brain (Watson et al. 1978a; Bloom et al. 1978; Rossier et al. 1977a). However, Watson et al. used an antiserum to human β-LPH to demonstrate that β-LPH and β-endorphin exist in the same fiber or cell in the rat brain. We have not been able to detect LPH in rat brain when using an antiserum raised against ovine β-LPH. Krieger et al. (1977a) also found a lack of concordance between immuno-assayable β-lipotropin and ACTH concentrations in different regions of bovine brain and spinal cord. Moreover, the functional roles of these separate and apparently discrete system may be more diverse than the general term "opioid peptides" implies.

POSSIBLE MECHANISM OF ADDICTION

Most people consider opiate addiction to comprise three major components : tolerance, physical dependence, and compulsive craving. Morphine withdrawal systems in the human elicits completely opposite effects of morphine, such as pupillary dilatation, diarrhea and central excitation.

Over the years many theoretical models have been proposed (Lochin 1974; Collier et al. 1966; Goldstein et al. 1961; Shuster 1961; Snyder 1979) to explain opiate addiction. They are listed

and discussed as follows:

(1) Change in opiate receptor, such as fewer drug receptors, proliferation of inactive receptors, change of number or conformation of receptors. However, opiate receptor assays both in vivo and in vitro have failed to show systematic changes related to addiction (Pert et al. 1973c; Klee et al. 1974a).

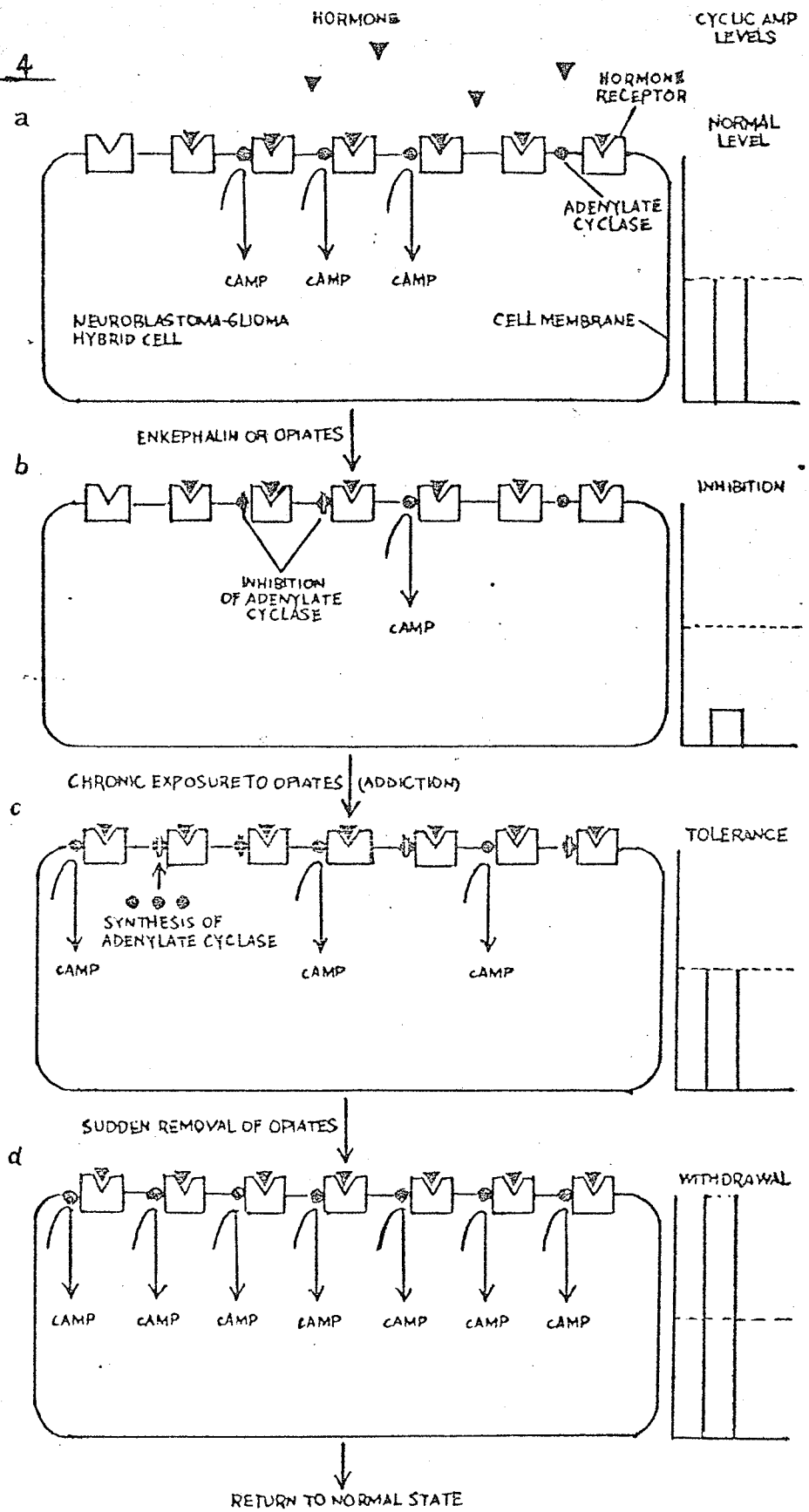
(2) Compensatory change in a "second messenger" in neurons containing opiate receptors. In cells in which opiates acutely reduce the activity of adenylate cyclase, compensation may involve increases in adenylate cyclase activity. Some evidence of this phenomenon was found in recent studies of opiate effects on a neuroblastoma-glioma hybrid in cell culture. Opiate agonists in this clone, decrease adenylate cyclase (Klee et al.1974b; Sharma et al. 1975) and enhance the accumulation of cyclic GMP (Traber et al. 1975). Opiates reverse the stimulation of adenylate cyclase by Prostaglandin-E_1 and adenine (Sharma et al. 1975). This effect is not only found in cancerous cells of the nervous system in tissue culture, but Collier and Roy (1974a) have also decribed a prostaglandin-stimulated adenylate cyclase in mammalian brain which is inhibited by morphine and other opiates. Moreover phosphodiesterase inhibitors which elevate brain cyclic AMP levels elicit in rats behavioral changes resembling opiate withdrawal syndrome and which are enhanced by as little as 0.03 mg/kg of naloxone (Collier et al. 1974b; Francis et al. 1975). A model of these

events is depicted in Fig. 4.

(3) Role of the central noradrenergic neurones

Specific noradrenergic neurones containing opiate receptors may account for symptoms of opiate withdrawal. The locus coeruleus nucleus in the brain stem consists almost entirely of norepinephrine cells whose axons project widely throughout the brain. Opiate receptors are more highly concetrated in the locus coeruleus than in almost any other part of the brain, and opiates selectively slow the firing neurones in the locus coeruleus (Bird et al. 1977). The α-noradrenergic agonist drug clonidine also slows the firing of locus coeruleus cells by stimulating their α-adrenergic "receptors". Morphine tolerance and dependence in rats is accompanied by tolerance of locus coeruleus neurons to the slowing effects of opiates but not of clonidine (Aghajanian, 1978). Morphine withdrawal is associated with a considerable acceleration of locus coeruleus firing that can be suppressed by clonidine. Piperoxan which accelerates the firing of locus coeruleus neurons by blocking their autoreceptors reproduces many of the symptoms of opiate withdrawal such as anxiety and hypertension. Moreover, low doses of clonidine have recently been demonstrated to alleviate dramatically the symptoms of opiate withdrawal in human methadone addiction (Gole et al. 1978). Thus, of the multitude of opiate receptors distributed throughout the brain, those concentrated in the

Fig. 4



Reference: Snyder, et al. 1977.

small locus coeruleus may largely account for opiate withdrawal effects.

(4) Compensatory increase in activity of enkephalin neurones other than the receptor-bearing neurones. Chronic opiate treatment might produce a slow rate of enkephalin neurons with less release of enkephalins and secondarily slowed biosynthesis and turnover of enkephalins (Kosterlitz et al. 1975b; Simantov et al. 1976e). Unfortunately, technics to measure firing of enkephalin neurones or enkephalin turnover have not yet been developed. However, one can measure the total enkephalin levels. Radioreceptor assays of the amount of substance in the brain that could compete for opiate receptor binding suggested increased enkephalins levels in the brain of morphine addicted rats (Simantov, 1976e). However, direct radioimmunoassay of brains of rats chronically exposed to morphine failed to reveal any alteration in enkephalin levels (Fratta et al. 1977; Childers et al. 1977).

(5) Increase in activity of a specific enkephalin degrading peptidase

Recently Malfroy et al. (1978) identified a specific enkephalin degrading enzymes that may be the physiologic regulator of enkephalin synaptic action, much as acetylcholinesterase physiologically regulates acetylcholine activity. This "enkephalinase" enzyme is localized in brain membranes, has a unique high affinity for enkephalin. Chronic morphine

treatment of rats results in a 60% increase in enzyme activity. One might speculate that with less endogenous enkephalin, the receptors would become "tolerant" to opiate-like substances and that after their withdrawal, the receptors would be "abstinent".

Loh et al.(1976), Wei and Loh (1976) showed that continuous infusion of Met-enkephalin or β-endorphin into the periaqueductal gray fourth ventricle region of rat brain over a period of 70 hrs. produces physical dependence as evidenced by the production of withdrawal signs after naloxone administration. Incubation of Met-enkephalin or Leu-enkephalin with neuroblastoma x glioma hybrid cells showed that the increased adenylate cyclase activity associated with opiate tolerance and dependence is elicited.

POSSIBLE PHYSIOLOGICAL ROLES OF ENDOGENOUS OPIATES

(1) Neurotransmitters

A list of seven criteria has been proposed for assessing the likelihood that a substance is a neurotransmitter in brain (Werman 1966; Phillis 1970). These are not easily satisfied and few neurotransmitters have as yet been unequivocally established in brain. Of the endorphins which have been decribed to date only the enkephalin pentapeptides can be seriously considered

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as neurotransmitter. The evidence is summarized briefly in the Table 10.

(2) Nociception regulation-analgesia

β-endorphin is a potent analgesic agent when injected either intracerebroventricularly (Loh et al. 1976) or intravenously (Tseng et al. 1976). Focal application of enkephalins (Met or Leu) directly into periaqueductal gray regions of rat brain results in only transient behavioral effects without analgesia due to rapid degradation (Jacquet et al. 1976). Electrically produced or induced analgesia is partly naloxone reversible (Akil et al.1976a). Morphine-tolerant animals respond less well to electrostimulation than a naive animal(Mayer et al. 1975). In man, pain relief produced by intracerebral stimulation has been obtained in patients with severe chronic pain (Adams 1976; Gybels et al.1976; Richardson et al. 1977) using electrodes chronically implanted into sites adjacent to the third ventricle. Adams (1976) and Meyerson et al. (1977a) report that in some patients the analgesic response is antagonized by naloxone. Furthermore, in two of three patients where the analgesia was naloxone reversible, CSF levels of endorphins rose after stimulation (Meyerson et al. 1977a). Similar results were reported by other laboratories (Hughes, 1977; Hosohuchi et al. 1978). The evidence for involvement of endorphin in electrically induced analgesia is therefore very strong. Acupuncture-like electrostimulation produces naloxone-reversible

Table 10 : Evidence to Suggest that Enkephalins May Act as Neurotransmitters

(A) The enkephalins are present in brain using bioassay, RIA, RRA, and immunochemical techniques. Furthermore they are present in specific areas of brain in association with stereospecific receptors (Hiller et al. 1973; Simantov et al. 1976b; Smith et al. 1976; Kuhar et al. 1973), and are apparently localized in nerve terminals (Kuhar et al. 1973; Simantov et al. 1976d; Queen et al. 1976).

(B) The enzymatic machinery has not been identified but synthesis from labelled precursor has been observed in brain (Clouet et al. 1976).

(C) While specific enzymes have not been identified on synapses, a highly effective system for inactivation of enkephalin exists in brain (Pert et al. 1976d; Malfroy et al. 1978; Frederickson et al. 1976b; Hambrook et al. 1976).

(D) Evidence, direct and indirect, has been obtained for release of enkephalins both from isolated guinea-pig ileum (Puig et al. 1977; Neuten et al. 1976; Schulz et al. 1977) and from brain e.g. rat striatal slices in vitro and in vivo (Akil et al. 1976a, 1976b; Wahlström et al. 1976; Jacob et al. 1974; Frederickson et al. 1977a; Sjölund et al. 1976; Lal et al. 1976; Terenius et al. 1976). Release has been

demonstrated either at rest or with potassium stimulated and calcium-dependent conditions.

(E) The enkephalins have a predominatly depressant action except at the Renshaw cells in feline spinal cord and pyramidal cells in hippocampus, apparently at a postsynaptic site, on single neurons in particular brain regions where they and opiate receptors occur and this can be antagonized by naloxone (Zielgansberger et al. 1976a, 1976b; Bradley et al. 1976; Bramwell et al. 1974; Gent et al. 1976; Hill et al. 1976; Frederickson et al. 1976c; Davies et al. 1976).

analgesia (Sjölund et al. 1976), in some patients undergoing such analgesia and lumber CSF endorphin levels increase (Sjölund et al. 1976). The analgesia produced by classical acupuncture in man is also naloxone reversible (Mayer et al. 1977). Several papers also report that the level of endorphin is decreased in CSF in some patients with trigeminal neuralgia (Terenius et al. 1975b) and patients with severe chronic headaches (Sicuteri et al. 1978). However, analgesia produced in rat mesencephalon by electrostimulation is not (Pert et al. 1976a; Yaksh et al.1976) or only partially (Akil et al. 1976a) naloxone-reversible. Naloxone also has essentially no effect upon hypnotic analgesia in man (Goldstein et al. 1975b), analgesia by high frequency stimulation, shock escape threshold in the rat (Goldstein, 1976) or pain perception or threshold in human (El-Sobky et al. 1976). It is therefore probable that mechanisms other than endorphin activation may cause analgesia.

(3) Influence complex mood and behavior

Peripheral administration of enkephalin or β-endorphin was found to increase markedly the activity of mice in the DOPA-potentiation test (Kastin et al. 1976a; Plotnikoff et al. 1976) and of rats in a model of depression (Kastin et al. 1978), facilitate the running of a complex maze by hungry rats (Kastin et al. 1976b), immobilize goldfish in a habituation paradigm (Olson et al. 1978), affect openfield behavior, increase grooming and sexual arousal (Veith et al. 1978), and reduce distress vocaliza-

tion of chickens accompanying social isolation (Panksepp et al. 1978). Intracerebroventricular injection of β-endorphin in rats, in addition to analgesia, leads to disorganization of normal behavior, inducing, in lower doses, an opiate withdrawal syndrome (such as stiffly arching tail or Straube sign, wet dog shakes, excessive grooming, etc.), whereas higher doses result in akinesia, which was described as a catatonia-like state (Bloom et al. 1976; Havlicek et al. 1975, 1976). Also β-endorphin in higher doses induced EEG hypersynchrony with significant increase in power in all frequencies (Havlicek et al. 1979). Other effects have also been reported such as : drive reduction by self-administration (Bellussi et al. 1977), increased multiple-unit electrical activity in periaqueductal gray matter (Urca et al. 1977), tranquillizing effect of α-endorphin, the violent effect of γ-endorphin (Guillemin et al. 1976b), influencing sex behavior of male rats (Meyerson et al. 1977b). A dose dependent suppression of lever pressing for food by hungry rats was found after β-endorphin administered intracerebroventricularly as well as peripherally (Lichtblau et al. 1977). The immobility observed after central administration of β-endorphin has been related to similar states observed in schizophrenia. Moreover, increased levels of endorphin-like materials have been reported in the spinal fluid of chronic schizophrenic patients (Terenius et al. 1976), but the effectiveness in schizophrenia (Gunne et al.

1977) of opiate antagonists has not been confirmed (Davis et al. 1977; Mielke et al. 1977). The causative material may be β-leu^5-endorphin which appears to have been isolated from dialysates of schizophrenia patients (Palmour et al. 1977).

(4) Thermoregulation

Endogenous opiates may be a negative determinant in thermoregulation (Bloom et al. 1976), α- and β-endorphin can lower body temperature, but γ-endorphin elevates it. These actions of endogenous opiates are blocked by naloxone and these effects are probably mediated by opiate receptors. Naloxone itself has essentially no effect upon body temperature in rats, but naloxone antagonized conditioned hyperthermia (Lal et al. 1976). Injection of only 0.74 nM β-endorphin into the preoptic/anterior hypothalamus (POAH) can cause hyperthermia (Martin et al.1978). β-Endorphin evoked a hypo- or hyperthermia depending on the dose and the route of administration. Moreover, in emotional hyperthermia of rats, β-endorphin-like immunoreactivity in the plasma increased dramatically, and the stress-induced hyperthermia can be antagonized by naloxone (Blässig et al. 1978).

(5) Food intake

β-endorphin may stimulate food intake in satiated rats when injected into the ventromedial hypothalamus, this can be blocked by naltrexone or phentolamine (Grandison et al. 1977a). Naloxone at doses as low as 0.25 mg/kg selectively depresses

food intake in genetically obese mice c57BL/6J ob/ob (Beatriz et al. 1978). Intraventricularly injections of β-endorphin resulted in a significant and substantial increase of liquid diet intake in mild deprived rats (Kenney et al. 1978). These experiments suggest that β-endorphin may be involved in the regulation of food intake. Moreover, elevated concentrations of β-endorphin were found in the pituitaries of both obese mice (ob/ob) and rats(fa/fa), and in the blood plasma of obese rats. Brain levels of β-endorphin and Leu-enkephalin were unchanged (Margules et al. 1978). These data suggest that excess pituitary β-endorphin may play a role in the development of overeating and obesity syndromes. However, Rossier et al. (1979b) found that the increase in hypophyseal immunoreactive β-endorphin does not occur until several months after the onset of obesity and thus seems, if anything, to be a consequence of hyperphagia rather than its cause. On the other hand, immunoreactive Leu-enkephalin levels in the pars nervosa of obese mice were elevated almost 2 fold at 1 month of age, and this relative increase persisted throughout life. The increase was also highly correlated with increases in ob/ob body weight and, therefore, may warrant further investigation as a possible factor in the obesity syndrome (Rossier et al. 1979b).

(6) Sexual maturation

Subcutaneous injections of the opiate antagonist-naloxone

lead to an increase in serum luteinizing hormone concentrations in female but not in male rats before puberty. In addition, estradiol benzoate specifically blocks the LH response to naloxone in prepubertal female rats (Blank et al. 1979), suggesting that opioid peptides may have a physiological role in the endocrine events leading to sexual maturation. The intraventricular injection of D-alanine-methionine-enkephalinamide (D-Ala^2-Met-enkephalinamide), a synthetic analog of Met-enkephalin that is resistant to enzymatic degradation, inhibits copulatory behavior in sexually vigorous male rats in doses which do not influence motor activity or feeding behavior. This effect is prevented by naloxone. In addition, injections of naloxone induce copulatory behavior in sexually inactive male rats. These results suggest that endorphins play an important role in the regulation of sexual behavior (Gessa et al. 1979).

(7) Regulation of pituitary hormones

Like morphine and morphine analogues, the opiate peptides can affect the release of pituitary hormones. Both enkephalins and the endorphins affect the release of growth hormone (GH) and prolactin (PRL) when exogenously administered.(Bruni et al. 1977; Dupone et al. 1977a, 1977b; Shaar et al. 1977; Rivier et al. 1977). It is worth noting that when dose response studies were attempted with opioid peptides on pituitary hormone secretion, PRL was released by doses considerably lower than those necessary to induce

a GH response (Dupont et al. 1977a, 1977b). Consistent with this greater sensitivity of the PRL response to endogenous opiates, higher doses of naloxone are necessory to inhibit PRL than GH release induced by endogenous opiates (Dupont et al. 1977a, 1977b). When a different approach is attempted, i.e. to influence the activity of the endogenous opiates by the administering specific antagonists such as naloxone or naltrexone, interesting insights into the role of the endogenous opiates are obtained. In such experiments, naloxone does not influence the release of GH in the free-moving, unstressed rat, indicating that endogenous opiates are not involved physiologically in the release of this hormone (Tannebaum et al. 1979). A similar study for PRL has not yet been conducted. However, data obtained in rats killed by decapitation in normal condition, during lactation or after stress (Meites et al. 1979) indicate that under these experimental conditions, naloxone induces a prompt and profound inhibition of PRL release, indicating that endogenous opiates are involved in the control of PRL release (Meites et al. 1979). Some evidence is also cumulating that endogenous opiates are involved in the regulation of luteinizing hormone (LH) release. Both enkephalins and β-endorphin inhibit LH release when exogenously administered (Bruni et al. 1977). The administration of naloxone consistently increases LH concen-

trations in adult male rats and ovariectomized or orchiectomized rats (Blank et al. 1978; Meites et al. 1979). A possible role of endogenous opiates on TSH release has also been proposed (Bruni et al. 1977; Meites et al. 1979; Yamauchi et al. 1978). Met-enkephalin and morphine can depress serum level of TSH in the male rat (Bruni et al. 1977). In pituitary cell culture, some investigators also found a significant increase of basal TSH release after adding β-endorphin (Yamauchi et al. 1978). Injection of naloxone reversed the effect of Met-enkephalin on serum TSH (Meites et al. 1979). The level at which endogenous opiates interfere in the regulation of pituitary hormone secretion is still a matter of discussion. Most evidence indicates that their effects on the pituitary, like those of morphine, are mediated through the central nervous system. They may associated with dopaminergic, serotonergic or cholinergic systems. (Meites et al. 1979; Tache' et al. 1979; Dupont et al. 1979). Moreover, some evidence shows β-endorphin increases dopamine and acetylcholine turnover in striatum, and serotonin turnover and release in brain stem and hypothalamus (VanLoon et al. 1978; Biggio et al. 1978), but decrease serotonin turnover and release in hippocampus (VanLoon et al. 1978). However, the possibility of a direct effect on the pituitary has not been completely ruled out yet (Lien et al. 1976). Most of the studies have been conducted in the rat. Somewhat different

results have been obtained in the human. Endogenous opiates do not seem to be involved in GH or PRL release in basal conditions in the human. Naloxone however can induce a clear and long lasting release of both LH and cortisol (Blankstein et al. 1978). Further sthdies have to be conducted in conditions of PRL or GH stimulation, before a role of endogenous opiates can be unequivocally established.

(8) Regulation of hypothalamic hormones and pancreatic hormones

In hypothalamic organ culture, β-endorphin increased TRF release. This effect was inhibited by naloxone. However, a complex biphasic response to β-endorphin on SRIF release was observed with significant release at 10^{-9}M but inhibition at 10^{-8}M (Yamauchi et al. 1978). Opiate receptors have been identified in the Islets of Langerhans, and the presence of endocrine cells with enkephalin-like immunofluorescence have been reported in Islets of the pancreas (Forsmann et al. 1977), Ipp et al. (1978) found that infusion of porcine β-endorphin into isolated pancreas can increase the release of insulin and glucagon and inhibit somatoststin release. This action was completely abolished by the opiate antagonist-naloxone. However, in vivo, β-endorphin antiserum, naloxone or naltrexone, fail to alter the basal GH or insulin secretion (Tannebaum et al. 1979). Morphine injections are known to release vasopressin into the blood (DeBodo 1944), and opiate receptors

are also concentrated in the pars nervosa. Because dehydration decreased both the enkephalin and the vasopressin content of the pars nervosa, pituitary enkephalin fibers may have a role in the regulation of vasopressin or other magnocellular hormonal secretion (Rossier et al. 1979). Although some contradictory reports have appeared (Greidances et al. 1979), some laboratories (Tseng et al. 1978; Bisset et al. 1978; Haidobro-Toro et al. 1979) have suggested antidiuretic effects of β-endorphin and the natural or synthetic enkephalins.

(9) Others

Opiates and endogenous opiates inhibit acetylcholine release from the guinea-pig ileum and cortex (Jhamaudas et al. 1973). Opioid peptides may affect memory (Gallagher et al. 1978) and regulation of respiration (Moss et al. 1978). β-Endorphin stimulates corticosterone synthesis in isolated rat adrenal cells (Shanker et al. 1979).

FACTORS AFFECTING ENDORPHIN RELEASE, SYNTHESIS OR ENDORPHIN-MEDIATED BEHAVIOR

(1) Endocrine glands or hormones

(a) Pituitary gland

Rossier et al. (1977a) and Cheung et al. (1976) have

reported using opiate receptor assay (Cheung et al. 1976) or radioimmunoassay (Rossier et al. 1977a) that hypophysectomy fails to change the amount of β-endorphin in the brain. By using Met-enkephalin and Leu-enkephalin radioimmunoassay (Kobayashi et al. 1978), brain enkephalin distribution is unaltered by hypophysectomy. Pert et al. (1976) also reported significantly lowered opiate-like activity in blood after hypophysectomy. Their results are doubtful in view of the poor sensitivity of opiate receptor assay. Hypophysectomized rats are supersensitive to the hypothermic effects of morphine and β-endorphin injected intraventricularly as early as 1 week after surgery. At two weeks after surgery, there is a significant increase in the antinociceptive potency for these opiates (Holaday et al. 1977). The results of studies on the effect of hypophysectomy on alternating responsiveness to electric footshock (Gispen et al. 1970, 1973; Gibbs et al. 1973) have been contradictory and inconclusive. However, Pomeranz et al. (1977) found that hypophysectomized rats did not respond to electroacupuncture induced analgesia as well as normal controls. Likewise, reports on changes in morphine toxicity in hypophysectomized animals are contradictory (Tanabe et al. 1958; Ziekind et al. 1975).

(b) Adrenal gland

Rossier et al (1977) found a significant increase of both

β-endorphin and enkephalin immunoassayable materials in the adenohypophysis after adrenalectomy. Only β-endorphin immunoassayable substances were detected in the intermediate lobe-neurohypophysis. No change in the amount of β-endorphin immunoreactive substances in the brain occurred after two months of adrenalectomy (Rossier et al. 1977). However, Tseng et al. (1979) recently reported that 9 days after adrenalectomy a reduction of immunoreactive β-endorphin in the rat pituitary of 38.8% while β-endorphin in hypothalamus was not altered. β-endorphin-like immunoreactivity in rat plasma was substantially increased after adrenalectomy (Akil et al. 1978; Guillemin et al. 1977). Following adrenalectomy, subcutaneous morphine injections but not intracerebroventricular injections enhance the opiate antinociceptive potency, especially the potency of intravenous β-endorphin. Furthermore, this supersensitivity to intravenous β-endorphin was completely reversed by dexamethasone, suggesting that corticosteroids may function as an antagonist to opiates through a mechanism peripheral to the neuroaxis (Holaday et al. 1977).

(c) Thyrotropin releasing hormone

β-endorphin decreases general motor activity after intracerebroventricular administration, but thyrotropin releasing hormone pretreatment completely abolished the depressant effect of β-endorphin. In addition, thyrotropin releasing hormone

enhanced the prolactin secretion induced by β-endorphin and antagonized the slight elevation of plasma GH levels observed in β-endorphin treated rats. These results do not seem to be related to an interaction with opiate réceptors (Tache' et al. 1977). Thyrotropin releasing hormone also antagonized β-endorphin hypothermia and catalepsy in intact and hypophysectomized rats. These effects of thyrotropin releasing hormone appear to be independent of pituitary-thyroid involvement. Thyrotropin releasing hormone did not alter the antinociception produced by β-endorphin on either sham-control or hypophysectomized rats.

(d) Tumors of pituitary origin

In female rats bearing a prolactin and growth hormone secreting tumor(MtTW15), the concentrations of β-endorphin in the hypothalamic area are greatly decreased compared to controls (1.3 vs 11.1 μg/g tissue). A significant decrease in β-endorphin concentrations also occurs in the midbrain. However, no change was found in the hindbrain and pituitary. Similarly, in female rats bearing a prolactin and adrenocorticotrophin secreting tumor (7315a), the concentrations of β-endorphin in the hypothalamus are markedly decreased (2.3 vs 12.6 μg/g tissue). A comparable decrease of β-endorphin concentrations also occurs in the pituitary (Panerai et al. 1979).

(2) Effect of Age : discussed in the previous "ontogenic study" section.

(3) Factors affecting ACTH secretion

The opiate-like peptide β-endorphin and ACTH are concomitantly secreted in increased amounts by the adenohypophysis in response to acute stress or long-term adrenalectomy. In monolayer cultures of adenohypophysial cells both ACTH and β-endorphin secretion increases in response to purified ovine corticotropin releasing factor and other secretagogues like BrcAMP, Arg-Vasopressin, Norepinephrine (Vale et al. 1979). Hypophysectomy abolishes the increase in plasma concentrations of β-endorphin and ACTH to stress, indicating that these peptides are of hypophysial origin. Administration of synthetic glucocorticosteroid dexamethasone inhibits the secretion of both ACTH and β-endorphin (Guillemin et al. 1977b). See Table 11. In vitro, pretreatment of adenohypophysial cells with glucocorticoids or progesterone inhibits the secretion of β-endorphin-like substances as well as ACTH-like substances. Prostaglandin (PG E_2) partially suppresses the CRF-mediated release of both β-endorphin-like substances and ACTH-like substances. However, there is some difference between the regulation of secretion of corticotropes in the anterior and intermediate lobe. In contrast to adenohypophysial corticotrophes, neurointermediate lobe cell cultures do not secret β-endorphin-like substances in response to purified CRF preparations or elevated medium $[K^+]$. Also they were not inhibited by glucocorticoids, but they inhibited by dopamine and its

Table 11 : Pituitary Concentrations of ACTH and β-Endorphin as Modified by Adrenalectomy, Administration of Dexamethasone

Treatment	Whole Pituitary µg/gland		Adenohypophysis µg/gland	
	ACTH	β-Endorphin	ACTH	β-Endorphin
None(controls)	4.8±0.3(3)	2.6±0.2(11)	2.7±0.5(3)	1.1±0.2(3)
Adrenalectomy	9.9±1.1(3)	10.8±0.5(3)	8.3±1.1(3)	5.4±0.7(3)
Dexamethasone	2.1±0.1(6)	1.2±0.1(7)		

Reference : Guillemin et al. 1977b.

agonists (e.g. apomorphine) (Vale et al. 1979).

(4) Diurnal rhythm

In the human plasma β-LPH levels parallel the diurnal variations of plasma ACTH increasing in the early morning and falling in late afternoon and evening (Krieger et al. 1977b). Naloxone decreases diurnal variation in pain sensitivity and somatosensory evoked potential (Davis et al. 1978). Procacci et al. (1972) found increased pain thresholds in human volenteers in the morning and Frederickson et al. (1977b) noted that the diurnal peak in pain tolerance (as measured by jump latency in mice) was dampened by naloxone. Frederickson (1979) also found a diurnal rhythm of enkephalin secretion.

(5) Others

Depolarization of AtT20 mouse pituitary tumor cells with high K^+ concentration (56mM) increased the release of endorphins 2-3 fold. The K^+ (Simantov 1978) evoked release was Ca^{2+} dependent. Electrical stimulation of periaqueductal and periventricular gray matter produced a two to four fold increase in the levels of β-endorphin-like substances in the CSF (Hosohuchi et al. 1978).

PROHORMONE THEORY AND ENDOGENOUS OPIATE PRECURSORS

The prohormone theory was proposed by Chrétien and Li in 1967. They said, "β-LPH now appears to be a unique molecule comprising the structures of two biologically active peptides : β-MSH and γ-LPH". As yet there is only limited direct evidence, but the following experimental findings support this theory. (1) β-LPH is a relatively stable molecule, and isolated β-LPH fragments are not likely to be degradation products due to the isolation procedure as shown by Chrétien and Gilardeau (1970). (2) Chrétien et al. (1976b) using in vitro pulse-labelling techniques have already demonstrated the transformation of β-LPH into γ-LPH. (3) β-LPH has much lower melanocyte stimulating activity than β-MSH and much lower morphine-like activity than new peptides. This would be an analogous situation to the proinsulin-insulin model (Li et al. 1966; Steiner et al. 1974). β-LPH, γ-LPH and β-endorphin are consistently isolated from pituitaries of different species. (5) By immunocytochemistry of normal pituitary tissue, ACTH. β-LPH, β-endorphin, α-endorphin are all present in the same cells in the anterior and intermediate lobes of the pituitary gland (Pelletier et al. 1977; Watson et al. 1977, 1978a). ACTH and β-endorphin were shown by Mains, Eipper and Ling (1977) originally to be part of a much larger precursor glycoprotein (31 dalton, known as 31 K precursor, pro-opiocortin, big-ACTH), as synthesized by the cloned pituitary cells of the (mouse) cell

line At T-20/D-16V. Giognoni et al. (1977) reported that the parent At T-20 cell line produces opioid peptides as well as ACTH. In vitro studies have also shown that under numerous experimental or pathological circumstances, the secretory rate of ACTH-like substances are modified in parallel with the secretory rates of β-LPH-like and recently β-endorphin-like immunoreactivity (Guillemin et al. 1977b; Vale et al. 1979; Rossier et al. 1977b). Yoshimi et al. using RIA for β-endorphin also found the presence of "big-big" β-endorphin with an apparent molecular weight of 37,000 and 31,000 in human and rat pituitaries respectively (Yoshimi et al. 1978). Characterization of the 31 K precursor also was done by several investigators (Rubinstein et al. 1978; Robert et al. 1977), ACTH and β-LPH are produced from this high molecular weight precursor by trypsin or trypsin-like enzymes (Rubinstein et al. 1978; Robert et al. 1977). In human placental extracts, Odagiri et al. (1979) found that there were at least two high MW components (48,000 and 36,000), both of which had ACTH, LPH and β-endorphin immunoreactivities. They suggested these human placental proteins are larger than mouse ACTH precursor molecule. Liotta et al. (1979) found that the higher molecular weight immunoreactive ACTH, β-endorphin-like ^{3}H-labeled product derived from the bovine hypothalamic culture is similar to the pituitary and placental derived precursor in containing the dual antigenic

determinants in its gel filtration characteristics. Recently, Kimura et al. (1979) sucessfully purified the pro-opiocortin with an apparent molecular weight of 33,000 daltons from camel pituitaries. Thus the precursor relationship of the pure protein to the opioid peptides and to corticotropin was confirmed. Several studies, have demonstrated major differences in the precursors of enkephalins both in brain and pituitary. Striatal extracts of guinea-pig, rats and cattle were found to contain two large proteins (>40,000 and >100,000 daltons) that on treatment with trypsin yielded opioid peptides - Leu-enkephalin and Met-enkephalin, but not endorphins and β-LPH (Lewis, Udenfriend et al. 1978). Also after trypsin treatment of the soluble fraction of rat brain, a high molecular weight enkephalin immunoreactive peak is present which yields a peak with the same apparent molecular weight as enkephalin. The molecular weight of this precursor ia 50,000 to 70,000 (Childers et al. 1979). These putative enkephalin precursors are all different from 31 K precursor of β-LPH and β-endorphin which was found in the rat pituitary.

ENDOGENOUS OPIATES IN THE BLOOD, PLACENTA, AND CSF

Immunoreactive β-LPH, β-endorphin (Nakai et al. 1978),

enkephalin (Jansen et al. 1979) -like substances can be detected in extracts of hyman placenta by using RIA (Nakai et al. 1978) or bioassay (Jansen et al. 1979). Gel filtration studies revealed the total β-endorphin immunoreactivity consists of two fractions with elution positions compatible with β-LPH and β-endorphin respectively, and a fraction of larger molecular weight, possibly their precursor. Recently, Odagiri et al. (1979) using sodium dodecyl sulfate/polyacrylamide gel electrophoresis on the immune affinity-purified placental extract, found that there were at least two high MW components (MW≈48,000 and 36,000), both of which had ACTH, β-LPH and β-endorphin immunoreactivities. However, the two largest placental immunoreactive ACTHs migrated more slowly than did the ACTH glycoprotein of MW 31,000 purified from mouse AtT-20/D-16V pituitary tumor cells applied to parallel gels, suggesting that the human placental proteins are larger than this mouse ACTH precursor molecule (Odagiri et al. 1979). Several studies (Wahlström et al. 1976; Terenius et al. 1975b; Shibuga et al. 1977; Jeffcoate et al. 1978; Gautry et al. 1977) also revealed endogenous opiates in the CSF. Jeffcoate et al.(1978) found 90% or more of total β-endorphin immunoreactivity in CSF was accounted by β-endorphin. He found three peaks, one coincident with β-LPH, one with β-endorphin, another in the void volume (Jeffcoate et al. 1978). Akil et al. (1978a) using a Met-

enkephalin RIA and RRA, found one peak of Met-enkephalin which appears to resemble fraction II in Wahlström's experiment (Gautry et al. 1977). However, Jeffcoate et al. found no correlation between CSF and plasma β-endorphin or β-LPH. Only one paper (Gautry et al. 1977) reports the presence of immuno-assayable β-endorphin in human amniotic fluid with elevated levels in cases of fetal distress. Several laboratories have reported on endogenous opiates or opioid activity in the plasma. However, there are several discrepancies in these reports. Wardlaw et al. (1979) found 15% by weight of the total β-LPH-βendorphin immunoreactivity is attributable to human β-endorphin in basal plasma. Nakao et al. (1978) reported on human β-endorphin in human basal plasma, but the immunoreactive species was mainly β-LPH (over 90%, by weight).

Table 12 : Possible Involvement of Endogenous Opiates in Pathogenesis of Following Syndromes

(1) Schizophrenia and Amnesia (Henk 1978; Watson et al. 1978b)

(2) Morphine addict

(3) Hypotension (Moladay et al. 1978; Lemaire et al. 1978) : Naloxone reversal of endotoxin hypotension suggests role of endorphins in shock (Moladay et al. 1978). Presence of decreased concentrations of enkephalins in genetically hypertensive rats.(Diginlio et al. 1979).

(4) Parkinsonism (Barbeau 1978).

(5) Diabetes

(6) Obesity syndromes (Margules et al. 1978)

(7) Others

OBJECTIVES

The objectives of these studies were (1) To develop optimal procedures for obtaining accurate and reproducible estimates of the content of β-endorphin in rat brain regions. (2) To determine the changes in brain β-endorphin immunoreactivity after a variety of experimental endocrine and pharmacological manipulations.

These studies involved an examination of β-endorphin in rat brain (1) by comparing different methods of rapidly killing rats by microwave irradiation or by decapitation using guillotine. (2) by comparing several methods of extraction of brain tissues. (3) after removal of selected endocrine glands e.g. thyroid, pituitary, ovary, testis, pineal, adrenal. (4) after administration of certain hormones e.g. T_4. (5) after the administration of several drugs, e.g. naloxone, pentobarbital, morphine. (6) in rats of different ages and sex.

MATERIALS AND METHODS

I. Hormones and drug preparations

Camel β-endorphin, human β-endorphin, Met-enkephalin, Bombesin were purchased from Peninsula Laboratories, San Carlos, CA. α-endorphin was a gift from Dr. R. Guillemin (Salk Institute Lajolla, Ca.). Ovine LPH was a gift from Dr. M. Chrétien. L-Thyroxine and bovine albumin fraction V were obtained from Sigma Chemical Company. Naloxone was from Endo Drug Ltd. Sodium pentobarbital was obtained from Abbott Laboratories. Morphine was obtained from BDH, Poole, England. Trasylol was produced by Bayer AG. Ethylene diamine tetraacetic acid, carbon decolorizing alkaline Norit-A, H_2O_2(30%, V/V, Solution), POPOP (1,4-Bis(2-(5-Phenyloxazolyl)Benzene) and Ethylene Glycol Monoethyl Ether were all obtained from Fisher Co. Freund's complete adjuvant was from Difco Lab.. Lactoperoxidase was obtained from Calbiochem., La. Jolla. Sephadex G-50 (Medium), Dextran T 70 were from Pharmacia. PPO (2,5-Diphenyloxazone), ^{3}H-Naloxone and $Na^{125}I$ were from New England Nuclear Co.

II. Protein Measurement

All protein determinations were made by the method of Lowry et al. (1955) using bovine serum albumin (BSA) fraction V as standard.

III. Immunization of Rabbits to Generate Antiserum

Synthetic camel β-endorphin was conjugated with bovine serum albumin (BSA) using glutaraldehyde as decribed by Reichlin et al. (1968) for ACTH. This complex (500 µg of β-endorphin equivalent per rabbit) was emulsified in Freund's complete adjuvant and injected into two young rabbits at multiple intradermal sites. Booster injections were given similarly every 3-4 weeks. Twelve days after the third immunization, the animals were bled and sera tested for immunoreactivity. All the radioimmunoassays were performed using antiserum coded [1-6].

IV. Iodination of β-Endorphin

^{125}I-β-endorphin was prepared by a modified lactoperox-

idase method of Thorell and Johannson (1971), using 1 mCi (25 µl) of $Na^{125}I$, 5 µg (5µl) of β-endorphin in 0.05 M phosphate buffer, pH 7.4, 5 µg (5µl) lactoperoxidase in 0.05 M phosphate buffer, pH 7.4; 25 µl 0.5 M phosphate buffer, pH 7.4; and 5 µl of 30% hydrogen peroxide at 1:15,000 dilution, after reaction for 3-4 minutes, another 5 µl of 30% hydrogen peroxide at 1:15,000 dilution was added. The reaction was terminated after 3-4 minutes by diluting the reactants with excess cold buffer (1 ml ice cold phosphate buffer, pH 7.4). Unreacted iodide and damaged hormone were separated from intact iodinated hormone by gel filtration on Sephadex G-50 column (Medium, 1.8x60 cm) or Sephadex G-25 column (1x50 cm) using 0.05 M phosphate buffer pH 7.4 as eluting buffer. This separation step must be done at 4^0C. The Sephadex G-50 column was pretreated immediately before use with 1-2 ml of 0.05 M phosphate buffer, pH 7.4, containing 2.5% bovine serum albumin (BSA) in order to minimize the loss of iodinated proteins. In order to determine the specific activity of iodinated hormone, 10 µl of the iodination reaction mixture was removed prior to its application to the Sephadex column. This aliquot was diluted with 1 ml of 0.01M phosphate buffered saline, pH 7.4, containing 0.1% BSA. A 0.1 ml aliquot of this solution was diluted further (1:10) with 0.1% BSA in PBS, pH 7.4. Finally two 0.1 ml aliquots were removed to determine the radioactive counts. To each was added

0.1 ml 2.5% BSA in PBS and 2 ml of 10% trichloroacetic acid (TCA). After 3 hours of incubation at 4°C, the total radioactivity in each sample was determined and then each tube was centrifuged at 2000 g for 30 minutes, the supernatant was decanted and the precipitate counted in an LKB autogamma counter. The incorporation of radioactivity into protein is expressed as a percentage by dividing the TCA precipitable radioactivity by the precount of the TCA reaction mixture and multiplying by 100. The specific activity of the iodinated hormone is defined as the total TCA precipitable counts divided by the amount of protein used for iodination (5 µg). The percentage of incorporation of radioactivity into camel ß-endorphin was approximately 70-80% and its specific activity was 80-100 µCi/µg. An alternate method for estimating the percentage of incorporation is by a charcoal test which is performed as follows. Take 10 µl of the reaction which is diluted with 1 ml 0.14 M phosphate buffer containing 0.5%BSA, then take 0.1 ml of this solution and add 0.9 ml phosphate buffer. Remove 0.1 ml of this solution and add 0.1 ml Trasylol, 0.2 ml phosphate buffer with 0.5% BSA and 1 ml dextran coated charcoal and incubate at 4°C for 2-3 hours. The suspension is centrifuged at 2000 g for 30 minutes, the supernatant aspirated and the pellet counted in a LKB autogamma counter.

V. Preparation of Dextran Coated Charcoal

Wash 250 mg charcoal with distilled water 20 ml, spin the mixture at 2000 g for 15 minutes, decant the supernatant, then add phosphate buffer (assay buffer for β-endorphin RIA) to 100 ml, then add Dextran T-70 25 mg and mix well.

VI. Radioimmunoassay for β-Endorphin

A double antibody radioimmunoassay was used. The diluent for all reagents was 0.14 M sodium phosphate buffer containing 25 mM EDTA and 0.5% BSA, pH 7.4 (PB). The radioimmunoassay was carried out in glass tubes (10x75 mm) containing 0.1 ml of PB, 0.1 ml of Trasylol (5,000 K.I.U./ml), 0.1 ml of camel β-endorphin standard or sample, 0.1 ml of ^{125}I-β-endorphin (approximately 30,000 cpm/0.1 ml), and 0.1 ml of anti-β-endorphin rabbit serum (1-6, diluted 1:10,000). This reaction mixture was incubated at 4^{0}C for 48 hours, then 0.1 ml of sheep anti-rabbit gamma-globulin serum(1:20 dilution) and 0.1 ml of normal rabbit serum (1:350 dilution) were added and the mixture was incubated 24 hours at 4^{0}C, the supernatant decanted and the pellet counted in an automatic LKB gamma-counter. When β-endorphin standards or samples were added in 0.1 N acetic acid (0.1 ml), the results

of standard curves were identical to those obtained when samples were added in neutral solutions.

VII. Preparation of Brain Samples

Rats were weighed and then inserted into a brass cylinder with only the head exposed. The cylinder contains the rats were placed in a microwave oven (Philips model HN 1124) and microwave irradiation was applied at a power of 2.2 KW for 4 seconds followed by 10 seconds at half power. The brain was removed, and when necessary dissected according to Glowinsky et al. (1966). The weight of each rat brain or different regions of rat brain were determined on a Mettler H51 balance. All tissues were homogenized in 0.1 N acetic acid with a polytron set at 6. When the whole brain was homogenized, 5 ml of acetic acid were employed, while 1 ml was used for the midbrain, hindbrain, hypothalamus, and the pituitary. For the other regions of the rat brain (except midbrain, hindbrain, hypothalamus, and pituitary) 3 ml of acetic acid were employed. The homogenates were centrifuged at 2,000 g for 60 minutes, the supernatants removed and recentrifuged under the same conditions to obtain a second supernatant referred to as extract 1. The pellet obtained after the first centrifugation was reextracted using

the same conditions and then centrifuged at 2,000 x g for 60 minutes. The supernatant obtained was recentrifuged as before and final supernatant obtained was designated extract 2. The two extracts were assayed separately and the amount of endorphin in each supernatant was added to obtain the total endorphin present.

VIII. Gel Filtration

Extracts of the whole brain of intact or hypophysectomized (hypox) male rats and extracts of hypothalamus, hindbrain and midbrain of intact rats obtained as described were applied to a Sephadex G-50 column (2x100 cm). The column was equilibrated and eluted with 0.01 M phosphate saline buffer, pH 7.4, collecting 3.0 ml aliquots per fraction. Dextran blue 2000 was used to determine the void volume of the column. To calculate the partition coefficient (Kd) of β-endorphin, the standard formula was used, Kd=Ve-Vo/Vf-Vo in which Ve is the elution volume of the fraction, Vo is the void volume (blue dextran peak), and Vf is the elution volume of the smallest molecule (free iodide peak). The elution volume of β-endorphin was determined by measuring it in aliquots by radioimmunoassay (RIA). Alternatively the following radioactivity labeled

hormones were used to calibrate the column : ^{125}I-labelled β-endorphin, ^{125}I-labelled β-LPH and ^{125}I.

IX. Opiate Receptor Assay

(1) Receptor Preparation

Rats were killed by decapitation, the brains were quickly removed, the cerebellum was discarded and the remainder of the brain tissue was kept on ice. The brain tissue was homogenized in 0.05 M Tris buffer solution pH 7.4, 30 Vol./gm. using Elvejhen-Potter Teflon Pestle. The homogenate was centrifuged at 44,000 x g at 4°C for 20 minutes in a Beckman 60 Ti rotor. The supernatant was immersed in a water bath at 37°C for 1 hour. The supernatant was centrifuged again in a 60 Ti rotor at 44,000 x g at 4°C for another 20 minutes. The pellets were resuspected in 0.05 M Tris buffer pH 7.4 to make a final concentration of 30 mg brain tissue (wet weight)/ml.

(2) Opiate Receptor Assay

To each tube (glass 10x75 mm) was added 0.3 ml 0.05M Tris buffer pH 7.4, 0.1 ml of standard or unknown sample and 0.5 ml receptor. The suspension was incubated at 0°C(in ice bath) for 15 minutes, then 0.1 ml of ^{3}H-Naloxone (specific

activity 19.45 Ci/mmoles, precount : 15,000-18,000, about 5 nM in each tube) was added and the incubation continued for another 60 minutes at 0^0C (ice bath). The mixture was centrifuged at 2000 g for 30 minutes and the supernatant discarded. To each tube containing the remaining the pellet, 0.3 ml 2 N KOH was added and the tubes were placed in a boiling water bath for 10 minutes, until the solutions became clear. Then 0.2 ml of the solubilized extract was added to 10 ml scintillation fluid and the samples were counted in a Scintillation counter. (Scintillation fluid : Mixture of 2 liters of toluene, 1 liter of ethyleneglycol monoethyl ether, 15 grams of PPO and 1.5 grams of POPOP).

X. Preparation of Animals

In the experiments in which the effects of thyroid function were examined on brain β-endorphin content, male Sprague Dawley rats (Canadian Breeding Farms, MOntreal, Que.), 30 days of age were used. These rats were treated with daily injections of 10 µg L-thyroxine T_4/100g body weight administered intraperitoneally for 1 month (The T_4 preparation was prepared by making up a 1 mg/ml solution of L-thyroxine using 0.06 N NaOH as diluent. This stock solution was further diluted with 0.9 % NaCl to make

a final concentration of 25 µg L-thyroxine/ml. After 1 month treatment, all rats were killed by microwave irradiation between 10:30 A.M. and 12:00 noon. Two or three days before sacrifice using retroorbital blood samples were obtained for determination of T_3 and T_4 levels by radioimmunoassay. Hypox rats were killed $7\frac{1}{2}$ months after hypophysectomy. Adrenalectomized, pinealectomized, ovariectomized, and orchiectomized rats were killed one month after operation. After adrenalectomy, rats were offered 0.9% saline in their drinking water. The body weights of these animals were between 250 to 300 grams. The completeness of operations was examined at autopsy by gross inspection. In the experiments on the influence of ovaries, vaginal smears were taken daily and these were examined after staining with 1% Toluene Blue in 3% acetic acid for 1 minute. Pinealectomy was done using a slight modification of Kuzak's method (Kuzak et al. 1977). Samples were obtained by orbital bleeding technique as described by Riley (1960). Approximately 15 minutes after intraperitoneal injection of morphine (10 mg/kg of rat body weight) or naloxone (5mg/kg of rat body weight), rats were killed by microwave irradiation. Pentobarbital (35 mg/kg of rat body weight) was given intraperitoneally to another group of rats and 45 minutes later the animals were killed by microwave irradiation. All three groups of rats receiving drugs were compared with the control group of rats

which received an injection of saline 1 ml intraperitoneally 15 minutes before microwave irradiation.

XI. Conversion Factors

1 nanomole β-endorphin = 3.5 µg β-endorphin
1 nanomole enkephalin = 0.6 µg enkephalin
1 nanomole β-LPH = 9.5 µg β-LPH
1 nanomole ACTH = 4.5 µg ACTH
1 nanomole 31K =31 µg 31K

1 µg β-endorphin = 286 picomoles β-endorphin
1 µg enkephalin = 1666 picomoles enkephalin
1 µg β-LPH = 105 picomoles β-LPH
1 µg ACTH = 222 picomoles ACTH
1 µg 31K = 32 picomoles 31K

XII. Statistical Analysis

Statistical calculations were done by using a one way analysis of variance, followed by the Dunnet's or Tukey's tests for multiple comparisons. The level of significance is indicated

as follows :

"**" $p<0.01$ in Dunnet's test or Tukey's test.

" *" $p<0.05$ in Dunnet's test or Tukey's test.

RESULTS

OPIATE RECEPTOR ASSAY

Fig. 5 shows the relative potency of human and camel β-endorphin in competing with ^{3}H-naloxone for binding sites on the opiate receptor. This opiate receptor was slightly modified from the method described. The rat brain receptor was prepared by resuspending the 100,000 g pellet obtained from centrifuging rat brain extracts, such that each tube contains 10 mg protein and using ^{3}H-naloxone as tracer (precount : 25,000 cpm). Human β-endorphin is more potent than camel β-endorphin in displacing ^{3}H-naloxone from rat brain opiate receptors.

In Fig. 6, the opiate receptor assay was done exactly as described in the "Materials and Methods" section. This method provided greater sensitivity compared to the method used to obtain the data shown in Fig. 1, moreover, a smaller amount of receptor was required. As shown in Fig. 6, camel β-endorphin is more effective in competing for naloxone binding sites than Met-enkephalin or α-endorphin.

CHARACTERIZATION OF ^{125}I-β-ENDORPHIN

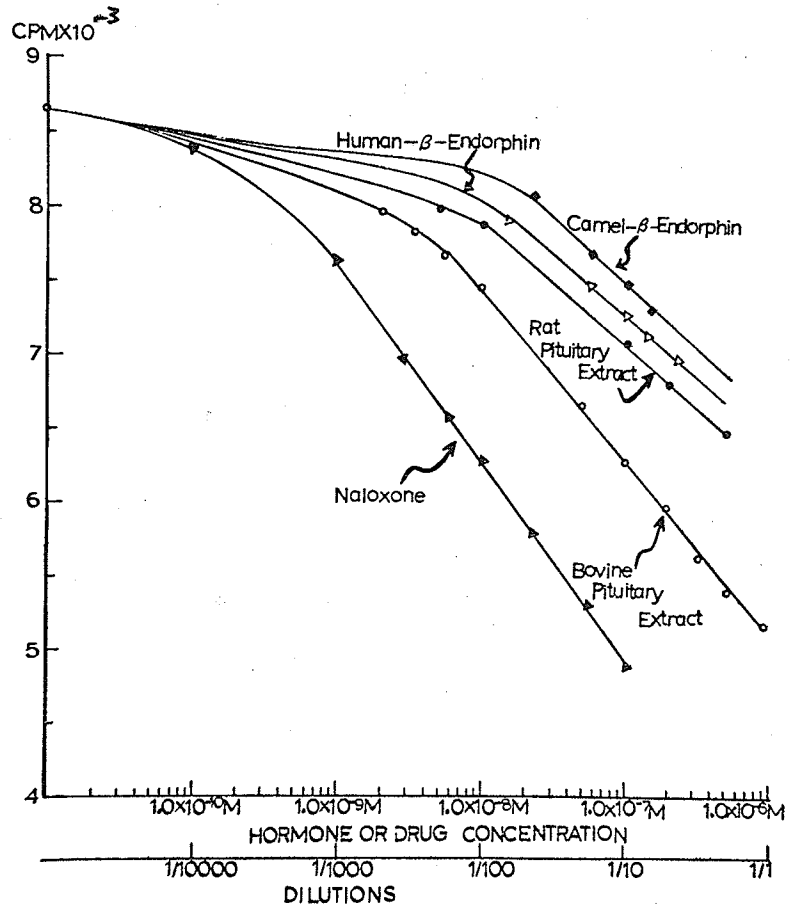

Fig. 5 : Radioreceptorassay for Opiates and Endogenous Opiates Using Crude Rat Brain Membrane Preparation as Receptor. (Pituitary extracts were diluted as indicated).

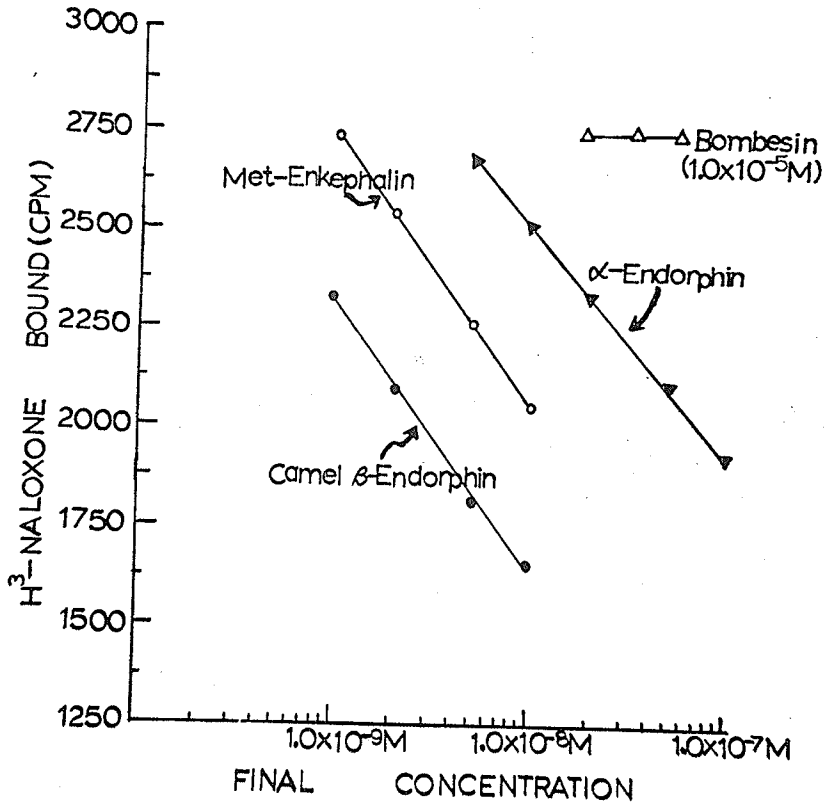

Fig. 6 : Competition of Camel β-Endorphin, Met-enkephalin and α-Endorphin to Naloxone Binding Sites in Rat Brain.

The opiate receptor assay was done as described, using ^{3}H-Naloxone as tracer. All standards were diluted in Tris HCl containing 0.1% BSA. Each point represents the mean of triplicates. Bombesin does not compete for binding to opiate receptors even at 16.2 µg/ml (1.0×10^{-5} M, final concentration).

(1) Iodination profile

As shown in Fig. 7, the iodination mixture when passed through Sephadex G-50 column (1.8x60 cm) is resolved into three peaks. The major peak is the iodinated camel-β-endorphin which is well separated from free unreacted iodide. Generally, the incorporation of radioactivity into camel β -endorphin is around 75%.

(2) The identification of a "satisfactory tracer"

A satisfactory tracer will exhibit 85% specific binding and less than 5% nonspecific binding in charcoal test when excess antibody is present. Similarly greater than 80% specific binding and about 2% nonspecific binding is observed in a double antibody test with excess 1st antibody (1:100). Aliquots of the tubes containing the ^{125}I-β-endorphin after gel filtration are used to determine which fraction contains the most immunoreactive tracer. When using an antibody titer of 1:10,000 in double antibody test, a satisfactory tracer generally will have more than 40% specific binding and less than 5% nonspecific binding.(here specific binding = 0 count-blank /total count, 0 count : without cold hormone, blank : without cold hormone and antibody).

(3) Testing of antisera for proper dilution

When serial dilutions of camel β-endorphin antiserum coded [1-6] were used, dilution of 1:10,000 produced more than 30%

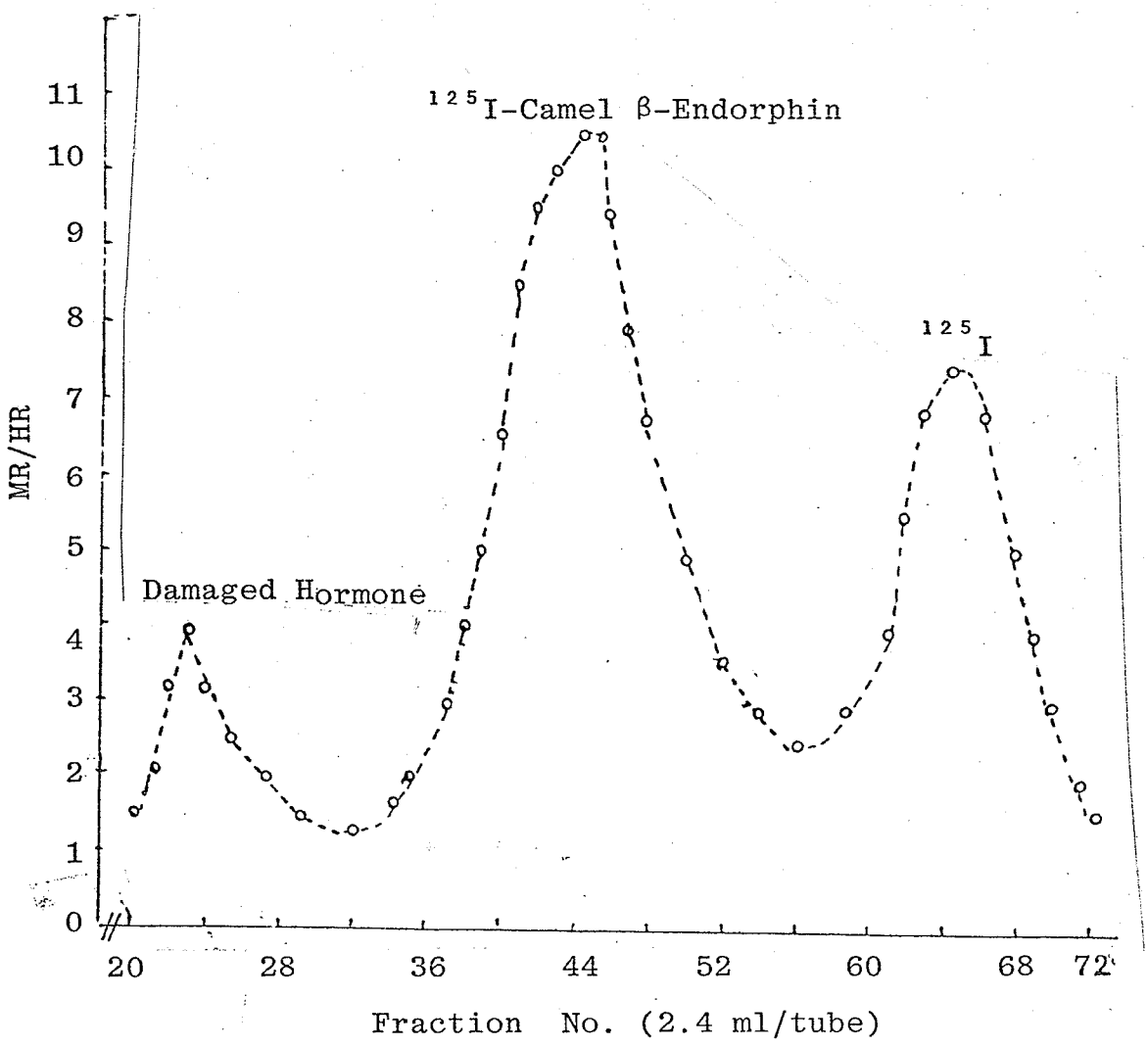

Fig. 7 : Distribution of Radioactivity After Iodination of Camel β-Endorphin.. The elution position of ^{125}I-camel β-endorphin and its separation from "damaged hormone" and free ^{125}I is shown. The iodination mixture was passed through G-50 column (Medium 1.8 x 60 cm) using 0.05 M phosphate buffer pH 7.4 as eluting buffer.

specific binding and less than 5% nonspecific binding with a sensitivity around 0.8 ng/ml to 1 ng/ml.

CHARACTERIZATION OF RADIOIMMUNOASSAY FOR β-ENDORPHIN

(1) Sensitivity : The sensitivity of the assay is 0.8 ng/ml to 1 ng/ml as shown in Fig. 8-12. and the standard curve is linear between 1 ng/ml and 40 ng/ml.

(2) Specificity : No cross reactivity with α- and γ-endorphin, both enkephalins, α-MSH, ACTH, vasopressin, insulin, glucagon, TRH, LH-RH, bombesin, myelin basic protein, growth hormone, prolactin, morphine and naloxone. However, it has a 50% cross reactivity with ovine β-lipotropin (as shown in Fig. 8), and 30% cross reactivity with human β-endorphin on a molar basis (data not shown). Moreover, some cross reaction with the 31K precursor and its breakdown products may also occur.

(3) Reproducibility : The interassay variations using camel β-endorphin standards dissolved in PB are summarized in Table 13. For a concentration of 3.7 ng/ml and 9.7 ng/ml, the coefficient of variation was 10% and 17% respectively. The interassay variation using a pituitary extract is 12% at 16± 2 ng/ml and using a hypothalamic extract is 14% at concentration of 2±0.3 ng/ml and 12% at 1.2±0.1 ng/ml.

(4) Precision : The intraassay variation is 3.8% at 65.5±2.5ng/ml

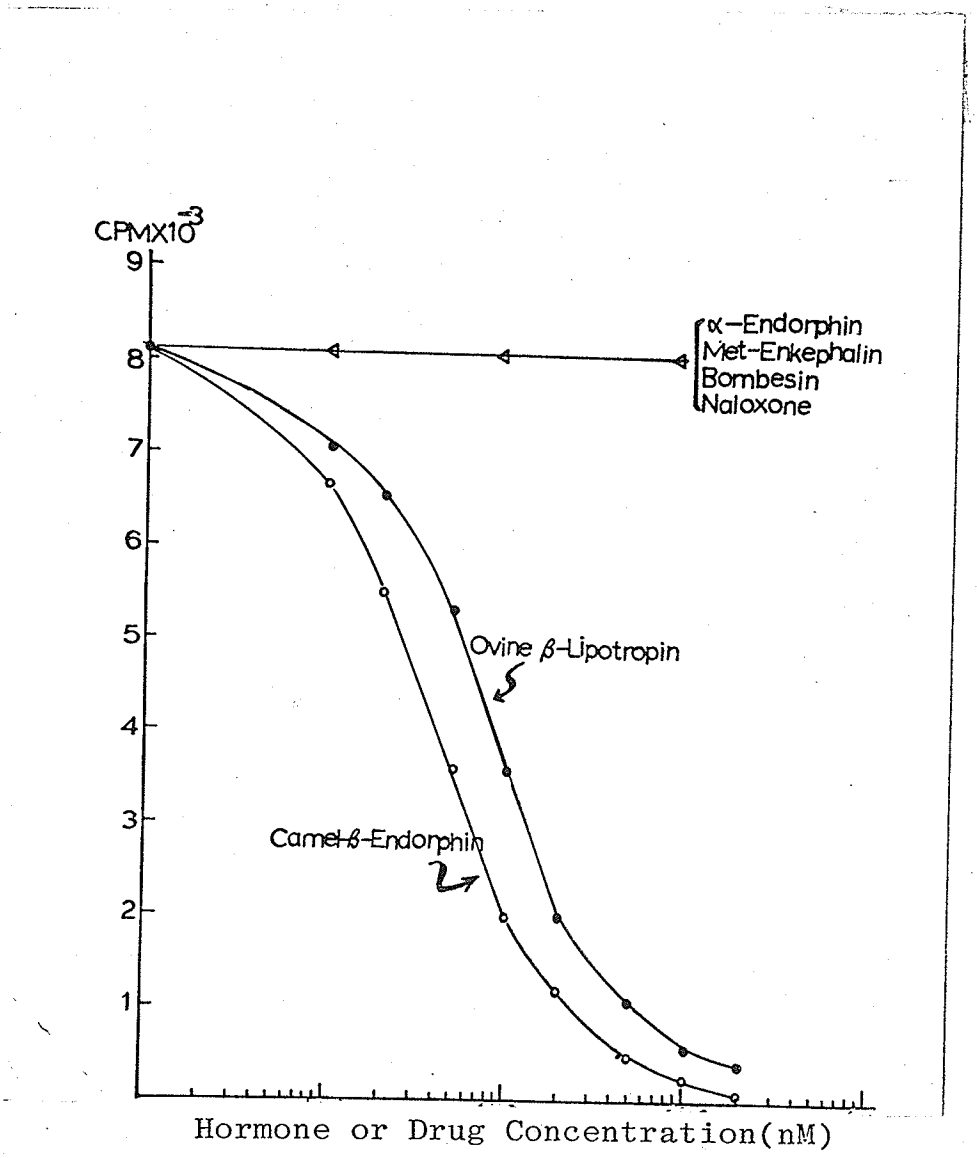

Fig. 8 : Characterization of Camel β-Endorphin Antiserum (1-6).

and 5.5% at 10.0±0.6 ng/ml.by using the camel β-endorphin standard.

DEVELPOMENT OF OPTIMAL PROCEDURES FOR OBTAINING RAT BRAIN AND EXAMINATION OF EFFICIENT EXTRACTION METHODS TO MAXIMIZE YIELD OF ENDORPHIN

As shown in Table 14, increased concentrations of acetic beyond 0.1 N did not increase the yield of β-endorphin. When the rats were killed by decapitation, followed by rapid removal and boiling brain tissue, the concentration of β-endorphin in these extracts was about 3 times less than when an identical extraction procedure was used but the rats were killed by microwave irradiation. Similar results were obtained when rats were killed by decapitation and the head immediately exposed to microwave irradiation (Table 8). If the rats were killed by decapitation but the brain tissue was not boiled prior to extraction, the value of immunoreactive β-endorphin was even lower(about 5 times) than when rats were killed by decapitation and the brain tissue boiled (data not shown). When rats were killed by decapitation with a guillotine it didn't matter whether the brain was kept on ice bath, liquid nitrogen or dry ice, the estimates of β-endorphin in extracts from these tissues were uniformly low. As shown in Table 15,

Table 13 : Interassay and Intraassay Variation of Radioimmunoassay for β-Endorphin

Concentration of β-Endorphin (ng/ml)						
Individual Values in the Same Assay		Individual Values in Different Assays				
β-Endorphin Standard		β-Endorphin Standard		Pituitary Extract	Hypothalamic Extract	
60	9.3	4.1	10.5	18.0	2.8	1.3
66	9.6	3.8	10.5	17.0	2.2	1.0
64	9.8	3.3	7.2	14.0	2.2	1.2
68	11.1	2.9	8.1	19.8	2.3	1.4
66	9.6	4.5	10.0	14.3	1.7	1.1
68	10.3	3.5	7.6	15.3	2.5	1.0
66	10.4	3.5	8.2	19.0	2.0	1.2
66	9.7	3.7	12.2	14.5		
	10.1	3.4	9.1	17.2		
	10.3	3.6	10.8	14.1		
		4.1	10.4			
		4.3	11.0			
		3.8	10.9			
Mean ± S.D.	65.5 ± 2.5	3.7 ± 0.4	9.7 ± 1.6	16.o ± 2.0	2.0 ± 0.3	1.2 ± 0.1
	3.8%#	10%*	17%*	12%*	14%*	12%*

#:Intraassay Variation
*: Interassay Variation

TABLE 14: COMPARISON OF DIFFERENT METHODS OF KILLING RATS AND OF DIFFERENT ACETIC ACID EXTRACTIONS ON β-ENDORPHIN CONCENTRATIONS

No. of rats	Method of Killing Rat	Extraction Buffer	Method of Processing	Camel β-endorphin Immunoreactivity ng/g wet weight of Brain Tissue Mean ± S.D.
5	Microwave (3"FP,6"HP)	0.1 N Acetic Acid	2000g, 30' Neutralization then 15,000g,20'	237 ± 84
5	Microwave (3"FP,6"HP)	0.5 N Acetic Acid	2000g, 30' Neutralization then 15,000g,20'	190 ± 74
5	Microwave (3"FP,6"HP)	1 N Acetic Acid	2000g, 30' Neutralization then 15,000g,20'	87 ± 58
4	Guillotine Decapitation then Boiling Brain Tissue 15' at 95°C 1 N Acetic Acid	1 N Acetic Acid	2000g, 30' Neutralization then 2000g, 30'	29 ± 9.6

FP : Full Power.

HP : Half Power.

TABLE 15 : POSTMORTEN CHANGE IN RAT BRAIN β-ENDORPHIN CONTENT

Rat	Treatment	Wet weight of Rat Brain(grams)	β-endorphin (ng) 1st Extract	β-endorphin (ng) 2 nd Extract	Sum of 1st + 2 nd Extracts ng/g
1	Without Boiling	1.92	2.8	5.8	4.48
2	Without Boiling	2.0	2.9	7.0	4.95
3	With Boiling	1.73	5.1	2	4.11
4	With Boiling	1.88	6.1	2.2	4.41

when the rats were decapitated and the brain was kept at 4°C for 1½ hours before extraction, the value of β-endorphin found in extracts is about 1/6 the values obtained when similar extractions were carried out immediately after decapitation.

As shown in Table 16, a second extraction of rat brain tissue led to a greater yield of β-endorphin and also decreased the coefficient of variation. Approximately 80% of total immunoreactive β-endorphin in total rat brain was found in the first extract and the remaining 20% in the second. The recovery of exogenously added endorphin using a double extraction method as described in "Preparation of Brain Samples" is 93%. The power of mocrowave irradiation needed to kill the animals varied with the size and body weight of the rats.

Having established the conditions which appeared to provide maximal yields of β-endorphin in whole brain extracts, I proceeded to examine regional brain concentrations of β-endorphin using these methods.

MEASUREMENT AND CHARACTERIZATION OF β-ENDORPHIN IN BRAIN REGIONS OF MALE RATS

Apart from the pituitary, the highest concentrations of β-endorphin were found in the hypothalamus, followed by the

TABLE 16: DOUBLE EXTRACTION OF BRAIN TISSUE SAMPLES FOR β-ENDORPHIN

Rat	Wet Weight of Total Brain (Including Cerebellum) g	β-endorphin (ng) (1st Extract)	β-endorphin (ng) (2nd Extract)	Sum of 1st & 2nd Extracts ng	ng/g
1	1.82	305	82	387	212
2	1.78	1046	246	1292	726
3	1.67	1131	189	1320	790
4	1.87	586	165	752	402
5	1.75	746	246	992	567

hindbrain and midbrain, with the lowest concentrations of β-endorphin in the cerebellum, striatum, septal nuclei and pineal body (Table 17).

Fig. 4 shows the distribution of β-endorphin immunoreactivity after gel filtration of brain extracts of one intact and four hypox rats. A major peak representing 98% of the immunoreactivity coelutes with synthetic β-endorphin in both extracts with only a minor peak eluting immediately after the void volume. No immunoreactivity is detectable in fractions which coelute with β-LPH. The same pattern is observed when extracts of hypothalamus, midbrain or hindbrain are applied to the same column (Fig. 10).

Serial dilutions of brain extracts of intact and hypox rats yield parallel curves to the standard curves for camel β-endorphin in the radioimmunoassay (Fig. 11).

In Fig. 12, serial dilutions of extracts of hypothalamus, midbrain, pituitary and hindbrain also yield curves parallel to the standard curve for camel β-endorphin.

CHANGES IN β-ENDORPHIN CONCENTRATIONS OF RAT BRAIN REGIONS WITH ALTERATION OF ENDOCRINE STATUS

(1) Hypophysectomy

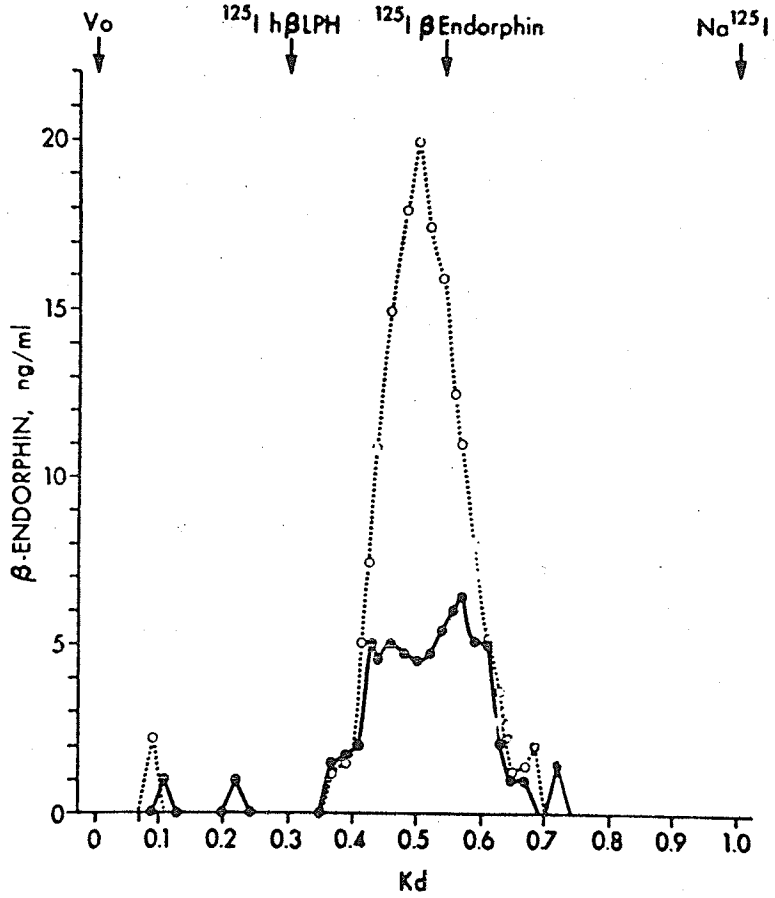

Fig. 9 : Gel Chromatography of Brain Extracts from One Intact (o--o) or Four Hypox Rats.(o—o) on Sephadex G-50 Column (2x100 cm) using 0.01 M Phosphate Saline pH 7.4 as Buffer.

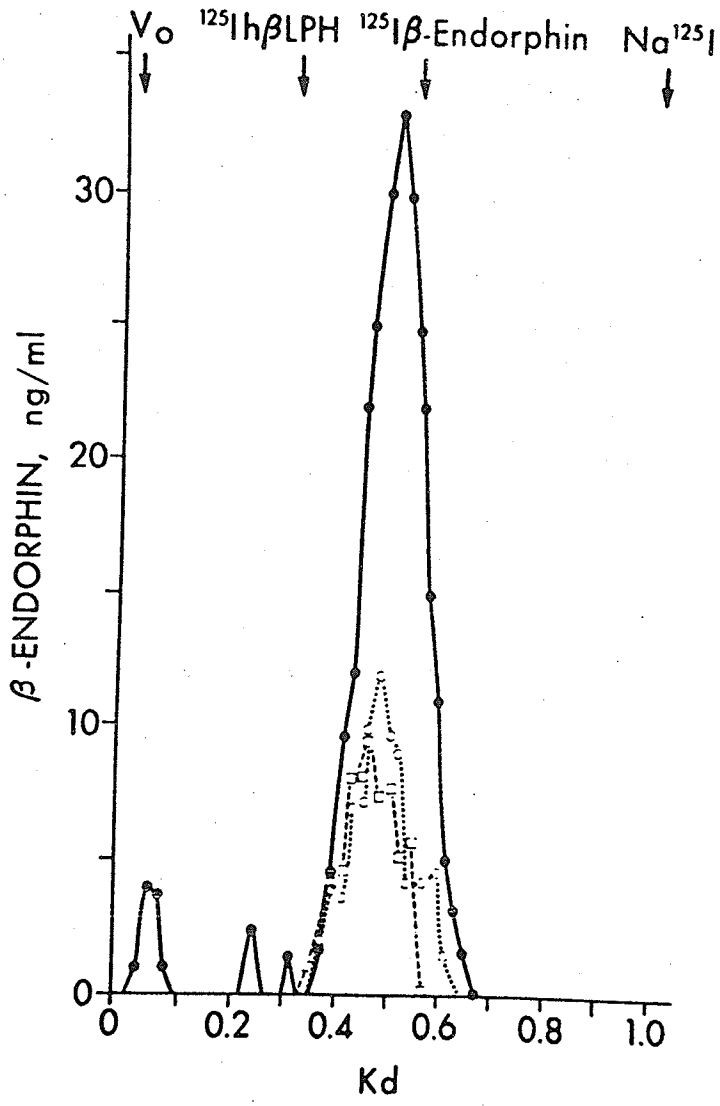

Fig. 10 : Gel Chromatography of Hypothalamic (o—o), Midbrain (o--o) and Hindbrain (□--□) Extracts.

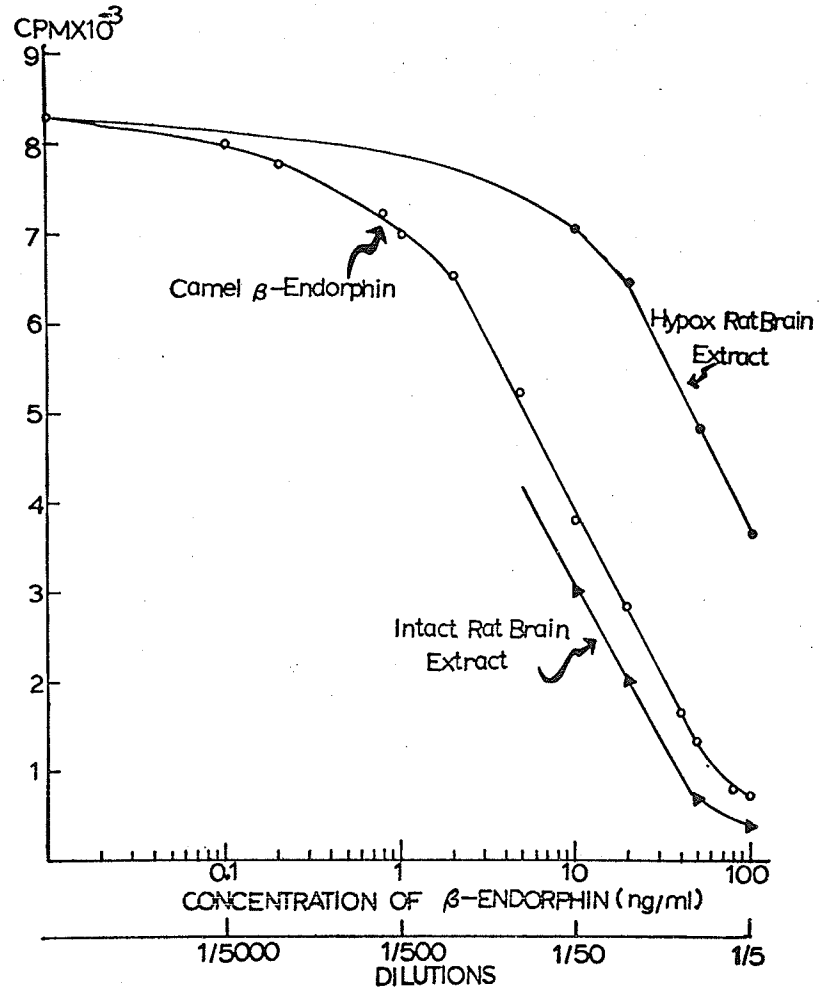

Fig. 11 : Serial Dilutions of Brain Extracts from Intact and Hypox Rats Yield Curves Parallel to the Standard Curve for Camel β-Endorphin

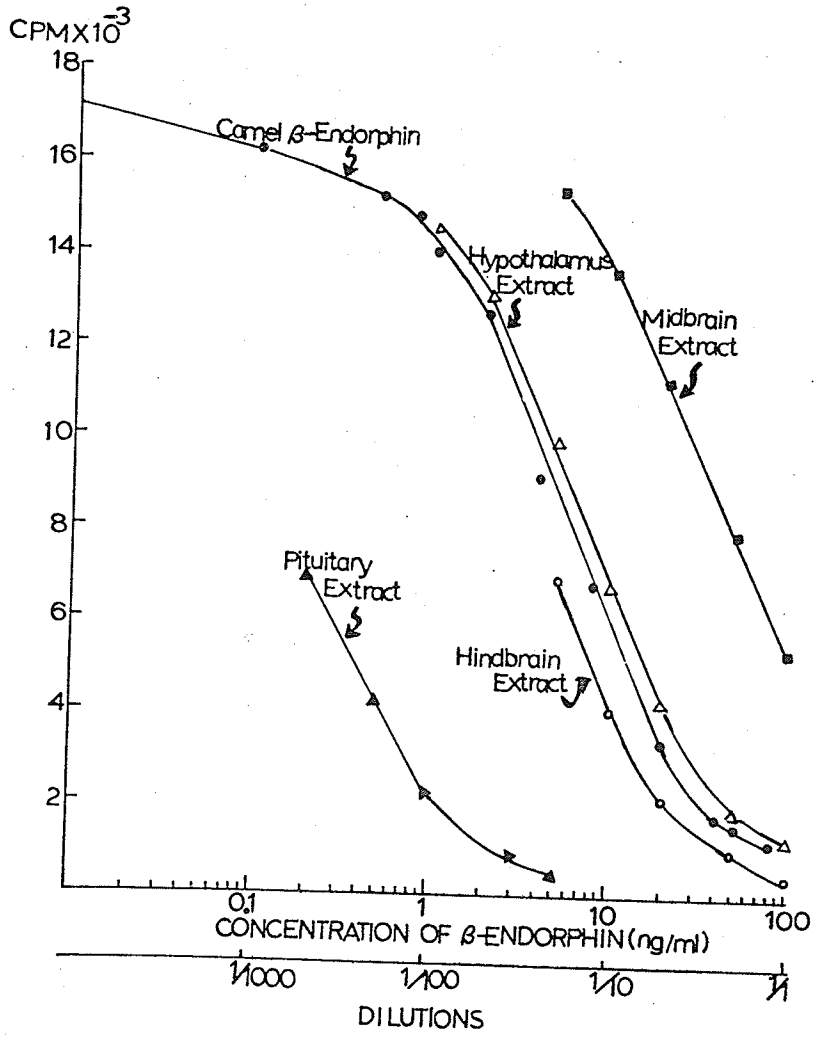

Fig. 12 : Serial Dilutions of Extracts of Midbrain, Hindbrain, Hypothalamic and Pituitary Yield Curves Parallel to the Standard Curve for Camel β-Endorphin.

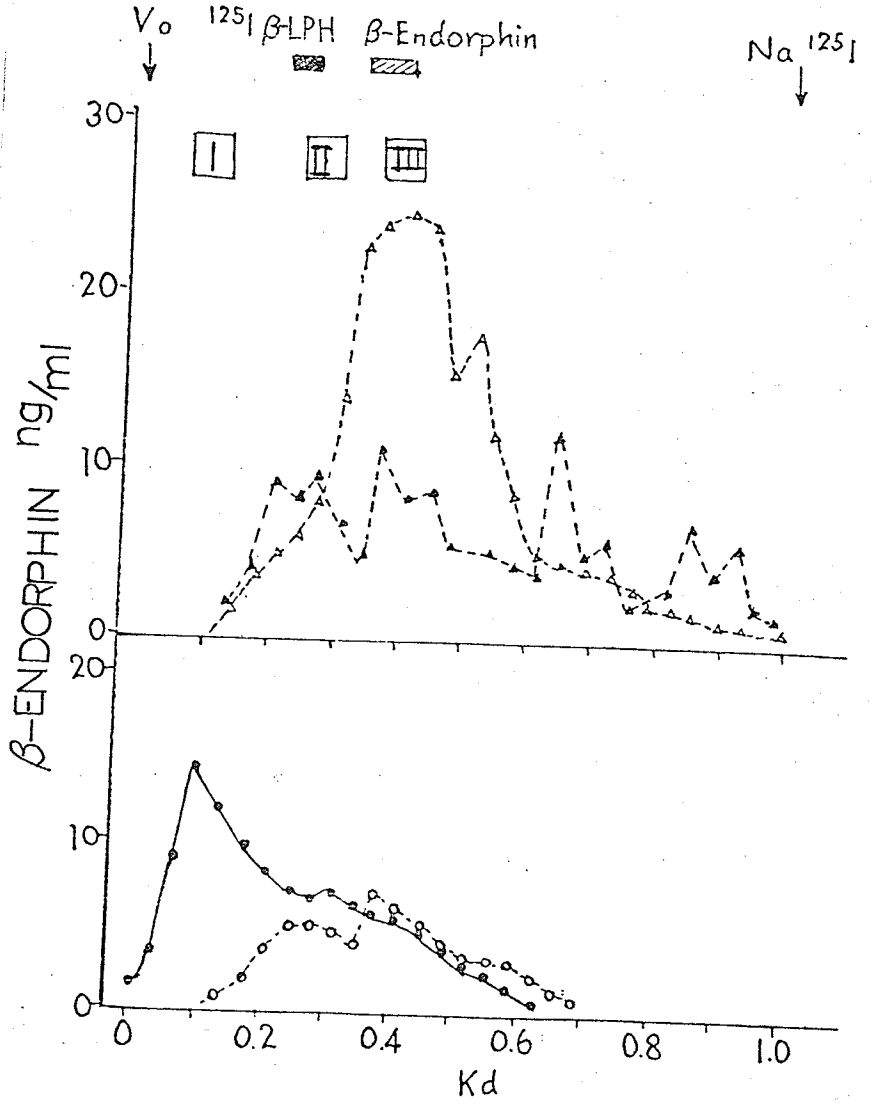

Fig. 13 : Elution Pattern of Immunoreactive β-Endorphin of Incubation Media and Acid-boiled Extracts of Incubated Pituitary Gland. Upper panel: (▲--▲) extract of incubated neural intermediate lobe; (▲---△) incubation medium from intermediate lobe. Lower panel : (●--●) extract of incubated anterior pituitary lobe; (○---○) incubation medium from anterior lobe. (Ogawa N. et al. 1979).

TABLE 17 : β-ENDORPHIN IMMUNOREACTIVITY IN RAT REGIONS

	β-Endorphin		
	*ng/Region	*ng/gTissue	*ng/mg Protein
Cerebellum(4)	22±6	77±20	1.1±0.2
Hindbrain(5)	142±15	628±73	12.8±1.7
Midbrain(5)	92±38	573±243	9.5±3.9
Hypothalamus(5)	252±39	4945±796	70.6±9.8
Thalamus + Subthalamus	14±3	260±52	3.5±0.8
Striatum, Septal Nuclei & Pineal Body (5)	9±1	87±12	1.5±0.4
Hippocampus + Cortex (5)	67±14	71±24	3.9±1.7
Whole Brain(5)	601±103		

( ) : No. of rats

* : Mean±S.E.M.

Table 18 shows that hypophysectomy causes a dramatic ten fold reduction in brain β-endorphin concentration (540 ng/g vs 57 ng/g). These results are similar to the differences observed by Ogawa et al. who also used microwave irradiation to kill the rats (Table 19), but who used somewhat different methods to prepare brain extracts. As early as 7 days after hypophysectomy, there has already been a 50% reduction in the concentration of β-endorphin in the hypothalamus. The hindbrain, midbrain, hippocampus, cortex and cerebellum also show a significant decrease in β-endorphin concentration 28 days after hypophysectomy. At two months after hypophysectomy, very little further change in β-endorphin concentration is noted in different brain regions.

(2) Thyroidectomy and T_4 Treated Rats

Rats 30 day old were thyroidectomized or treated with T_4 and killed one month later. As shown in Table 20, thyroidectomized rats gained less weight than controls, but had significantly greater β-endorphin in the hypothalamus (4.8 μg/g vs 11 μg/g, $p<0.01$). The completeness of thyroidectomy was confirmed by low plasma T_4 levels 0.1 to 1.5 μg/100 ml, compared to the normal level in control rats of 6 to 10 μg/100 ml. In T_4 treated rats, significant increases of β-endorphin were found in the midbrain (0.35 μg/g vs 0.60 μg/g, $p<0.05$), hypothalamus (4.8 μg/g vs 7.6 μg/g, $p<0.05$) and pituitary (187 μg/g vs 290 μg/g,

TABLE 18: EFFECT OF HYPOPHYSECTOMY ON BRAIN CONCENTRATIONS OF β-ENDORPHIN IMMUNOREACTIVITY

			β-endorphin	
	No. of Rats	Average Wet Wt. of Rat Brain (Including Cerebellum) g	*ng/Region	*ng/g Tissue
Control	5	1.8	1026 ± 178	540 ± 106
7½ Months After hypophysectomy	4	1.5	88 ± 12	57 ± 4

* : Mean ± S.E.M.

TABLE 19 : EFFECT OF HYPOPHYSECTOMY ON BRAIN CONCENTRATIONS OF β-ENDORPHIN IMMUNOREACTIVITY

	* ng/mg Protein			
	Days After Hypophysectomy			
	Control (6)	7 (6)	28 (6)	60 (6)
Cerebellum	0.38±0.01	0.38±0.01	0.1±0.1	0.2±0.11
Hindbrain	1.95±0.44	2.2 ±0.67	0.6±0.09	0.9±0.03
Midbrain	7.6 ±3.3	2.6 ±0.9	0.9±0.2	1.2±0.4
Hypothalamus	46.7 ±8.6	23.5 ±1.5	7.4±0.4	7.5±1.9
Thalamus & Subthalamus	1.3 ±0.2	5.8 ±1.1	1.4±0.2	1.6±0.5
Striatum	0.1 ±0.03	1.9 ±0.04	0.2±0.02	0.4±0.08
Hippocampus	0.6 ±0.3	0.5 ±0.08	0.3±0.03	0.4±0.03
Cortex	0.5 ±0.1	9.3 ±0.05	0.3±0.04	0.2±0.02

* : Mean±S.E.M. ( ) : No. of rats

(By Ogawa N. et al. 1979)

TABLE 20: EFFECT OF THYROIDECTOMY OR T_4 TREATMENT ON THE CONCENTRATIONS OF RAT BRAIN β-ENDORPHIN

	β-endorphin ng/g Tissue		
	Control (9)	Thyroidectomy (13)	T_4 Treated (10)
Midbrain	354 ± 57#	320 ± 63	595 ± 80*
Hindbrain	561 ± 72	498 ± 108	833 ± 148
Hypothalamus	4835 ± 493	10991 ± 1819**	7642 ± 1101*
Pituitary	187313 ± 14159	215796 ± 27730	289845 ± 36231*
Remaining Regions	54 ± 13	39 ± 6	99 ± 29

: Mean ± S.E.M. ( ) : No. of rats

* : $p < 0.05$ ** : $p < 0.01$

The average body weight of control rats is 250 grams, of thyroidectomized rats is 190 grams and of T_4 treated animals is 300 grams.

$p<0.05$).

(3) Pinealectomy

All rats were killed at 120 days of age one month after pinealectomy at which time average body weights of both groups were comparable. No significant difference was noted in β-endorphin in midbrain, hindbrain, hypothalamus or pituitary in both groups (Table 21). A significant difference between the two groups was seen in the remaining brain area.

(4) Adrenalectomy (Table 22)

One month after adrenalectomy very significant increases in β-endorphin were found in the pituitary (359 μg/g vs 540 μg/g, $p<0.01$), hindbrain (0.7 μg/g vs 1.5 μg/g, $p<0.01$), hypothalamus (3.9 μg/g vs 8.2 μg/g, $p<0.01$), and midbrain (0.53 μg/g vs 0.86 μg/g, $p<0.05$).

(5) Orchiectomy and Ovariectomy

A significant decrease of β-endorphin immunoreactivity was found in the pituitary one month after orchiectomy from 359 μg/g to 189 μg/g ($p<0.01$). Table 23.

No significant difference in β-endorphin was seen between

TABLE 21 : EFFECT OF PINEALECTOMY ON BRAIN CONCENTRATIONS OF β-ENDORPHIN

	β-endorphin ng/g Tissue	
	Control (4)	Pinealectomy (4)
Midbrain	326 ± 70#	507 ± 176
Hindbrain	553 ± 70	508 ± 104
Hypothalamus	7370 ± 437	6200 ± 409
Pituitary	274010 ± 20286	233740 ± 40107
Remaining Regions	61 ± 14	151 ± 33*

: Mean ± S.E.M.

* : $p < 0.05$

( ) : No. of rats

TABLE 22: EFFECT OF ADRENALECTOMY ON BRAIN CONCENTRATIONS OF β-ENDORPHIN

	β-endorphin ng/g Tissue	
	Control (13)	Adrenalectomy (10)
Midbrain	529 ± 76#	863 ± 196*
Hindbrain	680 ± 101	1535 ± 331**
Hypothalamus	3870 ± 920	8194 ± 1307**
Pituitary	358869 ± 28573	539648 ± 51203**
Remaining Brain Regions	105 ± 32	138 ± 44

: Mean ± S.E.M.

* : $p < 0.05$
** : $p < 0.01$

( ) : No. of rats

control and ovariectomized rats Table 23.

The male rats were 100 days of age when killed while the female rats were 165 days old.

(6) Naloxone, Morphine or Pentobarbital Administration

The results of administering each of these agents on brain endorphin concentration are shown in Table 24. After naloxone injection a significant increase of β-endorphin was found in the hypothalamus (4.7 μg/g vs 10.5 μg/g, $p<0.05$). No difference in β-endorphin were noted after morphine, but after pentobarbital administration a significant increase of β-endorphin was found in the pituitary. (390 μg/g to 607 μg/g, $p<0.05$).

(7) Effect of Sex and Age on β-Endorphin

As shown in Table 25, no significant sex differences in brain β-endorphin immunoreactivity was seen among 8, 24, and 60 day old male and female rats except for the midbrain where at 60 days of age a significant difference was found ($p<0.05$). However, β-endorphin immunoreactivity exhibits a very significant increase between day 24 and day 60 old rats in almost all regions in rat brain, but no significant difference was found

in β-endorphin between day 8 and 24 old rats.

TABLE 23 : EFFECT OF OVARIECTOMY AND ORCHIECTOMY ON BRAIN CONCENTRATIONS OF β-ENDORPHIN

	β-Endorphin ng/g Tissue			
	Female		Male	
	Control (6)	Ovariectomy (6)	Control (13)	Orchiectomy (10)
Midbrain	265 ± 75#	428 ± 152	529 ± 76	713 ± 181
Hindbrain	657 ± 183	333 ± 124	680 ± 101	468 ± 78
Hypothalamus	9734 ± 2053	9987 ± 1041	3870 ± 920	5158 ± 620
Pituitary	170816 ± 33601	165775 ± 11528	358869 ± 28573	189130 ± 24498**
Remaining Brain Regions	64 ± 16	101 ± 35	105 ± 32	89 ± 20

: Mean ± S.E.M.

** : $p < 0.01$

( ) : No. of rats per group

TABLE 24 : EFFECT OF NALOXONE, MORPHINE, PENTOBARBITAL ON BRAIN CONCENTRATIONS OF β -ENDORPHIN

	β -endorphin ng/g Tissue			
	Control (5)	Naloxone (5)	Morphine (4)	Pentobarbital (5)
Midbrain	718 ± 188#	771 ± 247	1268 ± 419	
Hindbrain	829 ± 242	414 ± 104		
Hypothalamus	4748 ± 852	10585 ± 2978*	3529 ± 1080	3599 ± 1063
Pituitary	390496 ± 47839	436801 ± 54167	425086 ± 7261	607903 ± 98600*
Remaining Brain regions	103 ± 20	128 ± 24	184 ± 43	183 ± 67

: Mean ± S.E.M.

* : $p < 0.05$

( ) : No. of rats

TABLE 25 : AGE AND SEX DIFFERENCES IN BRAIN CONCENTRATIONS OF β -ENDORPHIN

	β -endorphin ng/g Tissue					
	Age of Animals (days)					
	8	24	60	8	24	60
	Male			Female		
	(5)	(6)	(6)	(5)	(5)	(6)
Midbrain	N.D.	74 ± 9#	537 ± 147**	N.D.	79 ± 21	188 ± 34
Hindbrain	N.D.	230 ± 44	533 ± 65**	N.D.	188 ± 32	719 ± 289
Hypothalamus	1810 ± 519	2892 ± 565	5660 ± 1003*	1636±423	2712±303	6105 ± 1044*
Pituitary	214066 ±26714	117722 ±25160	334853** ±29522	257016 ±36981	118462 ±25185	364082** ±65575
Other Brain Regions	N.D.	23 ± 5	54 ± 12*	N.D.	15 ± 2	36 ± 4
Whole Brain (Cerebellum)	107 ± 30 (6)			53±16 (6)		

: Mean ± S.E.M.
( ) : No. of rats
* : $p < 0.05$
** : $p < 0.01$
N.D. : Not done

Significant differences were observed only when comparisons were made between different age groups within each sex.

TABLE 26 : AVERAGE VALUES OF ALL THE CONTROL MALE RAT GROUPS (SUMMARY)

Table No.	Age or Body Wt.	No.of Rat	Brain	Midbrain	Hindbrain	Hypothalamus	Pituitary	Remaining Regions
14		5	237#	N.D.	N.D.	N.D.	N.D.	N.D.
17		5	601	573#	628	4945	N.D.	N.D.
18	250-300 g	5	540	N.D.	N.D.	N.D.	N.D.	N.D.
20	250 g, Day 60	9	N.D.	354	561	4835	187313	54
21	430 g, Day 120	4	N.D.	326	553	7370	274010	61
22	430 g	13	N.D.	529	680	3870	358869	105
24	320 g	5	N.D.	718	829	4748	390496	103
25	271 g, Day 60	6	N.D.	537	533	5660	334853	54
Mean±S.D. of All the Controls			459 ± 246	500 ± 326	633 ± 303	4898 ± 2475	308345 ± 108825	79 ± 76

: ng/g of β-Endorphin Concentration (Mean)

N.D. : Not Determined

TABLE 27 : DATA COMPARISON BETWEEN AVERAGE VALUES OF CONTROL RATS AND DIFFERENT ENDOCRINE MANIPULATION, DRUG ADMINISTRATION AND AGE (SUMMARY)

Treatment	Age or Body Wt.	No.of Rat	Brain	Midbrain	Hindbrain	Hypothalamus	Pituitary	Remaining Regions
Thyroidectomy	190g, Day 60	13		320#	498	10991**	215796*	39
T_4 Treated	300g, Day 60	10		595	833	7642	289845	99
Pinealectomy	410g, Day120	4		507	508	6200	233740	151
Adrenalectomy	360g	10		863*	1535**	8194*	539648**	138
Orchiectomy	410g	10		713	468	5158	189130**	89
Naloxone	310g	5		771	414	10585**	436801*	128
Morphine	320g	4		1268**	N.D.	3529	425086	184
Pentobarbital	320g	5		N.D.	N.D.	3599	607903**	183
Day 8 Rats	17g	6	107**	N.D,	N.D.	1810	214066	
Day 24 Rats	57g	6				2892	117742**	23*
Hypophysectomy		4	56.8**					

: ng/g of β-Endorphin Concentration (Mean)

N.D. : Not Determined

* : $p<0.05$

** : $p<0.01$

DISCUSSION

RADIOIMMUNOASSAY OF β-ENDORPHIN

The radioimmunoassay system employed an antiserum raised against synthetic camel β-endorphin. Our assay can be considered homologous for rat β-endorphin since Seiteh has recently shown that the amino acid sequences of rat, ovine and camel β-endorphin are identical. No cross reactivity was evident with α- and γ-endorphin, both enkephalins, α-MSH, ACTH, vasopressin, insulin, glucagon, TRH, LH-RH, bombesin, myelin basic protein, growth hormone, prolactin, morphine and naloxone. However, a 50% cross reactivity with ovine β-lipotropin, 30% cross reactivity with human β-endorphin on a molar basis is observed, and it is also possible that there may be a cross reaction with 31K precursor or undiscovered opiates. In view of the increasing complexity of processing of the 31K precursor and its breakdown products (Mains et al. 1977; Lewis et al. 1978; Odagiri et al. 1979; Liotta et al. 1979; Kimura et al. 1979), as well as the likely presence of precursor forms for "leu"-enkephalin and "leu"-endorphin, it is possible that a number of additional opioid peptides and their precursor forms may cross react in this assay. Extracts of rat pituitary, midbrain, hypothalamus,

hindbrain and intact or hypox rat whole brain at different dilutions produced a curve parallel to the standard curve for camel β-endorphin, as shown in Fig. 11 and Fig. 12, so nonspecific interference in this radioimmunoassay by relatively crude tissue extracts seems unlikely. For further characterization of the cross reacting material, we have fractionated brain extracts by gel filtration to try to distinguish among these various components. In all brain regions examined, the major immunoreactive species had an elution profile very similar to β-endorphin. In the case of the pituitary, however, the situation was very different as shown by Ogawa et al. (1979) (see fig. 13).

EXAMINATION OF IMMUNOREACTIVE β-ENDORPHIN CONCENTRATIONS IN RAT BRAIN AFTER KILLING BY DIFFERENT METHODS

As shown in Table 14, the immunoreactive β-endorphin concentrations found in brain extracts of rats killed with microwave irradiation are at least three fold greater than in rats killed by decapitation. This pattern is similar to the result obtained by Yang et al. (1977) who compared the enkephalin content of rat brain when animals were killed with microwave irradiation or by decapitation. However the question might be raised whether the high content of immunoreactive β-endorphin might be an artifact

caused by the microwave irradiation itself producing an accelerated breakdown of precursor forms. In order to examine this possibility, we have irradiated frozen brains or brains just after decapitation and compared their β-endorphin content with brains from rats which were decapitated. We found no difference in β-endorphin levels among these samples. Moreover, β-endorphin levels in brain homogenates after microwave irradiation were similar whether β-LPH was added or not. Thus, the possibility that immunoreactive β-endorphin was generated by microwave irradiation seems unlikely. Since there are several reports that various neuropeptides were inactivated or degraded rapidly by proteolytic enzymes in brain (Meek et al. 1977; Jean et al. 1977; Marks et al. 1977), the high levels of β-endorphin found after microwave irradiation may be the consequence of almost instantaneous cessation of degradation by proteolytic enzymes. Evidence supporting this possibility is the fact that animals which were exposed to an insufficient dose of irradiation had much lower values of immunoreactive β-endorphin, values which in fact were similar to those found in brains obtained after decapitation alone. The power of microwave irradiation needed to kill the animals depends on the size and the body weight of the animals. If a constant power was used, the anatomy of brains of smaller and lighter rats were severely distorted because fragments of brain were extruded through the

orbital cavities or ear canals making dissection more difficult if not impossible.

Proteolytic degradation of endorphin occurs very rapidly. No matter how quickly rat brains were removed after rats were killed by decapitation using a guillotine and regardless whether tissues were kept in the ice bath, liquid nitrogen or dry ice, the results were similar. Moreover as shown in Table 15, if brain tissue was kept at 4^0C for $1\frac{1}{2}$ hours before extraction, the value of β-endorphin was about 1/6 of the values obtained if extraction was carried out immediately after decapitation. This may explain why the estimates of β-endorphin which were obtained in human brain extracts from autopsy specimens were so low (Liotta et al. 1978; Dowling et al. 1978). Our findings may also explain discrepancy between our results and those reported by others(Rossier et al. 1977a). In these studies, immunoreactive β-endorphin concentrations were much lower than our estimates, especially in rat pituitary and hypothalamus. These results also raise some doubts about using immunofluoresence or immunohistochemical techniques to localize immunoreactive β-endorphin or enkephalins in brain regions. With these techniques rats are killed by decapitation and then either fixed or cryostat sections are used. Our data would suggest that by this time only a small portion about 10-25% or less of the original β-endorphin content remains in the various brain regions.

EFFECTS OF EXTRACTION PROCEDURES ON IMMUNOREACTIVE β-ENDORPHIN CONCENTRATIONS IN RAT BRAIN

Liotta et al. (1978) reported that homogenization of rat anterior pituitaries with acetic acid yielded substantially greater concentrations of β-endorphin and decreased concentrations of β-lipotropin and ACTH than when 0.2 M HCl was used. They also found that homogenization with 0.2 M acetic acid extracted more β-endorphin than 1 M acetic acid. Their result is comparable to the data shown in Table 14. My data showed that 0.1N acetic acid extracted more β-endorphin-like substances than 0.5N or 1.0N acetic acid. Moreover, 0.1 ml samples of 0.1 N acetic acid extracts do not affect the radioimmunoassay of β-endorphin. However, as shown in Table 16, I found that extractions of rat tissue twice with 0.1 N acetic acid improved the extraction and also decreased the coefficient of variation. A Polytron homogenizer provides similar results to a teflon homogenizer. However, the former is advantageous in that much less time and energy is required to prepared the brain homogenates.

EFFECTS OF HYPOPHYSECTOMY ON IMMUNOREACTIVE β-ENDORPHIN CONCENTRATIONS IN RAT BRAIN

Table 18 shows that hypophysectomy causes a 90% loss of β-endorphin immunoreactivity in rat brain. This result is at variance with previous reports using opiate receptor assays (Cheung et al. 1976) or radioimmunoassays (Rossier et al. 1977a). In both these studies no significant changes in whole brain β-endorphin was observed after hypophysectomy. The following factors may account for the different results : (1) Different methods of killing the animals. Rossier et al. (1977a) and Cheung et al. (1976) both killd the rats by decapitation while we used microwave irradiation to kill the rats. As discussed the two methods in our hands yields strikingly different results. Because β-endorphin concentrations were much lower in brain samples obtained after decapitation, a decrease after hypophysectomy might be difficult to detect. (2) Different methods of extraction. Rossier et al. (1977a) used 1 N acetic acid, while we used 0.1 N acetic acid to extract the brain tissues. Again we found the latter to extract more β-endorphin.

The 90% decrease in β-endorphin concentration that we observed in the brain $7\frac{1}{2}$ month after hypophysectomy suggests that the pituitary might be a major source of brain β-endorphin or that pituitary hormones might secondarily influence endorphin synthesis by the brain. The first possibility is enhanced by the recent demonstration of a retrograde blood flow from the pituitary (Oliver et al. 1977). Since β-endorphin is found in

the cerebrospinal fluid (Jeffcoate et al. 1978; Akil et al. 1978), it is possible that this compartment provides a link between pituitary and brain. The presence of low, but constant levels of β-endorphin in the brain 7½ months after hypophysectomy suggests either that hypophysectomy was incomplete as indicated by Moldow for ACTH (Moldow et al. 1978) or that β-endorphin is synthesized in the brain. The finding of β-endorphin in cells with potential hormone synthesizing and secreting properties e.g. the ependymal cells of the third ventricle and in the chorid plexus (Salih et al. 1979). Recently Liotta et al. (1979) also found that the hypothalamus has the potential to synthesize immunoreactive 31 K precursor support the concept that β-endorphin may be synthesized in the brain. Moreover, the observation that enkephalins can be synthesized in cultured spinal cells (Neale et al. 1978) suggest that isolated neuronal cells can synthesize opioid peptides. However, the 90% loss of immunoreactivity after hypophysectomy seems largely a reflection of changes in hypothalamic levels. We cannot exclude the possibility that during hypophysectomy damage to the hypothalamus, especially the median eminence might have occurred. However the fact that in the midbrain a similar decrease was observed makes this less likely.

The distribution of β-endorphin after gel filtration of extracts of intact or hypox rat brain, hypothalamus, midbrain,

and hindbrain was in some respects similar to that of Rossier's report (Rossier et al. 1977a). They found two peaks of β-endorphin immunoreactivity, one major peak coincided precisely with the location of ^{125}I-labeled synthetic β-endorphin, while another minor peak eluted in a broad zone containing proteins of larger MW. (10,000-30,000) which did not coincide closely with the elution pattern of either β-LPH or the 31,000 MW prohormone. Thus, in the rat brain, β-endorphin is the principal immunoreactive species detected while β-LPH represents only a small fraction of the immunoreactive species.

EFFECTS OF ENDOCRINE MANIPULATION OR DRUG ADMINISTRATION ON IMMUNOREACTIVE β-ENDORPHIN IN RAT BRAIN

After adrenalectomy, the concentration of β-endorphin immunoreactivity significantly increased in pituitary, hypothalamus, hindbrain and midbrain. Rossier et al. (1977a) also found significant increases of both β-endorphin and enkephalin in the adenohypophysis, increases of ACTH and β-endorphin in whole pituitary gland, and increase in β-endorphin in the intermediate obe-neurohypophysis. However, he failed to find any significant change in β-endorphin in the brain (Rossier et al. 1977a). The reasons for the discrepany between his results and ours may

be due to the fact that in his study Rossier measured the β-endorphin concentrations in the whole brain, while we have shown that the increases are present in discrete regions. There is other evidence to suggest that β-endorphin-like immunoreactivity in rat plasma (Akil et al. 1978b; Guillemin et al. 1977b) increased after adrenalectomy. Furthermore, administration of the synthetic glucocorticosteroid dexamethasone, inhibits the secretion of both ACTH and β-endorphin (Guillemin et al. 1977b). The opiate-like peptide β-endorphin is secreted in increased amounts in response to purified ovine corticotropin releasing factor in monolayer cultures of adenohypophysial cells with glucocorticoids or progesterone. This evidence suggests that corticosteroids may function as an antagonist to opiates through a mechanism acting directly on the pituitary (Holaday et al. 1977). Moreover, in human's infusion of naloxone also induces a clear and long lasting release of both LH and corticol (Blankstein et al. 1978).

In my results, the concentrations of β-endorphin immunoreactivity are significantly increased in the hypothalamus after thyroidectomy, or after T_4 treatment for one month. The content of β-endorphin immunoreactivity in pituitary, midbrain and hypothalamus increased as compared to the control. In the literature, there are only a few reports concerning thyroid hormones and opioid peptides. Met-enkephalin and morphine can depress serum level of TSH in the male rat (Bruni et al. 1977) and this

effect is reversible by naloxone (Meites et al. 1979). TRH can antagonize some actions produced by β-endorphin (Tache' et al. 1977). Moreover, in organ culture of the hypothalamus β-endorphin increased TRH release. With this limited information, no firm conclusions can be drawn at present. We also don't know whether thyroid hormone affects immunoreactive β-endorphin concentration through neurotransmitters or by affecting the metabolic rate or other mechanisms.

In pinealectomized rats, there is a significant increase in β-endorphin in "the remaining regions" of the brain. Because I didn't subdivide this region, I don't know which particular area was affected. I also couldn't rule out the possibility of damage to the cortex or the structures around the pineal gland that might have occurred during pinealectomy.

It is known that Met-enkephalin and morphine depress serum levels of LH in the male rat (Bruni et al. 1977) and this effect is naloxone reversible (Blank et al. 1979). We also know that the castration and ovariectomy-induced rise in LH release was partially blocked by morphine administration, and increases above control values after naloxone administration, suggesting that the endogenous opiate peptides chronically depress LH release and prevent a maximum rise in LH in response to castration and ovariectomy (Meites et al. 1979; Blank et al. 1979).

As shown in Table 24, naloxone and pentobarbital can cause changes in immunoreactive β-endorphin concentration in rat brain. If pentobarbital is used to anesthetize the rats before doing an experiment, possible effects of the drug itself on the concentration of β-endorphin in the brain must be considered. Pentobarbital has been shown to inhibit the release of ACTH from the pituitary. Since the regulation of β-endorphin and ACTH secretion seems to be similar, this same mechanism might explain the increase in pituitary concentrations observed after pentobarbital treatment.

VARIATION IN β-ENDORPHIN

The great variation of β-endorphin concentrations in extracts of rat brain is evident if one compares the values of the control groups in each of the different experiments.

The following factors may contribute to this variability : (1) Age, sex and body weight. As shown in Table 25, there is a significant increase of immunoreactive β-endorphin from day 24 to day 60 old rats in almost all regions. The data reveals no major differences in β-endorphin concentration between male and female rats 8, 24 or 60 day old rats. I don't know whether at different stages of the estrous cycle

a significant difference of β-endorphin immunoreactivity might be found in the rat brain. The study of β-endorphin in rats of different ages may explain part of the variability. It is therefore necessary that all animals be of the same age and sex to obtain valid comparisons. (2) Method of killing the rat. By using decapitation the value of β-endorphin is much smaller than the values obtained by microwave irradiation. However, the variation also seemed smaller in decapitated groups as shown in Table 14. Stress can cause β-endorphin release and may affect β-endorphin concentrations in the brain. Every animal has different susceptibility to stress and different behavior patterns,which in these rats ranged from extreme panic to apparent indifference when putting the animal into the microwave. This procedure involved placing rats in a cotton bag with the head on the outside and the bag loosely tied around the neck. Then the rat is placed into a brass cylinder. The rats have to be pushed forward to expose their heads outside the brass cylinder and finally plugs are placed in the entrance of the cylinder to immobilize the animal to prevent it from withdrawing its head. It is obvious that this is a stressful event. Perhaps with training rats might be conditioned to enter the cylinder with minimal stress or fear or perhaps more importantly redesign of the device for microwave irradiation is desirable. Because of the diurnal

rhythm of β-endorphin or endogenous opiates, the time of killing in each experiment should be held constant. (3) Extraction method. As shown in Table 14, 0.1 N acetic acid is an efficient extraction procedure and produces lower variability in β-endorphin than when 0.5 N acetic acid is used. Double extractions also decreased the variability. (4) Assay system. The interassay and intraassay variation contribute to the variability in the results, but can be controlled and rigorously defined.

FURTHER STUDIES

In these studies, we have found a large number of changes in immunoreactive β-endorphin concentrations in rat brains after different manipulations. However, the assay system which we used is not specific for β-endorphin, it also measures other endogenous peptides e.g. 31 K precursor, β-lipotropin, etc. For further characterization, it is better to put each extract through gel filtration or other separation techniques. The brain is the most complex organ within the human body and as such it receives information from each part of the body. We have studied brain concentrations of β-endorphin in discrete regions of the brain, obviously this approach doesn't allow

us to understand the exact mechanism producing the change or an integrated view of the factors contributing to the changes. Our studies do suggest that certain selected approaches might be made and do hint at a dynamic relationship between the endocrine status of the animal and brain endorphin. If we could improve the sensitivity of our assay and measure β-endorphin in the blood, we might obtain a better understanding of this interaction.

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