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IMMUNO- AND BIOASSAYABLE EXTRAPITUITARY

GROWTH HORMONE AND THYROID STIMULATING HORMONE

FROM DISCRETE AREAS OF THE CENTRAL NERVOUS SYSTEM

SALLY A. HOJVAT

A Dissertation Submitted to the Faculty of the Graduate School

of Loyola University of Chicago in Partial Fulfillment

of the Requirements for the Degree of

Doctor of Philosophy

ACKNOWLEDGEMENTS

LIFE.

Sally Ann Hojvat was born in Bangor, N. Wales, on
August 19th, 1942. After graduating from Queen Elizabeth's
Grammar School, Manchester, England, in 1960, she
received her Bachelor of Science with Honors in Microbiology
and Agriculture from the University of Wales, Aberystwyth,
in 1964. The next two years were spent completing a Master
of Science in Microbiology from the University of Alberta,
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was employed as a research assistant in the Departments of
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and the Univensité de Genève, Switzerland, and the Department
of Immunology, McGill University, Montreal, Canada. In 1977,
she began her doctoral thesis research at Loyola under the
supervision of Dr. Ann Lawrence, studying the characterization
of two pituitary peptides found in the rodent CNS.

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INTRODUCTION

1. STATEMENT OF PROBLEM

Although the formal study of neuroendocrinology is a relatively
new discipline, historical writings have long shown an awareness of
interactions between the nervous and endocrine systems. The writings of
Aristotle on pseudo-pregnancy described a relationship between hormones
and emotional states, and the practice of primitive husbandmen to cas-
trate bulls, thus altering aggressive behavior, are two such examples.

It was mainly the results of Geoffrey Harris's work in the late
1940's which ushered in the new era of neuroendocrinology. The anatomi-
cal studies of Green and Harris (1949) demonstrated that a direct
connection between the central nervous system (CNS) and the endocrine
pituitary gland existed in the form of the portal venous system. The
portal veins were shown to drain capillaries that have traversed the
median eminence region of the hypothalamus, an area of the central
nervous system having complex neural connections with other brain areas.
The veins themselves then break up into a capillary plexus in the pitui-
tary which, in its turn, is drained by veins into the general venous
circulation. Thus virtually all the afferent blood supply of the an-
terior pituitary was shown to have first been in contact with the
hypothalamus.

Specific examples of the interaction between the central nervous
and endocrine systems have been revealed by clinical observations

demonstrating that lesions of the hypothalamus can induce malfunction of the pituitary gland, such as suppression of the release of pituitary growth hormone, as observed by Frohman et al (1968). Other functions attributed to the pituitary gland which are modified by such lesions include the regulation of endocrine systems manufacturing steroids in the adrenals and thyroxine in the thyroid gland, as well as the regulation of other key metabolic processes more directly regulated through the pituitary gland's output of growth hormone and thyroid stimulating hormone.

The importance of the portal venous connection between hypothalamus and pituitary was shown by the subsequent work of Schally et al (1966), and Guillemin (1966) who demonstrated the existence of hypothalamic peptidergic hormones that act to control the release of pituitary hormones. These peptides are produced in the hypothalamus and are transported via the portal vascular system to the pituitary cells on which they exert their action. Gonadotropin releasing hormone (GnRH) appears to modulate the synthesis and release of two pituitary hormones, follicle stimulating hormone and luteinizing hormone; and thyroid releasing hormone (TRH), the secretion of two other pituitary hormones, prolactin and thyroid stimulating hormone (TSH). Both GnRH and TRH have been characterized chemically and synthesized by Enzmann et al (1971) and Matsuo et al (1971), respectively. The hypothalamus was also found to contain peptides that inhibited the release of pituitary hormones. The first of these to be isolated by Brazeau et al (1973), was the tetra-decapeptide, somatostatin, which was shown to inhibit the secretion

of pituitary growth hormone (GH), TSH, and prolactin as well as the secretion of glucagon and insulin from the pancreas.

It appears from studies such as those of Krulich et al (1977), Schneider et al (1969), and Müller (1973) that the hypothalamic secretory cells producing such regulatory factors are themselves activated by neurotransmitters of CNS origin such as dopamine and norepinephrine, which are released at synaptic connections from the various afferent neurons known to converge on the hypothalamic region from brain areas such as the hippocampus, amygdala, cortex and thalamus (Szentágothai et al, 1968).

A somewhat simplified picture of the neuroendocrine system can therefore be summarized as involving the stimulation of hypothalamic neurosecretory cells by neurotransmitters, produced in other higher centers of the brain, to produce releasing or inhibitory factors, which regulate pituitary hormone secretion, which in turn largely controls growth and development and functions connected with the adrenals, thyroid, and reproductive glands. On the other hand, the hypothalamic inhibitory and releasing factors have been shown to have roles which are not restricted to the control of pituitary function. As reviewed by Prange et al (1975) in the article "Brain Behavioral Effects of Hypothalamic Releasing Hormones," peptides identical to hypophysiotropic hormones have been found in extrahypothalamic areas of the brain; TRH has been demonstrated in the cerebellum, thalamus, brain stem and cerebral cortex (Winokur et al, 1974, and Oliver et al, 1974); somatostatin has been found widespread throughout the CNS (Brown et al, 1975), and

GnRH appears to be present in the mammillary body, thalamus, amygdala, and hippocampus (Kubek et al, 1979). These findings have led to the suggestion by Krieger et al (1980) that these peptides may also act as synaptic transmitters, i.e., convey information between adjacent nerve cells or neuromodulators, which amplify or dampen neuronal activity. Such a neuromodulator role may include functions in relation to sensory conduction (Renaud et al, 1975), pain mechanisms (Segal et al, 1975), and behavioral responses (Moss, 1979) in various brain regions.

Schaeffer et al (1977) and Arimura et al (1975) have reported the presence of hypophysiotropic hormones outside the central nervous system, most notably in the gastrointestinal tract where, for example, the finding of TRH has led to the speculation that this peptide may modulate the responses of feeding and drinking behavior (Moss, 1979). There is growing evidence that the reverse situation is also true. In addition to the presence of brain peptides outside the CNS, other hormones, classically thought to have a site of origin in, for example, the kidney, such as renin (Haulica et al, 1975), and in the gastrointestinal tract, such as glucagon (Conlon et al, 1979), gastrin (Vanderhaeglin et al, 1975), or vasoactive intestinal protein (Said et al, 1976) and Substance P (Hökfelt et al, 1975), have now also been isolated from the CNS.

More recently there have been reports giving evidence for the presence of the classical anterior pituitary hormones in discrete regions of the CNS. Krieger et al (1977[a]) have reported the finding of adreno-corticotrophin (ACTH) in extrapituitary locations such as the arcuate

nucleus of the hypothalamus and the amygdala. Fuxe et al (1977) have

shown prolactin containing cells in the hypothalamus, and Vaudry et al

(1978) have demonstrated melanocyte stimulating hormone (∝ MSH) in cells

of the arcuate nucleus.

Once thought only to contain monoaminergic neurotransmitters or

their precursors, the isolation of more than 20 different peptides in

the brain in the last ten years has led to a new concept of the function-

ing of the CNS. Speculation, such as that of Krieger (1980), has led to

the hypothesis that peptides, such as those of pituitary origin, exist

in the brain as part of a peptidergic neural system, present in addi-

tion to the classically described motor, sensory, and autonomic systems.

During this time when ectopic sites of origin for many peptide

hormones were being described, Lawrence et al (1974) reported the pre-

sence of a factor in human cerebrospinal fluid which actively promotes

the in-vitro and in-vivo release of pituitary growth hormone.

In the course of seeking to identify the origin of this growth hormone

releasing factor, Pacold et al (1976) found pituitary growth hormone-

like immunoreactive material in dispersed rat brain cell cultures.

It is the further characterization of this anterior pituitary-like

growth hormone in the CNS that will be the subject of this dissertation.

Similar studies will also aim to characterize a pituitary TSH-like

material which was also found during the course of this work in rat

brain cell tissue cultures. The purpose of this dissertation will

therefore first be to review data drawn from classical studies of the
two pituitary hormones, GH and TSH, with a view to characterizing their
presence in the rat brain. An attempt will be made using biochemical,
anatomical, and physiological methodology to determine whether these
particular peptides correspond to their pituitary counterparts or not.
The significance of these findings and the findings of other "brain
peptides" will be reviewed and discussed.

2. RELEVANT LITERATURE REVIEW

A. THE PITUITARY-HYPOTHALAMIC LINK

An understanding of the anatomy of the pituitary gland and its
hypothalamic connections is crucial to the analysis of its physiological
functions.

These functions include the secretion of at least 6 well-charac-
terized hormones from the anterior lobe of the gland. Growth hormone
(GH) and thyroid stimulating hormone (TSH) have important modulatory
effects on carbohydrate, lipid, and protein metabolism, whereas ACTH,
a linear polypeptide, once released from the pituitary, exerts its most
potent effect on the adrenal cortex, where it stimulates the conversion
of cholesterol to pregnenolone, a precursor of steroid hormones such as
cortisol. Follicle stimulating hormone (FSH) and luteinizing hormone
(LH) are glycoproteins produced in the pituitary and which act on the
Leydig cells of the testis to stimulate androgen secretion and on the
tubules to induce spermatogenesis in the male. In the female they
regulate the ovulatory cycle. In addition, the anterior pituitary
cells secrete prolactin, a polypeptide exhibiting a diversity of physio-
logic actions, including an effect on growth of mammary duct tissue and
the maintenance of progesterone output by the corpus luteum of the ovary.
Another group of pituitary peptides consists of α and β melanocyte stim-
ulating hormones (α and β MSH), β and γ lipotropins (β and γ LPH), and
the endorphins which, together with ACTH, originate from a common pre-
cursor molecule and exhibit a variety of physiological effects.

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Two hormones, vasopressin and oxytocin, are released from the posterior lobe of the pituitary gland and are concerned with the maintenance of volume and tonicity of body fluids, and influence pregnant uterine contractions and post partum milk ejection, respectively.

The pituitary gland, itself long considered the "master gland" for reasons obvious from the preceding discussion, is located at the base of the skull, covered by a tough connective tissue diaphragm and lies in a bony cup, the sella turcica. It is connected by the pituitary stalk, containing vascular and neural elements, to the floor of the hypothalamic region of the diencephalic brain. Fig. 1.

This intimate relationship between the pituitary gland and the hypothalamus develops during embryonic life. The origins of the pituitary can be traced to Rathke's pouch, which begins as a cul-de-sac protruding from the roof of the stomoderm, the posterior nasopharynx. During development, Rathke's pouch loses its connection to the stomoderm and becomes a hollow spheroid collection of cells forming the primitive anterior pituitary. The posterior pituitary evaginates downward from the base of the primitive brain diencephalon as the anterior lobe grows upward from the buccal endoderm, forming the mature pituitary gland linked to the diencephalon by the pituitary stalk. The distinct origins of posterior and anterior pituitary lobes are reflected in their cellular anatomy, the posterior lobe consisting of up to 42% nerve axons and axon terminals whereas the anterior lobe contains a variety of hormone secretory cells and an apparent absence of neural imput from the brain.

ANTERIOR HYPOTHALAMIC AREA

PARAVENTRICULAR NUCLEUS

DORSAL HYPOTHALAMIC AREA

MEDIAL PREOPTIC NUCLEUS

POSTERIOR HYPOTHALAMIC AREA

DORSOMEDIAL NUCLEUS

SUPRAOPTIC NUCLEUS

VENTROMEDIAL NUCLEUS

PRIMARY PLEXUS IN MEDIAN EMINENCE

PITUITARY STALK

ANTERIOR PITUITARY

POSTERIOR PITUITARY

LONG HYPOPHYSEAL PORTAL VEINS

PLANE OF FRONTAL SECTION. FIG. 2
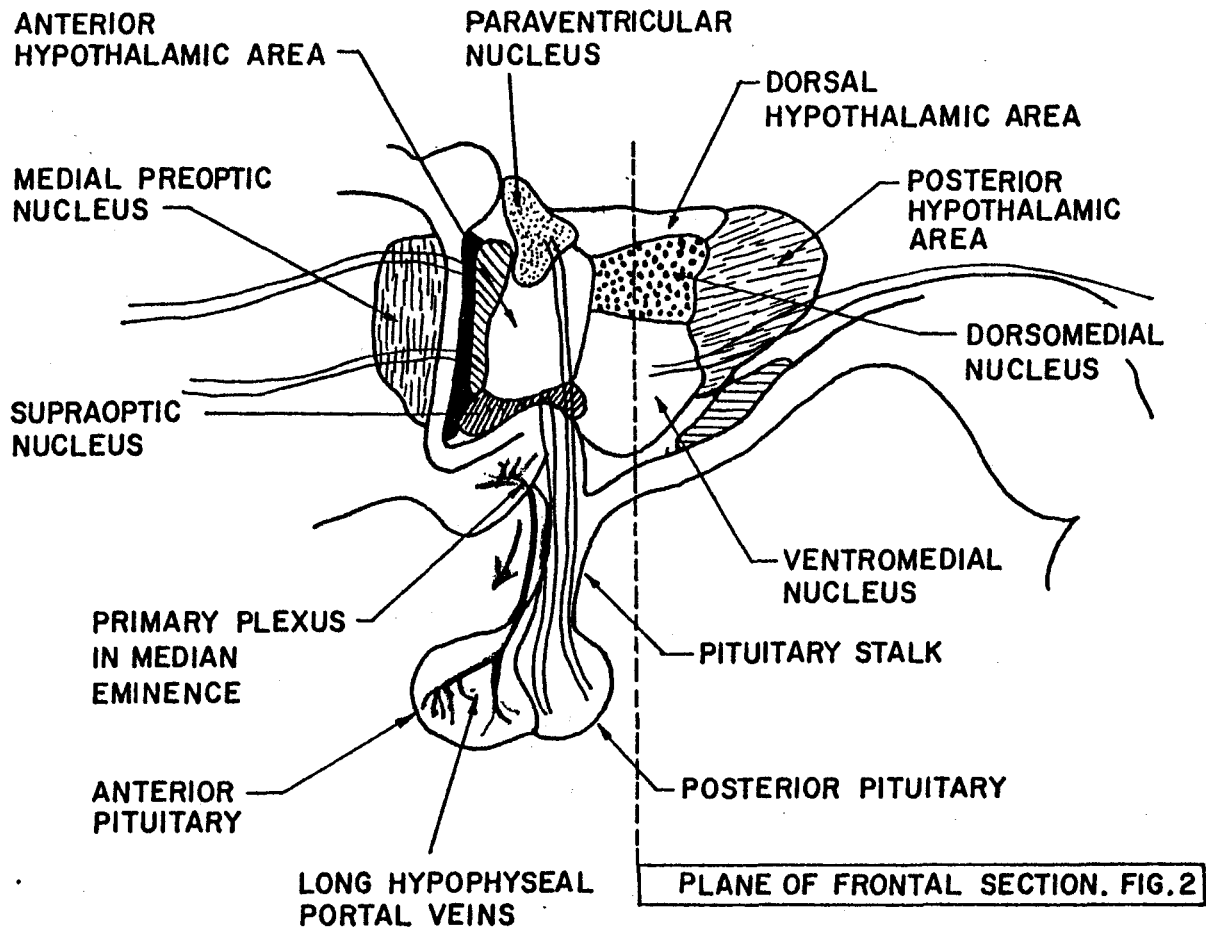
Figure 1. The major centers of the hypothalamus and its neural interconnections with the CNS.

The origin of the nerve terminals in the posterior pituitary are the supraoptic and paraventricular nuclei in the hypothalamus (Figs. 1 and 2). Nerve tracts descend from these hypothalamic nuclei via the pituitary stalk and the severance of this hypothalamic-pituitary connection causes atrophy and irreversible damage to posterior lobe function. This intimate anatomical link influencing the functioning of the posterior pituitary was defined by Scharrer in 1954, who showed that the two pituitary hormones, oxytocin and vasopressin, originate in the supraoptico- and paraventriculohypophyseal nuclei of the hypothalamus. Experiments in which the neurons of these nuclei were pulse-labeled with radioactive amino acids indicated that the two posterior pituitary hormones and their respective neurophysin-carrier proteins are synthesized as part of separate precursor proteins which are processed into the smaller biologically active molecules while being transported along the axons within the pituitary stalk (Sachs et al, 1969). A study of the development of this neural link by Diakoku in 1971 revealed that by the 18th day of fetal life, a rat already possesses some nerve fibers containing dense core, precursor-filled vesicles in the hypothalamus.

The link between the rat hypothalamus and the anterior lobe of the pituitary is vascular in nature. These connections via the pituitary stalk were first observed by Popa and Fielding in 1930. Although these early studies postulated that the flow of blood in the stalk vessel system was from pituitary to brain, Wislocki et al in 1936 demonstrated that the flow of blood was toward the pituitary. Green and Harris, in 1949,

DORSOMEDIAL NUCLEUS

THIRD VENTRICLE

DORSAL HYPOTHALAMIC AREA

LATERAL NUCLEUS

SUPRAOPTIC NUCLEUS

ARCUATE NUCLEUS

MEDIAN EMINENCE

AMYGDALA
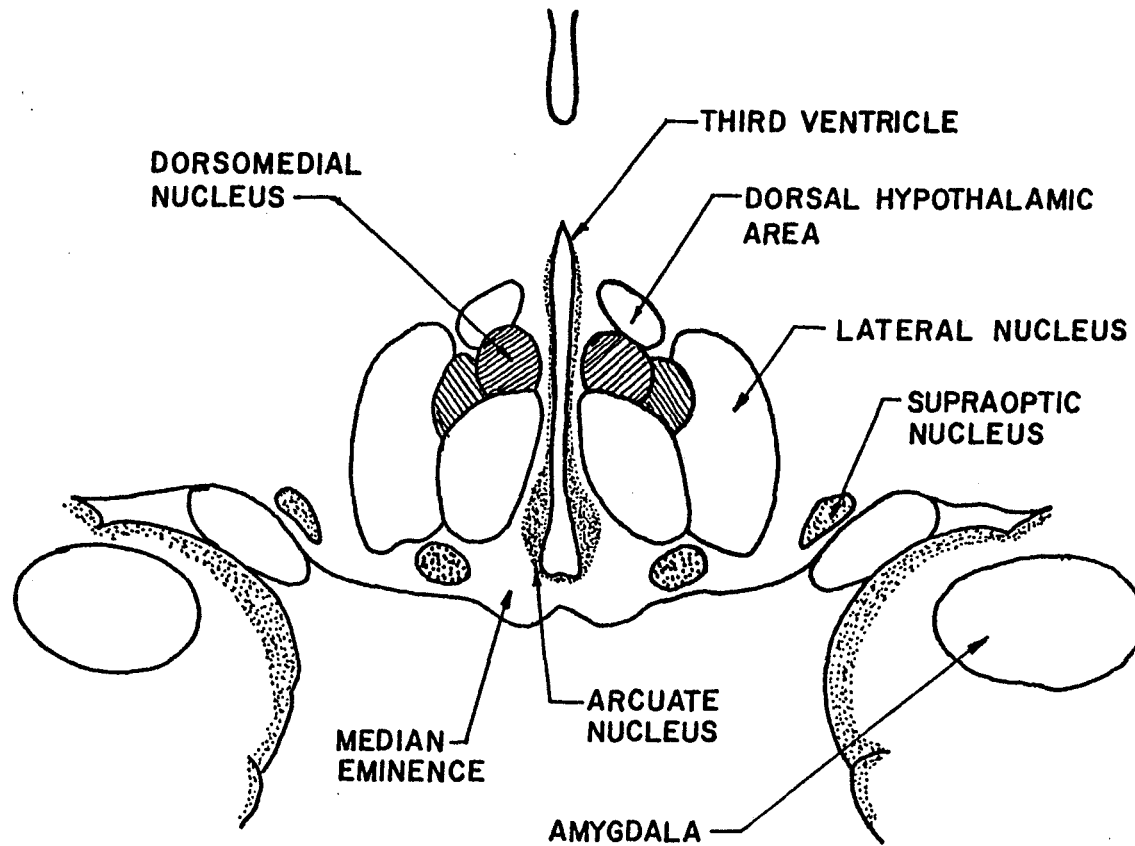
Figure 2. Frontal section showing relationships of nuclear centers of the hypothalamus.

confirmed this observation and their classic anatomical studies identi-
fied the vascular connections as the long portal vessels, which were
independent from the blood supply to the brain and upper hypothalamus
and which divided into capillary tufts within the floor of the third
ventricle of the diencephalon. The embryonic development of such a
hypothalamic-pituitary vascular link was examined in the rat by Glydon
in 1957, who showed that although the portal veins could be identified
in the pituitary stalk at the time of birth, the primary capillary plex-
us in the hypothalamus did not actually attain full development until
5 days after birth.

In adult animals, loss of this vascular connection leads to a
marked decrease in some of the anterior pituitary's functions such as
the secretion of the hormone TSH. Transsection of the rat pituitary
stalk by Cahane in 1936 led to a degenerative change in the histologi-
cal appearance of both the pituitary and thyroid, the latter being a
target organ for pituitary TSH. As already reviewed, the finding of
hypothalamic inhibitory and releasing factors controlling anterior pi-
tuitary hormone secretion by Schally (1966), Guillemin (1966), and
Brazeau (1973) supports this idea that the brain, particularly the
hypothalamus, is intimately involved in the functioning of the anterior
pituitary.

Mechanisms controlling anterior pituitary secretion, other than
those of hypothalamic origin, are those exerted by the target gland hor-
mones such as thyroid, adrenal, and gonadal hormones. Changes in circu-
lating levels of target organ hormones may either inhibit or encourage

the synthesis of pituitary hormones, and in this way an optimum level of

hormone is maintained in the general circulation. These "feedback loops",

as they are known, are also thought to affect the levels of hypothalamic-

hypophysiotropic hormones on exposure of the hypothalamus to changing

levels in circulating hormones such as T_4 and the steroids.

This section has dealt with the hypothalamic-pituitary link and

its role in the control of pituitary hormone release. The two anterior

pituitary hormones, growth hormone, and thyroid stimulating hormone will

be discussed in greater depth in the sections which follow, with respect

to their molecular structure, normal levels found in the pituitary and

serum, mechanism of action on target tissues or metabolic systems, and

the regulation of their release from the normal pituitary gland.

B. PITUITARY GROWTH HORMONE

Synthesized and secreted by the eosinophylic somatotroph cells of

the anterior pituitary, growth hormone cells account for 4% - 10% of the

wet weight of the adult anterior pituitary. The hormone is produced in

the Golgi area of the somatotroph, and the cell's microtubular system is

involved in the movement of hormone-containing granules from this area

to the plasma membrane, whose release into the circulation is associated

with fusion of the secretory granules with the plasma membrane.

Circulating GH is needed for longitudinal growth of the skeleton,

has both insulin-like and anti-insulin like effects on the metabolism of

glucose and enhances the incorporation of amino acid into protein. The hormone also induces lipolysis of stored fats as shown by Raben in 1959, who observed a rise in plasma-free fatty acids after administration of growth hormone intravenously to man.

a. Molecular Structure of Growth Hormone.

Rat growth hormone is a polypeptide with a molecular weight of 46,000 Daltons, an NH_2 terminal phenylalanine, and a sedimentation coefficient, S_{20} of 3.21, as shown by Ellis et al (1968). Complete sequencing of rat growth hormone has not been reported; however, it is known from the work of Li et al (1971) that all mammalian growth hormones contain 2 disulfide bridges, one spanning 110 residues and joining cysteine residues 53-164; the other spans only 6 residues and joins residues 181 and 189. There is evidence that pituitary growth hormone contains 50% of α helix, and all known species, such as human, porcine, and rodent, undergo similar structural transitions in acid or base. Of interest in this regard, two other peptide hormones, placental lactogen and prolactin, show remarkable structural similarities with growth hormone. This is reflected in their similar placement of disulfide bridges and residue homology, and has led to the suggestion of a common evolutionary origin for the 3 hormones by Niall et al (1971), which may have arisen from repeated tandem duplication of a gene coding for a relatively small peptide. These similarities in structure are not, however, reflected in the major biological activities of the 3 hormones. Growth hormone alone possesses the ability to increase the length of the skeleton, whereas

prolactin is found to be essential for such processes as mammary duct development, lactogenesis and, together with luteinizing hormone, for the maintenance of progesterone output of the corpus luteum. Metabolic actions resembling those of GH have been demonstrated for prolactin, but only after relatively large doses, and it is questionable whether prolactin makes any significant contribution to somatotrophic activity under normal circumstances. Placental lactogen, produced by the placenta, is highly lactogenic but cannot induce growth of the long bones. (Wallis et al, 1976 and Kelly et al, 1976) All three hormones have been found to exist in several forms with varying molecular weights in the serum. (Hambley et al, 1972, and Gordon et al, 1973)

The suggestion has been made by Sigel and Lewis (1979) that the predominant form of growth hormone in pituitary extracts is itself a prohormone, and that after limited proteolysis by plasmin (Ellis et al, 1968), smaller molecular weight sequences still retain biological activity. (Li et al, 1974) This heterogenicity with respect to size of circulating human growth hormone (hGH) has been studied by Gordon et al (1973) and Bala et al (1970), these groups showing forms with molecular weights both larger and smaller than monomeric hGH. Some of the larger species dissociate when treated with urea, whereas others require disruption of the interchain disulfide bonds for conversion to a molecular weight comparable to monomer hGH. (Beneviste et al, 1978) Bioassay discloses hGH-like activity in blood which does not correspond to immunoreactivity, indicating the presence of circulating forms with heterogenous immunogenicity. Enzymatic modification of the major polypeptide

chain by chymotrypsin, papain, or subtilisin results in proteolytic cleavage occurring in the large disulfide loop to produce two acidic isohormones. These exhibit a several-fold higher biological activity than other cleavage products, as shown by Singh et al (1974); however, the mechanism of this enhanced activity is unknown. Frigen and Lewis (1980) have shown that a 20,000 Dalton variant hGH lacks various insulin-like and anti-insulin-like effects found with the principle form of hGH as reported by Goodman (1965); yet it is fully active in promoting growth by the GH bioassay measuring rat tibia.

Human, and perhaps rat growth hormone as usually isolated, can therefore be considered as a complex of proteins, perhaps serving different functions. The diversity of effects exhibited by growth hormone on metabolism and growth may perhaps be accounted for by this heterogeneity. However, as shown from Sigel's (1980) latest work, the specific physiological activities required to make a protein a growth hormone are essentially unknown.

Levels of circulating radioimmunoassayable growth hormone vary depending on such factors as age of the animal and the time of day the sample is taken. These factors are discussed in the next section on rat growth hormone.

b. Growth Hormone Levels in Plasma and Pituitary.

Birge et al (1967) found that GH was undetectable in the rat fetus in either plasma or pituitary before day 19 of gestation. Rieutort, in

1972, speculated that the origin of circulating GH after day 19 until birth at day 21-22 was the fetal pituitary, as growth hormone was absent in hypophysectomized animals, i.e., rats which had their pituitaries removed. Jost (1959), working with encephalectomized fetuses with intact pituitaries, showed a lowering of plasma GH in the fetus, suggesting that the CNS, and in particular the hypothalamus, have an early role on somatotroph development and growth hormone secretion. Hypothalamic control, in terms of that found in the adult, does not seem likely in the fetal rat, as somatostatin, the GH-inhibitory hypothalamic peptide, is undetectable in the CNS until the fifth day after birth. There is evidence that fetal and not maternal levels of circulating GH control the release of pituitary hormone, as Grumback, in 1974, has shown little or no interchange of any pituitary hormone transplacentally. Gluckman et al (1979), Cornblath et al (1965), and Blasquez et al (1974) have also shown a rise in fetal plasma growth hormone in the last days of gestation of the sheep, rat, and human to levels exceeding maternal plasma hormone levels, indicating that the two circulatory systems are independent in terms of growth hormone levels. Postpartum levels of plasma and pituitary hormone reach adult levels between 15 and 20 days as shown by Ojeda et al (1977).

In the adult rat, a circadian variation in plasma hormone level is observed. Evidence indicates that all the pituitary hormones studied to date are secreted in such an episodic manner throughout the 24-hour day, with both plasma and urine concentrations manifesting a circadian rhythm (Saunders et al, 1976). It is now clear that, in the absence of stress,

whether physiological or psychological, endocrine activity, particularly the secretion of ACTH, GH, and prolactin, is extremely variable and under the control of some CNS mechanism other than a stimulus response and "feedback" system. Because of the absence of confirming evidence for a "steady state" system of neuroendocrine activity, a new hypothesis has developed that proposes that hormone secretion is temporally ordered and that the dominant determinant of this activity in man and other animals is the 24-hour sleep-wake rhythm (Weitzman, 1980). Studies by Weitzman's group have shown that, in man, the release of growth hormone coincides with the onset of definitive sleep, characterized by slow, synchronous electrocortical activity, REM sleep, suggesting that the same mechanism precipitates both events. Saunders et al (1976) showed a more regular pattern of episodic release of GH with intervals 3 hours apart in the rat. Peak levels occurred in late morning, indicating that species-to-species differences to occur.

In the female rat, the amplitude of pulses does not change during pregnancy (Willoughby et al, 1978) or with variation in temperature. This unresponsiveness to external stimuli is typical of circadian rhythms in both man and animals (Zucker, 1980). The significance of circadian rhythms to endocrine systems lies in the need for the synergistic effects of hormones on the precise timing of secretory activity.

Several CNS areas have been implicated in such rhythms, especially the suprachiasmatic and preoptic nuclei and the pineal gland. Destruction of the suprachiasmatic nucleus appears to render animal behavior

aperiodic, and adrenal coricosterone rhythms are also eliminated. Pineal
dysfunction has been implicated in cases of infertility where abnormal
variations in the ovulatory cycle were the underlying cause (Wurtman, 1980).
While these are certainly not the only mammalian circadian clocks, the
suprachiasmatic nucleus in particular probably functions to integrate the
activity of other oscillating systems that in turn regulate the periodic-
ity of numerous physiological and behavioral systems.

c. Biological Effects of Growth Hormone.

Initial recognition of the pituitary's relationship to growth came
from the correlation of growth hormone producing pituitary tumors and
gigantism with the pathology of the gland. Pituitary deficiency of growth
hormone secretion was also found to be associated with dwarfism. The
regulation of growth by growth hormone was first examined in the 1930's
when pituitary extracts containing growth hormone became available (Sil-
berberg, 1935). Growth of the mammalian skeleton was shown to occur at the
epiphyseal plate where chondrocytes proliferate and synthesize a matrix
composed of collagen and sulfated polysaccharides. Kibrick et al (1941)
found that narrow epiphyseal plates in hypophysectomized rats became wider
after growth hormone was given in vivo, a property which has since been
used as a bioassay for GH. Other workers such as Murphy and Daughaday
(1956) observed that cartilage metabolism could be measured by the

incorporation of radioactive sulfate into cartilage chondroitin sulfate. Cartilage from hypophysectomized rats had low sulfate uptake, which was increased by growth hormone administration given in vivo.

The classic experiments of Salmon and Daughaday in 1957 examined the mechanism by which growth hormone might stimulate growth. The in vitro addition of growth hormone to rodent excised rib cartilages in tissue culture did not accelerate radioactive sulfate incorporation; however, incorporation was stimulated by a component of normal serum. This sulfation factor was absent in the serum from hypophysectomized animals and was restored to serum by the administration of growth hormone to these hypophysectomized animals. From these observations, it was hypothesized that growth hormone stimulated skeletal growth indirectly through generation of a circulatory "sulfation factor" or somatomedin, as it was later termed, which acted directly to cause cartilage proliferation.

Van Wyk et al (1972) found initially that this active component in whole serum behaved as a large protein. After acid ethanol extraction, the active material had a molecular weight between 4,000 and 12,000 D., suggesting that somatomedin was aggregated or bound to a larger carrier protein. Two such carrier proteins were isolated by Kauffman et al (1977), and "somatomedin" was found to be not one but a family of small peptides. Three of these were named Somatomedins A → C, with molecular weights ranging from 5,000-7,000 Dalton by Van Wyk et al in 1974. Two other insulin-like growth factors have also been shown to stimulate cartilage proliferation by Ginsberg et al (1979). These growth factors resemble

proinsulin in amino acid sequence and tertiary structure but have limited
cross-reactivity with insulin in binding to receptor sites on target
tissues, as demonstrated by Blundell et al (1978).

Structurally similar to proinsulin, the somatomedins have also been
shown to have anabolic, insulin-like actions on fat and muscle by Clemmons
et al (1974) and Uthne et al (1974), as well as their property to enhance
cartilage proliferation.

It is thought that growth hormone stimulates skeletal growth, in
vivo, indirectly by regulating the level of the somatomedins. A contra-
diction to this idea is found in the observation that somatomedin levels
are lower in the young animal than in the adult. This has led to specu-
lation that growth may be less dependent on somatomedins or that somato-
medins may be more available in the young due to reduced binding to a
specific serum carrier protein or to an increase in somatomedin receptor
sites as shown by Rosenfeld et al (1979). However, growth hormone excess
in humans and rats is associated with an increase in somatomedins as shown
by Firlanetto et al in 1977. Regulation of somatomedin production appears
to occur mainly in the liver and reflects nutritional (Phillips et al,
1978) and hormonal status, possibly insulin and GH levels (Hintz et al,
1978), of the animal. Levels of somatomedins in perfusates of livers
from hypophysectomized animals are below levels in normal animals and are
restored to normal by treatment with growth hormone in vivo, as shown by
Schalch et al (1977). Thus, the liver may respond to available hormone
levels in altering the levels of circulating somatomedins.

In addition to somatomedin being a mediator of growth hormone's effects on collagen synthesis, it appears to mediate other actions of growth hormone, specifically, its anabolic effects on skeletal muscle. Salmon and DuVall (1970) have provided evidence that somatomedin is more potent than native growth hormone in increasing leucine incorporation into muscle protein. Earlier work by Kostyo et al (1966) had shown that leucine incorporation into protein, in excised diaphragm muscle from normal rats, to be twice that of tissue removed from hypophysectomized rats. Treatment of these animals without pituitaries with growth hormone restored amino acid incorporation into new protein. Korner et al (1965) showed increases in the total number of ribosomes in rat liver cells, an increased capacity of ribosomes for incorporating amino acids into protein, and an elevation in messenger RNA in vivo after growth hormone administration. Wikland et al (1980) have studied the response to growth hormone on amino acid transport and protein synthesis in vivo in the rat diaphragm and find that stimulation is transient and followed after about 3h by a period of refractoriness. In contrast, they found a much longer lasting stimulatory effect of growth hormone on protein synthesis in tissues of hypophysectomized rats. These "insulin-like" effects of growth hormone suggest that changes in responsiveness of tissues to growth hormone, or more likely to somatomedin, play a physiological role in the regulation of protein metabolism in muscle tissue.

As with effects on amino acid transportation, so also with respect to plasma free fatty acid levels, there is an early "insulin-like" action shortly after intravenous injection of growth hormone. Birnbaum and

Goodman (1979) showed that levels of free fatty acids fall within the

first 1/2h of injection before showing the secondary rise typical of the

lipolytic action of the hormone. Somatomedins have been shown to stimu-

late incorporation of free fatty acids into lipids. (Clemmons et al,

1974) This early antilipolytic effect of growth hormone could be

mediated by somatomedin, or, Birnbaum and Goodman (1976) postulate, it

may result from a decreased activity of protein kinase in the cytosol.

The delayed rise in plasma free fatty acids after growth hormone

administration can be blocked by administration of glucose. Also giving

GH simultaneously with insulin, a potent inhibitor of free fatty acid

release from adipose tissue, obliterates the lipolytic action. (Merimee,

1979) It is possible that the inhibiting effect of glucose is mediated

via stimulation of endogenous insulin release. Fain et al (1971), using

isolated fat cells in the presence of dexamethasone, have demonstrated

a requirement for RNA synthesis and for new protein synthesis in the li-

polytic action of the hormone.

Growth hormone also has both an "insulin-like" and "anti-insulin"

effect on the metabolism of glucose. An "insulin-like" effect such as a

fall in blood glucose levels has been seen within 10 minutes of an I.V.

injection of bovine growth hormone into a normal rat by Goodman (1967).

An increase in glucose uptake and glucose oxidation due to an increase in

cell permeability is postulated to account for this fall in serum glucose;

however, Martin and Young (1965) have shown that very small concentrations

of growth hormone depress glucose uptake when added to preparations of

excised rat diaphragm. The "insulin-like" effect of growth hormone on glucose metabolism may therefore be merely a pharmacological effect.

There is greater unanimity with regard to the less immediate effects of growth hormone administration on glucose metabolism, namely, its "contrainsulin" and its "insulinotropic" effect, i.e., enhancement of pancreatic release of insulin in response to a variety of stimuli as shown by Martin et al (1967). Daughaday and Kipnis (1966) compared the rate constant for glucose disappearance from human serum under control conditions with that observed when an I.V. glucose load was administered 2h after the administration of growth hormone (5 mg. I.V.). The clearance rate of glucose from the serum fell from control values after GH administration, substantiating a "contra-insulin" effect for the hormone. Precisely in what manner this "contra-insulin" effect is mediated is not clear.

A second aspect of the action of growth hormone on glucose metabolism is the "insulinotrophic" action of the hormone. Pretreatment of normal subjects with human growth hormone results in an increase of insulin release in response to a large number of secretogogues such as glucose and arginine, as shown by Rimoin et al (1968). In vitro data from Martin et al (1967) also supports a direct effect of growth hormone on the β cell of the pancreas. Isolated islets from hypophysectomized rats have a significantly reduced insulin content, output, and synthesis, which can be corrected by growth hormone treatment.

The biological effects of growth hormone can therefore be viewed as the result of a dual action: those of growth hormone per se, and those actions of growth hormone dependent on somatomedins. Factors controlling the secretion of growth hormone by the pituitary are unusually complex; however, most stimuli appear to act via a neural mechanism through the hypothalamus to the pituitary. Such stimuli will be reviewed in the next section.

d. Regulation of Growth Hormone Release.

The direct release of growth hormone appears to be under the control of two substances: a growth-hormone-releasing factor, which has yet to be isolated, and an inhibitory agent, somatostatin.

While searching for the releasing factor, Brazeau (1973) observed that the addition of crude hypothalamic extract to cultures of rat pituitary cells decreased the secretion of growth hormone. Further purification resulted in the isolation of the linear tetradecapeptide, somatostatin. This peptide has been located by Sétaló et al (1978) in the external layer of the median eminence around the capillary loops of the portal vessels which eventually lead to the pituitary. Somatostatin has also been located in substantial amounts by similar immunohistochemical methods in the ventromedial nuclear area of the hypothalamus by Hökfelt et al (1977[a]), as well as other areas of the CNS.

Evidence for some form of "short-loop feedback" control mechanism for growth hormone involving somatostatin comes from experiments with

hypophysectomized rats performed by Baker et al (1976). A reduction in somatostatin levels in the median eminence was observed after pituitary removal, suggesting that this fall in inhibitory factor levels could be directly due to insufficient circulating growth hormone, and Hoffman et al (1977) claim to have been able to restore, partially to normal, levels of somatostatin by administration of exogenous growth hormone to hypophysectomized animals. Whether GH acts directly on somatostatinergic neurons in the hypothalamus to restore protein synthesis (Karatsuka et al, 1979), or indirectly through somatomedin has not been determined. Baker et al (1976) also caution that the partial recovery of somatostatin levels could be due to the regeneration of neurons destroyed during hypophysectomy.

The mechanism by which somatostatin inhibits the release of growth hormone, induced by prostaglandins, dibutyril cyclic AMP, and theophylline, is possibly due to inactivation of adenyl cyclase. A rise in cyclic GMP due to somatostatin administration has also been observed in rat somatotropes. Takahara et al (1980) have recently proposed that the release of growth hormone induced by γ-aminobutyric acid (GABA), a neurotransmitter, may also be partly due to the inhibition of hypothalamic somatostatin release.

Evidence linking other neurotransmitters such as norepinephrine, dopamine, and serotonin with the release of GH has been reviewed by Martin (1973). These more indirect mechanisms of controlling growth hormone release are currently thought to be mediated via any of three

neural centers: the ventromedial nucleus (VMN) and arcuate nucleus (AC) of the hypothalamus, and the limbic system which includes the hippocampus and amygdaloid nuclei of the brain.

Although somewhat of an oversimplification, norepinephrine can be thought of as the dominant neurotransmitter for stimuli reaching the VMN, dopamine for the AC, and serotonin for stimuli mediated via the limbic system. One can thus divide stimuli for growth hormone release on the basis of which of these three systems they may be activating.

When lesions are placed in the VMN, suppression of growth hormone release has been observed to occur by Frohman et al (1968). If the neuro-transmitter content in this area of the hypothalamus is norepinephrine, then excitation can be said to be via α adrenergic mechanisms. Vaso-pressin (Heidingsfelder, 1968), exercise (Hansen, 1971), cholecystokinin, and renin (Vijayan, 1979) are thought to induce growth hormone release at the level of the VMN via such α adrenergic mechanisms. Black-ard et al (1968) also showed that GH release, mediated by insulin-pro-duced hypoglycemia, could be prevented by blockage of α-adrenergic receptors by phentolamine, an inhibitor of α adrenergic transmission. The exact method by which norepinephrine and adrenergic blocking agents act to regulate GH secretion is unknown but could be due to an effect on the production of releasing factors in the hypothalamus. Ascending nore-pinephrine inputs to the hypothalamus have been shown by Jonsson and Hökfelt (1972) to terminate directly on the arcuate nucleus in the rat, and could therefore have some effect on somatostatinergic neurons.

This same group showed, by histochemical means, that dopaminergic nerve terminals surround the primary capillary plexus of the portal vascular system, and that the arcuate nucleus is rich in dopamine. Conflicting results are found in the literature concerning the effect of dopamine on GH release. Administration of L-dopa by Kansal et al (1972) to rhesus monkeys led to the release of GH from the pituitary; however, no such release was observed if L-dopa was administered to rats (Kato et al, 1973). Müller et al (1967) and Boyd et al (1970) thought that L-dopa is taken up by the monkey dopaminergic terminals in the arcuate nucleus from the circulation and converted to dopamine by dopa decarboxylase. Increased storage of the neurotransmitter could then be postulated to lead to the release of some growth-hormone-releasing hypothalamic factor into the portal system. However, more recent work by Steiner et al (1978) argued against the view that GH release by L-dopa was mediated by a dopamine receptor mechanism in the hypothalamus. Their work and the observation by Brown et al (1976) suggest that dopamine receptors are not found in the median eminence of the hypothalamus but only in the pituitary. This suggested to these authors and others such as Ben-Jonathan et al (1977) that dopamine in the median eminence may serve as a neurohormone carried by the portal vessels to the pituitary rather than as a neurotransmitter acting on the hypothalamic releasing and inhibitory factors.

In summary, the present data on the effects of the catecholamines, norepinephrine, and dopamine on GH release suggest that α-adrenergic receptors in the hypothalamus stimulate the release of GH, whereas dopamine receptors may inhibit GH, this area being somewhat controversial, and

wide species differences may also exist.

The third area of the brain associated with growth hormone release
is the limbic system. Having a role involving the maintenance of wake
emotional and behavior states, the limbic system also appears to play an im-
portant role in slow wave sleep. As previously noted in humans, this
sleep state is associated with a rise in GH levels. Since nerve termin-
als of the limbic system terminate in the hypothalamus and are, in general,
serotoninergic, the neurotransmitter serotonin may mediate the growth
hormone release seen with slow wave sleep in man.

There are a number of stimuli to GH release from the pituitary,
whose locus of action is unclear. These include stress, protein deple-
tion, and the administration of morphine derivatives as shown by Greenwood
et al (1966), Schalch (1969), and Pimstone et al (1966). As with stimu-
lation, there are other conditions that inhibit growth hormone release
such as obesity, elevation of free fatty acid levels, glucocorticoids,
and hypothyroidism, whose mechanism of action is likewise unknown. (Pim-
stone, 1966; Unger, 1965; and Frantz, 1964) Fragments of ACTH (4-10) and
ACTH (18-39), α MSH and β MSH also can be shown to inhibit growth hormone
secretion in the rat. Here, the mechanism of action studied by Bruni et
al (1977) appears to be mediated via the hypothalamus, not the pituitary,
as they reported a failure to elicit hormones in vitro from pituitaries.

Evidence also exists for a "short loop feedback" mechanism of growth
hormone itself on both the hypothalamic and pituitary levels. For example,
growth hormone pellets implanted into the hypothalamus can cause a

reduction in circulating pituitary GH levels   (Voogt et al, 1970). Exo-
genous growth hormone can diminish the growth hormone response to insulin,
exercise, or arginine in the rat   (Müller et al, 1966). Similarly, Hagen
et al (1972) observed a dampening of GH release to known stimuli such as
arginine infusion in normal volunteers administered human growth hormone.

In summary, factors that affect the release of growth hormone from
the pituitary are complex, some being mediated via hypothalamic inhibi-
tory and possibly releasing factors, and others whose mechanisms of action
remain unknown.

The next section will review the relevant literature relating to
thyroid stimulating hormone (TSH), another hormone which, like growth
hormone, is derived from the pituitary gland.

C. PITUITARY THYROID STIMULATING HORMONE (TSH)

Thyroid stimulating hormone (TSH) is the principal hypophyseal
hormonal regulator of thyroid growth and the biosynthesis and secretion
of the thyroid hormones, thyroxine (T_4) and triiodothyronine (T_3).

Using immunohistochemical methodology, Moriarty (1973) showed that
thyrotrophs constitute 3%-5% of the pituitary cell population. Polygonal
cells, often appearing in clusters predominantly in the anteromedial
portion of the gland, they contain small cytoplasmic granules. In the
absence of the thyroid, these cells show a marked ballooning of the rough
endoplasmic reticulum cisternae and a decrease in secretory granules,

implying a disruption of the transport processes of granules through the Golgi apparatus. (Farquhar et al, 1954) The concentration of TSH, which is synthesized on ribosomes and stored in the granules, may only be 5% of normal in a hypothyroid rat. The molecular structure of this pituitary hormone will be dealt with in the next section.

a. Molecular Structure of Pituitary TSH.

Thyroid stimulating hormone is a glycoprotein containing 15% carbohydrate and having a molecular weight of 23,300 Daltons. Treatment of the molecule with dilute acid by Cornell et al (1973) resulted in its dissociation into two dissimilar subunits or peptide chains, designated alpha (α) and beta (β). The α subunit consists of 96 residues, which are almost identical to α chorionic gonadotrophin (CG), α luteinizing hormone (LH), and α follicle stimulating hormone (FSH) subunits. The β subunit is larger than the α, being composed of 110 amino acid residues, and is structurally dissimilar from the β units of CG, LH, and FSH. Both subunits contain many half cystine residues, assumed by Pierce (1974) to be in a covalent-disulfide bond form.

Each TSH molecule contains three oligosaccharide moieties attached to the peptide via asparagine residues. The sugars and amino sugars include fructose, mannose, galactose, glucosamine, and galactosamine. Two of the three carbohydrate moieties are associated with the α subunit, where sialic acid residues are similarly located. The significance of the carbohydrate constituents, in terms of biological expression on TSH

metabolism, has not been determined. The native structure of the molecule depends upon noncovalent interactions between the two subunits. A high glycine and proline content and the intrachain bonding preclude any long distances of α helix. An isolated α chain has no biological activity, and a separated β chain only a minimal ability to bind and activate TSH receptor sites. Recombination of α and β chains fully reactivates the hormone. Studies by Vaitukaitis et al (1973), generating antisera to both intact molecules and to both subunits, revealed that most immuno- logical determinants reside on the hormone-specific β chain. Although TSHβ, LHβ, and CGβ share extensive sequence homology, little immunologi- cal cross reactivity has been encountered. A common ancestral molecule for the four different hormones has been proposed to account for the similarities between individual α and β subunits.

Levels of TSH present in the circulation vary with age and are dependent in the young rat on the development of the hypothalamic pitui- tary-thyroid axis.

b. <u>Thyroid Stimulating Hormone Levels in Plasma and Pituitary</u>

The development of the hypothalamic pituitary thyroid axis has been examined in the rat by several groups: Dussault et al (1973), Cons et al (1975), Kieffer et al (1976), and Friedman et al (1979). In all cases, pituitary TSH was found in low levels just before birth and postnatally reached a maximum at day 15 before falling slightly to adult levels.

At term, fetal rat serum TSH is found to be slightly higher than maternal serum levels. There is a falloff in hormonal levels of serum after birth followed by a surge to reach adult levels by day 12. A rise in human serum TSH is also observed at birth and is sustained for 2-3 weeks postpartum (Conklin et al, 1973). In this study and those of the rat, maternal TSH was found not to traverse the placenta.

Adult rats have been reported to show diurnal variation in their levels of serum TSH, a similar situation to that reviewed for GH except that Parket et al (1976) do not believe a link exists with sleep and TSH. Leppäluoto et al (1974) have reported diurnal variations in serum, peak levels being attained by late evening, the lowest levels in the afternoon, and a relatively high plateau between 8-10 a.m. The mechanisms responsible for this rhythm are not completely understood, although Niles et al (1979) have suggested that melatonin or the precursor of melatonin, n-acetyl serotonin, in the pineal, may have a role in the diurnal rhythm of TSH. Pinealectomy was found to increase circulating TSH in animals kept under one-hour-light/23-hour-dark lighting conditions, and a similar increase in TSH levels was observed after active immunization against melatonin and n-acetyl serotonin.

The overall concentration of TSH in the periphery reflects a dynamic equilibrium between TSH secretion and TSH distribution, degradation, and excretion. This regulated level of circulating TSH is dependent on many factors; however, the condition of the thyroid gland, which requires TSH for the maintenance of euthyroidism, is one of the most important factors.

c. Biological Effects of TSH

Effects of TSH on the thyroid gland include hyperplasia and hyper-
trophy, stimulation of I^- transport into thyroid cells and its incorpora-
tion into the thyroid hormones T_4 and T_3, and the hormonogenesis and
secretion of these two hormones. In order to comprehend the importance
of pituitary TSH to the thyroid, a brief description of the thyroid and
the production of T_4 and T_3 will be given.

The thyroid gland consists of two lobes connected by a narrow
isthmus crossing the rat trachea. These lobes are composed of follicles,
which are spheroid structures consisting of a single layer of epitheleal
cells enclosing a cavity or follicle lumen which is filled with a viscous,
proteinaceous solution, the colloid. The shape of the follicle cells
vary; however, in any one follicle, the cell height is almost uniform. In
general, low cells are characteristic of hypoactive follicles, as seen
in hypophysectomized animals, whereas high cells are hyperactive follicles.
Administration of pituitary TSH to hypophysectomized animals can restore
the height of thyroidal follicle cells to normal (Tice, 1974).

The specific physiological processes of the thyroid include trapping
of iodide required for the thyroid hormones, the synthesis by the follicle
cell of a specific thyroid glycoprotein, thyroglobulin; storage of thyro-
globulin in the follicle lumen, reabsorption of thyroglobulin into the
cell, degradation of this protein, and release of the thyroid hormones T_3
and T_4 into the circulation.

The kinetics of TSH action appear to be biphasic on iodide transport. An early depressive phase lasts 1-2h and is followed by overall enhancement, as shown by Williams et al (1975) using mouse thyroid tissue. The depressive phase is thought to be due to an acute increase in iodide efflux from the follicular cells, possibly reflecting a general increase in membrane permeability, and the enhancement phase by an increase in the unidirectional clearance of iodide by cells presumably reflecting the induction of iodide carriers.

The binding of iodide to protein and the formation of iodothyronine is a rapid process. Two enzymatic systems are involved in this step, the generation of H_2O_2 and the oxidation of I^- by a peroxidase (Taurog, 1970). As no effect of TSH on peroxidase activity has been observed, it is probable that the H_2O_2-generating system is the hormone's site of activation as proposed by Ahn et al (1970).

Thyroglobulin is synthesized in the rough endoplasmic reticulum and passes via the Golgi apparatus to the cell surface in vesicles before reaching the follicular lumen by exocytosis. The influence of TSH in this process is observed in hypophysectomized animals where a decrease in the size and contents of the lumen is noted. Ekholm et al (1975) have demonstrated this phenomenon using radioautography to show that a rapid stimulation by TSH discharged radio-labeled thyroglobulin into the colloid space.

The first step in the release and secretion of thyroid hormones is marked by pseudopod formation by the apical plasma membrane protruding

into the follicle lumen and by endocytosis followed by portions of colloid reappearing in follicular cells. Pseudopods are formed within two minutes of TSH addition to incubated thyroid slices, and 10 minutes later T_4 can be measured in the external medium (Ekholm, 1979).

Degradation of thyroglobulin in the follicle cell requires that hydrolytic enzymes gain access to the content of the colloid droplets; the source of these enzymes is the lysosomes, as shown by Wollman et al (1964). Lysosomes are found in all parts of the cell but accumulate towards the lumenal cell surface after TSH stimulation (Seljelid, 1967). The consensus, therefore, is that proteolytic enzymes reach the thyroglobulin by colloid-droplet/lysosome fusion, whereby degradation of the protein and release of thyroid hormones are achieved.

The precise way in which TSH achieves this regulation of thyroid protein synthesis is not clear. Adiga et al (1971) showed that certain effects of TSH on the thyroid such as the stimulation of RNA synthesis and RNA polymerase in porcine thyroid could be inhibited by protein or RNA synthesis inhibitors, indicating the possibility that TSH acts at the level of transcription. Although a direct effect on the synthesis of thyroglobulin messenger RNA has not been observed by Larry et al (1971) in rats, the continuous presence of TSH seems to be required to sustain the expression of the thyroglobulin gene, as postulated by Pavlovic-Hournac et al (1971).

The stimulation of incorporation of amino acids into thyroid proteins in vivo (Pavlovic-Hournac et al, 1973), and in vitro (Lecocq et al,

1972) is thought to be an effect at the translational level by such authors. However, a direct stimulatory effect of cyclic adenosine monophosphate (cAMP), the postulated intracellular mediator of TSH actions (Rapaport, 1976), on protein synthesis in an acellular system, devoid of nuclei, has only been observed at unphysiological concentrations of the cyclic nucleotide by Wagar (1973).

With regard to thyroid metabolism, TSH enhances thyroid cell respiration, mainly mitochondrial, as shown by Dumond (1971), and enhances glucose uptake and its overall metabolism (Dumont et al, 1965). Dumont's group also showed an augmentation in the overall pentose phosphate cycle and the incorporation of glucose into protein after TSH administration. Glycogen and fatty acid levels in lipids decrease, reflecting an activated catabolism. TSH has been shown to enhance the incorporation of precursors into most phospholipids, as demonstrated by Scott et al (1970) using ^{32}P incorporation into monophosphatidylinositol, phosphotidates, phosphatidylethanolamines, phosphatidylserine, and in some cases phosphatidylcholine. No effect of TSH was observed on ^{32}P incorporation into sphingomyelin or phosphatidylglycerol in isolated cells or slices from pig thyroid.

Extrathyroidal actions of TSH, the significance of which are not well understood, include the ability to stimulate lipolysis (Hartree et al, 1972) and intracellular levels of prostaglandins (Vale et al, 1972) in human adipose and rat pituitary tissue.

The hormone can therefore be seen to have a wide range of effects both on the thyroid and other tissues. The secretion of TSH from the

pituitary gland can be regulated by several factors which will be dis-
cussed in the next section.

d. Regulation of TSH Release from the Pituitary.

The hypothalamic-pituitary-thyroid axis has been shown to be a
dynamic "negative feedback" control loop, the activity of which is in-
fluenced by the peripheral consumption of thyroxine (T_4) and modulated
by hypothalamic control of pituitary responsiveness, by thyrotropin re-
leasing factor (TRH).

Early experiments with pituitary stalk sectioning by Boler & Schally
et al (1969) and Burgus & Guillemin et al (1969) led to the identification
and synthesis of thyrotropin releasing hormone (TRH), a small peptide
consisting of the 3 amino acids, Glu-His-Pro-NH_2, which was inactivated
within minutes by serum. Secreted into the portal blood vessels by hypo-
thalamic cells, TRH travels to the pituitary and binds to the thyrotroph
membrane on specific receptor sites with high affinity, stereospecificity,
and reversibility, as shown by Wilber et al (1973[a]). The magnitude of TSH
release from the pituitary cells is dependent on the increase in membrane
binding of TRH (Wilber, 1973[b]). TRH stimulation also causes augmented
TSH synthesis, as judged by the incorporation of ^{14}C alanine or ^{14}C gluco-
samine into pituitary slices (Wilber, 1971). An interesting immunohisto-
chemical report of Moriarty et al (1978) suggests that TRH may also be
located in the thyrotroph secretory granule within the pituitary gland.
Whether this material is of hypothalamic origin is not known.

Control of TRH secretion is not well understood. Ablation of supra-optic and paraventricular nuclei results in suppression of thyroid func-tion, and destruction of the median eminence and arcuate nucleus results in a fall of TSH and T_4 levels (Grear, 1951; and Hefco et al, 1955[a]). Attempts to correlate the hypothalamic content of TRH with metabolic status indicate minimal or no variation (Montoya et al, 1975), although possibly the augmented TSH secretion induced in rats by cold exposure is via an elevation in TRH production (Hefko et al, 1975[b]). The synthesis of TRH is stimulated also by dopamine and norepinephrine and reduced by serotonin, as shown in vitro using mouse hypothalamic tissue by Grimm and Reichlin (1973). Further evidence of monoaminergic transmitter control of TRH comes from the work of Krulich et al (1977), who showed that systemic injection of α methyl-p-tyrosine and inhibitors of dopa-mine β hydroxylase cause decreases in serum TSH, as does blockade of α adrenergic receptors. Stimulation of dopamine receptors with apomorphine and L-dopa will similarly cause decreased release of TSH, leading to the conclusion that central noradrenergic systems have a stimulatory effect on serum TSH via TRH. The opiate peptide, leu-enkephalin, also appears to inhibit TSH via TRH as shown by May et al (1979).

Primary control of TSH secretion is, as described, from the posi-tive input of TRH as well as the negative control exerted by the thyroid hormone (T_4). The inability of pharmacological quantities of exogenous T_4 to prevent an increase in TRH in response to cold exposure suggests that T_4 does not alter TRH secretion (Montoya et al, 1974), but rather has an effect on the pituitary thyrotroph. Reichlin et al (1973), however,

showed that intrahypothalamic implants of T_4 do depress thyroid function; the controversy of where T_4 exerts its negative feedback control over TSH release still exists.

T_4 stimulates in the pituitary thyrotroph the synthesis of nucleic acids and protein which, in turn, affects the secretion of TSH. Observation by Samuels et al (1973) has shown that the effects of T_4 on the pituitary are mediated by effects on transcription of genetic material to increase protein synthesis. Thus, a metabolic effect of thyroid hormone, rather than actual circulating level of hormone, modulates pituitary responsiveness.

Other regulators of TSH secretion than TRH and T_4 are also known.

Knigge (1964) has shown that infusion of TSH itself into the cat hypothalamus will inhibit thyroid hormone release, and Motta et al (1969) have shown decreases in TRH after exogenous TSH was administered to thyroidectomized rats. Although TSH levels are not modified by activity, starvation, circulating free fatty acids, or amino acids, hypoxia can cause a lowering of TSH in rats, which is probably a stress phenomenon (Curbelo et al, 1979). This group also showed a decrease in circulating TSH after adrenalectomy.

The last two main sections of this literature review have dealt with the characterization of the two pituitary hormones, GH and TSH, whose presence in the brain is the subject of this dissertation. In order to introduce the next section, which reviews the present knowledge

on peptides present in the brain, it is necessary at this point to intro-
duce the "amine precursor uptake and decarboxylation theory" of A. Pearse.
Thyroid releasing hormone has been located in a number of other locations
besides the hypothalamus, in particular, the pancreatic islet D cells.
Known to contain primarily the hormone somatostatin, they are also known
to contain TRH-like immunoreactive material (Morley et al, 1978). The
finding of this and other peptides in both brain and gut has led to the
"APUD" theory of Pearse (1966), Pearse (1977), and Pearse and Takor (1979).

D. THE AMINE PRECURSOR UPTAKE AND DECARBOXYLATION THEORY (APUD)

The idea that cells widely dispersed in the nervous and gastroin-
testinal system share the same ability to secrete similar peptides has
been examined by Pearse. These cells, known as "APUD" cells, were pos-
tulated to be derived originally from the embryological neural crest.
Such cells included thyroid C cells, ultimobranchial body C cells, supra-
renal medullary cells, melanocytes, mast cells, pituitary corticotrophs,
melanotropes, somatotropes, pinealocytes, and a wide range of endocrine
cells in the gastrointestinal tract and pancreas. These regulatory mole-
cules produced by such cells may enter the bloodstream to reach and alter
the activity of distant target cells, an endocrine action; diffuse local-
ly through interstitial spaces to reach and influence groups of neighbor-
ing cells, a paracrine action; or cross a synaptic junction to activate or
inhibit the postsynaptic cell, a neurocrine action (Zimmerman, 1979).

Although the APUD-neural crest theory suggested the existence of a widely dispersed neuroendocrine system, offering a unifying concept for understanding many tumor-associated endocrine syndromes and the phenomenon of ectopic hormone production by a wide range of tumors; it seems evident from more recent studies that the possession of APUD characteristics by cells does not imply necessarily a neural crest origin. Cheng et al (1974) conclude that APUD endocrine cells of the pancreas and intestine arise from the endoderm of the developing gut wall, and that the development of "APUD" characteristics appears to be one of several pathways of differentiation available to primitive and uncommitted epitheleal cells.

However, APUD characteristics do seem to be associated with tissues producing small polypeptide hormones, and it was therefore not surprising to find such hormones in both brain and gut. The next section of this review of literature discusses in more detail such findings, and demonstrates that many of the peptides found in brain and gut appear to have significant roles to play in the functioning of the brain.

E. PEPTIDES FOUND IN THE CENTRAL NERVOUS SYSTEM

The idea of chemicals being involved in neuronal communication is credited to Elliot (1904). His theories foresaw later results from microelectrode recordings of single cells and the microiontophoretic application of peptides to neurons which showed that results could not be explained completely by the presence of purely electrical junctions.

The concept of the presence of chemical neurotransmitters, which convey information between adjacent nerve cells, and chemical neuromodulators, which amplify or dampen neural activity but which are not responsible for direct transfer of a nervous signal from the pre- to post-synaptic element, has developed over the last 60 years. Monoaminergic catecholamines such as dopamine and serotonin are known to be among the class of substances known as neurotransmitters. Whether peptides recently found in the brain such as renin and the hypothalamic peptides act as neurotransmitters or neuromodulators is not as yet known. It is clear that many such peptides affect the ionic potential of a receptive membrane as will be seen in the following review, and that these effects are not identical to those produced by conventional neurotransmitters.

Hökfelt et al in 1979 proposed that the peptide receptor is restricted to specialized areas of the post-synaptic membrane. Several theories have been discussed by this group, using the evidence available in 1979, for a neuromodulator role for neuropeptides. One such theory entertains that a peptidergic nerve terminal in the brain could release its product into the extracellular spaces adjacent to fenestrated capillaries. The peptide is then carried via the vascular system to a target, where it interacts with a receptor. Another possibility would be a synaptic arrangement, where the peptide and possibly a neurotransmitter are released into the synaptic cleft where interaction occurs with receptors on the post-synaptic membrane. A third possibility would be a parasynaptic arrangement, whereby the peptide, on release from the nerve terminal, would diffuse and interact with receptors at sites other than the post-

synaptic membrane, possibly nearby neural membranes, or on the peptidergic terminal itself. These are some of the ideas at present being discussed to explain the presence of the large number of peptide hormones being discovered in the CNS.

They could also be present in the brain due to incomplete suppression of the genome or as receptor-bound hormone originating from secretory tissue in areas other than the brain. In the following discussion of present-day knowledge of brain peptides, beginning with a review of the presence in extrahypothalamic areas of hypothalamic hormones, some of these points will be discussed.

HYPOTHALAMIC HORMONES

The localization of peptidergic neurons in the CNS had indicated an emerging pattern with respect to distribution. Thyroid-releasing hormone, somatostatin, and gonadotropin-releasing hormones have been found in cell bodies and nerve terminals, both intra- and extrahypothalamically. It appears that besides mediating anterior pituitary secretion, these peptides may also play a role in brain function by mediating extrapituitary events. They will be reviewed individually here.

a. Gonadotropin-Releasing Hormone (GnRH).

GnRH is a decapeptide with a blocked aminoterminal composed of pyroglutamic acid, which stimulates pituitary gonadotropins and was found in the hypothalamus by Matsuo and Schally (1971), and Burgus and

Guillemin (1971). Kubek et al (1979) have identified immunoreactive
GnRH in extrahypothalamic areas such as the mammillary body, thalamus,
amygdaloid nucleus, and hippocampus. Very small amounts or no hormone
are found in the human cerebellum and cortex. Baker et al (1975) re-
ported the hormone to be present in fibers leading to extrahypothalamic
areas of the rat brain. Extraction and analysis of this material showed
molecular similarities to the original hypothalamic hormone. Studies by
Parker et al (1978) showed the presence of neurons, the terminals of which
end in the median enimence, which contained GnRH-like material associated
with dense cored granules. On fractionation, 52%-65% of the hormone was
associated with the synaptosomes. In hypophysectomized, ovarectomized
rats, the intraventricular application of synthetic GnRH produces a
specific action on mating behavior in estrogen-primed animals (Foreman
and Moss, 1977).

    b.   <u>TRH (Thyroid-Releasing Hormone)</u>.

TRH-like material has been found in varying concentrations in the
rat brain extrahypothalamically by several groups including Oliver et al
(1974) and Winokur et al (1974). Although the greatest concentration
for any one brain part is found in the hypothalamus, over two-thirds of
the total brain content of TRH is found outside of the hypothalamus.
Areas rich in TRH include the cerebellum, thalamus, brain stem and cere-
bral cortex. Little or no hormone is found in the pineal, amygdala,
hippocampus, or caudate. These two groups differ as to whether TRH is
present or absent in the pituitary; however, Moriarty et al (1978), as

previously reported, have shown histochemically, TRH associated with pituitary thyrotrophs.

The TRH-like material found extrohypothalamically possesses the same physiochemical and biological properties as the hypothalamic peptide (Nemeroff et al, 1979). The content of TRH in the CNS is not affected by hypophysectomy, thyroidectomy, or adrenalectomy (Nemeroff et al, 1979), whereas hypothalamic levels of TRH fall after such treatment. It was found by Nemeroff (1979) that hypothalamic TRH levels could be restored with a combination of T_4 plus TSH treatments. In the rat brain, it appears that the synthesis of this tripeptide occurs in situ, independent of the possible transport of the hormone from the median basal hypothalamus (Jackson et al, 1977). Studies by Bennett et al (1975), Parker et al (1978), and Schaeffer et al (1977) indicate the presence of TRH in rat median eminence and hypothalamic synaptosomal fractions, and that depolarizing procedures can release the hormone from such preparations. Burt et al (1975) have shown the presence of binding receptors in rat brain membrane preparations. These receptors occur ubiquitously throughout the regions of the brain, except for their absence in the cerebellum, and give credence to a possible neurotransmitter, rather than neuromodulator, role for TRH.

A study by Renaud et al (1975) on single neuronal activity in various brain sites showed that microiontophoretic application of TRH, somatostatin, and GnRH reduced neuronal activity in the rat. Studies by Brown et al (1975) show that, although this effect may be similar for all

three hormones, the biological effects on the CNS suggest opposing mechanisms for the individual peptide. However, the independence of all these effects from those on the pituitary suggest a direct action on the CNS by the hypothalamic hormones.

Pentobarbital toxicity is reversed by TRH but enhanced by somatostatin (Prange et al, 1974), whereas a strychnine effect on convulsions is potentiated by TRH but antagonized by somatostatin (Moss, 1979). It has been shown that TRH not only increases norepinephrine turnover in the brain (Keller et al, 1974), but also potentiates the behavioral effect of L-dopa (Plotnikoff et al, 1972). Plotnikoff showed that this latter ability was reduced by castration or pinealectomy. It appears from the work of Metcalf (1974) that TRH markedly antagonizes the sedation and hypothermia induced by a wide variety of centrally acting depressants such as ethanol, and that adrenergic blocking agents such as propanolol do not alter this antagonistic effect. Cott et al (1976) have further shown that these effects of TRH are independent of TSH and T_4 influences, since hypophysectomy and T_4 administration failed to reverse these responses. Prompt emergence from ether anesthesia is also characteristic in rats following TRH treatment (Moss, 1979). Other behavioral effects seen after intraventricular injection of TRH by Schenkel-Hulliger et al (1974) include muscle tremor, excitation, tail lifting, and pilo erection. A noted resemblance of these effects to those induced by morphine withdrawal has been noted, and it is of interest that brain areas where naloxone, a competitor for morphine binding sites, precipitates withdrawal shaking, parallel the sites of TRH-induced shaking and endogenous

sites of TRH in the brain. Another behavioral effect by TRH administration was shown by Vijayan et al (1977) to be an apparent suppression of feeding and drinking behavior. Most catecholamine levels remain unchanged after TRH treatment; however, Nemeroff et al (1979) report an increase in norepinephrine release from presynaptic terminals.

The range of CNS effects which TRH exhibits is therefore large. Apart from the CNS, thyroid releasing hormone has been identified in the spinal cord (Hökfelt et al, 1975[a]), retina (Schaeffer et al, 1977), in the islet cells of the pancreas (Morley et al, 1978), and in the placenta (Shambaugh et al, 1978). Recent evidence from Engler et al (1980) has shown that circulating TRH in the neonatal rat is derived from the pancreas and GI tract.

The third hypothalamic peptide to be isolated extrahypothalamically is the GH-inhibiting factor or somatostatin.

c.   Somatostatin.

This linear tetradecapeptide has been found widespread throughout the CNS using histochemical and radioimmunoassay techniques. Although the highest concentration is in the hypothalamus, other areas contain substantial amounts (Parsons et al, 1976). Subcellular studies by Epelbaum et al (1977) indicate that 70% of the somatostatin is localized in the synaptosome fraction. This peptide has also been isolated in both cells and fibers of the hypothalamus as well as from cell bodies throughout the rat brain, in particular the limbic system and cortical areas as

shown by the immunohistochemical studies of Hökfelt et al (1977[a]).

Somatostatin's presence in synaptosomes and its ability to act on single neuron units to inhibit spontaneous electrical activity imply a role in neural function (Brown et al, 1975). Tan et al (1977) have suggested that somatostatin inhibits the release of a pool of Ca^{++} taken up by synaptosomes. As Ca^{++} influx is closely related to nerve depolarization, neurosecretion in nerve cells, and secretory processes in endocrine cells, it is possible that somatostatin influences neuronal excitation through modification of Ca^{++} transport at the synaptic membrane level. Injection of somatostatin into the amygdaloid nucleus has been studied by Rezek et al (1977). They revealed a dose-dependent dissociation of effects on behavior and motor control. Lower doses induced a behavioral excitation accompanied by a variety of tremors, whereas higher doses prevented the expression of a normal behavioral pattern due to the severity of disturbances in motor control and the sleep-waking cycle. The effect on behavior is almost opposite to that of TRH. This includes a reduction in locomotion (Segal et al, 1974), prolongation of pentobarbital sedation (Prange et al, 1974), a decrease in the duration of strychnine-induced seizures, and sedation and hypothermia induction (Brown et al, 1975). In general, therefore, somatostatin exhibits a CNS-depressant form of action as shown by Segal et al (1975) and Prange (1974). Somatostatin also potentiates the behavioral effects of L-dopa and can act as a partial antagonist-agonist on opiate receptors.

From this review it is apparent that, as with the other hypothalamic peptides, a dual role exists for somatostatin. Extrahypothalamically, it

acts as a modulator of nervous function and hypothalamically as a peptide inhibiting the release of growth hormone, TSH, prolactin, glucagon, insulin, gastrin, and cholecystokinin.

Somatostatin-like immunoreactivity has also been located in thyroid C-cells (O'Brian et al, 1979), and in the stomach (Arimura et al, 1975) and pancreas of the rat by Luft et al (1974). In the pancreas, the anatomical positioning of islet D-cells between α and β cells of the islets suggests that they may be involved in some form of control over insulin and glucagon secretion (Dubois, 1975) and (Goldsmith et al, 1975). Hökfelt et al (1977[a]) have suggested the possibility that somatostatin is present in a population of peripheral sympathetic noradrenergic neurons, a fact which would violate Dale's principle of "one neuron, one transmitter" to suggest concomitant storage of a small biologically active peptide and an amine in the same neuron.

Another group of hormones found in the CNS are those originally detected in the GI tract.

d. Bombesin.

Originally detected in frog skin and mammalian gut by Wharton et al (1978), bombesin has also been isolated from mammalian hypothalamus by Villareal et al (1978). The "brain" hormone is able to stimulate the secretion of growth hormone, glucagon, and prolactin, and is chemically and immunologically distinct from frog skin bombesin. If injected intraventricularly, a lowering of body temperature is noted and an

elevation in blood sugar levels also occurs (Brown et al, 1977).

A second GI tract hormone to be found in the CNS is glucagon.

e. Glucagon.

This pancreatic islet hormone has recently been detected in several areas of canine brain by Conlon et al (1979). Found in concentrations 100× the level in plasma, the glucagon-like peptide seems to be a variety of gut glucagon.

A second pancreatic hormone recently found in the CNS is insulin.

f. Insulin.

Insulin has been demonstrated in the brain in concentrations higher than plasma by Havrankova et al (1979). This "brain" insulin is indistinguishable chemically and immunologically from pancreatic insulin and does not vary with extreme changes in plasma insulin, indicating that brain insulin and its receptors are probably regulated independently of their counterparts elsewhere. Whether this hormone is synthesized locally is not known. Specific binding sites for insulin are localized on axons and axon terminals in the external median eminence and hypothalamic arcuate nucleus, suggesting that a discrete population of nerve terminals exist as potential targets for the direct effects of insulin (Van Houten et al, 1980). This group suggests that, after the initial binding, in the median eminence, insulin may be internalized and transported by retrograde flow to the arcuate nucleus.

Highest concentrations of insulin receptors are found extrahypo-
thalamically in the cerebral cortex and olfactory bulb. Other areas such
as the hippocampus and amygdala have fairly high numbers of receptors,
whereas the retina, spinal cord, and cerebellum have the least. Specu-
lation that insulin receptors are preferentially located within elements
of the limbic system leads to an association between emotional behavior,
feeding, and defense, characteristically limbic system functions, with
insulin as a possible regulator of these actions.

The hormone has been shown to promote, in cells of the hypothalamus,
local glucose uptake (Smith et al, 1972) and subsequent metabolism (Panks-
pepp, 1973), to be capable of altering the electrical activity of hypo-
thalamic neurons (Oomura, 1976), and to stimulate hypothalamic norepine-
phrine release (McCaleb, 1979).

A note of caution before assigning insulin as a peptide indigenous
to the CNS is that apparently no close correlation between insulin levels
and receptor content exists in the different regions so far studied.

Two other GI tract peptides, gastrin and cholecystokinin, are also
found in the CNS.

g.   Gastrin and Cholecystokinin (CCK).

Vanderhaeglin et al (1975) first reported the finding of a peptide
in hog, dog, and human CNS which reacted with antigastrin antibodies.
From chromatographic elution patterns, they concluded that the molecular
weight of this peptide was less than gastrin (2-17) and more like that of

cholecystokinin. Since then, it is apparent that both gastrin-and chole-
cystokinin-like activity are present, but that they exhibit distinct
distribution patterns in the CNS.

As reported by Rehfeld et al (1979), gastrin is found in the
hypothalamus, pituitary, and peripheral nerves. Cholecystokinin distri-
bution has been studied histochemically by Innis et al (1979), Larsson
et al (1979[a]), and Strauss et al (1977). This peptide was found to be
widely distributed in nerves throughout the body. In the CNS, neocorti-
cal as well as hippocampal, amygdaloid nucleus and hypothalamic regions
are richly endowed with cholecystokinin-containing nerves and cell bodies.

Rehfeld et al (1979), using ^{35}S methionine incorporation, have
shown in vivo biosynthesis of cholecystokinin in human and hog cortical
cells. Brain CCK has been shown by Dockray (1980) to be mainly the COOH-
terminal octapeptide (CCK 8) of the GI hormone. Cholecystokinin and CCK 8
are present in amounts comparable to those found in the GI tract. Mal-
esci et al (1980) have shown that the brain contains at least two enzymes
involved in the conversion of CCK to CCK 12 and CCK 8; the twelve residue
peptide appears to be more biologically active than CCK 8.

In vivo and in vitro effects of CCK have been reported on gonado-
tropin, prolactin, growth hormone, and TSH release in the rat by Vijayan
et al (1979). Intraventricular injections, followed by assay of plasma
hormone levels, showed an increase in growth hormone and a decrease in
TSH levels. The mechanism of release of pituitary hormone appears to be
mediated via a hypothalamic intermediary. Gastrin exhibits similar

properties in the release of growth hormone and TSH (Vijayan et al, 1978). A possible role for CCK in appetite regulation, as proposed by Gibbs et al (1973), is more controversial, as results may be due to the peripheral effects of the peptide.

Evidence for serotoninergic and opiate modulation of brain cholecystokinin has been presented by Morley et al (1980).

Vasoactive Intestinal Polypeptide (VIP), another GI tract peptide, has been isolated from CNS tissue.

h. Vasoactive Intestinal Polypeptide (VIP).

Vasoactive Intestinal Polypeptide (VIP) is a 28-amino-acid residue peptide originally found in porcine duodenum by Said et al (1972). It possesses similar amino acid sequences and pharmacology to secretin and glucagon.

Its actions peripherally, as shown by Said and his co-workers, are systemic vasodilation, stimulation of myocardial contractility, glycogenolysis, lipolysis, stimulation of insulin secretion, and inhibition of gastric acid production.

Said et al (1976) reported the presence of VIP in intra- and extra-hypothalamic sites in the CNS, in neural tumor cell lines, as well as in the GI tract. A recent paper by Sims et al (1980) has demonstrated histochemically the presence of four major VIP-containing systems in the brain. A major intracerebral cortical system, another innervating the

central amygdala and nucleus of the stria terminalis, a pathway originating in the suprachiasmatic nucleus of the hypothalamus, and another in the central gray of the midbrain. Specific cell body staining was seen in the limbic and neocortex, the suprachiasmatic nucleus of the hypothalamus, and the central gray of the midbrain. Giachetti et al (1977) have shown VIP's localization in synaptosomes, and a K^+-evoked, Ca^{++}-dependent release has been demonstrated by Emson et al (1978). Specific binding of VIP and stimulation of cAMP release has been demonstrated in brain synaptic membranes by Taylor et al (1979).

Intraventricular administration of VIP has been shown to stimulate the release of growth hormone, prolactin, and luteinizing hormone (Borghi et al, 1979) from the pituitary. Direct application of VIP to the pituitary has no effect, but Samson and McCann et al (1980) have shown that VIP receptors are present in the hypothalamus and that VIP can act to stimulate LH release from hypothalamic synaptosomes obtained from ovarectomized female rats. They propose a neuromodulator role on GnRH in the hypothalamus, as the mechanism for release of LH from the pituitary.

The heavy VIP innervation of the central nucleus of the amygdala and central gray of the midbrain is similar to the reported distribution of the opiate, enkephalin, and for neurotensin (Basbaum et al, 1978). As hypothesized for these two peptides, VIP may also have a role in pain sensation.

Evidence for synthesis within certain brain areas comes from the work of Besson et al (1979), who studied the effects of deafferentation

of the hypothalamus on levels of VIP. Two weeks after deafferentation,

the VIP concentration of the caudal hypothalamus and stalk were lowered

by 40%. In contrast, no change was shown in cortical VIP levels, sug-

gesting that the peptide is derived from neural cell bodies mainly out-

side the median basal hypothalamus.

The next peptide to be discussed, Substance P, was known to be

present for many years in other tissues before its recent isolation

from the CNS.

i.   Substance P.

Von Euler et al (1931) first isolated from equine tissues, other

than brain, a factor which they designated Substance P and which caused

intestinal contractions and lowered blood pressure by vasodilation.

Substance P was not, however, more thoroughly characterized until 1971

with the establishment of the amino acid sequence of a sialogogic peptide

isolated from bovine hypothalamus (Chang et al, 1971).

Using immunohistochemical techniques, Hökfelt et al (1977[b]) reported

Substance P to be present in the central and peripheral nervous system,

showing an uneven distribution in the CNS except in the cortex, cerebel-

lum, and in the sympathetic ganglia of the guinea pig, cat, and rat.

Emson et al (1978) have reported the presence of Substance P in the medial

and central nuclei of the amygdala and the bed nucleus of the stria ter-

minalis. Efferents lead from the amygdala to the hypothalamus via the

stria terminalis. Highest concentrations are found in the substantia

nigra. The occurrence of Substance P in neurons of the medullary raphe nucleus projecting to the spinal cord is intriguing, since these same nuclei also have been shown to contain serotonin by Hökfelt's group, yet another possible violation of Dale's Law of "one neuron, one transmitter."

The levels of Substance P in the brain stem and spinal cord are higher in fetal than adult tissues, as shown by Gilbert et al (1979). Other areas of the brain such as the cortex, caudate, and hypothalamus show a rise in peptide level at 14-28 days postpartum and demonstrate a similar developmental pattern to that of VIP and noradrenaline.

Substance P has been shown to have some neuroendocrine functions, particularly those mediated by dopamine. It has been shown by Fisher et al (1974) that Substance P can release LH and FSH but not GH, PRL, or TSH, in vitro, from pituitary slices, whereas Euler et al (1979) showed stimulation of prolactin, growth hormone release, and inhibition of the release of insulin, in vivo.

This peptide fulfills several criteria to support its postulated role as a transmitter of pain impulses (Otsuka et al, 1975); however, it has yet to be shown to be synthesized in sensory nerves mediating pain transmission. A close analogy exists between Substance P and the opiate metenkephalin. They both are present in the substantia gelatinosa of the spinal cord, and it may be that Substance P is a transmitter of brain signals carried by sensory nerves into the spinal cord and then relayed to the brain thalamus, where pain sensing terminals are located. Because

of their immediate proximity, it has been postulated that the endorphins may produce their analgesic effects by suppressing Substance P release in the spinal cord (Marx et al, 1979).

Evidence that Substance P modulates synaptic transmission of cat motoneurons comes from the work of Krivay et al (1979). This effect appears biphasic: low doses inhibiting and high doses facilitating synaptic transmission, suggesting a neurotransmitter role. However, the slow time-course of some Substance P responses (neurotransmitters usually induce rapid responses), and the finding that the peptide depresses responses to other putative neurotransmitters, suggests that the peptide may not be a true neurotransmitter (Otsuka et al, 1975). Krnejevic et al (1977) have proposed two operationally distinct actions of Substance P to explain these functional differences: a neurotransmitter-like action mediating excitatory synaptic events which are both rapid in onset and brief in duration, and actions of Substance P such as those involving the depression of glutamate-activated events, more of a neuromodulator effect. The presence of Substance P, but not its exact role in the CNS, has clearly yet to be completely characterized.

During the course of the purification of Substance P from bovine hypothalamic extracts, a new tridecapeptide was detected, sequenced, and synthesized by Carraway and Leeman (1973) and is reviewed below.

j. Neurotensin.

Neurotensin has been found by Kobayashi et al (1977) to be in the

highest concentrations in the median eminence, but also to be present in

the amygdala, nucleus accumbens, and caudate. Low levels are found in

the cortex, hippocampus, and pineal, and trace amounts are present in

the cerebellum. Carraway et al (1976) showed that receptor binding con-

centrations differ from this hormonal distribution pattern in that highest

concentrations are in the thalamus and cortex as well as the hypothalamus.

Adrenalectomy or hypophysectomy does not seem to alter overall levels of

the hormone in the brain, and the intestine, stomach, and pancreas have

higher levels than those found in brain tissue (Leeman et al, 1977).

Intraventricular doses of neurotensin were given by Carraway et al

(1976) and found to lower body temperature, accentuate barbiturate seda-

tion, cause hypotension, increase vascular permeability, cyanosis, and a

marked hypoglycemia.

Disagreement exists as to its role in modulation of the secretion

of the hormones of the pituitary. Rivier et al (1977[a]) have shown that

neurotensin is able to stimulate the pituitary secretion of growth hor-

mone, prolactin, luteinizing hormone, follicle stimulating hormone, and

ACTH, postulating the effect is via a hypothalamic intermediate. However,

in a recent abstract by Nemeroff et al (1980), intraventricular neuro-

tensin modulated neurotransmitter activity in the mesolimbic but not the

nigroneostriatal dopamine system and reduced the secretion of TSH, GH,

and prolactin.

Neurotensin can therefore be summarized as being present in both

the CNS and GI tract and appears to have some biological effects on brain

tissue.

The next peptides to be reviewed are those originally associated with the renin-angiotensin system of the kidney which have been isolated from CNS material over the last five years.

k.   Renin-Angiotensin.

Angiotensin II is an octapeptide formed by the action of converting enzymes on the decapeptide Angiotensin I, which is normally the product of the enzymatic action of renin from the kidneys on angiotensinogen. Almost all parts of this renin-angiotensin system have now been isolated from brain homogenates.

A renin-like activity was found in dog, human, and rat brain by Haulica et al (1975), exhibiting molecular and immunological similarities to kidney renin. Renin levels were measured in hog brain by Hirose et al (1980), who showed that the pineal was the richest source of renin-like activity, followed by the hypothalamus, cerebellum, and amygdala.

Reid et al (1977) reported that the pH optimum of brain renin differed from that of kidney renin and that the renin-like brain peptide behaved more like Cathepsin D, a nonspecific acid protease. However, more recent studies have shown that "brain renin" is indistinguishable from renal renin both biochemically and serologically (Slater et al, 1980, and Inagami, 1978). Studies also demonstrated that the distribution of renin and Cathepsin D were distinct and showed no overlap, as shown by Govindrajan et al (1974) and Daul et al (1975). Renin has been identified by Slater and Zimmerman (1980) in many neurons throughout the human

brain by immunohistochemical methods.

Angiotensinogen, renin's natural substrate, was first located in
brain tissue by Ganten et al (1976) and shown to have a molecular weight
of 40,000-60,000 D, presumably too large to cross the blood brain barrier
and, therefore, it was concluded by the authors to be endogenous to the
brain. Wallis et al (1980) have shown a wide distribution for angioten-
sinogen in the rodent brain and that these levels can be modulated by
glucocorticosteroids. The hypothalamus, preoptic area, and periaqueductal
gray regions showed the most consistent reduction in brain angiotensino-
gen after 14 days' adrenalectomy, other brain regions showing little or
no effect. Treatment with corticosterone reversed the effect of adrenal-
ectomy in the four brain regions.

The "converting enzyme" for Angiotensin I to Angiotensin II is
present in rat and human brain in large amounts and is widely distributed
(Yang et al, 1972, and Poth et al, 1975).

Fuxe et al (1976) found positive fluorescence for angiotensin II-
like material in the median eminence and amygdaloid nucleus. The octa-
peptide is localized in neuronal cell bodies, fibers, and terminals in
the paraventricular and perifornical area of the hypothalamus, with
highest concentrations in the terminals of the hypothalamic dorsomedial
and basal nuclei.

Bennet et al (1976) and Siret et al (1977) have identified high
affinity binding sites for ^{125}I angiotensin II on rat brain membranes
which were saturable and reversible. Specific receptors exist only in

the midbrain, thalamus, and hypothalamus. Baxter et al (1980) have shown that these binding sites reach maximum numbers, ten times those found in adult animals, in the first week of post-natal life. Levels of renin and angiotensin II are also higher at birth than those found in adult brains.

Nephrectomy does not change the levels of angiotensin II in the brain, indicating an independence from the renal system. This peptide has been shown to exert several actions on the CNS after intracerebral injections. These include stimulation of drinking, a rise in blood pressure, and vasopressin and adrenocorticotropin (ACTH) release after intraventricular injection (Fitzsimon, 1972). The sites of action of intracerebral angiotensin II appear to be the same as for the circulating peptide, that is, close to the ventricles in the septal region, anterior hypothalamus, and preoptic areas (Johnson and Epstein, 1975). Destruction of tissue at these sites abolishes the rise in blood pressure and increase in water uptake, stimulated by angiotensin II (Andersson et al, 1975). Intracarotid injection of saralasin acetate, an inhibitor of angiotensin II activity, decreases blood pressure in genetically hypertensive rats (Phillips et al, 1977).

In this review, the site and mode of action of components of the brain iso-renin-angiotensin system have been discussed. Vasopressin, the posterior pituitary hormone, will now be reviewed.

1. <u>Vasopressin</u>.

Traditionally a posterior pituitary hormone, vasopressin is produced by neuronal cell bodies in the supraoptic and paraventricular nuclei of the hypothalamus and has recently been reported in extra-hypothalamic areas of the brain. Kovács <u>et al</u> (1979[a]) have studied the stimulatory effect of minute quantities of vasopressin injected into certain limbic-midbrain structures such as the hippocampus, and showed facilitation of memory consolidation processes. Oxytocin, another posterior pituitary hormone, was shown to attenuate passive avoidance behavior in a time-dependent manner, suggesting that memory consolidation is influenced by this peptide (Kovács <u>et al</u>, 1979[b]).

Whether the presence of these peptide hormones extrahypothalamically represents <u>in-situ</u> synthesis or axonal transport from the hypothalamus is still unresolved, but results suggest that limbic-midbrain areas are involved in the mediation of action of both posterior pituitary neuropeptides on memory consolidation.

Not only posterior pituitary but anterior pituitary hormones are among those peptides being found localized in the CNS. The discovery of a family of related peptides in both brain and pituitary demands an introduction to their relationships in terms of molecular characteristics.

m. <u>A common precursor for some pituitary hormones</u>.

For many years it was known that the bioactive form of ACTH was only 39 amino acids long and yet antibodies to this peptide also reacted with a protein six times larger. The nature of this large precursor revealed new structural relationships among a group of peptide hormones.

Eipper <u>et al</u> (1975) and Mains <u>et al</u> (1977) found by studying the synthesis of ACTH, using pulse chase experiments with pituitary tumor cells incubated in ^{3}H tyrosine-containing media, that the biologically active ACTH 4.5K fragment was cleaved from the middle of a 31K.ACTH precursor molecule (Fig. 3). At the same time, a close relationship had been observed between ACTH and the β lipotropin (β LPH) families of peptides. They had been shown to be released in roughly equivalent amounts by normal pituitary tissue, and also Moriarty (1973) and Pelletier <u>et al</u> (1977) had shown that the two peptides were localized in the same secretory granules in pituitary cells. It had also been noted that the 31K precursor ACTH molecule contained peptide segments similar to both ACTH and the opiate β endorphin. Further work by Mains <u>et al</u> (1977) showed that the peptides of an ACTH biosynthetic intermediate, plus the peptides from β LPH, summed to equal the sequences from pro-ACTH/endorphin, thus establishing a common precursor relationship.

The β LPH can further be broken down to yield β endorphin and also β melanocyte-stimulating hormone (β MSH). At the other end of the large precursor, a sequence of seven amino acids corresponded to the structure of α MSH, being the breakdown product of the ACTH part of the molecule,
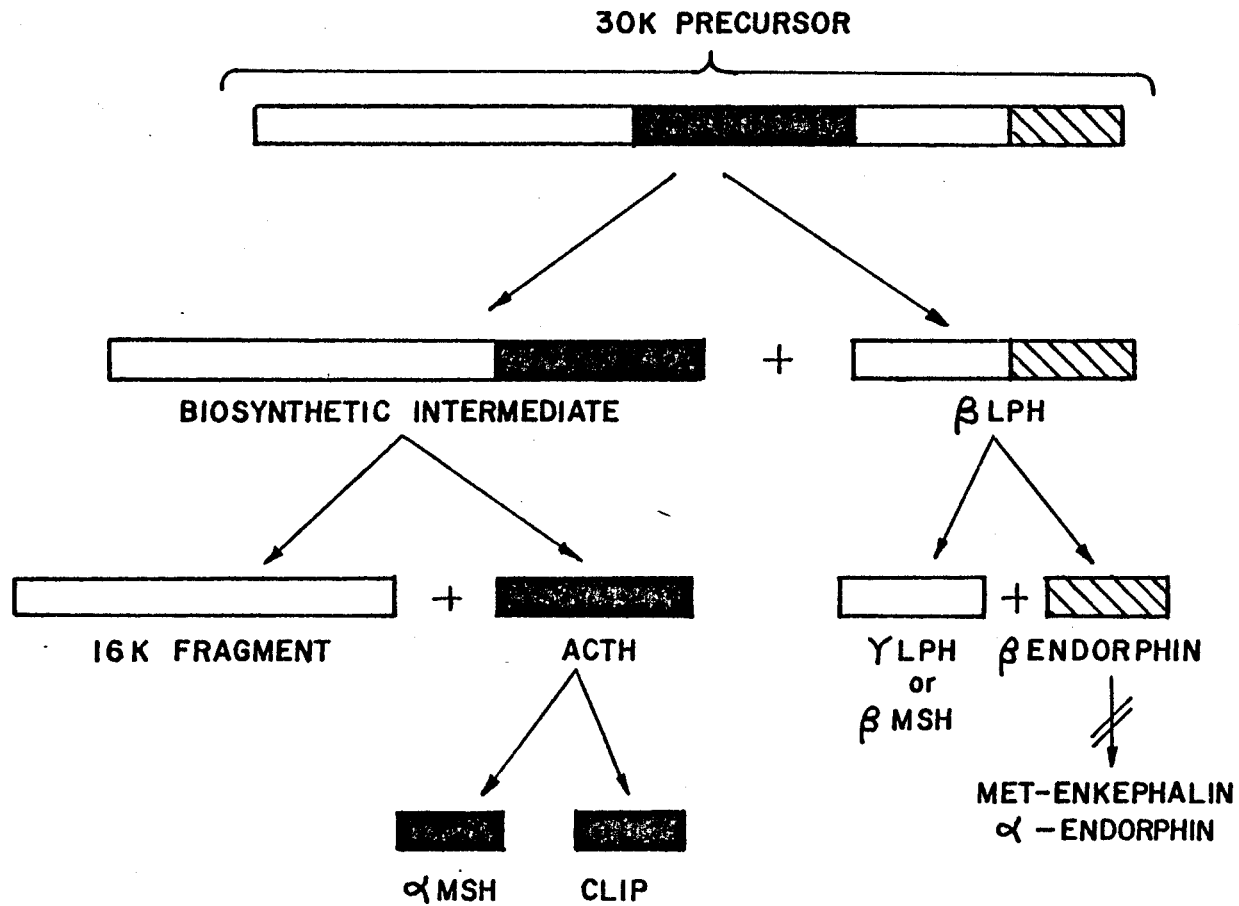
Figure 3. Proteolytic processing of the 30K precursor in pituitary tissue.

together with a 21-amino-acid peptide known as "CLIP." In summary, ACTH, the lipotropins, and the endorphins all originate as controlled cleavage products from a common 31,000 D glycopeptide.

The two lobes of the pituitary, anterior and intermediate, which contain the common precursor, exhibit differences in the levels of ACTH and β-lipotropin-related peptides, apparently due to different cleavages and other post-translational processing steps (Eipper and Mains, 1980). These peptides are not only found in the pituitary, but the precursor protein and many of its breakdown products have been found in the hypothalamus.

Highest concentrations of ACTH, α MSH, β lipotropin, and the opiate peptide β endorphin have been found in the hypothalamus. However, significant amounts are also found in the limbic system and to a lesser extent in the midbrain, cortex, and cerebellum (Krieger et al, 1977[b]).

Cell bodies containing these peptides have been isolated, histochemically, lateral to the arcuate nucleus of the rat hypothalamus, and positively staining fibers have been shown projecting from this area to the amygdala, thalamus, and the stria terminalis by Zimmerman et al (1978) and Watson (1977 and 1978). Similar immunohistochemical studies on rat pituitaries, using antisera directed against β-lipotropin, β-lipotropin/β-endorphin, ACTH and α MSH showed immunostaining of the same or similar cells in the anterior lobe of the gland (Jacobowitz et al, 1978, Martin et al, 1979.

Parallel synthesis of ACTH, β lipotropin, and β endorphin has been reported by Krieger and Liotta (1980) in the pituitary, and Gramsch et al (1980) have shown parallel patterns of distribution of β lipotropin, β endorphin, ACTH, and α MSH in the pituitary and brain. Gramsch also showed that whereas β endorphin and α MSH are predominant in the brain, in contrast, their respective precursors, β LPH and ACTH, were predominant in the pituitary of both rat and man. Unlike the pituitary, no cells or fibers in the CNS appear to contain both ACTH and β lipotropin together (Krieger et al, 1977[b]).

Local differences for concentrations of β LPH and ACTH have also been noted by Krieger et al (1979) in the regions of the CNS, such as lower β LPH than ACTH levels in hypothalamus, hippocampus, and amygdala, and higher β LPH than ACTH levels in the midbrain, pons, spinal cord, and medulla.

These data may be indicative of important differences in post-translational processing of the common precursor molecule between hypothalamus and pituitary tissues, and even between brain regions. Since the pituitary and hypothalamus are on opposite sides of the blood brain barrier, these two separate sources of peptides are therefore possibly regulated separately and exhibit differences in rates of synthesis, biological half-lives, and types of precursor present. Mains (1980) speculates that this compartmentalization may have evolved to allow the pituitary to stimulate glucocorticoid production by means of ACTH without over-producing opiate activity, and to allow the hypothalamus to achieve the maximum brain effect of its common precursor, derived opiate

peptides, without causing cortisol release.

Even though it seems from this discussion that the two pools of hormones are separate entities, the presence of pituitary-like hormones in the brain such as ACTH has been further investigated to prove this situation.

n.   Brain ACTH.

The pattern of distribution of ACTH in the brain, as reported by Krieger (1977[a]), persists after hypophysectomy, adrenalectomy, and dexamethasone suppression. Immunohistochemical studies by Pelletier et al (1980) also confirm this observation. After long-term hypophysectomy, the level of hormone initially falls slightly in the median eminence but not in the median basal hypothalamus or other tissues (Liotta et al, 1978). An enhancement in ACTH concentration is, in fact, observed after removal of both pituitary and median eminence in the remaining hypo-thalamic tissue. This data by Liotta tends to eliminate a pituitary source for brain ACTH, whether by contact with hypophyseal tissue or retrograde transport from the pituitary. Other lines of evidence to demonstrate that ACTH present in the brain does not have a pituitary source comes from the work of Allen (1974), who showed that the hormone is incapable of penetrating the blood brain barrier. Other evidence that indirect transport via the systemic system can be eliminated as a contaminating source of ACTH is that, in cases where a high level of ACTH is present in the circulation, such as following adrenalectomy, no

corresponding rise in "brain ACTH" has been observed (Krieger et al, 1979).

There is a possibility, not proven, of retrograde transport of hormone up the portal vessels. Thus, brain ACTH could reflect both the contribution of a pituitary source, perhaps mediating a short-loop feedback suppression of pituitary ACTH and a brain source. Moldow et al (1978[a]) have suggested that all extrapituitary sites of ACTH are pituitary-derived via such retrograde flow. Evidence for this comes from their observations that the distance from the pituitary at which ACTH is found and the concentration gradient in brain tissues are roughly comparable. However, this would seem to be invalidated by the biosynthesis, in vitro, of an immunoreactive 3,000 D ACTH/endorphin-like material in bovine hypothalamic cells maintained in culture, which was measured by Liotta et al (1979[a]) by showing ^{3}H-labeled amino acid incorporation into a high molecular weight material. The suggestion was made in this paper that cells within the hypothalamus, but not cortex, synthesize material with the immunological characteristics of the 31D ACTH precursor. Characterization by Liotta et al (1979[b]) confirmed such similarities, which included apparent identical size, presence of oligosaccharide units, the basic nature of the protein, and the ability to be processed by trypsin-like enzymes to smaller molecular weight products. The same group has shown that rat arcuate nucleus cells, maintained in culture for 17 days, release twice the amount of ACTH and β endorphin than is present in the tissue on day one.

Other lines of evidence for a brain origin include studies by Krieger's group where destruction of the arcuate nucleus in newborn rats by monosodium glutamate was associated with a decrease in brain ACTH but no change in pituitary ACTH levels. Immunohistochemical studies by Watson et al (1978), using colchicine to block axonal transport along nerve fibers, have shown an increased density of staining of arcuate nucleus cell bodies after such treatment, indicating accumulation of the hormone in the cells and therefore probable in-situ synthesis. Ether stress and chronic immobilization, which normally are associated with changes in ACTH pituitary levels, appears to have no effect on the levels of the brain hormone (Krieger, 1979).

These studies tend to weaken the hypothesis that the presence of ACTH extrapituitarily is due to diffusion from the pituitary alone, and strengthens the idea that the hormone may have specific functions on nervous tissue.

ACTH and ACTH fragments are reported to play a role in motivation, learning, and memory (Gispen and de Wied, 1977). Other fragments are reported to induce excessive grooming, stretching, and yawning when administered intraventricularly (Gispen et al, (1976[a]). These activities are not mediated by adrenal steroids. Sufficient evidence is available from Dunn et al (1977) to indicate that ACTH affects cerebral protein synthesis, RNA synthesis, protein phosphorylation, and cyclic nucleotide metabolism. It has been reported by Terenius et al (1975) that ACTH interacts with opiate receptors. This could possibly interfere with the analgesic action of morphine on the CNS. Naloxone can suppress the

excessive grooming seen after intraventricular administration of ACTH (Gispen et al, 1976[b]).

ACTH also affects the acetylcholine, norepinephrine, and serotonin content or turnover of various brain regions, selectively modifies unit activity in midbrain limbic structures, and enhances the dephosphorylation of membrane proteins leading to membrane permeability changes, properties reviewed by Gispen et al (1977).

These changes in behavior and the general metabolism of nervous tissue brought about by ACTH in the brain indicates its probable importance as a neuromodulator.

Another important product of the large precursor protein associated with ACTH synthesis is α melanocyte-stimulating hormone or α MSH. The primary function of pituitary α MSH is known to be the regulation of pigmentation changes in many vertebrates.

o.   α Melanocyte-Stimulating Hormone (α MSH).

Before the work concerning the existence of a large precursor protein for α MSH in both brain and pituitary; the sole source of α MSH, a basic tridecapeptide amide, was thought to be the pituitary gland.

Dube et al (1978) reported finding α MSH-containing nerves in both rat and man. The source of α MSH fibers appeared to be the α MSH-containing perikarya of the arcuate nucleus in the hypothalamus (Vaudry et al, 1978), and the peptide has been localized within vesicles of nerve

endings by Barnea et al (1977). Warburg et al (1977) demonstrated the release of α MSH from a synaptosome-enriched fraction prepared from hypothalamic tissue.

Over 40% of the immuno-assayable total brain content has been found in the hypothalamus, the limbic system containing substantial amounts also (Eskay et al, 1979[a]). Surgical isolation of the arcuate nucleus of the hypothalamus resulted in a significant fall in extrahypo-thalamic α MSH levels, whereas no reduction in hypothalamic peptide content was noted. This data gives further evidence that α MSH-containing cells in the arcuate region send projections to extrahypothalamic areas and are the source of α MSH in the CNS. Treatment of rats with mono-sodium glutamate (MSG) results in destruction of the neurons in the arcuate nucleus (Eskay et al, 1979[b]). This study measured the levels of α MSH in the hypothalamus and the pituitary after treatment with MSG and showed, again, that levels of peptide fell in brain tissue, whereas pituitary α MSH levels remained constant. This same treatment by Hughes et al (1979) also resulted in a reduction of ACTH-like and endorphin-like concentrations in the hypothalamus, substantiating the theory of a common precursor origin of α MSH, ACTH, and β endorphin.

Barnea et al (1977) examined the cosequestration of ACTH and α MSH in both hypothalamic and hypophysial neuronal granules. The ratio of one peptide to the other found in the granules was dependent on loca-tion. In the hypothalamus, 5 times more α MSH than ACTH was found, whereas 20 times more ACTH than α MSH was seen in the pituitary. The peptide granules for each peptide were distinguishable, and the peptides

are likely to be released simultaneously. There must be differences in the intragranular conversion of ACTH and α MSH, as has been alluded to previously, to account for the differences in hormone levels in pituitary and hypothalamic neurons. Similar data has recently been reported for human brain tissue (Kleber et al, 1980).

On the basis of chromatographic, electrophoretic, and biological characteristics, α MSH present in the rodent brain has been shown by Oliver et al (1978) to be identical to synthetic α MSH.

In support of the hypothesis that α MSH is a neurotropic substance, its effect in vitro on neural tissue has been shown to alter electrical activity (Krivoy and Guillemin, 1961, and Denman et al, 1972); to alter biochemical events such as the metabolism of biogenic amines (Leonard et al, 1976); and to increase the levels of c-AMP in the occipital cortex (Christensen et al, 1976). Administration of α MSH has also been shown to have an effect on various behavioral tasks such as avoidance behavior (Wimersma, 1977, and Greven et al, 1977).

Significant diurnal variation in α MSH has been observed by O'Donohue et al (1979), and the hypothesis that the hormone may participate in the manifestation of the diurnal rhythms of such behavioral effects as arousal, attention, and vigilance has been proposed. Panksepp et al (1976) have also reported an α-MSH effect on other obvious circadian rhythm patterns such as sleep-waking activity.

α MSH, therefore, appears to be a peptide found both in brain and pituitary, similar in structure, but each pool of hormone being independently regulated. Its importance as a neuromodulator has been discussed.

The next section deals with a group of endogenous substances which bind to opiate receptors and mimic the biological effects of morphine, the endorphins, which include α and β endorphins, and the enkephalins.

p. The endorphins.

Endorphins have been isolated from various tissues including the brain by Hughes et al (1975), the pituitary by Teschenmacher and Goldstein (1975), and the GI tract (Elde et al, 1976).

Historically, opiate receptors were found using immunohistochemical techniques by Kuhar et al (1973) to be concentrated in the mesolimbic system of the brain. It seemed unlikely that such highly stereospecific receptors should be developed merely to interact with alkaloids from the opium poppy. Early experiments by Akil et al (1972) showed that analgesia produced by electrical stimulation of the periaqueductal gray region of the brain stem of rats could be reversed by the administration of naloxone, a pure opiate agonist. Jacob et al (1974) found that mice could be made more sensitive to pain by injection of small amounts of naloxone. These observations indicated that there existed an endogenous analgesic opiate, the action of which could be blocked by naloxone.

By 1975, Hughes et al (1975[b]) isolated from pig brain a peptide with a molecular weight of 1000 D, which could combine as an agonist at opiate receptor sites and whose actions could be blocked by naloxone. Cox et al (1975) isolated a similar opiate peptide from human cerebral spinal fluid and pituitary extracts. Furthermore, in the brain, these opioid peptides

were localized in the same areas anatomically as Kuhar's opiate receptors, as shown by Pert and Sneider (1976).

Two related pentapeptides with opiate activity were isolated by Hughes (1975[a]). The most abundant, in pig brain, was named methionine-enkephalin and the other, leucine-enkephalin. As previously noted (Fig. 3), their sequences were contained within the structure of β lipotropin (Fig. 4). Low levels were also found in the pituitary. Evidence for separate populations in the brain of the met- and leu-enkephalin have been reported by Larsson et al (1979[b]) using histochemical techniques. The enkephalin-containing nerves also showed a distribution distinct from nerves containing β endorphin, a larger opiate peptide (Guillemin et al, 1976), ACTH, and β lipotropin (Bloom et al, 1978).

At the present time, there is no definitive evidence that the enkephalins in the brain are derived from β lipotropin (Guillemin, 1980).

The perinatal development of the endorphin- and enkephalin-containing systems has been studied in the rat brain by Bayon et al (1979). Endorphin levels were much higher than enkephalins in the 16-day fetus, dropping to lower levels by the 20th day of gestation. The enkephalin content rose at day 20 and continued to increase at a faster rate than the endorphins from the 6th to the 25th day postnatally to achieve adult levels. This would suggest an independent development of the endorphin and enkephalin systems due to the lack of correlation between rates of increase; however, the authors suggest that certain endorphin-like systems could develop differentially into enkephalin systems during gestation.
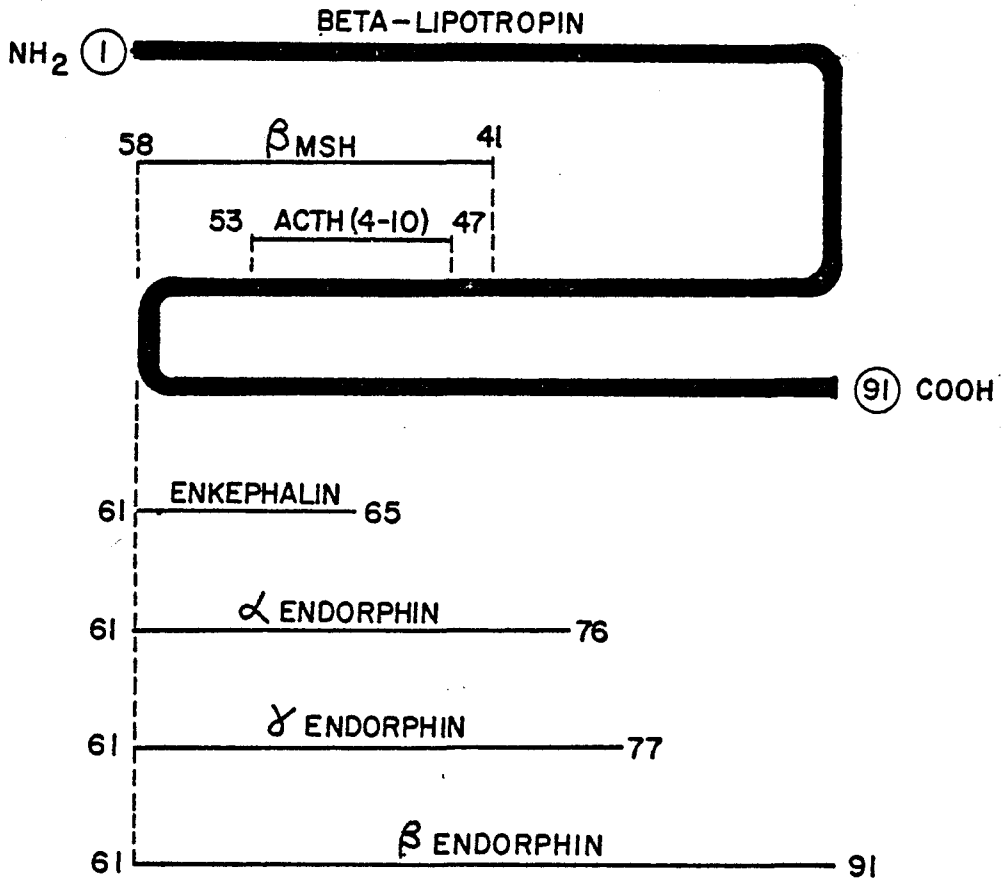
Figure 4. Relationship between β Lipotropin amino acid
sequence and other peptides of the brain and
pituitary.

This concept is in agreement with observations that small local inter-neurons, like the enkephalin-containing cells, frequently migrate and differentiate more than larger neuron-forming pathways.

Evidence for distinct pools of brain endorphins and pituitary endor-phins comes also from the work of Kobayashi et al (1978) and Cheung et al (1976). These groups showed that hypophysectomy failed to alter the brain content of opioid peptides after as long as 34 days without a pituitary. Guillemin et al (1977) also demonstrated that β endorphin levels in the brain did not change in experiments causing marked variations in pitui-tary β endorphin levels.

Two functions have been suggested for opiate peptides in brain. They may act as hormones or neuromodulators transported to their target organs by CSF or the vascular system; or, they may act as neurotransmitters, liberated from nerve terminals and binding to postsynaptic membranes.

Several studies suggest a role as neurotransmitters. In support of this role, they have been found concentrated in the synaptosomal fraction of brain homogenates and are localized in nerve endings (Simantov et al, 1976[b]). They appear to be able to inhibit neurotransmitter release, a response apparently related to changes in the Ca^{++} flux. It has been shown that morphine can decrease acetylcholine (Yaksh et al, 1975), and norepinephrine release (Arbilla and Langer, 1978), an effect antagonized by Ca^{++}. Evidence by Guerrero-Munoz et al (1979) indicates that a single dose of β endorphin causes a decrease in Ca^{++} concentration in synapto-somal fractions, attributable to reduced Ca^{++} binding and uptake.

Sustained administration of β endorphin showed a reversal of this effect and the development of tolerance and physical dependence or addition, supporting the concept that β endorphin may inhibit the release of transmitters by inhibition of Ca^{++} influx in a mode similar to that of morphine.

The rapid degradation of the pentapeptide endorphin in serum seems to exclude a hormonal function (Simantov and Snyder, 1976[a]), although Sarne et al (1978) have postulated that a larger enkephalin-like substance found in sera was resistant to degradation and could act as a neuropeptide stimulating pituitary GH and prolactin release, as shown by Rivier et al (1977[b]). Drouva et al (1980) have shown that this may be mediated by the inhibition of release of somatostatin in the hypothalamus by opiate peptides such as met-enkephalin. This inhibition of release of hypothalamic hormone was naloxone reversable and was consistent with the hypothesis that opiates interfere with Ca^{++} influx. Met-enkephalin and morphine were also shown to inhibit GnRH, and therefore LH release, from the pituitary, and opiate receptors were isolated on GnRH as well as somatostatin nerve endings in the hypothalamus. β endorphin has been reported to promote the secretion of prolactin, the exact mechanism of this being unclear (Rossier et al, 1980, and Spies et al, 1980).

Dawson et al (1979) have studied another aspect of the effect opiate peptides may have on nervous tissue. The presence of opiates in cultured mouse neuroblastoma cells modified the biosynthesis of both membrane glycosphingolipid and glycoprotein. These effects seem to be mediated by

opiate receptors, of which this cell line has a prolific quantity, as they were stereospecific and naloxone reversible. Neuroblastoma cells treated with α-AMP are known to undergo morphological differentiation and show enhanced glycosylation of a number of secretory proteins (Truding et al, 1975). As other studies have demonstrated that opiates and enkephalins inhibit PGE_1-stimulated adenylate cyclase activity (Klee et al, 1976), it appears that the modifications of membrane glycosphingolipid and glycoprotein seen by Dawson et al (1979) may represent a c-AMP mediated event.

Interest in the endorphins stems from the possibility that they or their analogues may be non-addictive pain killers to replace opiates such as morphine. It has been demonstrated that they are distributed in the pain-mediated pathways of the CNS. However, a lack of effect in producing analgesia has been observed when enkephalin is injected intraventricularly into rat brain. In vitro, indications are that enkephalins have a more profound opiate effect and bind to receptors about half as avidly as morphine. All these effects and those such as the induction of a state resembling catatonic schizophrenia by β endorphin (Bloom et al, 1976) can be blocked by the specific antagonist of opiate action, naloxone.

Evidence is opposed to the fact that the opiates are non-addictive, as both enkephalins and β endorphins are found to produce tolerance and dependence effects (Pert et al, 1976, and Blasig et al, 1976). β endorphin appears to resemble morphine pharmacologically. As an analgesic, it is 20-30 times more potent when given intraventricularly (Li et al, 1977).

Recent work by Pasternak et al (1980) supports the concept of multiple

opiate receptors and gives evidence for mediation of opiate analgesia by

a subpopulation of high affinity opiate receptors.

A role for enkephalins in the mediation of behavioral reinforcement

is supported by several lines of evidence. Central injection of enkepha-

lin serves to reinforce self-administered behavior, and electrical stimu-

lation of enkephalin-rich regions of the brain also serves to reinforce

self-stimulated behavior. Both effects are blocked by naloxone. Gunne

et al (1977) reported that chronic schizophrenics exhibit elevated levels

of endorphins in their CSF during symptom-rich phases of their disease.

When their condition improved, a return to low endorphin levels was ob-

served, implying that some psychological symptoms might be due to exces-

sive endorphin activity.

The endogenous opioids clearly have powerful effects, are involved

in behavior, and interact with other neuronal systems. While enkepha-

lins potentially appear to conform to definitions of a neurotransmitter,

β endorphin may be a neuromodulator. The enkephalins seem to be situated

in such a way to influence basic processes such as pain transmission, res-

piration, motor integration, endocrine responses, and limbic functions

involved in the elaboration of emotions. β endorphin seems more likely

to affect various structures by interacting with other local neuroregula-

tors.

Apart from ACTH and β LPH, there have also been recent reports of

other pituitary hormones, such as prolactin, in extrapituitary locations.

q.   <u>Prolactin.</u>

Evidence from Fuxe <u>et al</u> (1977) showed immunohistochemically that prolactin appears to be present in the nerve terminals of several hypothalamic nuclei such as the arcuate and ventromedial areas. Furthermore, Toubeau <u>et al</u> (1979) have recently noted the similarity in distribution of prolactin and ACTH immunoreactivity throughout the rat brain. Adrenalectomy and hypophysectomy have been reported to produce no effect on levels of prolactin in the hypothalamus.

A direct effect of prolactin on the CNS is suggested by reports that impotence present in males with hyperprolactinemia and normal testosterone concentrations may be corrected by lowering the prolactin concentrations by bromocryptene administration (Carter <u>et al</u>, 1977). Little else is known of the effect of prolactin on the CNS, as there is of the finding of TSH in extrapituitary tissues.

r.   <u>Thyroid stimulating hormone (TSH).</u>

As long ago as 1967, using a bioassay, Bakke <u>et al</u> reported the presence of TSH in the rat median eminence, which was apparently independent of circulating TSH levels. In more recent findings, Moldow <u>et al</u> (1978[b]) attributed the presence of TSH outside the pituitary to retrograde flow from the pituitary. Further investigation into these observations will be particularly considered in this dissertation as will the locating and biochemical characterization of radioimmunoassayable growth hormone-like material in the CNS.

s. Growth Hormone (GH)

Pacold et al (1976) were the first to report the finding that dispersed rat brain cells grown under standard tissue culture conditions produced an immunoassayable growth-hormone-like material, indicating the possibility that GH could be added to the growing list of brain peptides.

The continuance of these observations, together with the finding of an immunoassayable TSH-like peptide in the rat brain, represents the thrust of the independent investigations that form the basis of this thesis.

To reiterate, my research has been oriented to the further confirmation of the presence of pituitary-like GH and TSH within discrete areas of the rat brain. Having developed a substantial background literature review of recent developments dealing with the unusual, if not unexpected, finding of diverse peptides in the brain, subsequent consideration will deal with my independent investigations dealing with the distribution of GH- and TSH-like peptides in the rat brain, and with studies designed to ascertain the physiological meaning of these novel observations.

3. PROPOSED INVESTIGATION

Specific Objectives

The distribution in the rat and primate brain of the anterior pituitary hormones, growth hormone, and thyroid-stimulating hormone will be studied. Experimental work will involve the development of working, sensitive, and specific radioimmunoassays for two rat hormones, TSH and GH. Another line of approach will be the development of primary cell cultures of neuronal tissue from various brain regions and the use of immunohistochemical staining techniques to further characterize the distribution of pituitary-hormone-like material in the adult rat brain. Ultracentrifugation of brain homogenates will determine the association or lack of association of the hormones with a particular brain fraction, either nuclear, mitochondrial - synaptosomal, or microsomal. Also to be examined will be the ontogenetic developmental pattern of these hormones in fetal, neonatal, and adult brain regions.

In an effort to determine the similarities or dissimilarities between pituitary and brain hormones, a part of this study will attempt to characterize biochemically and biologically the brain hormones. Using gel chromatography, the molecular weights will be determined, parallelism studies with the radioimmunoassy will examine immunological similarities, and bioassays will show the extent of biological activity of brain hormones as compared to hormones of pituitary origin.

Although it appears unlikely that these brain-based hormones could be of pituitary origin, one aspect of this investigation will be to test that concern. This will be accomplished by examining the intactness of the blood-brain barrier to circulating radiolabelled pituitary hormones. Furthermore, the use of hypophysectomized animals will provide the opportunity to compare levels of brain hormones in animals with and without an intact pituitary gland. In other studies, neural cells from hypophysectomized rats will be cultured in monolayers to test their ability to release GH and TSH hormone in vivo.

If two distinct pools of hormone, brain and pituitary, exist, these may turn out to be regulated in different ways. In an attempt to discern these interrelationships, experiments such as endocrine target organ ablation studies will be undertaken in order to understand this problem and its ramification.

From these different study areas it is hoped that the objective of this work will gain some insight into the physiological implications involved in the finding of pituitary-like hormones in discrete areas of the rat brain.

4. MATERIALS AND METHODS

A. THE DEVELOPMENT OF RADIOIMMUNOASSAYS (RIA) FOR RAT GROWTH HORMONE AND THYROID STIMULATING HORMONE

The radioimmunoassay, as introduced by Yalow and Berson (1959) for the measurement of serum insulin concentrations, is a method of detecting trace concentrations of any substance that can serve as an antigen.

Based on competition for antibody; directed against the antigen; between a standard amount of radioactive antigen, C^*, and its unlabeled counterpart C, at unknown concentration in the test sample (Fig. 5). The higher the concentration of unlabeled competitor, the lower the ratio of bound C^* to unbound C^* which can be measured. Separation of bound from unbound C^* can be achieved by the precipitation of bound labeled and unlabeled antigen by a second antibody directed against the antibody moiety of the immune complex, and measurement of the radioactivity representing bound C^* in this precipitate. The higher the concentration of unlabeled competition, the lower the ratio of bound C^*, (B) to unbound C^* (B_0).

In order to determine the concentration of unknown C in a sample of blood, urine, or tissue extract, the amount of competitive inhibition observed in the unknown is compared with that obtained in separate solutions to which known amounts of purified C is added. A standard curve plotting the known concentrations of C against the ratio of bound to unbound C^* can thus be obtained (Fig. 6), and the level of unknown C in a sample determined by its ratio of bound C^* to unbound C^*.

85

$$C^* + AB \rightleftharpoons C^* - AB$$

(FREE)
or
UNBOUND

$$+$$

$$C$$

$$\updownarrow$$

$$C - AB$$
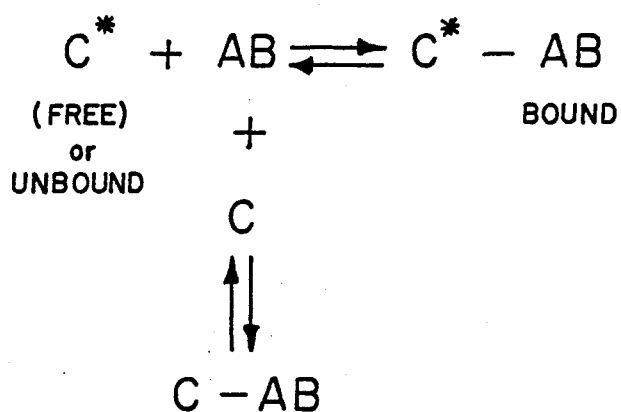
C =   COMPOUND TO BE MEASURED (ANTIGEN)

$C^* =$ SAME COMPOUND TAGGED WITH RADIOACTIVE TRACER (^{125}I)

AB =   SPECIFIC ANTIBODY TO C

Figure 5. Schematic diagram illustrating the principle
of Radioimnunoassay.
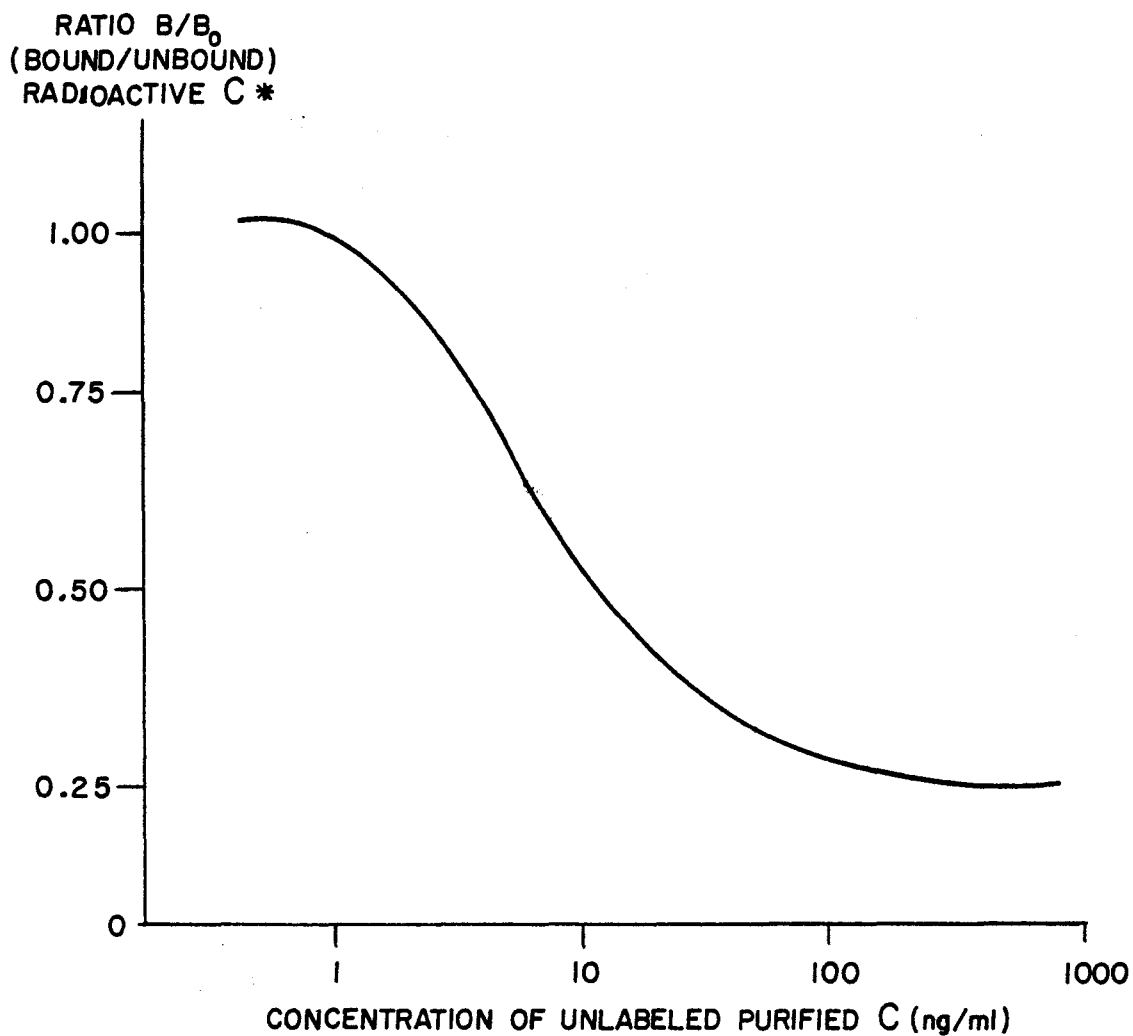
Figure 6. Standard calibration curve for
          radioimmunoassay.

Basic requirements for a radioimmunoassay therefore include a specific antigen-directed antiserum; a radioactive-labeled, purified antigen; appropriate standard reference preparations of the antigen, and a method for the clear, clean and consistent separation of antigen-antibody complexes from the unbound fraction.

In the double antibody method of separation, which was selected for the RIA's for rat GH and TSH in this study, the first antibody, which is specific to the antigen to be determined, is added at a level that enhances competition between labeled and unlabeled antigens or ligands. That is, the antibody/antigen ratio corresponds to the antigen-excess side of the equivalence zone in a liquid precipitin reaction (Fig. 7). The second antibody (anti-antibody) is introduced in excess to ensure complete precipitation of the first antibody and its complexes with antigen, that is, the antibody-excess side of the equivalence zone of the curve. The method of RIA is exceedingly discriminating because of the possibility to choose antisera with great specificity and sensitivity for the test activity.

I. THE RAT GROWTH HORMONE RADIOIMMUNOASSAY

a. Production of specific antiserum to rat growth hormone.

The larger molecular weight protein hormones, such as growth hormone (GH) and thyroid-stimulating hormone (TSH), generally elicit a good antigenic response, and it was therefore decided to produce our own rat growth hormone antibody from guinea pigs. Since a very pure antigen is
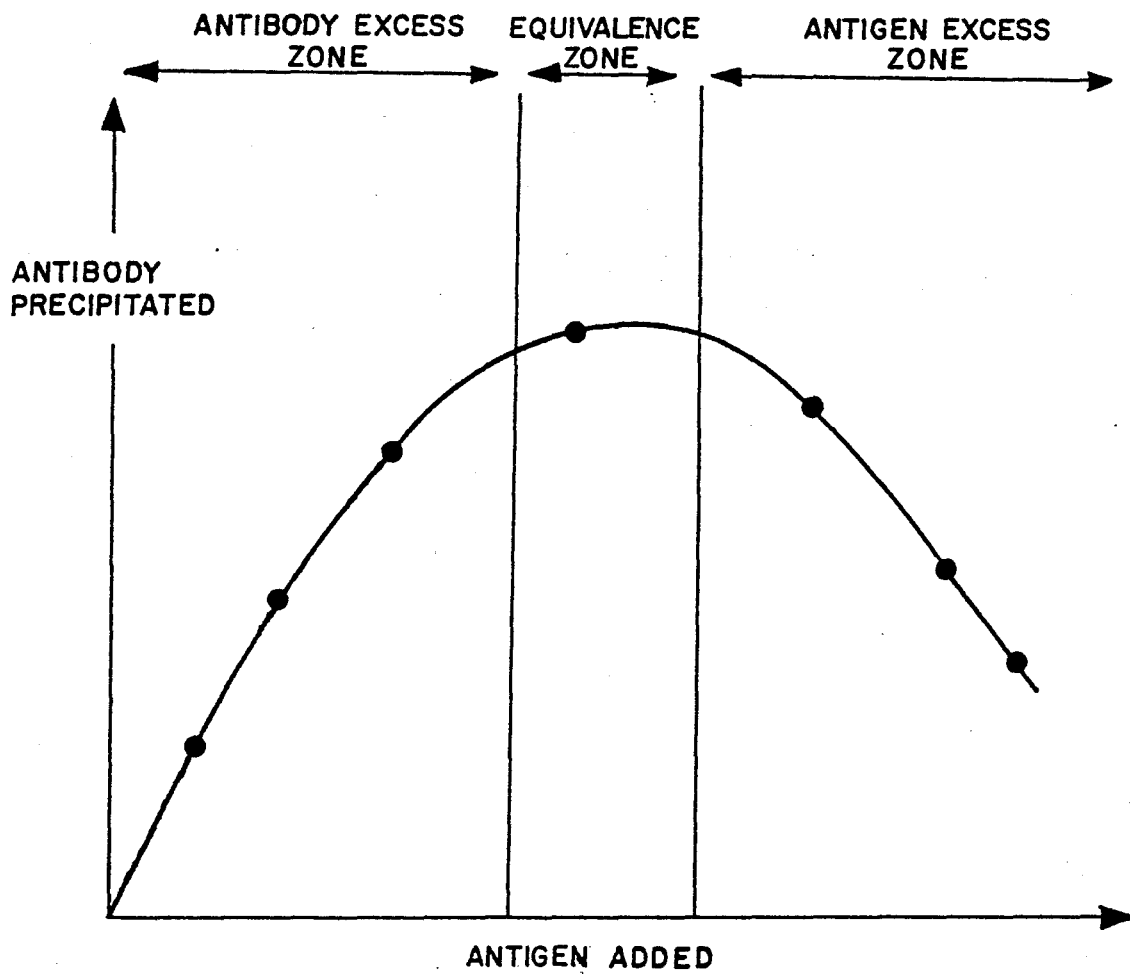
Figure 7. Precipitin curve for a monospecific system:
one antigen and the corresponding antibody.

radioactively labeled and used as the radioactive tracer in the RIA, the presence in the antibody preparation of other antibodies does not interfere with the reaction between labeled antigen and its antibody (Straus et al, 1977). It is therefore possible to use a relatively impure antigen, in this case growth hormone, for immunization. Extraction and isolation of crude rat pituitary GH was accomplished using a modification of the method of Stroud et al (1973). Approximately 50 rat pituitaries were removed from decapitated animals, weighed, and homogenized in a 3:2 mixture of 10% ammonium acetate and 96% ethanol. The mixture was stirred for two hours at 4° C, centrifuged for 20 minutes at 2000 rpm at 4° C, and the supernatant decanted off. The residue was stirred overnight in 30 mls of 0.1N NaOH, pH 10.0. After adjustment to pH 8.4 with 4N HCl, the slurry was stirred for 30 minutes and then centrifuged for 15 minutes at 3000 rpm at 4° C. The supernatant was reserved, and the residue re-extracted overnight with 0.1N NaOH, pH 10.0 and centrifuged at 4° C, after which the supernatants were pooled.

Using 4N HCl, the pH of the pooled supernatants was adjusted to 4.8 and stirred for 30 minutes. The heavy precipitate that formed was removed by centrifugation for 15 minutes at 3000 rpm at 4° C and the supernatant reserved. The precipitate was then dissolved in 0.1N NaOH and the solution adjusted to pH 4.8 by addition of 4N HCl and stirred for 30 minutes. After centrifugation, the precipitate was discarded and the supernatant combined with the previous pooled supernatant. The volume of the resultant solution was measured and 96% v/v alcohol, cooled to -30° C, was added slowly to give a final alcohol concentration of 25% v/v. After

stirring for a further 30 minutes, the precipitated crude growth hormone was collected by centrifugation for 15 minutes at 3000 rpm at 4° C. A total of twelve guinea pigs each received 0.5 ccs of crude <u>Bordatella</u> <u>pertussis</u> vaccine (E. Lilly Corp., Indianapolis, Indiana) intradermally into 30-50 sites over a small area of their backs.

Twenty-four hours later, each received, subcutaneously, approximately 1 mg of the crude rat growth hormone, prepared as described above, dissolved in 0.5 ccs of saline together with an equal volume of complete Freunds adjuvant (Difco). Five subsequent similar booster immunizations were given with this crude extract over a five-month period.

Within 10 days of each injection, the guinea pigs were bled from a leg vein and serum collected for evaluation of antibody "titre", which reflects the binding affinity of the antibody to labeled antigen. The "sensitivity" of the antiserum was judged as the ability of small amounts of unlabeled hormone to displace the radioactive labeled hormone, and the "specificity" or degree of cross-reactivity of an antiserum to one hormone with other closely related hormones was examined.

Effective competition upon addition of unlabeled hormone occurs only under conditions of limited antibody, and in the RIA, that amount of antibody which shows a binding of 35%-50% of labeled hormone is usually chosen, depending on the levels of unknown hormone to be measured. Where levels of unknown hormone to be measured are expected in the lower range, 35% binding conditions are used, as they are known to give a more sensitive standard curve for the lower concentrations of standard hormone.

In order to determine the best titre of antibody for a sensitive assay, a level of 35% binding of labeled hormone was therefore set for testing the individual test bleedings for antibody titre.

Dilutions of antiserum were prepared from 1:1000 to 1;1,000,000, and the binding and dissociation characteristics of radiolabeled purified growth hormone were examined, as shown for 5 different animals at one test bleeding (Fig. 8). Antibody binding characteristics not only varied between animals but between bleedings from the same animal. Serum from guinea pig 11, at a dilution of 1:65,000, was eventually selected for the growth hormone RIA on the basis also of the "sensitivity" of this particular antiserum.

The "sensitivity" of a particular antiserum was assessed by adding graded amounts of purified standard GH and testing their ability to displace the radiolabeled hormone under the conditions of our radioimmunoassay. If a standard curve was too flat, the antiserum was rejected as being "non-sensitive". A "sensitive" curve was one with a steep fall in the range of hormone concentration expected from our samples.

The "specificity" of the antibody from guinea pig 11 was evaluated by examining the degree of cross-reactivity with other molecules similar in their primary structure to GH, such as rat prolactin. A standard curve using growth hormone antiserum #11, radiolabeled GH, and known quantities of purified prolactin showed that less than 1% of the purified prolactin was being measured. The antiserum to GH from #11 guinea pig was therefore sensitive and specific and could be used in a radioimmunoassay. The second essential ingredient of an RIA is the radiolabeled hormone.
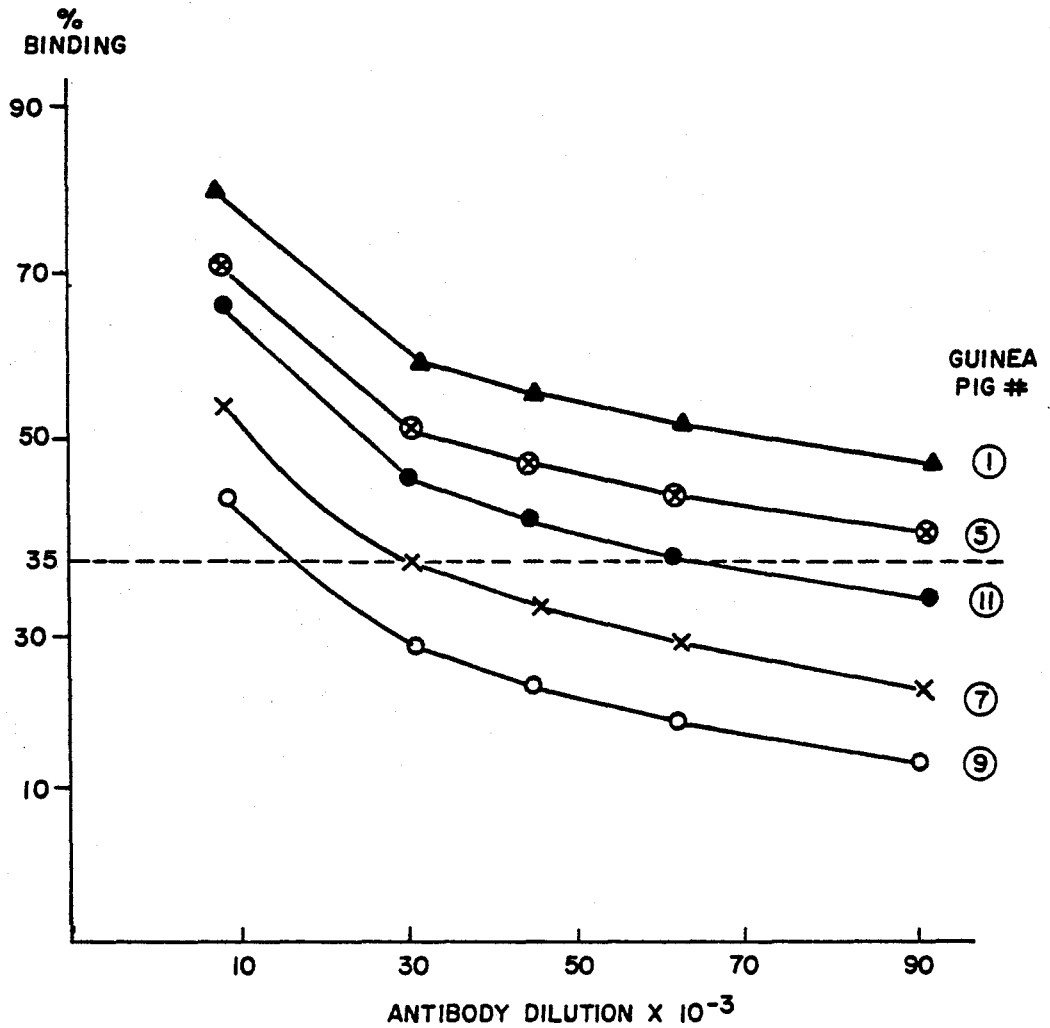
Figure 8. Determination of titer of G.H. antibody binding
35% of the total radiolabelled G.H. antigen from
one test bleeding of five guinea pigs (12/18/78).

b. <u>Radioiodine labeling procedure for rat growth hormone.</u>

^{125}I has proved to be the radioisotope most suitable for labeling

peptides. The labeling reaction consists of substitution of radioactive

iodine, produced by iodide oxidation in the presence of protein, for the

tyrosine in peptide linkages.

The chloramine-T technique of isotope labeling developed by Hunter

<u>et al</u> (1962) was used. This method produces radiolabeled GH with a high

specific activity, that is, with a high amount of radioactivity per amount

of hormone, while using smaller amounts of isotope than other techniques.

This is important, as to achieve maximum sensitivity the concentration of

labeled antigen must be kept below the minimum concentration of antigen

to be measured. Rat GH was obtained from the NIAMDD Pituitary Hormone

Distribution Program through Dr. A. F. Parlow.

In this method, radioiodide is oxidized at pH 7.6, with chloramine-

T to iodine, which in turn iodinates the protein's tyrosyl and histidine

residues, specifically.

The unreacted radioiodine is reduced to iodide by the addition of

sodium metabisulfite to the reaction mixture, and albumin is added to

protect the hormone from further radiation damage by a mechanism not

entirely clear. The labeled protein is immediately separated from un-

labeled iodide and damaged protein fractions to protect the labeled hor-

mone from further radiation damage. This separation is achieved using

gel filtration on coarse Sephadex G-50.

(i) <u>Labeling Procedure</u>.

Lyophilized, purified growth hormone was obtained from the NIAMDD,
Bethesda, and 100 µg was weighed out and dissolved in 10 µl 0.01N NaOH
and 90 µl distilled water. This was aliquoted in 20 µl amounts and stored
at -70° C.

At all times, adequate radiation safety procedures were ob-
served and the iodination procedure carried out under a hood to avoid
contamination of the surrounding area by volatilized material. Iodina-
tion was performed in a glass vial 5 cm x 0.5 cm by addition of reagents
in the following order:

1. 20 µl GH (purified from NIAMDD).

2. 50 µl Phosphate buffer. 0.5 M pH 7-6.

3. 10 µl ^{125}I, approximately 1 m curie (New England Nuclear,
Boston, Mass.).

4. Mixed well.

5. 10 µl Chloramine-T solution (35 mg Chloramine-T, Eastman
Kodak, Rochester, N. Y.) in 10 ml freshly prepared 0.05 M
PO_4 buffer, pH 7.6).

6. 45 seconds contact by swirling.

7. 15 µl Sodium metabisulfite solution (75 mg sodium meta-
bisulfite, Fisher Scientific, New Jersey) in 10 ml freshly
prepared 0.05M PO_4 buffer, pH 7.6.

8. 100 µl 2-5% bovine serum albumin (Miles Laboratories, Inc.,
Elkhart, Ind.) in 0.05 M PO_4 buffer, pH 7.6.

This mixture was then transferred to a freshly prepared Sephadex G-50 coarse 0.9 x 20 cm plastic column (Pharmacia Fine Chemicals, Uppsala, Sweden), which had previously been coated with a few drops of 22% bovine serum albumin (BSA). The column was eluted with 0.075 M veronal buffer, pH 8.6, and 15 drop fractions were collected in albumin-coated glass tubes. The two tubes showing the highest radioactivity in the first peak were saved, diluted with 10 ml 1% BSA, 0.01 M phosphosaline buffer, pH 7.4, and stored in 1 ml amounts at -20° C. The stability of this iodinated growth hormone could be maintained for approximately 4 weeks. After this, there was an increase in free radioactive iodide indicating a breakdown of the labeled hormone, perhaps due to radiation damage. An approximate specific activity between 90-110 mC/mg was obtained by this procedure.

Prior to setting up each radioimmunoassay, the labeled rat growth hormone was repurified using a Sephadex G-100 column 0.9 x 50 cm. The Sephadex was first equilibrated with 0.075 M veronal buffer and approximately 1 ml containing 1×10^{6} cpm labeled hormone applied. One ml fractions were collected and the top two tubes of the second peak used for the assay. The labeled hormone was immediately diluted in 0.01 M "working" phosphosaline buffer, pH 7.4, to approximately 12,000 cpm/100 μl.

Solutions Used in Labeling Procedure for Rat GH

Phosphate Buffer

Stock solution, 0.1 M

24.2 g Na_2HPO_4 $7H_2O$

2.62 g NaH_2PO_4 H_2O

Distilled H_2O to 1 litre, pH 7.6

Working solution: 1% BSA, phosphosaline buffer

(0.15 M   NaCl, 0.01 M PO_4), pH 7.6

5.14 gm $NaH_2PO_4H_2O$

26.6 gm   NaCl

22.7 mls   of 22% BSA/litre

0.1% Thimerosol (Sigma)

Final volume, 3.0 litres, pH 7.6.

Veronal Buffer 0.075 M , pH 8.6

292 g   Sodium diethyl barbiturate (Fisher)

52.44 g   Barbituric acid (Fisher)

4 liters distilled H_2O

Heat to dissolve

Make up to 19 litres

Stored at 4° C

c. Preparation of GH standards.

For validation of the RIA procedure, the unknown GH substance to be assayed must be immunochemically identical with the substance used as an antigen standard. For this purpose, lyophilized reference preparations of rat growth hormone were obtained from the NIAMDD, the stock standard solutions were prepared as follows:

   -- 100 µg was weighed using a Mettler balance, dissolved in
      100 µl 0.01 N NaOH, and made up to 1 ml with 0.01 M phos-
      phate buffer, pH 7.6.

   -- Aliquots of 50 µl containing 5 µg were then stored at -20°
      C for up to 6 months without loss of activity.

The standard curve for a RIA was made up using the following dilutions of stock hormone as growth hormone standards in working phosphosaline buffer, pH 7.6:

50 µl stock + 2.5 cc buffer	=	2000 ng/ml
Standard 9	=	100 ng/ml
Standard 8	=	50 ng/ml
Standard 7	=	40 ng/ml
Standard 6	=	20 ng/ml
Standard 5	=	10 ng/ml
Standard 4	=	8 ng/ml
Standard 3	=	5 ng/ml
Standard 2	=	4 ng/ml
Standard 1	=	2 ng/ml

d. <u>Separation of bound and free antigen in the GH RIA.</u>

In a radioimmunoassay, the labeled peptide-antibody complexes are
generally soluble, and some technique must be employed to distinguish
between the bound and the unbound labeled peptide. One of the most
accepted and least troublesome methods is that depending on precipita-
tion of the complex with an antibody directed against the γ globulin of
the first antibody in the complex, the "double antibody" method. The
precipitate formed is isolated by centrifugation. After several trial
runs, a 1:10 dilution of goat anti-guinea pig IgG (Pel-Freeze) was found
to give the optimum degree of precipitation in the RIA for GH.

e. <u>Assay protocol.</u>

The final protocol for the rat growth hormone double antibody RIA
was as follows, and the assay was incubated throughout at 4° C in 12 x 75
mm glass tubes. All samples were run in triplicate with individual non-
specific binding controls (NSB).

<u>DAY 1</u>   100 μl GH antibody 1:65,000, produced in guinea pig, made as
follows:

(1) 10 μl stock GH antibody in 6.5 ml working PO_4 buffer +
5 μl/ml normal guinea pig serum (Miles Laboratories,
Elkhart, Indiana)

or

100 μl non-specific binding buffer, made as follows:

5 μl normal guinea pig serum/1 ml working PO_4 buffer.

(2)   100 µl standard or unknown.

DAY 5

(3)   100 µl ^{125}I GH, 12,000 cpm.

DAY 7

(4)   100 µl goat anti-guinea pig serum, 1:10.

(5)   200 µl working PO_4 buffer.

Incubation for 4½ hours at 4° C.

(6)   1 ml ice cold saline.

Centrifugation for 30 minutes at 2,200 rpm using an

IEC 418 head in a refrigerated IEC Model PR-6000

centrifuge.

The supernatant was decanted and the precipitate counted in a Micromedic

System 2/200 automatic gamma counter.

f.   Treatment of data obtained from the GH RIA.

Calculation of data was handled using a Hewlett-Packard Clinical

Laboratory Radioimmunoassay computerized program cassette with a Hewlett-

Packard Model 7830A calculator and printout. This program performed a

weighted and unweighted least squares regression analysis using the log

of dose vs. % bound, logit of % bound, probit of % bound, and arcsin of

% bound. The "best fit" of these linearizing transforms was automatically

selected and all useful standard curve statistics and plots were outputted to verify acceptability of each dose response variable.

Typical data processing of standards and unknowns are seen in Figs. 9 - 13. In Fig. 9, the standard curve data is shown as the total amount of cpm of tracer present per tube, and nonspecific binding (NSB) as cpm/tube, representing the bound antigen. The concentration in GH ng/ml of each set of three tubes per standard is given plus the cpm in the bound fraction for each standard assayed. Fig. 10a illustrates the computer's assessment of the correlation, or how this data best fits a straight line, using different ways of handling the data such as "Logit," "Probit," and "Arcsin." In this case, Logit was chosen as the best fit, and a weighted regression was performed on the data of the standard curve as shown in Fig. 10b. The coefficient of variation for each point in this assay was acceptable, i.e. under 10%, and Logit transformation was used to plot the curve, shown in Fig. 12, from which the unknown samples were calculated, as in Fig. 12. Fig. 13 shows the standard curve plotted using the data B/B_0 vs. ng GH/ml. The "sensitivity" of this assay appeared good over the range of 2-100 ng of growth hormone.

g. <u>Validation of the GH radioimmunoassay.</u>

After the reagents were obtained and the method established, it was necessary to validate the RIA of GH in brain extracts. Primarily, assay validation consists of a demonstration that the assay is measuring what it is designed to be measuring, in this case, rat growth hormone. Multiple dilutions of brain extract and pituitary extract should give lines

```
RGH 68D
3/6/79    TECHNOLOGIST  S. HOJVAT

TOTALS (CPM)

 11586
 11599
 11725

TUBE NO.    CONCENTRATION         CPM
              IN NG/ML


   1          NSB             410
   2          NSB             333
   3          NSB             309

   4           0             4034  Bo
   5           0             4116
   6           0             4194

   7           2             3987  B
   8           2             3951
   9           2             3790

  10           4             3522
  11           4             3574
  12           4             3730

  13           5             3542
  14           5             3589
  15           5             3612

  16           8             3203
  17           8             3326
  18           8             3351

  19          10             3263
  20          10             3167
  21          10             3141

  22          20             2273
  23          20             2416
  24          20             2408

  25          40             1398
  26          40             1403
  27          40             1425

  28          50             1174
  29          50             1073
  30          50             1080

  31         100              764
  32         100              743
  33         100              705
```

Figure 9. Computerized output of G.H. standard curve.

Figure 10a.

ASSAY: RGH 68D
3/6/79   TECHNOLOGIST S. FOJVAT

MODEL	INTERCEPT	SLOPE	CORRELATION
RAW DATA	1.257956973	-0.599259588	0.989075873
LOGIT	4.084257617	-3.134433938	0.997675286
PROBIT	7.430118155	-1.876054213	0.996794585
ARCSIN	1.431518075	-0.699741408	0.972278768

BEST FIT FOR RGH 68D IS LOGIT

WEIGHTED REGRESSION

Figure 10b.

ASSAY RGH 68D
3/6/79   TECHNOLOGIST S. FOJVAT

DOSE	B/B0	STD. DEV.	VARIANCE	STD ERROR	COEFF OF VAR
0.00	1.0000000	0.0213562	0.0004561	0.0123300	2.1356199
2.00	0.9454481	0.0279642	0.0007820	0.0161452	2.9577765
4.00	0.8655685	0.0288450	0.0008320	0.0166536	3.3324872
5.00	0.8582182	0.0095649	0.0000915	0.0055223	1.1145030
8.00	0.7817924	0.0211258	0.0004463	0.0121970	2.7022286
10.00	0.7544279	0.0171477	0.0002940	0.0099002	2.2729378
20.00	0.5353348	0.0214009	0.0004580	0.0123558	3.9976582
40.00	0.2810840	0.0038443	0.0000148	0.0022195	1.3676850
50.00	0.2014701	0.0150043	0.0002251	0.0086627	7.4474094
100.00	0.1028162	0.0078120	0.0000610	0.0045102	7.5979878

X	LOGIT TRANSFORM
2	2.933497406
4	1.878566729
5	1.80200039
8	1.278911035
10	1.123850082
20	0.141728422
40	-0.939142958
50	-1.378810207
100	-2.168243684

Figure 10a. Best fit analysis for growth hormone standard curve data.

Figure 10b. Weighted regression analysis of G.H. standard curve.

ASSAY RGH 68D
3/6/79   TECHNOLOGIST S. HOUVAT

LOGIT Y = 4.884 - (3.134) LOG₁₀ X
R = 0.987675288

B/B0 VS X (NG/ML)

LOGIT VS X (NG/ML)
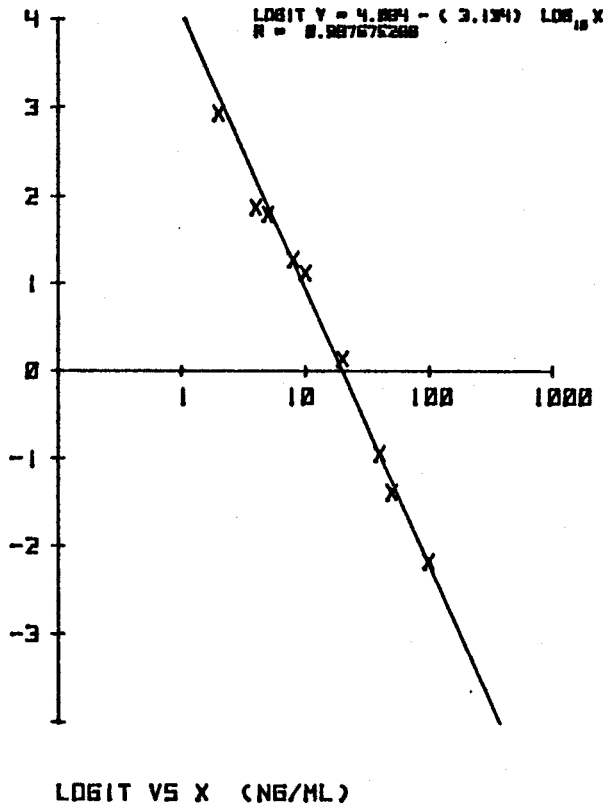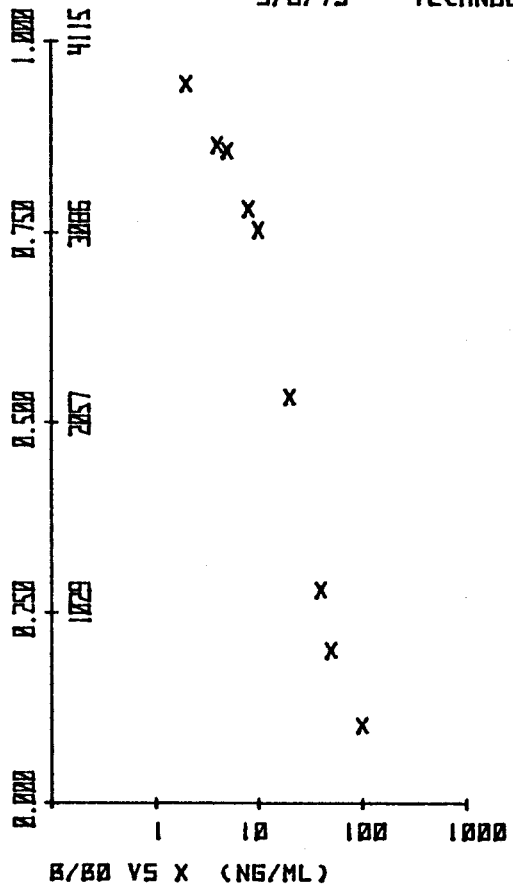
Figure 11. Growth hormone standard curve plotted as B/B_0 vs (ng/ml).

Figure 12. Growth hormone standard curve plotted as logit vs (ng/ml).

TUBE#	CPM	NSB.	CONCENTRATION IN NG/ML	(+ OR -) 95% CONFIDENCE
34		396		
35		395		
36	2804			
37	2850			
38	2931			
			12.5451	1.3877

39		448		
40		380		
41	2132			
42	2301			
43	1964			
			22.9888	6.0996

44		455		
45		440		
46	1423			
47	1436			
48	1414			
			43.4068	0.9736

Figure 13. Calculation of growth hormone content of
unknown samples.

parallel to the standard curve line of purified pituitary rat growth hormone, which would indicate immunological similarities between the brain and pituitary hormones. Physiological changes in the rat's circulating GH levels are brought about by hypophysectomy. These changes should be reflected in pre- and post-surgical serum samples, and were found to be distinguishable by this rat GH RIA. Another method of controlling and detecting interassay variation is the use of internal standards of pooled brain extract in each assay performed. A possible error source frequently encountered in RIA's are "false positive" measurements due to damage of the labeled antigen during the iodination process, or by enzymatic breakdown by tissue homogenates, which leads to a reduction in bound labeled hormone and a misinterpretation of these low counts, so obtained, as indicative of large amounts of growth hormone in the sample.

Recovery studies were therefore performed in order to test the assay for this type of error. Purified rat growth hormone was added in graded, known amounts to brain extracts in triplicate, each sample with its own nonspecific binding control (NSB). These NSB tubes, generally run in duplicate, contained no first antibody in order to detect the binding of labeled antigen nonspecifically to the walls of the assay test tube or to some protein, for which it has a nonimmunological affinity, and which is present in the sample.

As shown in Table 1, the detection of added known amounts of purified GH was approximately 100% from this growth hormone RIA, indicating no change in the hormone molecule or to the destruction of the labeled

TABLE 1

RECOVERY OF ADDED AMOUNTS OF REFERENCE PREPARATION GH

IN NORMAL BRAIN HOMOGENATE

	Recovery		
ng added GH	obtained (O)	expected (E)	O/E x 100
0	8.0 = amount of hormone indigenous to brain homogenate		
2	11.9	10.0	119.0%
4	16.6	12.0	138.3%
5	11.9	13.0	91.5%
8	16.2	16.0	101.3%
10	13.9	18.0	77.2%
20	24.3	28.0	86.8%
40	60.7	48.0	126.5%
50	63.2	58.0	109.0%
100	127.5	108.0	118.1%
		Average	107.5%

growth hormone by brain homogenate. From previous "specificity" studies and the "recovery" studies of purified GH, it seemed that this RIA, as developed for rat GH, was a valid assay and the results were not subjected to any artifactual problems.

The radioimmunoassay to detect thyroid-stimulating hormone in brain homogenates was developed in a similar way to that of GH and will be described in detail in the section to follow.

II. THE RAT THYROID STIMULATING HORMONE RADIOIMMUNOASSAY

a. Antibody to TSH.

For this assay, antiserum to rat TSH, prepared in rabbits, was obtained from Dr. Parlow, NIAMDD. This was stored as a 1:10 dilution at -70° C in a phosphosaline EDTA buffer (0.01 M PO_4, 0.15 M NaCl) with 0.05 M EDTA and 3% normal rabbit serum, pH 7.6. For the TSH RIA, a dilution of antibody 1:15,000 in the same buffer was found to give the most sensitive assay.

b. Radioisotope Labeling Procedure for Rat TSH.

A purified rat TSH was obtained from NIAMDD and stored in 20 μl aliquots containing approximately 5.0 μg of hormone in 0.01 M phospho-saline buffer, pH 7.6 at -70°C. The Chloramine-T oxidation method was again successfully employed for labeling of TSH with radioactive iodine (Hunter et al, 1962). The procedure was carried out using similar radiation safety measures as that of GH and was as follows:

1. 20 μl stock rat TSH (5.0 μg, NIAMDD).

2. 10 μl ^{125}I - approximately 1 m curie (New England Nuc.)

3. 25 μl PO_4 buffer, 0.05 M, pH 7.6.

4. 10 μl Chloramine-T (25 mg Chloramine-T in 10 ml freshly prepared 0.05 M PO_4 buffer, pH 7.6).

Mixed for 40 seconds.

5. 25 μl Sodium metabisulfite (25 mg sodium metabisulfite

in 10 ml freshly prepared 0.05 M PO$_4$ buffer, pH 7.6).

6. 50 μl 0.4% KI (Fisher).

This mixture was then transferred to a Sephadex G-50 coarse, 0.9 x 20 cm
plastic column, coated with bovine serum albumin, and eluted with 0.01 M
phosphosaline buffer, pH 7.6. Fifteen-drop fractions were collected
in 16 x 100 cm glass tubes, and those tubes showing the highest radio-
activity in the first peak were diluted in 0.01 M phosphosaline, 1% albu-
min buffer, pH 7.6, pooled and stored in 1 ml aliquots at -20° C. The
stability of this iodinated TSH was for approximately two weeks at this
temperature.

Repurification of labeled hormone was performed the day of an assay
using a Concanavalin-A, Sepharose column (Pharmacia Fine Chem.). The
principles behind this method of purification of TSH are described brief-
ly here.

"Affinity chromatography" using Con-A Sepharose is a well established
preparative technique which can be used for isolating glycoproteins which
contain αD-glucose or αD-mannose residues. In such a procedure, glyco-
proteins in a sample are washed into the column and are retained due to
their affinity for the lectin, Concanavalin-A, which has been previously
immobilized on the Sepharose beads. These glycoproteins can now be sep-
arated from other proteins in the sample which are not retained on the
beads. After a thorough washing of the column with a phosphosaline buf-
fer to remove any traces of non-glycoprotein, the bound glycoprotein may

be eluted off the column by 1-0 methyl ∝D-glucopyranoside, pH 9.0 (Shore et al, 1973). The glucopyranoside competes with bound glycoprotein for Con-A binding sites and at sufficient concentration can remove all glyco-protein from the ligand.

The following procedure, adapted from the method of Patritti-Laborde et al (1979), was employed to recover repurified ^{125}I TSH using a small Con-A Sepharose column, 0.9 x 19 cm. Glass wool was placed on the scin-tered glass disc at the base of the column and the gel poured to a height of 9 cm. The column was then washed continuously with 20 mls of 0.5 M phosphosaline, 1% BSA buffer, pH 7.6, and then approximately 1 ml of freshly ^{125}I labeled purified TSH applied. The column was then washed with 60 ml of 0.5 M phosphate buffer and the radiolabeled bound glyco-protein TSH, subsequently eluted with 0.2 M 1-0-methyl-∝D-glvcopyrano-side (Sigma Chemical Company, St. Louis, Missouri) in 0.5 M phosphosaline buffer, pH 7.6.

Three ml aliquots were then collected in 16 x 100 cm glass tubes and the two peak radioactive tubes pooled and diluted to yield approxi-mately 9,000 cpm/100 μl in "TSH buffer." The specific activity of the ^{125}I labeled TSH ranged from 80-95 mC/mg.

Solutions:

Phosphate buffer - 0.5 M

6.9 gm NaH_2PO_4 H_2O in 100 ml

7.1 gm $NaHPO_4$ in 100 ml

1:9 monobasic to dibasic, pH adjusted to 7.6.

0.05 M PO_4 = 0.5 M diluted 1:10 with distilled H_2O.

"TSH buffer" = 1% BSA, 0.01 M phosphate, 0.15 M saline.

    2 mls   0.5 M phosphate buffer

100 mls   0.155 M saline (9g NaCl/litre)

4.5 mls   22% BSA

          pH 7.6

c. Preparation of TSH Standards.

Rat TSH reference preparations from NIAMDD were stored as 10 µg TSH/100 µl of "TSH" buffer in aliquots at -20° C. Standards were prepared by double dilution of this stock solution in the same buffer, and ranged from Standard 1 = 39 ng/ml to Standard 8 = 5000 ng/ml.

d. Assay Protocol.

The rat TSH double antibody assay was run at room temperature and required 3 days of incubation in 12 x 75 mm glass tubes.

DAY 1   1. 200 µl TSH antibody, 1:15,000 rabbit anti-rat TSH (NIAMDD), made in 3% normal rabbit serum phosphosaline EDTA buffer;

or

            200 µl non-specific binding buffer (3% normal rabbit serum in phosphosaline EDTA buffer).

    2. 100 µl standard or unknown.

    3. 100 µl ^{125}I TSH, 9,000 cpm.

    4. 200 µl "TSH" buffer.

Mixed well.

DAY 2   5. 100 μl anti-rabbit IgG produced in goat (PelFreeze,

                Roger, Arkansas) 1:10 dilution in "TSH" buffer.

DAY 3   6. 1 ml saline.

The samples were spun for 30 minutes at 2,200 rpm, decanted, and
the antigen-antibody precipitate counted.

e. Treatment of Data Obtained from the TSH Radioimmunoassay.

Data was treated in a similar way to that described previously
for GH. Typical standard, and logit vs. conc. curves, for the TSH
radioimmunoassay are given in Figs. 14 and 15. The sensitivity of this
assay was in the range of 39-5,000 ng/ml.

f. Validation of the TSH Radioimmunoassay.

Attempts to validate the TSH assay were similar to those described
for GH. Cross-reactivity to FSH and LH was less than 0.14%, and recov-
ery studies using ^{125}I TSH added in varying amounts to brain homogenates
showed an average 97.3% recovery (Table 2).

ASSAY TSH 12E
10/29/79   TECHNOLOGIST   S. HOUVAT

LOGIT Y = 7.388 - (2.493) LOG₁₀ X
R = 8.990477292

B/B0 VS X (NG/ML)   LOGIT VS X (NG/ML)
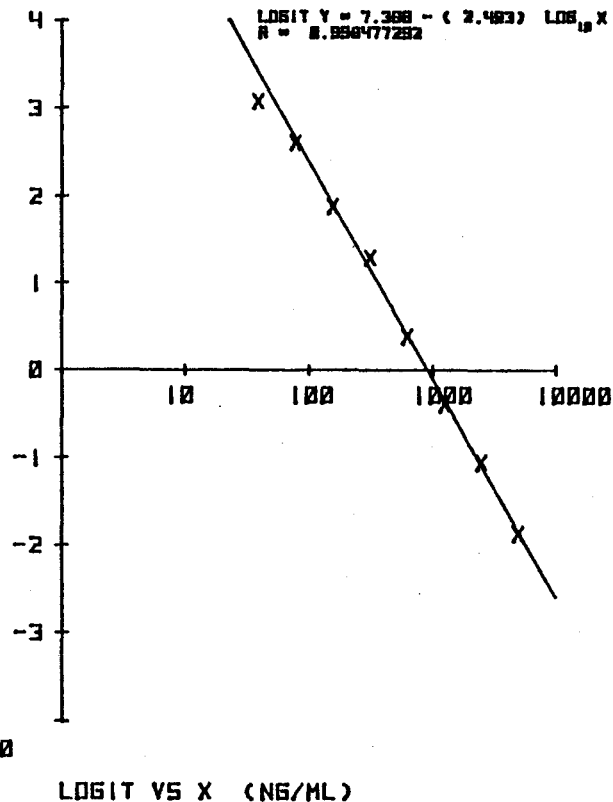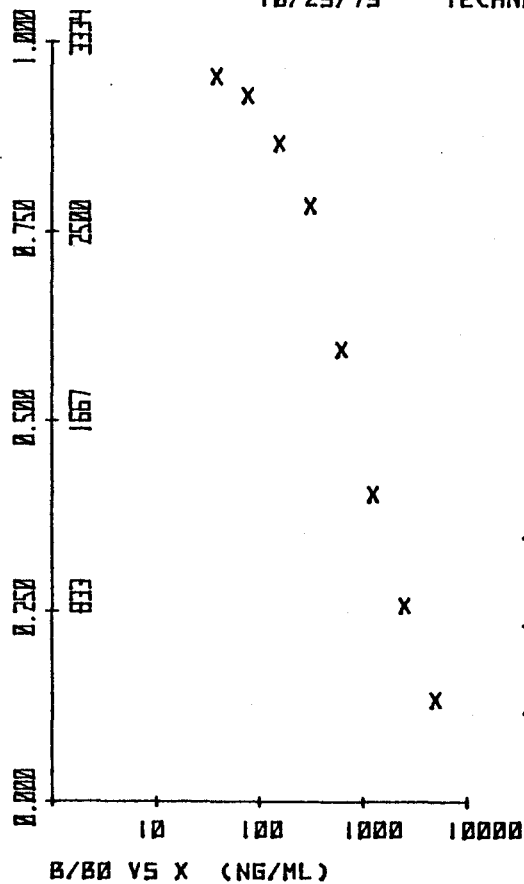
Figure 14. TSH standard curve plotted as B/B_0 vs (ng/ml).

Figure 15. TSH standard curve plotted as logit vs (ng/ml).

TABLE 2

RECOVERY OF ADDED AMOUNTS OF REFERENCE PREPARATION TSH

IN NORMAL BRAIN HOMOGENATE

	Recovery		
ng added TSH	obtained(O)	expected(E)	O/E x 100
0	266.8 = amount of hormone indigenous to brain homogenate.		
39	265.0	305.8	86.7%
78	348.2	344.8	101.0
156	389.4	422.8	92.1
312	548.1	578.8	94.7
625	850.6	891.8	95.4
1250	1577.3	1516.8	104.0
2500	2902.7	2766.8	104.9
5000	5248.4	5266.8	99.7

Average   97.3%

III. RADIOIMMUNOASSAYS FOR GROWTH HORMONE AND TSH IN PRIMATE BRAIN HOMOGENATES

a. Growth Hormone

A solid phase radioimmunoassay in antibody-coated tubes was set up after the method of Catt et al (1967). The principle of this solid phase radioimmunoassay is based on the ability of antibody-coated polymers, such as plastic test tubes, to bind radioactive tracer antigen. The use of antibody in this form is a technique, other than the "double antibody" method, for the rapid removal of unbound from bound radioactive tracer antigen by washing of the solid phase material with water on completion of the immune reaction. The solid phase consists at this point of bound radioactive tracer antigen-antibody complexes and competing bound unlabeled sample antigen-antibody complexes. This solid phase is then counted, after removal of unbound antigen by water, for the quantitation of bound tracer which varies inversely with the total quantity of antigen present in the original mixture.

In our assay, one ml of first antibody, anti-human growth hormone produced in rabbits and obtained from L. Kirsteins, was used at a 1:1500 dilution in 0.05 M bicarbonate buffer, pH 9.6, to coat the sides of 12 x 75 mm plastic tubes overnight at 37° C. An irreversible antibody-polymer complex was thus formed. After washing, to remove non-complexed antibody, radioiodinated growth hormone (Abbot) was added at a concentration of 10,000 cpm to each coated tube containing either standard growth hormone,

internal controls, or unknown tissue homogenate, plus bovine serum. This mixture was incubated at 37° C for 36 hours and, after rinsing, the tubes were counted for bound radioactivity. Levels of primate growth hormone were calculated for each tissue homogenate as pg/mg wet weight of monkey brain and pituitary tissue. The range of this assay was 1 to 100 ng of growth hormone.

b. <u>Thyroid Stimulating Hormone</u>

Samples of monkey brain were homogenized, and a double antibody radioimmunoassay for human TSH was carried out by the late Dr. A. Guansing, Wood Veterans Administration Center, Milwaukee, Wisconsin. The data obtained were calculated as pg/mg wet weight of tissue.

B. <u>DESCRIPTION AND CARE OF ANIMALS USED THROUGHOUT THIS STUDY.</u>

Sprague Dawley male and female rats were used for these studies. All animals were kept at day/night ambient light and temperature regimes and were fed Purina rat chow with water <u>ad libitum</u>. Pregnant female Sprague Dawley rats were housed in separate cages throughout pregnancy, and no more than three animals per cage were allowed for other studies.

Hypophysectomized and thyroidectomized rats were purchased from Hormone Assay Laboratories, Chicago, Illinois, and were fed a liquified rat chow and orange slice diet for the first week post-operatively.

Animal sacrifice was routinely performed between 8 a.m.-10 a.m. using a guillotine.

C. <u>BRAIN DISSECTION PROCEDURES.</u>

After allowing at least a one-hour period of acclimatization to a new locale, animals were killed by instantaneous decapitation using a guillotine, and trunk blood was collected by gravity flow from the severed neck. Handling prior to decapitation was kept to a minimum to prevent possible anomalous, stress-mediated rises in pituitary hormones. To minimize the possibility of retrograde portal blood flow after death, the occipital-parietal calvarium was cut away within two seconds of decapitation and the frontal lobes of the brain gently lifted in order to separate the brain from the pituitary stalk without disruption of the diaphragm sellae (Figs. 16 and 17).

CORTEX

HYPOTHALAMUS
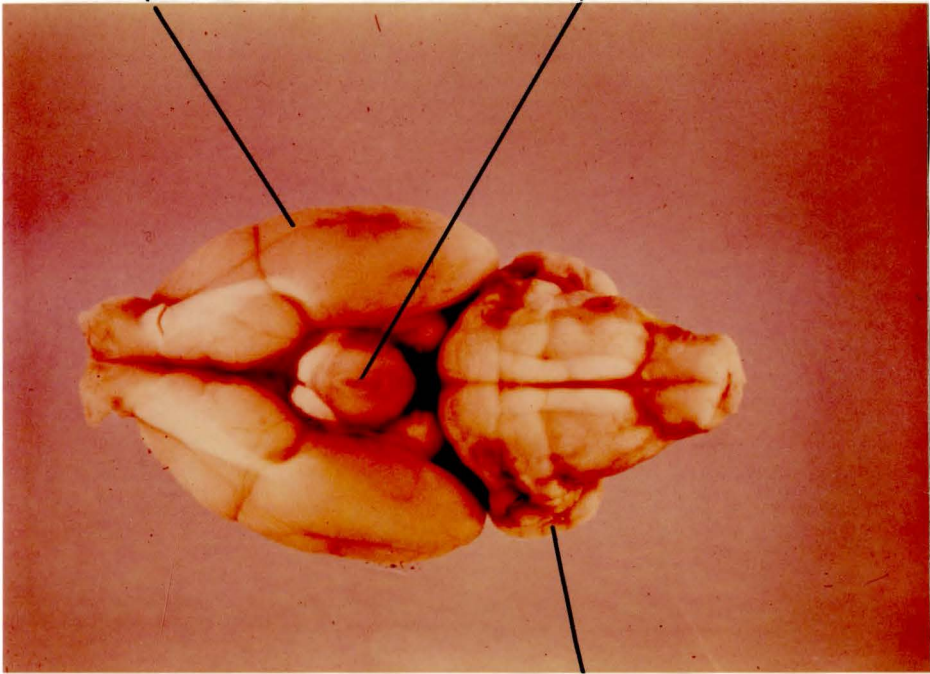
CEREBELLUM

Figure 16. Isolated rat brain.

CORTEX

HIPPOCAMPUS

AMYGDALA

THALAMUS

HYPOTHALAMUS
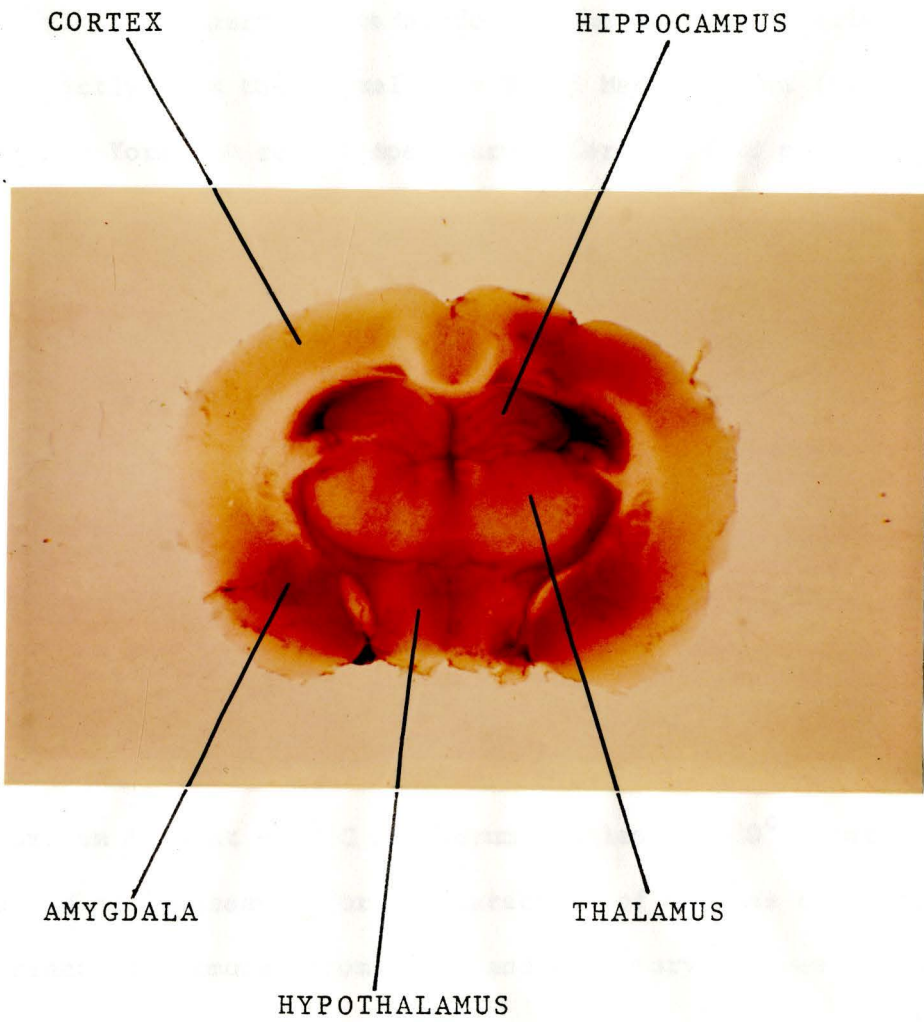
Figure 17. Coronal section of rat brain.

For tissue extraction studies, the brain was placed rapidly onto ice and, using separate instruments, individual brain parts were dissected free and immediately frozen on dry ice to minimize proteolytic enzyme activity. Brain parts intended for tissue culture experiments were placed directly from the animal into Basal Media Eagles (Gibco, Grand Island, New York) at room temperature before further processing.

Brains from the primate, Macaca mullata, were obtained from the Department of Dentistry, Loyola University of Chicago, after a lethal pentobarbital dose, dissected, and placed in acetone before extraction and hormone analysis.

For the harvesting of pituitary glands from intact rats and primates, the diaphragm sella was gently excised and the anterior hypophysis lifted from the sella turcica and placed on dry ice.

All separately dissected specimens of rat brain and pituitary were stored in aluminum foil at -70° C and serum samples at -20° C until radioimmunoassayed or bioassayed for the detection of hormone content. The method of extracting hormones from brain and pituitary tissues is outlined in the next section.

D. TISSUE EXTRACTION METHODS.

Individual rat brain parts and pituitaries were thawed at room temperature. The anterior pituitary, hypothalamus, and serum samples were assayed individually. However, GH and TSH concentrations in other brain regions required pooling tissue from at least five animals in order

to extract sufficient material to be measured within the sensitivity limits of the radioimmunoassay.

Fetal animals of appropriate gestational ages were obtained by maternal decapitation and hysterectomy. The brains of younger fetuses were frozen within six seconds of maternal death. The older fetuses were carefully removed from the uterine horns and kept warm and under as little stress as possible before decapitation.

Individual brain parts and pooled material were weighed and homogenized in 1 ml of ice cold buffer (PBS 0.01M, pH 7.6) for twenty seconds using a Tekmar homogenizer. The samples were spun at 3,000 rpm in a refrigerated centrifuge for 30 minutes, supernatants removed, and the pellets resuspended in 1.0 ml of cold buffer and stored overnight at 4° C. Tubes were recentrifuged in the cold and the two supernatants pooled. These were either assayed immediately or stored at -70° C until the time of assay.

In addition to being radioimmunoassayed for hormone content, materials for assay were thawed and a determination of protein content of tissue made on each sample using the method of Wadell (1956). Hormone levels for each brain area were determined using the radioimmunoassays of rat GH and TSH, and the results were expressed as either ng of hormone/ mg wet weight tissue, or ng of hormone/mg protein or ng hormone/total brain part. In this manner, the distribution of pituitary-like hormones was mapped for the rat brain. A rough mapping of the primate Macaca mulata brain was also determined.

E. TISSUE CULTURING OF BRAIN CELLS

Culture of tissue falls into two broad areas: the use of explant
cultures of small fragments, or single cell preparations from tissue dis-
sociated by mechanical or enzymatic procedures. The preparation and
culture of single cell suspensions was the method of choice for this study
in which distinct brain areas and pituitary tissue were further tested
for their ability to release hormone into the growth media. The principle
behind single cell suspensions, that of removing tissue from the influence
of the whole organism and placing it in a more defined environment, has
been validated by the demonstration that neurons in culture can develop
physiological properties similar to those seen in comparable cells in situ,
including the formation of new synaptic connections with one another and
small aggregates (Fischbach, 1972, and Dichter et al, 1975). Some latent
difficulties are, however, involved in the tissue culturing of nervous
tissue. Cell division is a limited phenomenon resulting in the inability
of primary cell cultures to remain viable indefinitely. Furthermore, long
term subculturing of nervous tissue generally yields a nonfunctioning
population of cells, as shown by Yasamura (1968). One alternative to
these problems has been to culture functioning differentiated neoplasms
which can be serially propagated in tissue culture indefinitely without
losing functionality, for reasons not fully understood.

To obtain successful cell separation from primary tissue for tissue
culture, it is necessary to begin with a suspension of single viable cells,
relatively free of damaged cells, cellular debris, and red and white blood
cells. Brain parts and pituitaries are dissected , using sterile instruments,

from 30-day-old male rats or 30-60-day-old males who have been hypophy-
sectomized for various time periods beforehand. Tissue from approximate-
ly 10 animals is placed in a petri dish containing sterile Basal Medium
Eagle plus 10% fetal calf serum (Gibco), and cleaned of any adhering
meninges or connective tissue. To remove debris and red blood cells,
the tissue is washed four times in sterile media before being cut into
small 1 mm cubed pieces using two sharp scalpels to avoid tearing. Dis-
sociation is carried out using 10 mls of 0.25% Pronase (Calbiochem-Behring
Corp., San Diego, California) in an Erlenmeyer flask shaken gently in a
37° C water bath. Freshly released cells are harvested at 10-minute in-
tervals and stored at 4° C with an equal volume of growth medium. The
separated cells are triturated with a Pasteur pipette approximately five
times and clumps of cells removed by passing the suspension through a
sterile nylon sieve of 35 micron pore size (Tetko, Inc., Elmsford, N.Y.).
The cells are spun gently for 10 minutes at 1,000 rpm in a Sorvall GLC-2
centrifuge at room temperature. After four washes with growth medium to
remove Pronase, the cells are counted in a Petroff Hauser counting chamb-
er. Viability is simultaneously checked by the trypan blue exclusion
method, where only dead cells take up the blue due and therefore can
be distinguished from live cells. From 90%-95% viability is generally
recorded with this methodology. After gentle mixing, the cells are
diluted up to 10^{6}/ml and 1 ml aliquots plated with 2 mls of growth medium
in plastic tissue culture flasks, 25 cm^{2} (Falcon, Oxnard, California).
These flasks and controls containing no cells are then placed in a hori-
zontal position and incubated at 95% humidity, 37° C, and 94% air/6% CO_2.

The medium is changed every 3-5 days and the spent medium removed and frozen to await radioimmunoassay for both GH and TSH. Cell viability is checked at this time in each culture. It was determined that results obtained from such cultures were markedly altered and variable with cultures over 30 days old, and therefore only media harvested by 30 days is generally used. Cellular morphology is examined throughout the entire 30 days. Initially, the nervous tissue is plated as a population of 5-10 μm spherical cells and after 3-4 days the cells are observed to adhere to the walls of the flask. By 8 days, a differentiated population of neurons and glia are present which resemble cells from the original tissue (see Fig. 18).

Pituitary cells remained as spherical cells throughout culture and became attached to the culture flask after 3-4 days.

A mouse neuroblastoma cell line, N4TGI, was obtained from Dr. R. Miller, University of Chicago, and cultured in a similar fashion to that described for primary tissue. The medium from such cultures was tested for immunoassayable GH and TSH. Cells from these cultures were also homogenized and extracted, as previously described for other tissues, and the supernatants analyzed for their hormone content by RIA.

Pooled samples of media from actively growing cultures of this cell line were lyophilized and reconstituted in 1 cc of 0.01 M phosphate buffer, pH 7.6, before elution on a Sephadex G-100 column to test the molecular identity of the immunoassayable TSH-like material.
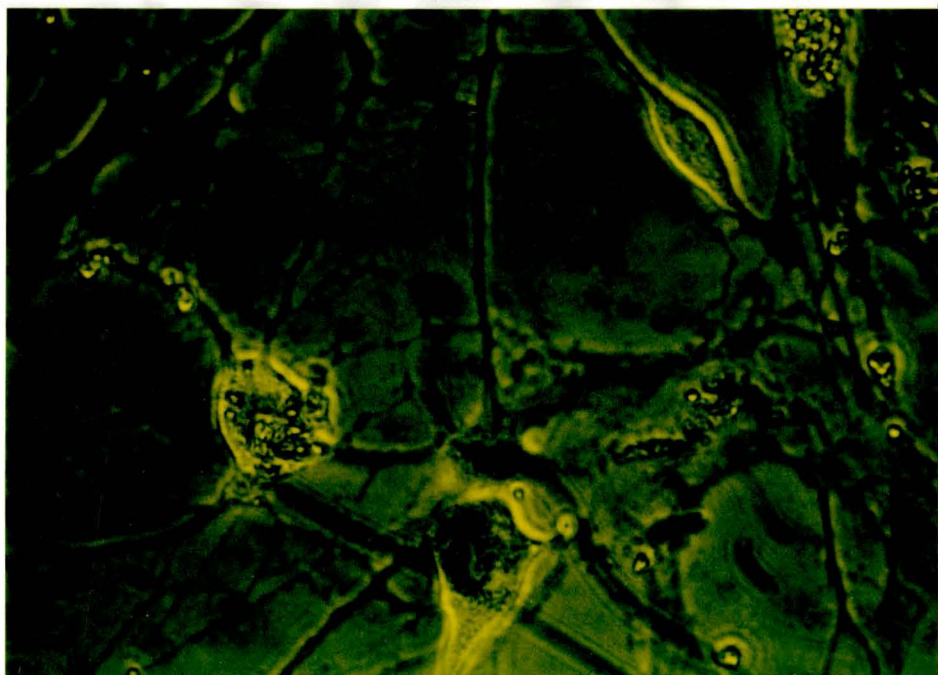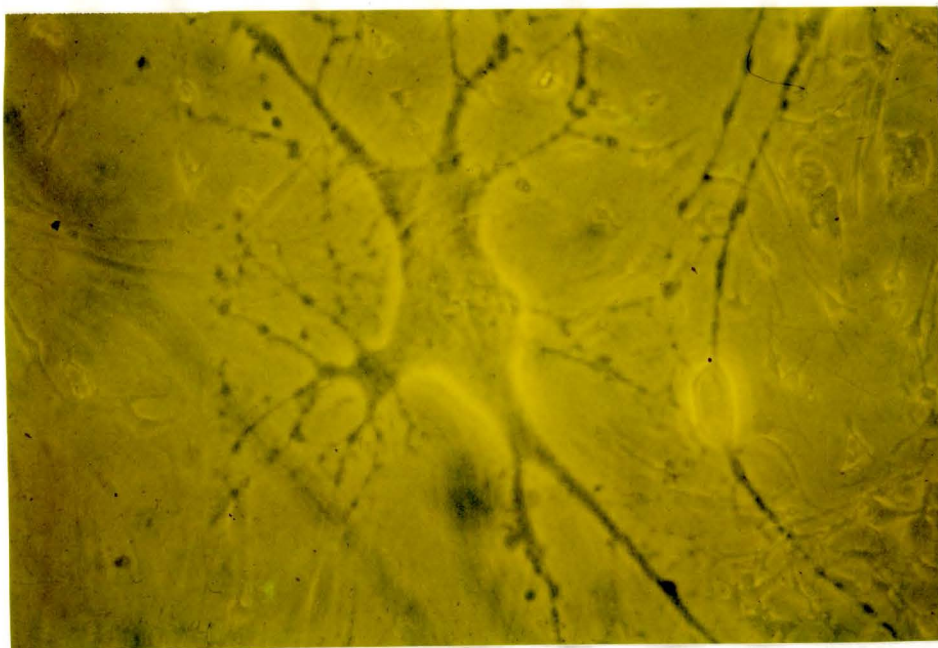
Figure 18. Representative samples of 10 day old nervous tissue culture obtained from the amygdala of adult rats.

Somatostatin, 1 μg/ml (Bachem Inc., Marina del Ray, California, 90201), and L-thyroxine, 1 μg/ml (Sigma) can be added to selected primary, neural, and pituitary cultures to observe any possible regulatory mechanisms governing GH and TSH release from monolayered cells in tissue culture.

Data from all tissue culture experiments are expressed as the average amount of hormone present/ml of medium taken from five individual Falcon flasks. The amount of hormone found intrinsically in the medium, due to the serum content, can be determined from the controls containing no cells and subtracted from all results obtained from flasks containing cells.

Solutions used for tissue culture experiments involving brain and pituitary tissue:

Phosphosaline buffer 7.4 (PBS)

8.0 g NaCl

0.2 g KCL

2.0 g Na_2HPO_4

0.4 g $K H_2PO_4$

pH adjusted with 7.5% sodium bicarbonate (Gibco)

Pronase solution

1.25 g Pronase (Calbiochem)

500 ml PBS

Mix and allow to stand 30' RT^o.

Centrifuged for 10 minutes and sterilized by filtration

(Millipore, Filter Corp., Bedford, Mass., 0.22 pore size).

Store at -20° C in aliquots.

Trypan Blue

    1 ml Trypan Blue (Gibco)

    3 ml Growth Medium

    Filter before use.

Growth Medium

    100 ml Basal Medium Eagle + 10% Fetal Calf Serum (Gibco)

    1 ml L-glutamine, 200 mM (Gibco)

    1 ml MEM non-essential amino acids, 10 mM (Gibco)

    0.1 ml Gentamicin Sulfate, 5 mg/ml (Sigma)

    pH adjusted with 7.5% sodium bicarbonate to 7.4.

F. SUBCELLULAR FRACTIONATION OF BRAIN HOMOGENATE TO DETECT THE LOCALIZATION OF PITUITARY-LIKE HORMONES IN THE BRAIN

Subcellular fractions of brain homogenates were prepared by differential centrifugation, as outlined in Fig. 19 .

In a typical preparation, 10 adult male rats are sacrificed by decapitation, their brains rapidly removed and the hypothalamus, amygdaloid nucleus, and thalamus immediately dissected and separated out at 4° C. Homogenization is accomplished for each pooled brain part in 1 ml ice-cold 0.32 M sucrose, 10 μM $CaCl_2$, pH 7.4, by 12-15 up and down strokes using a glass teflon homogenizer, 0.22 μm clearance, driven at 800 rpm. The homogenates are sedimented at 1,000 g for 15 minutes in a Sorvall RC 5B refrigerated centrifuge. Supernatant, S_1, is removed and the pellets, P_1, resuspended in 0.01 M phosphosaline buffer, pH 7.6, for

Brain Homogenate

0.32 M sucrose, 10 μM $CaCl_2$

1000 x g (10 minutes) 4° C

Precipitate

washed

washings added to supernatant

P_1

Nuclear

S_1

20,000 x g (20 min.) 4° C

Precipitate

washed

washings added to supernatant

P_2

S_2

Synaptosomal

150,000 x g (60 min.) 4° C

Precipitate

washed

washings added to supernatant

P_3

S_3

Microsomal

Soluble
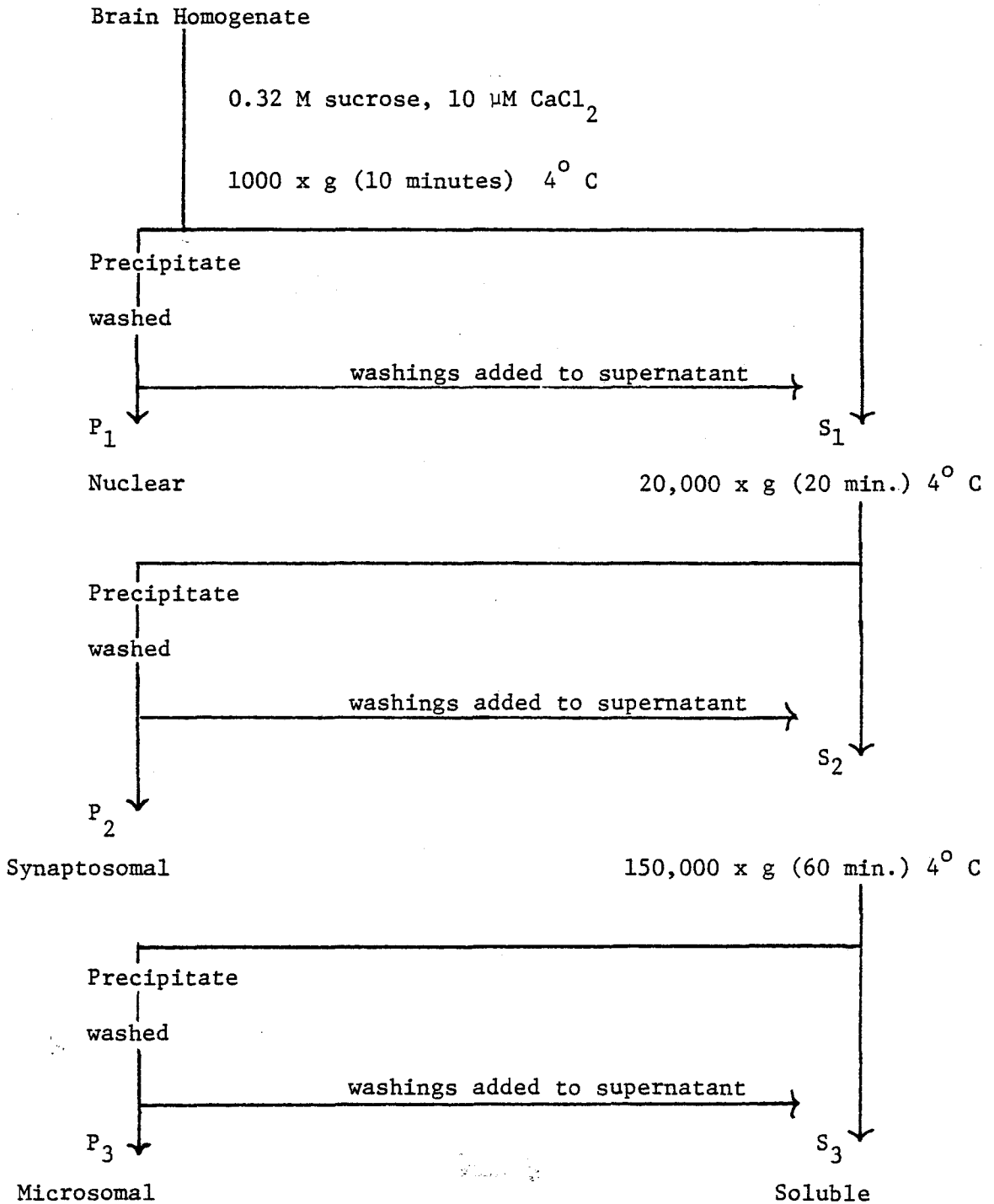
Fig. 19

Fractionation Scheme utilized to determine the growth hormone content of subcellular fractions of rat brain.

radioimmunoassay. Pellet, P_1, contains cellular debris, nuclei, and some myelin. Supernatants, S_1, are sedimented at 20,000 g for 20 minutes in a FA 65 rotor using a Beckman LS-65 preparative ultracentrifuge. The second pellet, P_2, primarily contains synaptosomes, mitochondria, and myelin and is resuspended for further analysis. Supernatant, S_2, is sedimented at 150,000 g for 60 minutes. The resulting pellet, P_3, consists of micro-somal and other membrane fragments.

Due to the possibility of contamination of low-speed pellets with high speed components, the pellets are washed 3-4 times before each step of the centrifugation scheme. Samples of all three pellets and superna-tants are then analyzed for both protein, TSH, and growth hormone content.

Furthermore, addition of ^{125}I GH and ^{125}I TSH during one subcellular fractionation can be used to check the possibility of hormone binding preferentially to a particular fraction and to determine whether pepti-dase activity is present in any fraction. No enhanced degradation of ^{125}I hormones and no selective binding to any particular fraction was observed using this fractionation scheme in this study, thus validating the data obtained.

The detection of antigenic material in cells of various tissues has been aided by the development of various immunohistochemical techniques.

G. IMMUNOHISTOCHEMISTRY OF PITUITARY AND BRAIN TISSUES

Microscopic sections of tissues whose cells contain various anti-
genic substances, such as hormone can, in the simplest case, be incubated
with specific antibodies to such antigens, and antigen-antibody complexes
will form within the sectioned tissue's cells. If this second antibody
is conjugated to a fluorescent or colored probe, these antigen-antibody
complexes will be easily detectable in antigen or hormone-containing cells
when examined under the microscope.

In order to study the tissues of various rat brain and pituitary
parts for the localization of the peptide hormones GH and TSH, such im-
munohistochemical techniques were employed. One of these, the unlabeled
antibody-enzyme-peroxidase method of Sternberger et al (1970), has pro-
vided a highly sensitive histochemical technique for the localization of
antibody-antigen complexes. Its permanent reaction products provide an
advantage over immunofluorescent histochemical techniques, in terms of
long-term study of preparations, as no rapid fading of the labeled complex
occurs with immunoperoxidase techniques as it does in immunofluorescence-
treated material.

The antibody-enzyme peroxidase method of Sternberger allows the
possibility of attaching an enzyme such as horseradish peroxidase to
tissue antigens via a specific antibody without the use of a covalent
labeling reaction. In this method, an antibody bridge system involving a
soluble peroxidase-antiperoxidase complex (PAP) is bound to the primary,
specific, tissue-bound antibody through a second antibody bridge. Cells

containing the specific bound-antibody are stained dark brown by the precipitated peroxidase. Schematically, the components of the peroxidase-antiperoxidase complex, unlabeled antibody stain for microscopic sections of tissue are given in Fig. 20 .

As a guide to the concentrations of antibody and times of incubation to be used in such a technique to detect TSH in tissue sections, the optimum conditions for the TSH radioimmunoassay were used. Sprague Dawley male rats, weighing approximately 200 g, were used for this study of the immunohistochemical localization of TSH in the CNS and pituitary. These animals were anesthetized using 0.1 ml/100 gm body weight, sodium pentobarbital (Holmes Serum Co., Springfield, IL). The thorax was quickly opened and the left ventricle of the exposed heart cannulated. The right atrium was cut and, using a large glass syringe, 50-75 ml of ice-cold calcium-free Tyrodes solution was very slowly perfused through the aorta. This was followed by a 30-minute perfusion of 300-500 ml ice-cold paraformaldehyde in Sorenson's buffer into the rat. The animal was kept on ice throughout this procedure. Following perfusion, the entire brain and pituitary were removed and immersed in the same fixative for 90 minutes, after which they were transferred to 5% sucrose in 0.1 M Sorenson's phosphate buffer and stored at 4° C. Before sectioning, the tissue was routinely dehydrated through a series of graded alcohols and xylene before embedding in paraffin.

Sections 6 microns in thickness were cut through the whole brain and pituitary and placed on gelatin-coated glass slides. After drying, these sections were gently heated to $40-45^{\circ}$ C to melt the paraffin and the slide-
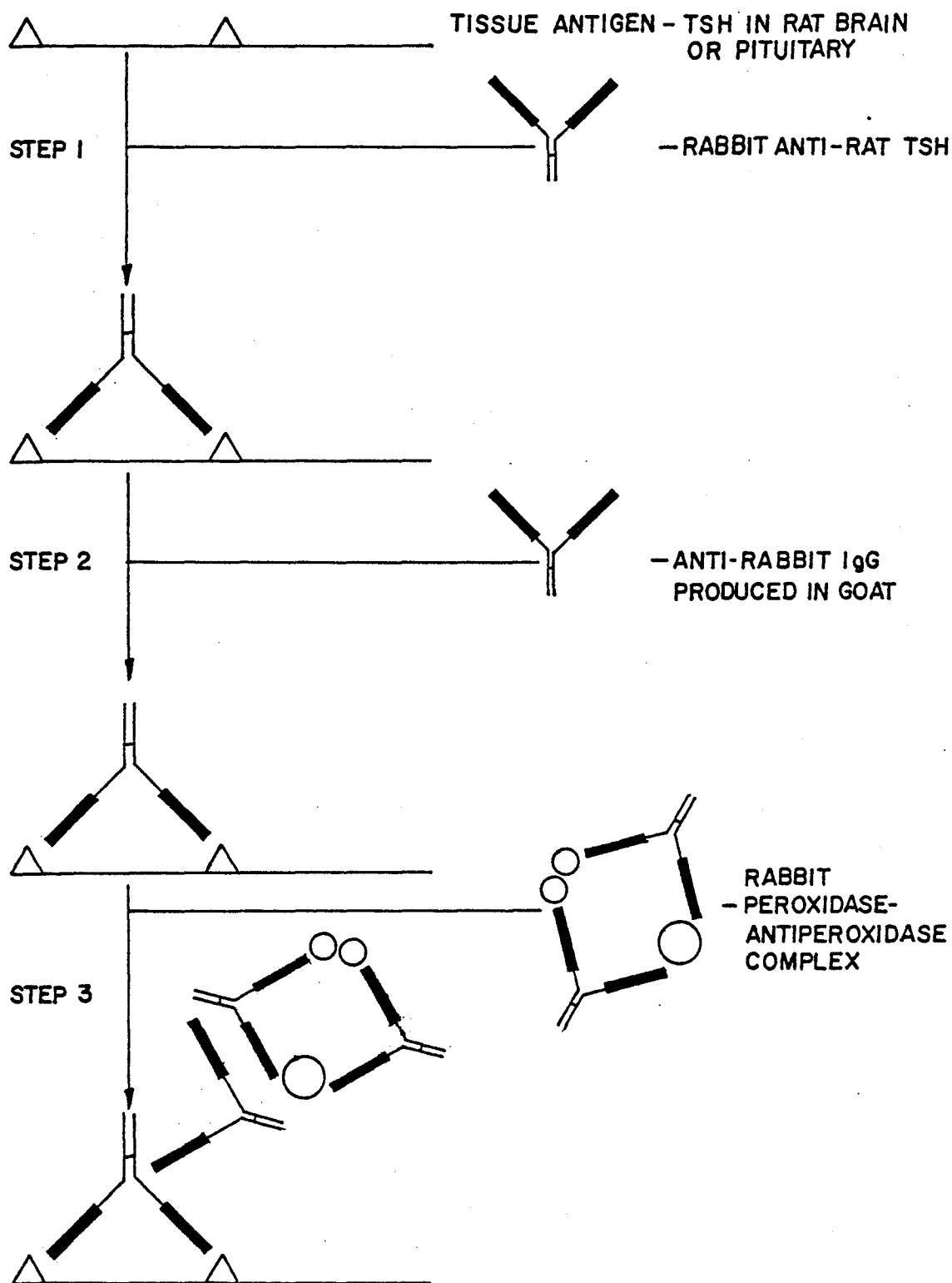
Figure 20. Components of the peroxidase-antiperoxidase complex unlabeled antibody stain used for the detection of rat TSH in brain and pituitary tissue.

bound tissue rehydrated through a series of xylene, alcohols (absolute – 70%), and finally in distilled water before commencing immunohistochemical staining. Staining procedures for TSH were modifications of histochemical protocols given by Moriarty (1973) and Moi Yoi et al (1979).

Deparaffinized sections were incubated in methanolic peroxide (1 vol. 3% H_2O_2/5 vol. methanol) for 30 minutes at room temperature to eliminate endogenous peroxidase activity. After washing in phosphate buffer (0.1 M), these sections were incubated for a further 30 minutes at room temperature with 5% aqueous egg albumin (Schwarz-Mann, Orangeburg, N.Y.) to minimize background nonspecific binding of antibody. Without washing, the sections were treated as in step 1 of the immunocytochemical staining procedure, by covering the tissue mount with the first antibody, rabbit anti-rat TSH antibody at a 1:15,000 dilution. Incubation of the slides was then carried out at room temperature for 48 hours in a moist chamber consisting of a sealed box containing dampened foam rubber so that the sections were never allowed to dry out throughout the entire procedure. After this two-day incubation, slides were thoroughly washed with 0.05 M phosphate buffer and then overlaid with the second antibody, a 1:10 dilution of goat anti-rabbit IgG. Incubation with this antibody was for 18 hours at room temperature, and after a wash step, sections were incubated for a further 30 minutes with a 1:50 dilution of rabbit peroxidase anti-peroxidase (PAP) complex in phosphate-albumin buffer. After rinsing in phosphate buffer (0.05 M), the peroxidase was localized by incubating the tissue in a solution containing hydrogen peroxide and 3.3' diaminobenzidene tetrahydrochloride for 30 minutes. Oxidation of the reagent formed a brown

precipitate at the site of the peroxidase – anti-peroxidase complexes, which was intensified by incubation in 1% OsO_4 for 30 seconds. The slides were then washed in phosphate buffer and mounted in Permount (Fisher) before examination microscopically using a Leitz Diavert inverted microscope. Sections were photographed using Kodak Ektachrome fast color 400 daylight film with a 35 mm microscope-mounted Leitz camera. Validation of the specificity of the reaction was tested via the following controls, which were stained for the same length of time for each tissue examined.

Negative controls were those in which the first antibody was replaced by buffer or by normal rabbit serum, diluted to 1:15,000. In some sections, the second antibody step was also replaced by addition of buffer. Immunabsorption of the anti-rat TSH serum before use in the procedure with purified TSH, overnight at room temperature at a concentration of 100 µg/ TSH/ml of diluted primary antisera, was also used to test the specificity of the reaction.

The methodology described so far has applied to the histochemical detection of TSH in slices of brain and pituitary. Histochemical detection of GH in brain slices was also attempted with less success, using a less sensitive method of immunoperoxidase staining than the method of Sternberger, the "indirect method of staining."

Although the method of section preparation was similar for GH as that for TSH detection, no PAP-complex was used in the immunohistochemical staining procedure. Instead, the immune reaction was terminated at the

point of formation of an antigen-antibody complex between the second anti-
body, labeled with peroxidase, and the initial complex formed between
first antibody and tissue-bound GH. Although cells containing a dark-
colored complex could be observed, this work will have to be repeated
using Sternberger's technique in an effort to obtain better histochemical
resolution.

Solutions used for the histochemistry study to detect TSH and GH in brain
tissue.

Sorenson's phosphate buffer 0.2 M

8.06 g KH_2PO_4

37.75 g Na_2HPO_4 7 H_2O

Made up to 1 litre with distilled water, pH = 7.2.

Paraformaldehyde in Sorenson's buffer

Dissolve 40 g paraformaldehyde in 450 ml distilled water.

Heat to 55-60° C.

Add 2 N NaOH dropwise until solution clears.

To the above paraformaldehyde, add 500 ml 0.2 M Sorenson's
phosphate buffer, pH 7.2.

Bring final volume to 1 litre.

Yields 1 litre of 4% paraformaldehyde in 0.1 M buffer.

Filter and store at 4° C.

Calcium (Ca^{++}) free Tyrodes

Add 800 mls distilled water to a 1-litre flask.

NaCl	6.80 g	Glucose	1.00 g
K Cl	0.40 g	NaHCO$_3$	2.20 g
MgCl$_2$	0.15 g	Swirl after each component.	
MgSO$_4$ 7 H$_2$O	0.10 g	Dilute to 1 litre. Gas with	
NaH$_2$PO$_4$ 2 H$_2$O	0.19 g	95% O$_2$/5% CO$_2$ on ice.	

Slide gelatinization technique

For 100 ml:

0.5 g swine skin gelatin (Sigma)

0.05 g chromium potassium sulphate 12 H$_2$O (Fisher)

Heat 100 ml water, dissolve gelatin and chromium potassium

sulphate.

Dip slides quickly, dry in open air.

Store at 4° C wrapped in Parafilm.

Phosphate buffer 0.1M

Used for wash after fixation.

Solution A:

27.80 g NaH$_2$PO$_4$. H$_2$O

1000 mls distilled H$_2$O

Solution B:

53.65 Na$_2$HPO$_4$ 7 H$_2$O

1000 mls distilled H$_2$O

(Solution B, continued)

19 mls of A + 81 mls B diluted to 200 mls, pH 7.4.

For wash between stains, diluted with water to 0.05 M.

For dilution of antisera and PAP: 0.05 M. phosphate buffer +

2.5 mg/ml bovine serum albumin.

The "PAP" complex

Made in rabbit from the method of Sternberger by Miles Laboratories,

Elkhart, Indiana.

An antigen-antibody complex dissociated and solubilized in excess

peroxidase antigen forming a stable soluble complex.

Maintained at -70° C in 0.1 ml aliquots.

Tris buffer 0.5 M

30.285 g THAM (Tris /Hydroxy-methyl/ amino methane)

250 ml H_2O

pH with 1 N HCl to 7.6

Add 250 ml H_2O

Diaminobenzidine – H_2O_2 solution

Always freshly made; good for 60-90 minutes.

6 mg 3 - 3' diaminobenzidine hydrochloride (Sigma)

10 ml Tris buffer

1-2 drops 3% H_2O_2 (not more than 2 months old)

Filtered and kept stirred throughout staining reaction.

Dehydration protocol for all tissues

2 x	80% alcohol	30 minutes
2 x	95% alcohol	30 minutes
2 x	absolute alcohol	30 minutes
2 x	chloroform	30 minutes
Paraffin		

Hydration protocol for all tissues

2 x	xylene	3 minutes
2 x	absolute alcohol	3 minutes
2 x	95% alcohol	3 minutes
2 x	80% alcohol	3 minutes
2 x	distilled H_2O	3 minutes

The next studies to be described were designed to determine the approximate molecular weight of the brain hormone in comparison to that of the hormone of pituitary origin.

H. COLUMN CHROMATOGRAPHY OF BRAIN EXTRACTS

A calibrated column of Sephadex gel provides a simple way of determining the molecular weights of proteins such as GH and TSH. Sephadex is a bead-formed gel prepared by cross linking dextran with epichlorohydrin. As a solute passes down a vertical column packed with such chromatographic beads, its movement depends on the bulk flow of the mobile phase and upon

the Brownian movement of the solute molecules. These movements cause the diffusion of molecules in the solute both in and out of the stationary phase of the beads. The separation of solutes in gel filtration depends on the different abilities of the various sample molecules to enter pores which contain the stationary phase. Very large molecules which never enter the stationary phase therefore move through the chromatographic gel bed faster than smaller molecules which can enter the gel pores, and thereby move more slowly through the column since they spend a part of their time in the stationary phase. Molecules are therefore eluted in order of decreasing molecular size. Results are usually expressed in the form of an elution diagram showing the variation of solute concentration in the eluent with the volume of eluent passed through the column. Sephadex G-100 was the gel of choice for these experiments, being able to separate clearly molecules with a molecular weight range between 4,000 - 150,000 Daltons. This range was chosen in order to detect the possibility of any larger prohormone-like molecules that may have been present in brain homogenates.

From a total of 40 male, 200 gm rats, brains were dissected into the amygdaloid nucleus, hypothalamus, and thalamus. Each brain region was pooled and kept on dry ice before processing. Extraction was as described in section D, except that the final supernatant was spun at 3,000 g for 10 minutes in a FA-65 rotor of a Beckman ultracentrifuge to remove nuclei and other debris. This supernatant was then lyophilized and reconstituted either in 1 ml veronal buffer, pH 8.6, for GH studies, or in 1 ml 0.01 M phosphosaline buffer, pH 7.6, for TSH column work.

Sephadex G-100, 0.9 x 50 cm columns were prepared, coated with albumin, and calibrated using blue dextran 2000 (Pharmacia) bovine serum albumin, mol. wt. 68,000 D, and ^{125}I insulin, mol. wt. 5,700 D, to give reliable calibration points.

A trace amount, approximately 7,000 cpm in 100 μl buffer of either ^{125}I rat pituitary GH or TSH, was applied to the column together with 1 ml of reconstituted brain homogenate. One-ml fractions were collected in a Gilford automatic fraction collector. These were analyzed for both radioactivity, using a Micromedic gamma counter, or for hormone content, using the radioimmunoassays for TSH or GH. In this way the elution pattern of the radioactive-labeled pituitary growth hormone could be compared to that of the radioimmunoassayable brain hormone. Similar studies were performed using material from fetal brain and pituitaries.

Another parameter of the brain "pituitary-like" hormones examined was their immunological similarity to their respective pituitary counterparts.

I. STUDIES TO DETERMINE THE IMMUNOLOGICAL IDENTITY OF "BRAIN" HORMONES

Immunological similarities between brain and pituitary hormones were determined by preparing serial dilutions of both tissue extracts and subjecting them to radioimmunoassay. Parallelism, or lack of parallelism, between logit log transformed displacement curves produced for each tissue was used as an indicator of probable similar or different interaction with antibody sites to a particular hormone.

The biological activity of brain hormones was also compared with that of pituitary GH and TSH.

J. BIOLOGICAL ACTIVITY STUDIES

a. Growth Hormone Bioactivity in Brain Extracts.

The traditional bioassay for the measurement of pituitary growth hormone has been that perfected by Greenspan et al in 1949. It had been shown earlier by Li et al (1945) that pituitary growth hormone extract stimulated growth of the bony epiphyseal plate of hypophysectomized rats. This observation formed the basis for the Greenspan assay which is based on the ability of growth-hormone-like material in tissue extracts to increase the width of the epiphyseal cartilage in hypophysectomized animals, a parameter measured quantitatively with a microscope micrometer. Li et al (1945) showed that a total dose of 100 µgms of pure growth hormone administered to 30-day post-hypophysectomized adult rats was necessary to produce a significant response in an increase in body weight bioassay for GH, whereas in the tibia epiphyseal cartilage test, as noted by Greenspan, a total dose of only 5 µgms would produce a significant response. Therefore without necessarily obtaining a growth hormone response in terms of weight gain, the use of the tibia bioassay provides a characteristic of growth hormone action which is able to be measured.

This experiment was repeated four times in hypophysectomized rats, twice using female and twice using male recipients. For each experiment, 250 donor rats were sacrificed by decapitation and the amygdaloid nuclei

and pituitaries harvested individually and placed immediately on dry ice.
Each tissue was then weighed, pooled, and homogenized separately, taking
care to avoid any possible contamination of brain homogenate with pitui-
tary tissue. The supernatants obtained from extraction of the tissues
with 0.01 phosphate buffer, pH 7.6, were spun at 5000 g to eliminate any
debris and nuclear material, dialyzed against 0.15 M saline, lyophilized,
and reconstituted in 20 ml of physiological saline (0.15 M, pH 7.4). The
final concentration of GH in both pituitary and brain extracts was measured
by the RIA described earlier and appropriate dilutions to give approxi-
mately 20-30 μg of immunoreactive GH/ml obtained. A more concentrated
pituitary extract was found to give no advantage over the lower pituitary
dosage.

For the actual bioassay, forty-two-day-old male and female rats,
which had been hypophysectomized at age 28 days, were kept in groups of
five in separate cages and fed a diet of oranges and rat chow mash.
Groups of five animals received twice a day for four days either: 0.5 ml
saline, 0.5 ml rat pituitary extract, or 0.5 ml of amygdaloid nucleus
extract intraperitoneally. Animals were weighed daily at the time of
injection to measure any weight gain. Twenty-four hours after the last
injection, all animals were weighed and sacrificed using sodium pento-
barbital injection. One or both tibias were dissected free of soft tis-
sue and split with a sharp razor in the mid-sagittal plane. The two-bone
halves were either stained immediately or fixed in 10% neutral formalin.
Thyroids, ovaries, and testes were similarly dissected from all control
and assay animals and fixed in 10% neutral formalin.

The staining procedure for the tibia epiphyseal cartilage halves is as described by Evans et al (1943) and is as follows:

1. Bone washed in H_2O - 30 minutes

2. Acetone - 60 minutes

3. Wash in H_2O - 30 minutes

4. Freshly prepared 2% silver nitrate - 1-3 minutes

5. Wash in H_2O and while under water, expose to a strong light until calcified portions appear dark brown.

6. 10% sodium thiosulfate - 25-30 seconds

7. Wash in running H_2O - 30 minutes

8. Store in 80% alcohol in dark at room temperature.

The width of the uncalcified epiphyseal cartilage, which remained unstained and easily observable, is measured under the low power of an American Optical microscope using a micrometer eye piece calibrated with a stage micrometer so that results can be expressed as micra units. A minimum of 10 measurements should be made along each epiphyseal cartilage in areas selected at random. This study was carried out "blind", and two independent observers used. The data between saline-, pituitary-, and amygdaloid-injected groups was statistically analyzed using the Student T test. The cartilage plate increases in width slightly from anterior to posterior, and there is therefore an inherent error in the measurement of mean width. For this reason, all readings should always be from narrow to wider areas and the variation accounted for by the multiple readings. There was usually no significant difference between left and right tibias, but only one tibia, usually the left, was used for the

measurements. A comparison in the total body weights and wet weights of testes and ovaries from both control and hypophysectomized rats was also statistically analyzed.

b. Thyroid stimulating hormone bioactivity in brain extracts.

The effects of TSH on the histology of the thyroid follicle cell were previously discussed in the Review of Literature section of this dissertation. Briefly, the presence of TSH influences the size and shape of the follicle epithelial cells and the distribution of colloid in the follicle lumen. Follicles from hypophysectomized rat thyroids contain cells which are flattened, and little colloid is found in the central lumen of the follicle, as compared to normal, euthyroid animal thyroid follicles.

A minimum dose of 20 ng immunoreactive pituitary TSH/hypophysectomized animal, given over a four-day period, is necessary to restore the histology of the thyroid to that of an intact rat. To demonstrate another biological effect of pituitary TSH on the thyroid, that of an increase in thyroidal radioiodide uptake, at least 10 μgm of TSH/rat are required.

For our experiments, aimed at assaying TSH-like activity of CNS extracts, thyroids from the three groups of animals used in the GH bioactivity experiments were used, i.e., hypophysectomized animals receiving saline, or CNS extract, or pituitary extract. A fourth group of hypophysectomized animals were also used in this TSH bioassay which received a higher dose of pituitary extract than the animals used in the GH bioassay.

In terms of levels of radioimmunoassayable TSH, these four groups of rats received 0 µg of TSH in the saline-injected group; 4.5 µg TSH, from amygdaloid tissue, in the CNS-injected group; 5 µg TSH, obtained from pituitary extracts, in the third group; and 100 µg TSH, from pituitary extracts, in the fourth group of rats.

Twenty-four hours after the last injection, the thyroids were removed, en bloc, with the trachea, fixed in neutral formalin, and subsequently paraffin-sectioned at a width of 6 microns and stained with hemotoxylin eosin. Sections were photographed in order to examine the follicle cells for any histological changes which could be related to the type of material injected. This method of bioassaying TSH represents a modification of the work of Tice (1974).

Another test of TSH's biological effectiveness was to examine this hormone's property to facilitate the uptake of circulating radioiodine by the thyroid. Hypophysectomized animals' ability to take up radiolabeled iodine into the thyroid with, and without, injections of immunoassayable TSH in extracts from brain and pituitary were compared. Similarly, the effectiveness of brain and pituitary extracts to increase the circulating levels of T_4 in hypophysectomized animals was examined as a measure of the bioactivity of brain TSH-like material.

In these two groups of experiments, i.e., measurement of iodine uptake and measurement of circulating T_4 levels, four groups of 5 adult, 14-day, post-hypophysectomized animals received one of the following treatments over a 4-day period: either an IP injection of 0.5 ml of

saline daily, or a total dose of 100 μg immunoassayable TSH from pituitary

extracts in 0.5 ml saline, or a total dose of 2 μg TSH from pituitary ex-

tracts, diluted saline, or 4.3 μg TSH obtained from amygdaloid tissue

homogenates diluted in saline. Eighteen hours before sacrifice, approxi-

mately 1.5 μ curies ^{131}I was injected intraperitoneally into all groups,

and after sacrifice, thyroid and muscle levels of radioactivity were

measured. Thyroxine (T_4) levels of samples of blood, taken from the

trunk by gravity after decapitation, were measured by means of a commer-

cial RIA kit (Tetra-Tab), which is described in detail in section K of

this Materials and Methods chapter.

Although the blood-brain barrier prevents the passage of a majority

of blood-borne materials into the brain, its effectiveness in preventing

the contamination of CNS tissue by pituitary-derived GH and TSH was tested

by methods described in the following section.

K. EXAMINATION OF RAT BLOOD-BRAIN BARRIER INTACTNESS TO PITUITARY GROWTH HORMONE AND THYROID STIMULATING HORMONE

Numerous chemical substances do pass from the bloodstream into the

brain at rates which are slower than for entry into all other organs in

the body. There are similarly slow rates of transport between the CSF

and the brain. The permeability barrier appears to be the end result of

numerous contributing factors that present diffusional obstacles to chem-

icals on the basis of molecular size, charge, solubility, and specific

carrier systems. The difficulty has not been in establishing the exis-

tence of these barriers, but rather in determining their mechanisms. When

the relatively small protein, 43,000 D, horseradish peroxidase is injected

intravenously into rats, its intracellular location within most tissues, such as muscle, can be demonstrated histochemically with the electron microscope. As opposed to the easy transvascular movement of horseradish peroxidase across muscle capillaries into muscle tissue, in brain the peroxidase molecule is unable to penetrate through the continuous layer of vascular endothelial cells constituting the blood brain barrier. This single layer of brain capillary endothelium differs from those of other tissue capillaries such as muscle and heart, in that the intercellular zones of cell membrane apposition are much more highly developed in the brain and are virtually continuous along the entire surface of these cells. They also show a lack of pinocytic vesicles. These vesicles, characteristic of capillaries found elsewhere throughout the body, have been related to transvascular carrier systems of both large and small molecules. Since the horseradish peroxidase enzyme marker can neither go through nor between the endothelial cells comprising the vascular system of the CNS, an operationally defined barrier is said to exist, i.e., the blood-brain barrier.

For a solute to enter the brain easily, it appears to have to be either highly lipid-soluble and be able to diffuse through cell membranes or have an affinity for certain specific and selective carrier-mediated transport systems present in the endothelial cell plasma membrane, as shown by Oldendorf (1977).

In order to test the possibility that circulating GH or TSH of pituitary origin could be reaching the CNS tissue via possible weak areas in the blood-brain barrier, systemically administered radiolabeled

hormones were used as markers of distribution, as described by Stern et al (1975). Using freshly iodinated GH or TSH, five adult animals per hormone were injected intravenously via the tail vein with approximately 1 x 10^6 cpm hormone in 200 μl phosphosaline buffer, ph 7.6. Thirty minutes later the animals were decapitated and the brain areas and various peripheral tissues dissected out. All tissue samples were weighed and counted in a gamma counter to detect the presence of radioactivity arising from the labeled hormone injection. Results were expressed as cpm/mg wet weight of tissue. Control rats injected with 200 μl of buffer alone were similarly killed and the tissues dissected out for detection of immunoassayable hormone, expressed as ng/mg wet weight of tissue.

For the studies involving fetuses, to test the possibility that the blood-brain barrier develops with age, the following protocol was used.

Pregnant females in the 20-21st day of pregnancy were decapitated and the fetuses in the uterine horns gently lifted out of the body cavity and kept warm. Once released from the uterus, these full-term fetuses, released from their attached placentas, were able to survive for several hours. To test the intactness of the blood brain barrier at this early age, 1 x 10^5 cpm of either labeled GH or TSH were injected intraperitoneally in 10 μl PBS into the fetuses. Thirty minutes later, the fetuses were sacrificed by decapitation with scissors and various brain parts and organs dissected out to determine the location of the radiolabeled tag. If there was the possibility of some breakdown in the blood brain barrier, and if the blood content of the brain is approximately 50 μ litres/gm of

brain tissue, by assaying the hormonal content of sera, it was possible to calculate the possible level of contamination from circulating hormone of brain tissue.

L. THE EFFECTS OF HYPOPHYSECTOMY ON BRAIN-BASED PITUITARY-LIKE GH AND TSH.

In this series of three experiments, the effect of removing the pituitary on levels of immunoreactive hormone in various areas of the brain and in serum were compared to levels obtained from intact, sham-operated controls of equivalent age. For each experiment, approximately 80 hypophysectomized animals and 80 controls were obtained from Hormone Assay Lab., Inc., Chicago. The hypophysectomized animals were operated on at 28 days of age, and batches of 20 animals were kept for 7, 14, 28, and 48 days post-surgery on a diet of oranges and mashed moist rat chow before sacrifice. Salinated water was found to be unnecessary for these animals.

Equivalent groups of intact controls, in terms of age, were sacrificed within one hour of the sacrifice of the hypophysectomized rats. Trunk blood was collected for hormone analysis and the brain parts dissected out on dry ice as previously described in section C. Extraction and homogenization were also performed as previously noted in section D of this dissertation, and levels of hormone were calculated, after radioimmunoassay, as ng hormone/mg protein or ng hormone/mg wet weight of tissue.

The effectiveness of hypophysectomy was determined by gross examination of the sella turcica with a dissecting microscope and also by measuring the levels of circulating hormones GH, TSH, and the thyroid hormone T_4,

as well as the "T_3 resin uptake," another indicator of thyroid and indir-
ectly pituitary function, which will be described in the next section.

M. THYROXINE (T_4) AND T_3 RESIN UPTAKE ASSAYS

The levels of circulating total thyroxine or T_4 were obtained using

a commercial T_4 diagnostic kit, "Tetra-Tab RIA" (Nuclear Medical Lab.,

Inc., Dallas, Texas), and the "T_3 uptake" estimation of unsaturated thy-

roid binding globulin capacity was also run using a kit, "Tri-Tab," from

the same company.

a. Measurement of T_4

Thyroxine circulates in the bloodstream as free (1%) and thyrobind-

ing globulin-bound hormone. In the T_4 radioimmunoassay, total thyroxine,

T_4, is released from endogenous thyroxine-binding proteins present in the

serum by the chemical inactivation of the binding protein by 0.025 N HCl.

The T_4 so released is then available for assay of total T_4. The principle

of the RIA is as described for GH and TSH. Specifically for T_4 detection

in the assay, a serum sample is mixed with 200 µl of 0.025 N HCl and a

constant amount of iodinated T_4 is added and the mixture incubated at

room temperature for 30 minutes with a highly specific antibody to T_4

prepared in rabbits. Free and antibody-bound fractions are then separ-

ated by ammonium sulfate precipitation and centrifugation of the antibody-

bound fraction. Following centrifugation, the supernatant was discarded

and the radioactivity in the precipitate measured directly in a gamma

counter. A standard curve was prepared calculating the percent of the

total radioactivity remaining in the bound fraction for the five serum standards ranging from 1 - 28 µg T_4/dl and plotted against T_4 concentration. Unknown T_4 values were calculated for the experimental serum samples directly from the standard curve as µgm/dl.

(i) Assay procedure for the detection of T_4 in serum

Assay carried out in triplicate in 12 x 75 mm plastic tubes, and reagents added in the following order:

1. 200 µl - 0.025 N HCl.

2. 10 µl - standard or serum sample.

   Standards = 1 - 28 µg T_4/dl.

All tubes shaken 10-15 seconds and incubated at RT for 60 minutes.

3. 400 µl ^{125}I T_4.

All tubes shaken and incubated for 60 minutes.

4. 2.0 ml - 35.5% ammonium sulfate containing 0.4% calf serum.

All tubes mixed gently and incubated 20 minutes before centrifugation for 10 minutes at 1000 g.

The supernatant was decanted and the precipitate counted, accumulating a minimum of 20,000 counts/tube. Reproducibility was assessed using frozen serum pools as internal standards for each assay.

   b. Measurement of the radiolabeled T_3 resin uptake in serum
       samples.

The radiolabeled T_3 resin uptake test which is not a measurement of circulating T_3 or T_4 hormones is designed to assess the unsaturated

binding capacity of certain serum proteins, primarily thyroxine-binding globulin (TBC). This assessment is accomplished in the assay by the addition of an excess of ^{125}I-labeled L-triiodothyronine (T_3) to serum in order to saturate all available TBG binding sites in the serum sample. If the endogenous T_4 level is increased, as in hyperthyroidism, the TBG will be relatively saturated. More ^{125}I-labeled T_3 remains in a free state to be adsorbed by a secondary silicate binder. Conversely, when T_4 output is low, as in hypothyroidism, more of the added labeled T_3 will bind to the serum TBG, yielding a comparatively low percentage of the remaining radioactivity to be adsorbed secondarily. For a secondary binding agent, the commercial kit uses an inorganic adsorbent, silicate, as binding of ^{125}I-labeled T_3 reaches over 99% of the maximum uptake within five minutes and remains constant for up to three hours. Basically, this test involves mixing a serum sample with excess ^{125}I-labeled T_3 which occupies all unsaturated binding sites on the TBG in the serum. ^{125}I T_3 not bound to TBG was removed by addition of the secondary silicate binder. Following centrifugation, the supernatant was discarded, and the radio-activity in the sample compared to the amount of radioactivity in a normal control serum, with an established percent uptake. Interassay reproduci-bility was assessed using a pooled, frozen internal serum standard.

(i) <u>Assay procedure for the measurement of the T_3 resin uptake in serum</u>

Assay carried out in triplicate in 12 x 75 mm plastic tubes at room temperature.

1. 100 µl standards or serum sample.

2. 2 ml - ^{125}I T_3 in 0.07 M barbital buffer containing 0.125% BSA.

Mixed thoroughly.

3. T_3 adsorbent tablet containing 10%-40% silicate by weight added.

Mixed for 5 seconds and incubated 10 minutes.

Centrifuged 5 minutes at 1000 g, supernatant discarded and the pellet

counted, in a gamma counter, each tube accumulating at least 20,000 counts.

The percent T_3 uptake is determined by dividing the test counts/the total

counts x 100. An accurate and practical assessment of thyroid status, and

therefore indirectly of pituitary TSH status, can be made by calculating

the product of the radiolabeled T_3 resin uptake and the total T_4 value,

the "Free Thyroxine Index."

N. ALTERATION OF FEEDBACK CONTROL MECHANISMS

    I. TARGET ORGAN REMOVAL

The effect on pituitary hormones of removing a target organ has

previously been alluded to in the discussion on feedback control mechan-

isms. For example, removal of the thyroid gland serves to remove the

"negative feedback" control which thyroxine, T_4, the major product of the

thyroid gland, has upon TSH production from the pituitary. Adrenalectomy

and gonadal removal also affect the levels of GH and TSH in the pituitary,

indicating that circulating steroid hormones have a certain amount of

influence in the release of hormones from the pituitary. The question

posed by this series of experiments was whether target gland removal would

affect the levels of pituitary-like peptide hormone found in the central nervous system in a similar way to effects of target gland removal on pituitary hormone content and release. If the answer was "no", it would be another indication supporting a separate and distinct pool of pituitary-like peptides in the brain.

a. Oophorectomy.

Groups of 20, sixty-day-old female rats were oophorectomized under light sodium pentobarbital anesthesia through bilateral paraspinal 1-cm incisions. A single ligature was made behind the ovary around the fallopian tube and the ovary with its surrounding capsule and fat cut away. Sham-operated animals were similarly handled without the tying of the fallopian tubes or removal of the ovaries. Both sham and operated animals were sacrificed either one or four weeks post-operatively.

b. Orchidectomy.

Groups of 20, 60-day-old male rats were lightly anesthetized and a longitudinal incision made in each scrotal sac. The testicle, along with the epididymus, were exposed and vascular structures were ligatured. The testicle was removed and the scrotal sac reanastomosed with 2 0 silk. Sham operated animals were similarly handled without ligaturing. As in the oophorectomized animals, both sham and castrated rats were sacrificed either one or four weeks post-operatively.

c. <u>Adrenalectomy</u>.

Under light anesthesia, groups of 20, sixty-day-old male rats were adrenalectomized or sham adrenalectomized. Bilateral paraspinal 1-cm incisions were made to expose the kidney and adjacent adrenal gland embedded in perirenal fat. Care was taken to remove the adrenal gland as gently as possible with curved forceps, in order to avoid disruption and dispersal of adrenal cells. Adrenalectomized animals were kept on 0.9% saline and normal rat chow. Both sham and adrenalectomized rats were sacrificed after one or four weeks post-operatively.

d. <u>Thyroidectomy</u>.

Thyroidectomized animals were obtained from Hormone Assay Labs, Inc., and kept for one or four weeks before sacrifice. Sixty-day-old male rats, equivalent to the thyroidectomized animals, were sham operated by making an incision over the trachea and gently parting the muscles overlaying the trachea to reveal the thyroid gland. Sacrifice was as for the thyroidectomized animals. Efficiency of thyroidectomy was confirmed by analysis of the levels of TSH, T_4, and T_3 uptake obtained from serum samples.

In all target organ experiments, the animals were sacrificed by decapitation, trunk blood collected, and brain parts and pituitary removed for tissue homogenization and radioimmunoassay. The levels of pituitary hormone and similar CNS peptides in intact and operated animals were compared after one week and four weeks of target organ absence.

II. THYROXINE

The effect of injecting intramuscularly 0.4 µg T_4/gm body weight daily for seven days into groups of 20 hypophysectomized or intact animals on levels of brain and pituitary hormones was also examined using saline-injected animals as controls.

Trunk blood was collected at sacrifice and TSH, T_3 uptake, and T_4 levels analyzed for all groups.

Brain parts were homogenized and treated as before with levels of immunoassayable hormone being detected by radioimmunoassay.

Preparation of thyroxine:

      9 mg   L-thyroxine/sodium salt-pentahydrate (Sigma)

     90 mls phospho-buffered saline 0.01 M, pH 7.6

The thyroxine required dissolving in a few drops of 1 N NaOH before addition of phosphosaline buffer to make a 0.1 mg in 1 ml solution. Appropriate dilutions with buffer were made to give the correct dosage for individual rats according to weight. Control rats were injected with buffer only.

O. ONTOGENEY STUDIES

Accumulating knowledge of the importance of the fetal endocrine system on morphogenesis, differentiation, and parturition in both man and other mammals prompted this study of the ontogenesis of brain pituitary-like hormones. It was also hoped that the presence of CNS peptides could

be detected earlier than pituitary peptides which would give further cre-
dence for their separate identities.

Eight pregnant rats were housed in separate cages from the day of
insemination, which was taken as day one of gestation. Staging of estrous
cycle periods and confirmation of fertilization was determined from daily
microscopic examination of vaginal scrapings suspended in saline and
viewed with a 10 x ocular. Fetal and neonate animals were decapitated at
various stages of development, pre- and post-partum. Trunk blood, amniotic
fluid, and samples of brain and pituitary tissue were frozen prior to
determination of GH and TSH levels by radioimmunoassay. Levels of hormone
in maternal blood and brain were also determined. Levels of hormone were
expressed as ng/mg protein. Immunologic and physiochemical characteristics
of the two fetal brain hormones, GH and TSH, were compared with those of
pituitary origin in a similar manner to adult hormones, i.e., by column
chromatography and radioimmunoassay parallelism studies. Attempts to
trace the origin of fetal brain hormones centered on the measurement of
placental, amniotic, fetal, and maternal sera levels of hormone, and the
intactness of the fetal blood brain barrier was examined as described in
section I. In order to confirm the presence of a placental barrier to
material pituitary hormones, 1×10^6 cpm of ^{125}I GH and TSH were injected
into the pregnant mothers at 21 days of gestation in 200-μl amounts via
the tail vein. Thirty minutes later, fetuses were removed and the kidney,
pituitary, and brain checked for radioactivity. The presence of radioac-
tivity would indicate the possibility of pituitary hormones crossing the
placental barrier; its absence would indicate barrier intactness.

P. <u>STATISTICAL ANALYSIS OF DATA</u>

Except for the target organ experiments, all data was analyzed by the two-tailed Students T Test which compared the mean and standard error of the mean to give the probability or "p" values of any difference between two groups being significant. This "p" value can be expressed, as for example $p = <0.05$, which would indicate that the two sets of data compared have less than a 5% chance of being the same, or if $p = <0.01$, in which case the two sets of data would have a less than 1% chance of being the same (Steel and Torrie, 1960).

As there was unexplainably wide variation in the data from the target organ experiments, it was decided to handle the data from these experiments using the Mann-Whitney μ-Two-Tailed Statistical Test in which the non-parametric confidence interval is based on the rank sum test (Mann and Whitney, 1947).

5. RESULTS

A. PITUITARY GROWTH HORMONE AND THYROID STIMULATING HORMONE-LIKE IMMUNOREACTIVE PEPTIDE DISTRIBUTION IN RAT BRAIN

Brain extracts from specific parts of the CNS of adult rats were examined for their hormonal content using the radioimmunoassays for rat growth hormone (GH) and rat thyroid stimulating hormone (TSH). The results are depicted in Tables 3 - 8 and Figures 21 and 22.

a. Growth Hormone-Like Immunoreactivity in the Rat Brain

The data in Tables 3 and 4 demonstrate the levels of immunoreactive growth hormone present in various brain regions and in the pituitary of the adult male rat expressed in terms of ng/mg protein or pg/mg wet weight of tissue, respectively. The amygdaloid nucleus was found to contain the largest amounts of this particular immunoreactive material per mg of protein, 5.28 ± 0.21 ng, and 284 ± 10 pg/mg wet weight of tissue. The hypothalamus, 3.16 ± 0.33 ng/mg protein, 119 ± 6 pg/mg wet tissue, and the caudate, 2.09 ng/mg protein, 117 ± 5 pg/mg wet tissue, had lower levels. Even smaller quantities were detected in the hippocampus: 1.35 ± 0.04 ng/mg protein, 95 ± 3 pg/mg wet tissue; cortex, 1.23 ± 0.13 ng/mg protein, 48 ± 7 pg/mg wet tissue; and thalamus, 1.59 ± 0.15 ng/mg protein, 99 ± 8 pg/mg wet tissue, with barely detectable amounts being found in the cerebellum--0.03 ± 0.01 ng/mg protein, and 5 ± 2 pg/mg wet tissue. These values are to be contrasted to the amount of GH found in the anterior pituitary gland, i.e., $1.3 \times 10^6 \pm 0.45$ ng/mg protein and $2.8 \times 10^5 \pm 0.26$ pg/mg wet tissue.

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TABLE 3

DISTRIBUTION OF IMMUNOREACTIVE GH IN ADULT RAT BRAIN

(ng/mg protein)**

	n	GH (ng/mg protein)**
Amygdaloid nucleus	13*	5.28 ± 0.21
Hypothalamus	50*	3.16 ± 0.33
Caudate	10*	2.09 ± 0.24
Thalamus	13*	1.59 ± 0.15
Hippocampus	11*	1.35 ± 0.04
Cortex	12*	1.23 ± 0.13
Cerebellum	13*	0.03 ± 0.01
Anterior pituitary	30	$1.3 \times 10^6 \pm 0.45 \times 10^6$

n = number of experiments.

*   represents a pool of 10 animals.

** Mean ± S. E. M.

TABLE 4

DISTRIBUTION OF IMMUNOREACTIVE GH IN ADULT RAT BRAIN

(pg/mg wet weight tissue)**

	n	GH (pg/mg wet wt.)**
Amygdaloid nucleus	13*	284 ± 10
Hypothalamus	50*	119 ± 6
Caudate	10*	117 ± 5
Thalamus	13*	99 ± 8
Hippocampus	11*	95 ± 3
Cortex	12*	48 ± 7
Cerebellum	13*	5 ± 2
Anterior pituitary	30	$2.8 \times 10^5 \pm 0.26 \times 10^5$

n = number of experiments.

* represents a pool of 10 animals.

** Mean ± S. E. M.

TABLE 5

DISTRIBUTION OF IMMUNOREACTIVE GH IN ADULT RAT BRAIN REGIONS

(ng/total brain region)**

n = 12	ng GH/brain region
* Cortex	38.40 ± 0.74
* Amygdala	14.20 ± 1.20
* Hippocampus	6.11 ± 0.56
* Caudate	5.40 ± 0.11
Hypothalamus	4.76 ± 0.27
* Thalamus	3.96 ± 0.63
* Cerebellum	1.00 ± 0.05
Anterior pituitary	$3.6 \times 10^{6} \pm 0.39 \times 10^{6}$

n = number of experiments.

* represents a pool of 10 animals.

** Mean ± S. E. M.

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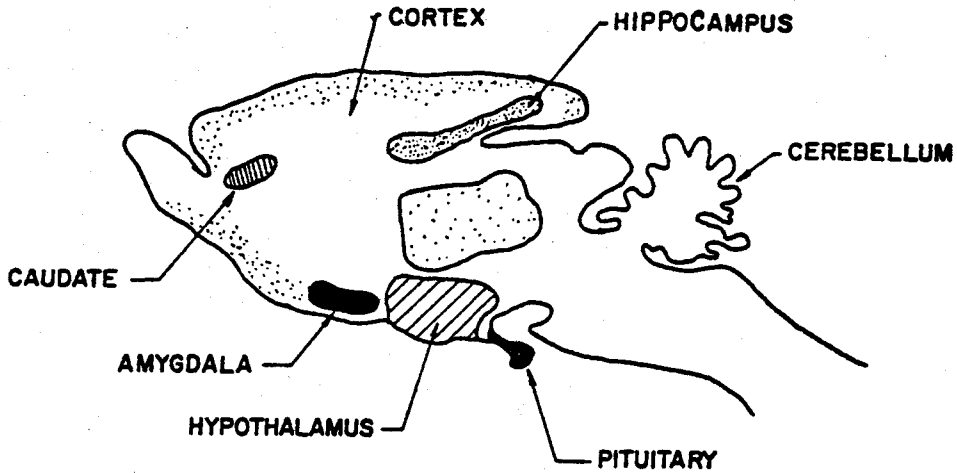
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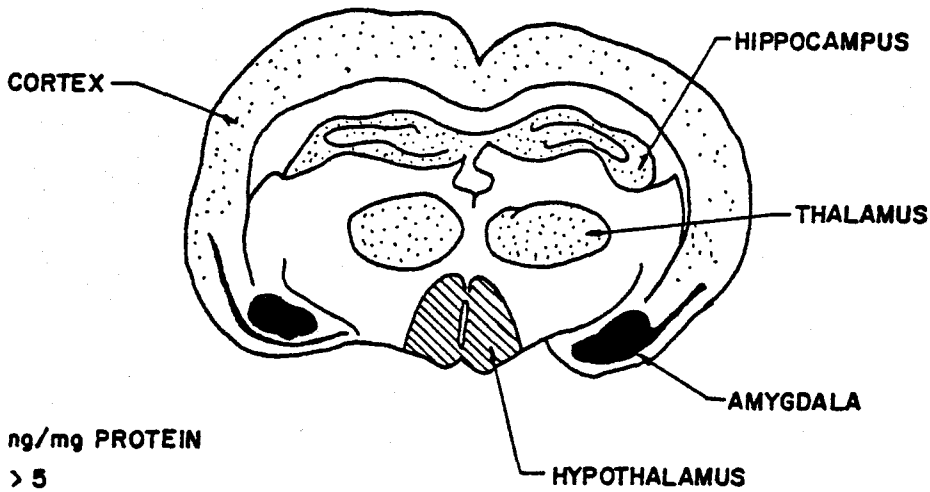
Figure 21. Distribution of immunoreactive growth hormone in the adult rodent brain.

When expressed as the total amount of hormone present in an entire brain region, as shown in Table 5, the cortex was shown to contain the largest overall amount of immunoreactive growth hormone-like immunoreactive material in the brain. Nevertheless, the total amount of growth hormone-like material present in an adult rat brain is still 1.5×10^4 less than that found in the adult rat pituitary.

The amount of immunoreactive growth hormone found in the systemic circulation was shown to be approximately 249.80 ± 35.32 ng/ml, a value similar to what has been previously reported (Ojeda et al, 1977). The amount of hormone per mg of brain tissue which could be attributed to circulating hormone contamination was determined to be approximately 5-12 pgs/mg wet weight of tissue, assuming the blood content of brain tissue to be 50 μl/gm. Taking into account this level of possible contamination and ignoring the blood-brain barrier effectiveness factor, the amount of hormone detected in cerebellar tissue could therefore be considered artifactual or merely the amount of immunoreactive GH present in the vasculature of the cerebellum. However, all other brain areas have immunoreactive material in amounts considerably greater than that possibly due to contamination of the tissue by blood-borne products.

b. Thyroid Stimulating Hormone-Like Immunoreactivity in the Rat Brain.

When the levels of immunoreactive TSH were similarly mapped for the brain areas of the adult rat, the results obtained are shown in Tables 7 and 6 for ng/mg protein or ng/mg wet weight of tissue, respectively. The distribution of TSH-like material/brain region was calculated as shown in

TABLE 6

DISTRIBUTION OF IMMUNOREACTIVE TSH IN ADULT RAT BRAIN

(ng/mg wet wt. tissue)**

	n	TSH (ng/mg wet wt. tissue)**
Hypothalamus	50	5.443 ± 0.56
Amygdala	17*	1.100 ± 0.42
Thalamus	10*	0.967 ± 0.10
Caudate	11*	0.810 ± 0.12
Cerebellum	12*	0.799 ± 0.05
Hippocampus	9*	0.798 ± 0.04
Cortex	13*	0.726 ± 0.09
Anterior pituitary	50	$1.9 \times 10^5 \pm 0.50 \times 10^5$

n = number of experiments.

* Represents a pool of 10 animals.

** Mean ± S. E. M.

TABLE 7

DISTRIBUTION OF IMMUNOREACTIVE TSH IN ADULT RAT BRAIN

(ng/mg protein)**

	n	TSH (ng/mg protein)**
Hypothalamus	50	204.20 ± 3.6
Amygdala	17*	29.42 ± 6.3
Cerebellum	12*	16.98 ± 2.5
Thalamus	10*	16.50 ± 1.4
Caudate	11*	15.21 ± 2.6
Cortex	13*	13.89 ± 0.5
Hippocampus	9*	11.88 ± 2.4
Anterior pituitary	50	$1.2 \times 10^{6} \pm 0.75 \times 10^{6}$

n = number of experiments.

* Represents a pool of 10 animals.

** Mean $\pm$ S. E. M.

TABLE 8

DISTRIBUTION OF IMMUNOREACTIVE TSH IN ADULT RAT BRAIN REGIONS

(ng/total brain region)**

n = 15	ng TSH/brain region**
* Cortex	580.21 ± 5.6
Hypothalamus	217.72 ± 7.3
* Cerebellum	158.03 ± 2.5
* Hippocampus	49.47 ± 3.0
* Amygdala	44.88 ± 3.6
* Thalamus	39.06 ± 4.1
* Caudate	37.42 ± 3.9
Anterior pituitary	$2.7 \times 10^{6} \pm 0.65 \times 10^{6}$

n = number of experiments.

* represents a pool of 10 animals.

** Mean ± S. E. M.

Figure 22. Distribution of immunoreactive TSH in the
adult rodent brain.

Table 8. If measured in terms of total amount of hormone/brain region, the cortex contains the highest level of any other brain region, 580.21 ± 5.6 ng/cortex, although the total amount of brain TSH is 10^3 times less than TSH found in the anterior pituitary, 2.7 x 10^6 ± 0.65 ng/pituitary. When calculated as per mg of protein or as wet weight of tissue, the hypothalamus shows by far the highest concentration of immunoreactive TSH, 204.20 ± 3.6 ng/mg protein and 5.443 ± 0.56 ng/mg tissue, followed by the amygdaloid nucleus, 29.42 ± 6.3 ng/mg protein and 1.10 ± 0.42 ng/mg wet tissue. Lesser amounts were detected in other regions of the brain and varied from the cerebellum, 16.50 ± 1.4 ng/mg protein, 0.799 ng/mg wet tissue, to the hippocampus, 11.88 ± 2.4 ng/mg protein and 0.798 ± 0.04 ng/mg wet tissue.

Assuming the level of TSH in serum to be approximately 489.67 ± 52.59 ng/ml, as measured by the RIA, and using the same factor as for growth hormone, the amount of hormone which could be contributed by possible blood borne contamination of brain tissue would be 24.5 pg/mg wet weight of tissue. Thus, significant amounts of a TSH-like material were found to be present in all brain regions examined.

B. GROWTH HORMONE AND THYROID STIMULATING HORMONE-LIKE IMMUNOREACTIVE PEPTIDE DISTRIBUTION IN PRIMATE BRAIN

These data are considered preliminary as only 3-8 brains were ex-amined; however, because they show findings comparable to those observed in the rodent brain and extend relevancy to these observations, they will be reported here. Variables beyond the control of the experimenter such

as prior treatment of the animals and the use of fixed material may have been present to alter these results.

a. Growth Hormone-Like Immunoreactivity in the Primate Brain

From a total of 8 adult monkeys, the amount of immunoreactive growth hormone in various brain regions was calculated and expressed in Table 9 as pg/mg wet tissue. In the primate, the hypothalamus appeared to contain the largest concentration of growth hormone-like material, 164.01 ± 9.5 pg/mg tissue, although fairly substantial amounts were also shown to be present in the amygdala, 56.20 ± 7.6 pg/mg tissue and many other brain regions. As with the rat, GH-like material in the monkey cerebellum showed the lowest amounts of GH/mg of tissue, only 19.66 pg/mg of brain tissue.

b. Thyroid Stimulating Hormone-Like Immunoreactivity in the Primate Brain

Measured as pg/mg wet tissue in Table 10, the hypothalamus and cortex were shown to contain the highest amounts of thyroid stimulating hormone-like activity, 133.92 ± 9.20, and 54.10 ± 2.36 pg/mg wet weight tissue, respectively. Several other areas of the primate brain showed little or no immunoreactive TSH activity such as the amygdala, cerebellum, thalamus, and putamen.

TABLE 9

DISTRIBUTION OF IMMUNOREACTIVE GH IN ADULT MONKEY BRAIN

(pg/mg wet wt. tissue)**

n = 8	GH (pg/mg wet wt. tissue)**
Hypothalamus	164.01 ± 9.5
Amygdala	56.20 ± 7.6
Pallidus	39.20 ± 2.5
Thalamus	36.80 ± 2.1
Cortex	35.50 ± 1.8
Hippocampus	30.88 ± 3.3
Caudate	25.80 ± 1.2
Putamen	23.80 ± 3.6
Cerebellum	19.66 ± 2.4
Pituitary	$1.78 \times 10^{4} \pm 0.51 \times 10^{4}$

n = number of experiments.

** Mean ± S. E. M.

TABLE 10

DISTRIBUTION OF IMMUNOREACTIVE TSH IN ADULT MONKEY BRAIN

(pg/mg wet weight of tissue)**

n = 3	TSH (pg/mg wet wt. tissue)**
Hypothalamus	133.92 ± 9.20
Cortex	54.10 ± 2.36
Mid Brain	13.28 ± 4.63
Hippocampus	5.92 ± 2.79
Amygdala	NM
Caudate	NM
Cerebellum	NM
Pallidus	NM
Putamen	NM
Thalamus	NM
Pituitary stalk	$1.5 \times 10^4 \pm 0.23 \times 10^4$

n = number of experiments.

NM = not measurable.

** Mean ± S. E. M.

C. GROWTH HORMONE AND THYROID STIMULATING HORMONE-LIKE IMMUNOREACTIVITY

IN EXTRAPITUITARY REGIONS OTHER THAN THE RAT CNS

a. Growth Hormone-Like Immunoreactivity in the Pineal Gland and GI

Tract

In Tables 11 and 12, the levels of immunoreactive GH, expressed as ng/mg protein and as pg/mg wet weight tissue, respectively, for extra-pituitary regions other than the CNS, show that substantial amounts of material exist in such areas. Certain areas of the gastrointestinal tract (GI) such as the small intestine, 1.23 ± 0.3 ng/mg protein, 42.6 pg/mg tissue, and the pancreas, 1.12 ± 0.05 ng/mg protein, 30.2 ± 7.6 pg/mg tissue, contain fairly high levels of a growth hormone-like material.

In an effort to determine whether these results were artifactual, due to enzymatic destruction of the labeled growth hormone in the RIA, a study of the recovery of added amounts of purified pituitary growth hormone to tissue extracts was carried out. Near to 100 % recovery of added hormone revealed no breakdown of the radioactive tracer, strongly indicating that these levels of immunoreactive growth hormone in the GI tract are probably not due to an artifact in the system. One month after hypophysectomy, levels of GH found in these areas did not alter or change.

The pineal gland was also shown to contain substantial amounts of immunoreactive growth hormone, 77.45 ± 8.3 ng/mg protein and 41.63 ± 2.7 pg/mg tissue. Of interest are preliminary studies which appear to show that these levels are subject to diurnal variation and that they drop significantly after removal of the pituitary.

TABLE 11

DISTRIBUTION OF IMMUNOREACTIVE GH

IN ADULT RAT TISSUES

(tissues perfused for 10 min. with phosphosaline buffer, pH 7.6)

(ng/mg protein)**

	n	GH (ng/mg protein)**
Muscle	3	0.02 ± .005
Kidney	3	0.40 ± .008
Liver	3	0.93 ± .02
Pancreas	16	1.12 ± .05
Small intestine	16	1.23 ± .03
Large intestine	16	0.05 ± .02
Pineal	30	77.45 ± 8.3

n = number of experiments.

** Mean ± S. E. M.

TABLE 12

DISTRIBUTION OF IMMUNOREACTIVE GH

IN ADULT RAT TISSUES

(tissues perfused with phosphosaline buffer, pH 7.6, for 10 mins.)

(pg/mg wet weight tissue)**

	n	GH (pg/mg wet wt.)**
Amygdaloid nucleus	13	284 ± 10.0
Muscle	3	3.0 ± 0.5
Kidney	3	4.5 ± 2.1
Liver	3	21.0 ± 5.2
Pancreas	16	30.2 ± 7.6
Small intestine	16	42.6 ± 3.5
Large intestine	16	3.2 ± 0.35
Pineal	30	41.63 ± 2.7

n = number of experiments.

** Mean ± S. E. M.

b. Thyroid Stimulating Hormone Immunoreactivity

Tables 13 and 14 demonstrate the amounts of immunoreactive TSH found in extrapituitary, non-brain tissues of the rat. As with immunoreactive GH, substantial amounts of TSH-like material were detected in GI tract tissues, especially in the pancreas, 77.0 ± 5.21 ng/mg protein, 12.42 ± ng/mg tissue, and in the small intestine, 23.8 ± 7.62 ng/mg protein and 12.42 ± 2.54 ng/mg tissue. Similar to the results obtained for GH, the enzymes of the GI tract did not appear to interfere with the RIA for TSH, as good recoveries of added pure TSH to intestinal extracts were obtained in recovery studies. Hypophysectomy did not cause any appreciable fall in levels of immunoreactive TSH in these tissues 30 days after pituitary removal.

The pineal gland also demonstrated high levels of immunoreactive TSH, 388 ng/mg protein, 1.49 ± 0.43 ng/mg tissue, which appears to be subject to diurnal variation being significantly higher in the afternoon than in the morning.

D. GROWTH HORMONE AND THYROID STIMULATING HORMONE IMMUNOREACTIVITY IN MEDIUM FROM CULTURES OF MONOLAYERED NERVOUS TISSUE

a. Growth Hormone Immunoreactivity Detected in Tissue Culture Medium

When the media from actively growing tissue cultures of amygdaloid tissue was examined for immunoreactive growth hormone-like material, the results, expressed as cumulative hormone present over a 30-day growth period, were determined and are shown in Fig. 23 .

TABLE 13

DISTRIBUTION OF IMMUNOREACTIVE TSH

IN ADULT RAT TISSUES

(tissues perfused for 10 min. with phosphosaline buffer, pH 7.6)

(ng/mg protein)**

	n	ng TSH/mg protein**
Muscle	3	5.2 ± 0.30
Kidney	3	4.3 ± 0.25
Liver	3	4.8 ± 0.32
Pancreas	16	77.0 ± 5.21
Small intestine	16	23.8 ± 7.62
Pineal	30	388 ± 25.1

n = number of experiments.

** Mean ± S. E. M.

TABLE 14

DISTRIBUTION OF IMMUNOREACTIVE TSH

IN ADULT RAT TISSUES

(ng/mg wet weight tissue)**

	n	ng TSH/mg wet wt.**
Hypothalamus	50	5.433 ± 0.560
Muscle	3	0.010 ± 0.002
Kidney	3	0.040 ± 0.01
Liver	3	0.063 ± 0.03
Pancreas	16	12.42 ± 2.54
Small intestine	16	10.78 ± 3.26
Large intestine	16	0.18 ± 0.02
Stomach	16	0.18 ± 0.02
Pineal	12	1.49 ± 0.43

n = number of experiments.

** Mean ± S. E. M.
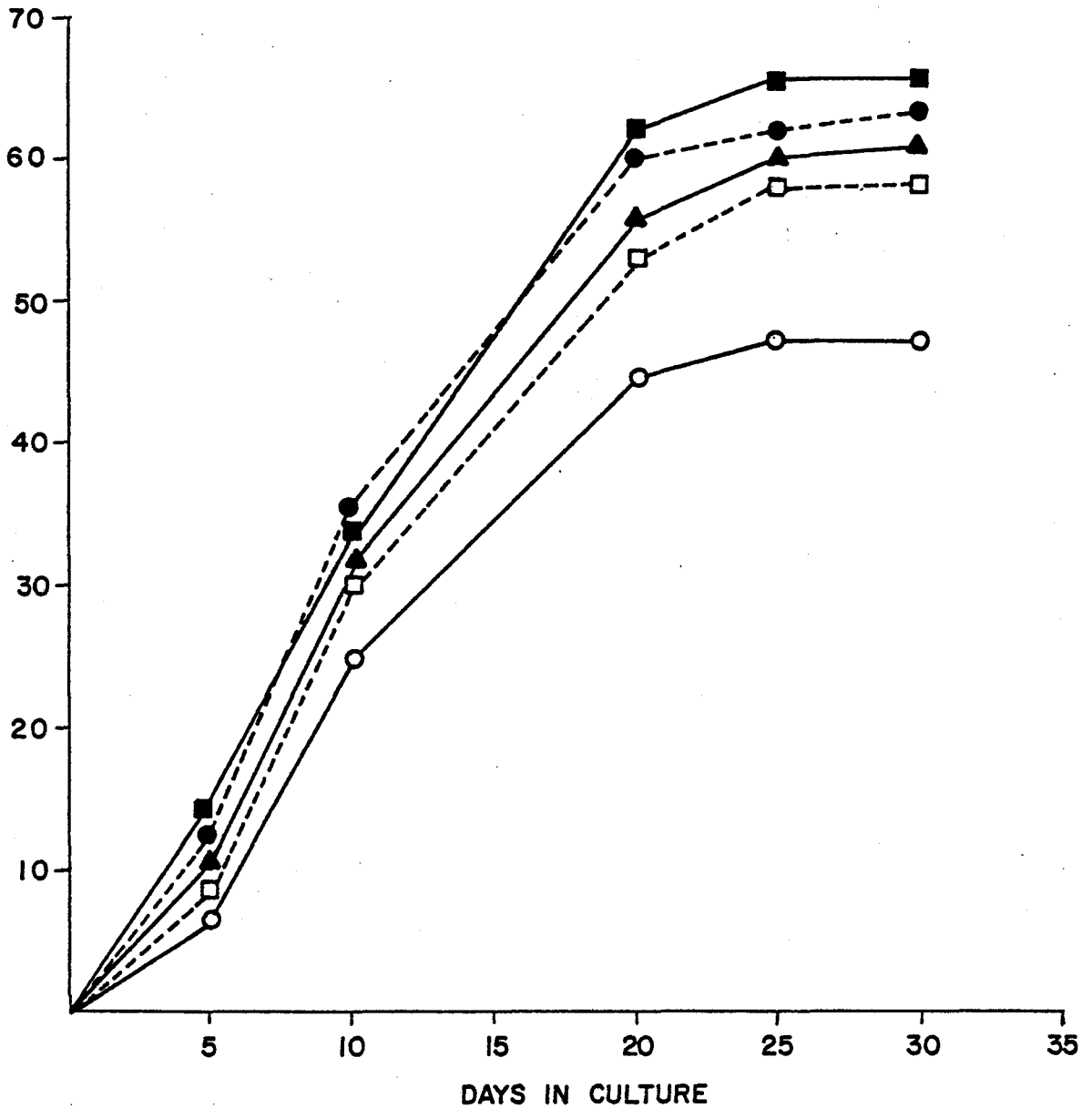
Figure 23. Cumulative immunoreactive growth hormone released from cells of the adult rat amygdaloid nucleus grown in tissue culture. In these five different experiments, medium was changed at 5, 10, 20, 25 and 30 days. Each symbol represents the average growth hormone released from approximately 2×10^6 viable cells plated in each of 5 Falcon culture flasks containing 3ml. media.

From a total of five experiments, in which the medium was changed every five days, approximately 2 x 10^6 viable amygdaloid cells released 47-65 ng of a growth hormone-like material into the tissue culture medium over the 30-day period of the study. Each symbol in Fig. 23 represents one experiment, and individual points, the mean levels of immunoreactive growth hormone present in the medium of five culture flasks obtained when a complete exchange of growth medium was carried out approximately every five days.

When brain tissue was obtained from 21-day post-hypophysectomized rats and plated out in monolayer cultures, the cumulative amount of immunoreactive growth hormone found in the media, over a 30-day period, was determined by RIA and is shown graphically in Fig. 24 . Four different brain areas are represented and each symbol shows the mean and standard error of the mean level of immunoreactive growth hormone released from a total of 15 plates from three different experiments. Medium was changed every five days and, in these studies using brain tissue from hypophysectomized rats, it was shown that substantial amounts of immunoreactive growth hormone were released from all brain tissues into the medium over 30 days of study. Little difference was observed between the amounts obtained from hypophysectomized amygdaloid tissue (Fig. 24) and amygdaloid tissue from normal intact animals (Fig. 23). The cumulative amounts of immunoreactive growth hormone released into the tissue culture medium from hypophysectomized brain tissue ranged from a mean of 20 ng in the cortex to 55 ng in amygdaloid cell cultures over three different experiments. Viability checks throughout all tissue culture experiments showed
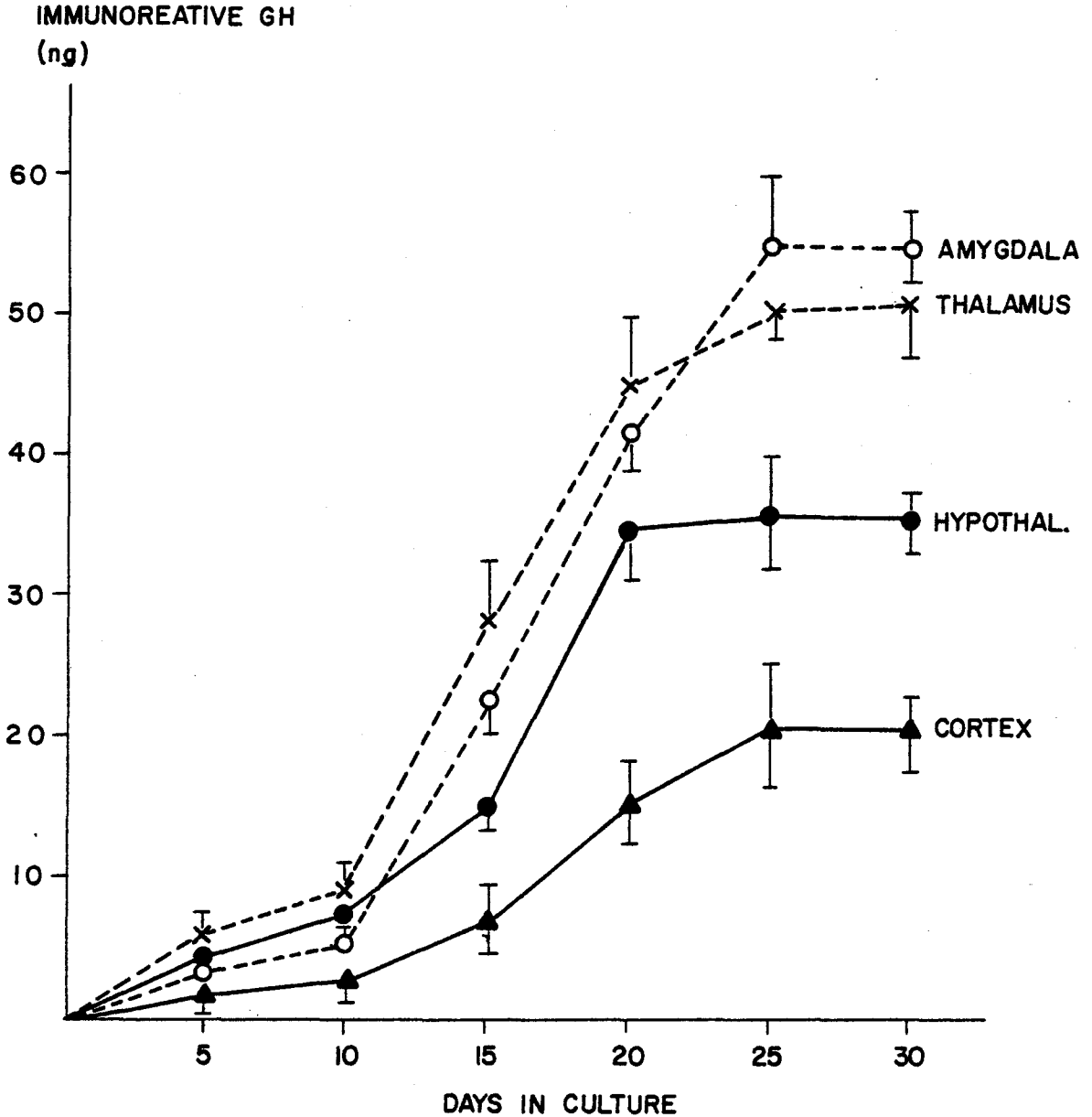
Figure 24. Cumulative immunoreactive growth hormone
released from cells of the 21 day post-
hypophysectomised, adult, rat brain regions
grown in tissue culture. Each symbol represents
the mean and S.E.M. of hormone released in 3
experiments from approximately 2×10^6 cells
plated in 5 culture flasks containing 3 ml.
media. In these experiments, medium was changed
at 5, 10, 15, 20, 25 and 30 days.

between 95%-98% viable cells present at each sampling date.

b. Thyroid Stimulating Hormone Immunoreactivity Detected in Tissue
   Culture Medium

Studies of brain tissue monolayers, in which the measurement
of TSH-like activity present in the growth medium was determined after a
certain time period, are plotted in Fig. 25 . Here cumulative TC medium-
immunoreactive TSH is plotted against days grown in tissue culture of dis-
persed cells obtained from specific brain parts from normal adult rat
brain and from anterior pituitaries. All tissues tested were shown to
release a TSH-like material into the medium over the 30-day period of
study, ranging from a total of 2,200 ng for the pituitary to 350 ng for
thalamus cells. Each symbol in Fig. 25 represents tissue from a different
brain area or anterior pituitary and the individual points, the mean and
standard error of the mean, cumulative value for immunoreactive TSH re-
leased from such monolayered cells. When cells were taken from 21-day
post-hypophysectomized adult rats and plated in tissue culture medium,
the amount of cumulative immunoreactive TSH released over a 30-day period
was also determined. The results are shown in Fig. 26 and ranged from a
total of 750 ng of TSH from hippocampal tissue to 550 ng of TSH from amyg-
daloid tissue after 30 days in culture. This demonstrates that even after
hypophysectomy, brain tissue possessed the ability to release immunoreac-
tive TSH into the medium, as long as the viability of the cultured cells
was maintained. Viability throughout these experiments was maintained
between 93%-98% of the original cell number.
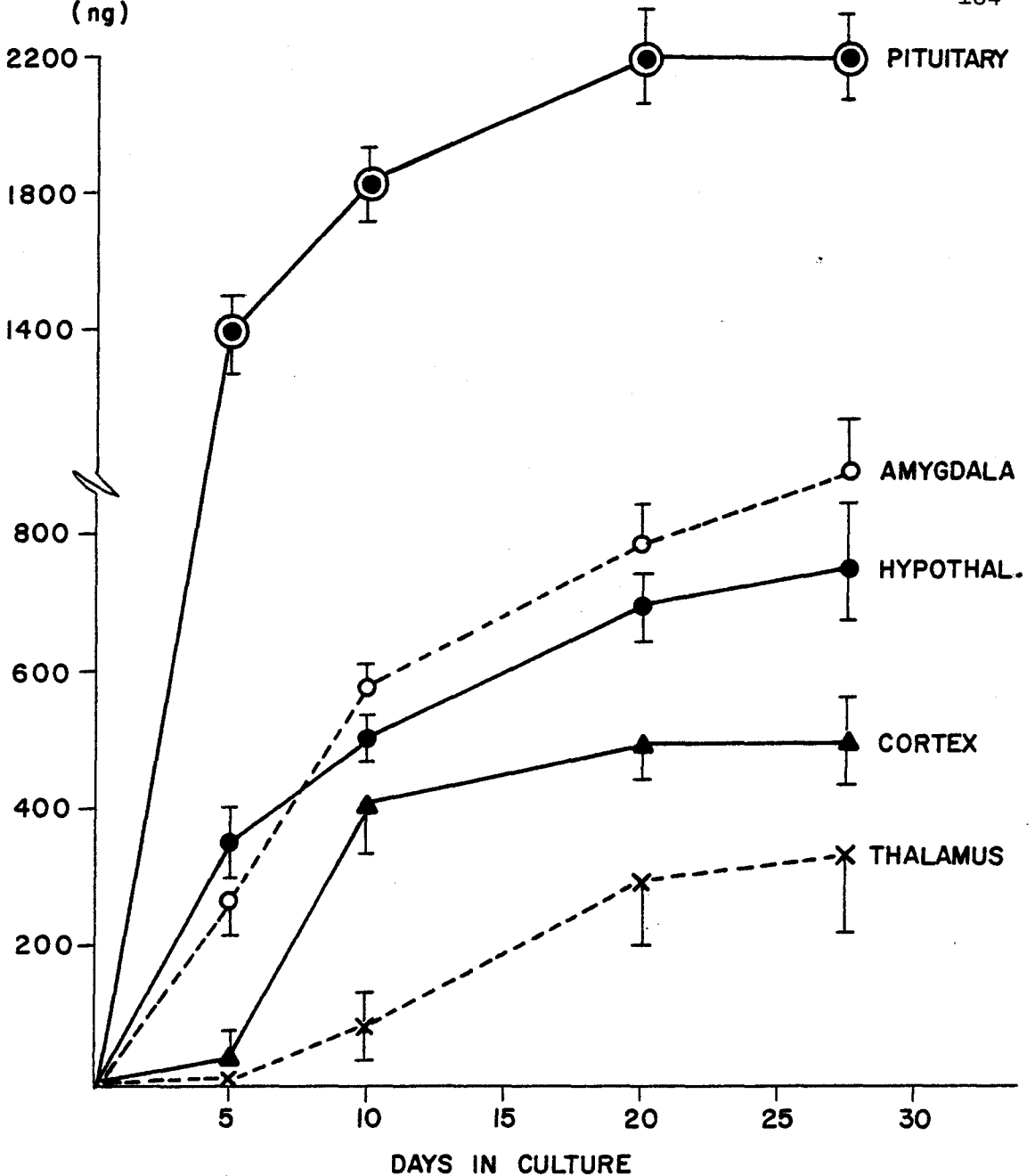
Figure 25. Cumulative immunoreactive thyroid stimulating hormone released from cells of the adult rat brain regions and pituitary grown in tissue culture. Each symbol represents the mean and S.E.M. of hormone released in 5 experiments, from approximately 2.5×10^6 cells plated in 5 culture flasks containing 3 ml media. In these experiments, medium was changed at 5, 10, 20 and 28 days.
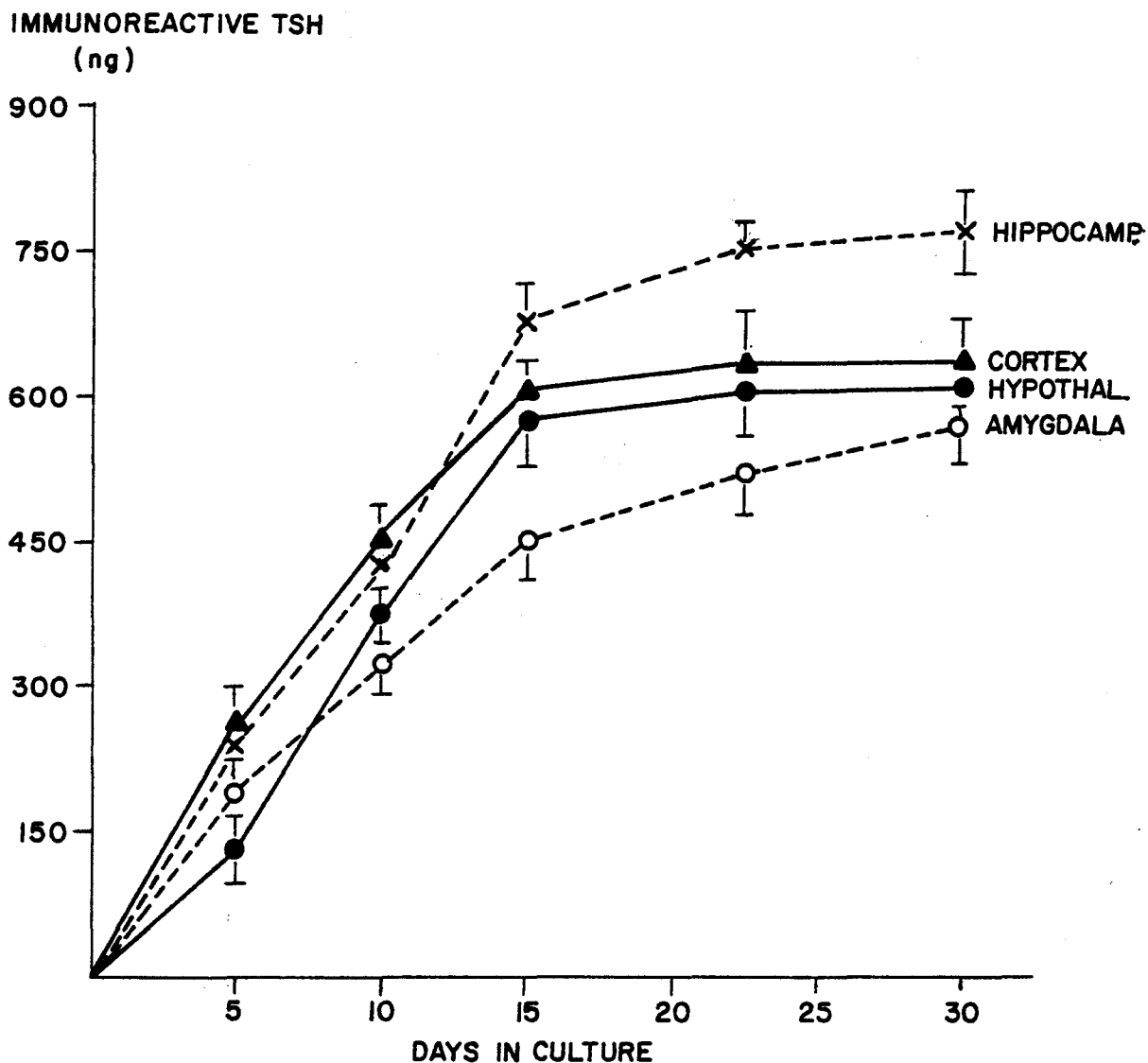
Figure 26. Cumulative, immunoreactive thyroid stimulating
hormone released from cells of the 21 day post-
hypophysectomised, adult, rat, brain regions
in tissue culture. Each symbol represents the
mean and S.E.M. of hormone released in 3 expe-
riments from approximately 2×10^7 cells plated
in 5 culture flasks containing 3 ml media. In
these experiments, medium was changed at 5, 10,
15, 22 and 30 days.

E. THE EFFECT OF KNOWN HORMONAL REGULATORS ON THE RELEASE OF IMMUNOREAC-
    TIVE GH AND TSH FROM TISSUE CULTURED CELLS OF THE BRAIN AND ANTERIOR
    PITUITARY

    a. Somatostatin Added to Tissue Cultured Brain and Pituitary Cells

        Somatostatin, a known inhibitor of growth hormone release from in-
    tact pituitaries (Brazeau, 1973) was shown to be effective at a concentra-
    tion of 1 μg/ml medium in significantly suppressing the release of immuno-
    reactive GH from both pituitary and various brain tissues grown in culture.
    In Fig. 27, the levels of growth hormone present in the medium of control
    and somatostatin-spiked tissue cultures are shown after a 24-hour period
    of incubation. The results delineated in Fig. 27 represent the mean, and
    standard error of the mean, of immunoreactive growth hormone levels from
    three different experiments each containing five separate tissue culture
    flasks, each containing approximately 6×10^6 viable cells.

    b. Thyroxine (T_4) Added to Tissue Cultured Brain and Pituitary Cells

        Levels of thyroid stimulating hormone-like activity in actively
    growing cultures of brain tissue (10^7 cells), incubated for 24 hours with
    and without 10^{-9} M L-thyroxine are shown in Fig. 28 . Although this con-
    centration of thyroxine (T_4) was able to significantly suppress the release
    of TSH from cultured pituitary cells from a mean of 1,700 ng/24 hours to
    a mean of 1,400 ng/24 hours, it was not, however, able to prevent the re-
    lease of immunoreactive TSH from any part of the brain grown in monolayers
    under similar conditions. Results in all cases were expressed as the mean,
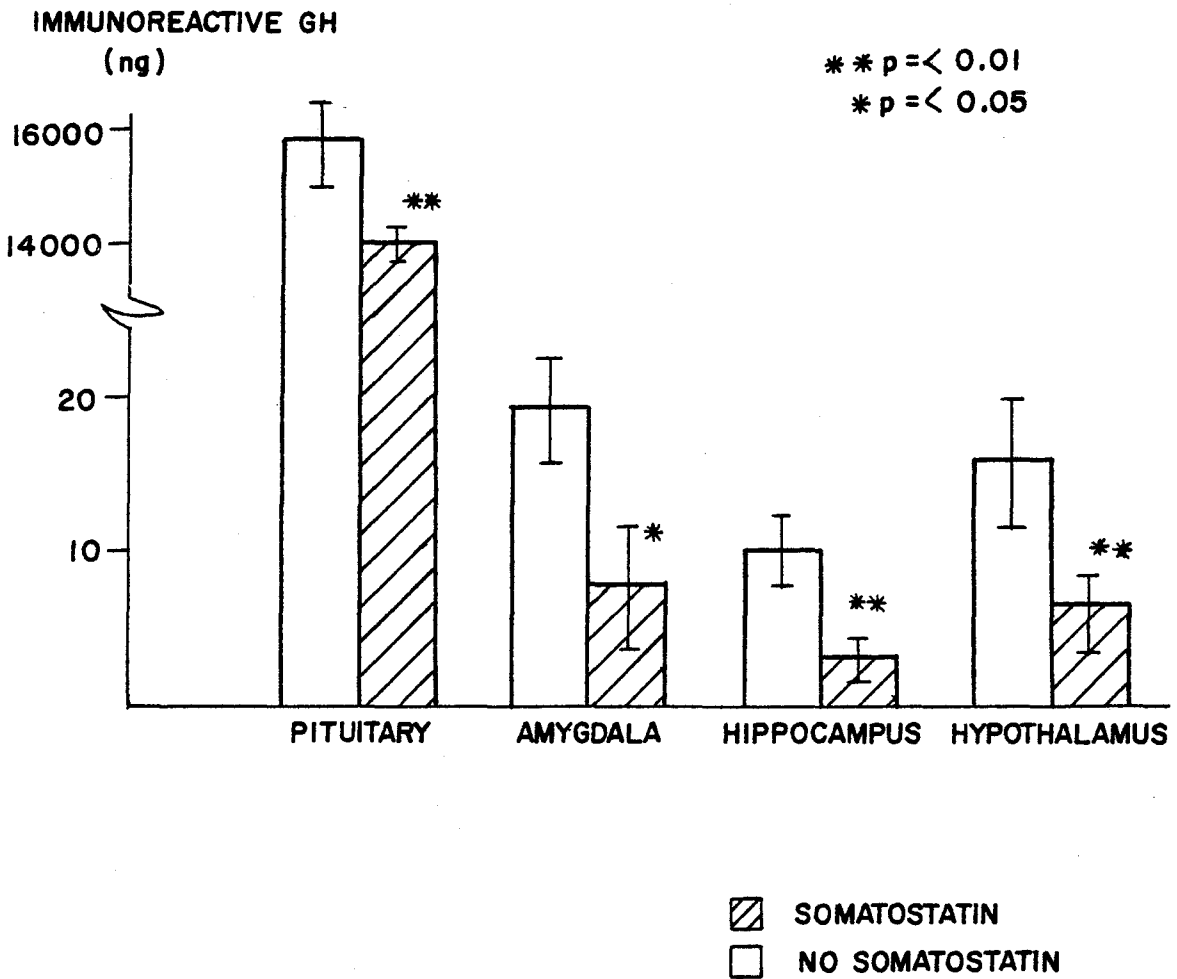
Figure 27. Immunoreactive growth hormone released from 6 x 10^6 cells of the adult rat brain and pituitary grown in tissue culture for 24 hrs with or without somatostatin (1 µg/ml). Results represent the mean and S.E.M. from 3 experiments.
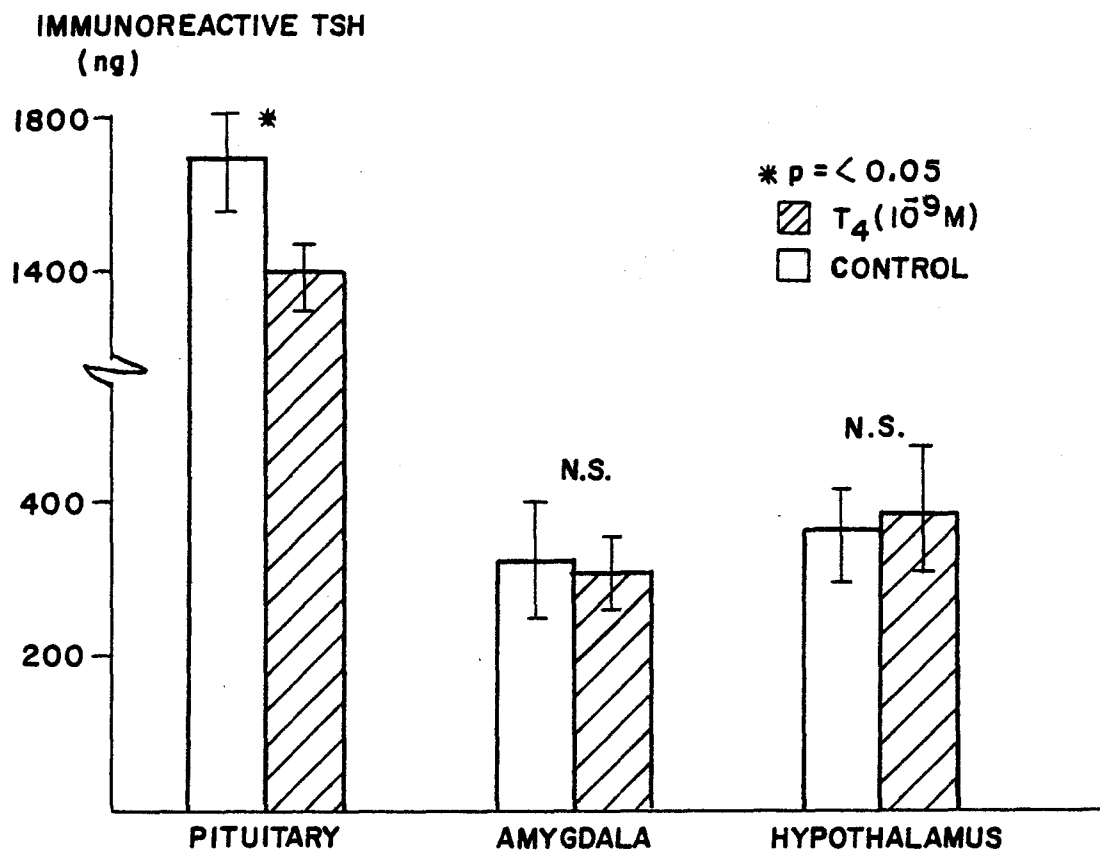
Figure 28. Immunoreactive thyroid stimulating hormone released from 10^7 cells of the adult rat brain and pituitary grown in tissue culture for 24 hrs with or without L-thyroxine, (T_4 10^{-9} M).

and standard error of the mean, levels of immunoreactive TSH detectable after 24 hours from at least five plates in three different experiments as shown in Fig. 28 .

F. IMMUNOREACTIVE THYROID STIMULATING HORMONE LEVELS DETECTED IN A
   CULTURED NEUROBLASTOMA CELL LINE N_4TGI

Over a ten-day growth period, in which media was changed at days 1, 4, and 9, the levels of immunoreactive TSH released into the growth medium from actively growing cultures of the neuroblastoma cell line N_4TGI were determined by RIA and are shown in Fig. 29 . The mean and standard error of the mean values for cumulative hormonal levels from 5 - 8 individual flasks in three different experimental runs are represented by each point. A total mean of 760 ng of immunoreactive TSH was found in the medium after 10 days growth of this cell line. Throughout the experiment, viability was checked by the trypan blue exclusion method and found to remain between 95% - 98% of the original number of viable cells plated at day one.

It was apparent that cells of this neuroblastoma cell line are capable of releasing a TSH-like immunoreactive material indefinitely, if one extrapolates, into the growth medium.

G. FRACTIONATION OF BRAIN HOMOGENATES BY ULTRACENTRIFUGATION

Assay of three pellets and three supernatants obtained by differential ultracentrifugation of brain extracts (as described in Materials and
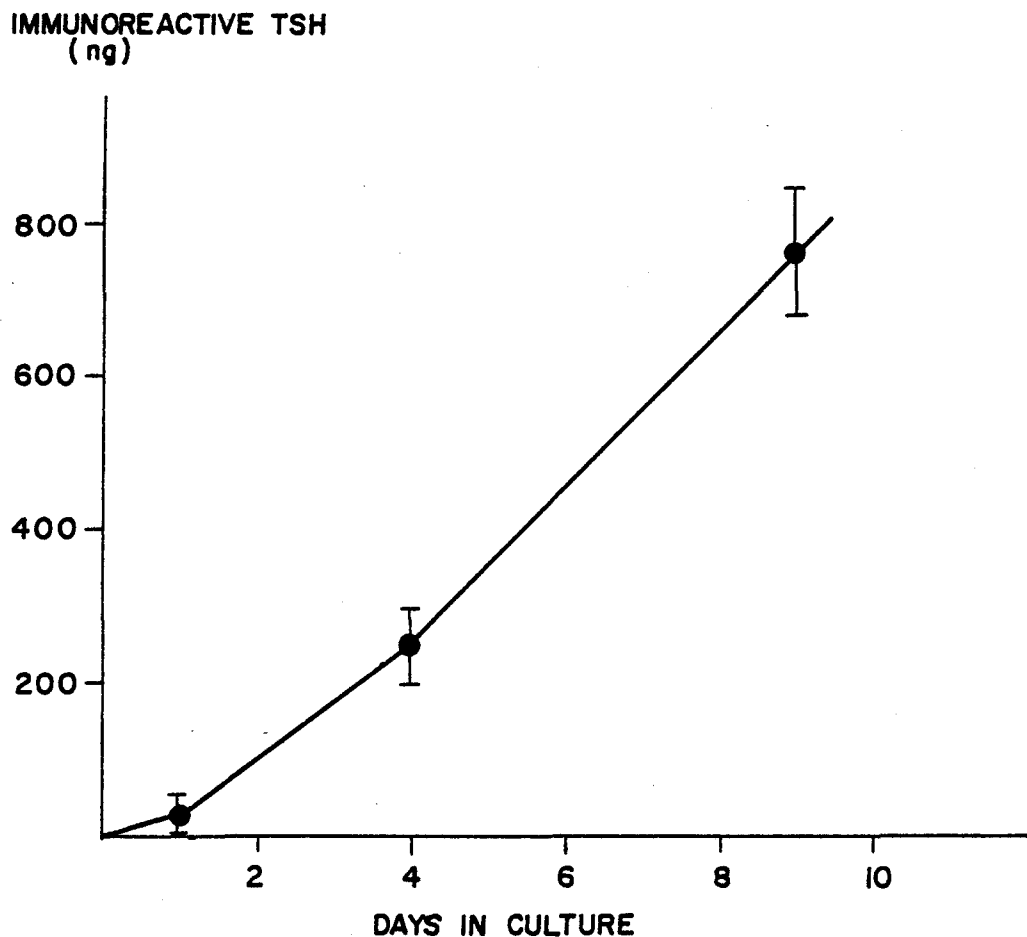
Figure 29. Cumulative immunoreactive thyroid stimulating
           hormone TSH released from 5 x 10^6 cells of
           the neuroblastoma cell line N$_4$TG1. Each point
           represents the mean and S.E.M. of hormone
           released from cells in 3 experiments. The media
           was changed at 1, 4 and 9 days.

Methods) for their content of either immunoreactive growth hormone or thyroid stimulating hormone, gave the data shown in Tables 15 and 16, respectively.

a. Growth Hormone: Distribution in Ultracentrifuged Homogenate Fractions

In Table 15, the quantity of GH is expressed either as ng/fraction, taking into account the volume of the fraction or as ng/mg protein, accounting for the amount of protein present. For amygdala and thalamic homogenates, the greatest concentration of immunoassayable growth hormone was clearly detectable in the second pellet P_2, i.e., $P_1 = 6.119$, $P_2 = 17.879$, $P_3 = 0.906$, $S_1 = 18.346$, $S_2 = 8.242$, and $S_3 = 5.477$ ng GH/fraction. This second pellet is known to represent the synaptosomal-mitochondrial fraction of the brain homogenate after ultracentrifugation. For the hypothalamic homogenate, however, there appeared to be an equal distribution of immunoreactive GH between the P_2 pellet and the final supernatant. This would suggest, that in the hypothalamus there is a fraction of immunoreactive growth hormone that is not associated with a specific structural entity, such as the synaptosomes or mitochondria but present instead in some free state, whereas a second fraction which sediments out is presumably associated with a specific entity present in the P_2 pellet preparation.

TABLE 15

FRACTIONATION OF BRAIN HOMOGENATES BY ULTRACENTRIFUGATION:

GROWTH HORMONE

(Content of fractions ng/fraction and ng/mg protein)

		ng GH/fraction		
AMYGDALA	P_1	6.119	S_1	18.346
	P_2	17.879	S_2	8.242
	P_3	0.906	S_3	5.477
THALAMUS	P_1	13.100	S_1	30.296
	P_2	21.602	S_2	6.663
	P_3	1.626	S_3	7.166
HYPOTHALAMUS	P_1	10.168	S_1	70.762
	P_2	36.661	S_2	41.237
	P_3	2.775	S_3	36.718

		ng/mg protein/fraction		
AMYGDALA	P_1	2.769	S_1	4.504
	P_2	4.659	S_2	2.601
	P_3	1.839	S_3	3.649
THALAMUS	P_1	1.160	S_1	4.483
	P_2	3.673	S_2	2.134
	P_3	0.797	S_3	2.020
HYPOTHALAMUS	P_1	0.664	S_1	24.824
	P_2	6.886	S_2	11.034
	P_3	4.462	S_3	5.669

P = pellet; S = supernatant

b. Thyroid Stimulating Hormone: Distribution in Ultracentrifuged Homogenate Fractions

Similar ultracentrifugal fractionation studies of brain homogenate for the detection of TSH-like material, by radioimmunoassay, gave the data shown in Table 16.

Here again, as shown for the amygdaloid homogenate, the greatest quantity of immunoreactive TSH was associated with the P_2 pellet whereas the hypothalamic fractionation pattern seemed to show two, rather than one, distinct pool of immunoreactive material--one associated with the P_2 pellet and the other with the final supernatant preparation.

Because of this heterogenous distribution of both immunoreactive GH and TSH in the hypothalamic homogenate, further fractionation of the P_2 pellet by sucrose density gradients might allow better discrimination of whether the two hormones are actually associated with a particular cellular entity such as the synaptosomes.

H. IMMUNOPEROXIDASE STAINING OF BRAIN TISSUE FOR PITUITARY-LIKE HORMONE DISTRIBUTION

a. Thyroid Stimulating Hormone

To further pinpoint the location of thyroid stimulating-like hormone in rat brain tissue, immunohistochemical staining of paraformalde-hyde-fixed tissue was attempted. The conditions noted in the "Materials and Methods" section revealed a dense uptake of peroxidase-stained

TABLE 16

FRACTIONATION OF BRAIN HOMOGENATES BY ULTRACENTRIFUGATION:

THYROID STIMULATING HORMONE

(Content of fractions ng/fraction and ng/mg protein)

ng TSH/fraction				
AMYGDALA	P_1	79.360	S_1	251.071
	P_2	225.632	S_2	52.768
	P_3	49.354	S_3	6.811
HYPOTHALAMUS	P_1	97.385	S_1	347.631
	P_2	126.783	S_2	210.295
	P_3	108.127	S_3	116.534

ng TSH/mg protein				
AMYGDALA	P_1	7.638	S_1	13.364
	P_2	9.999	S_2	4.713
	P_3	3.172	S_3	2.008
HYPOTHALAMUS	P_1	5.705	S_1	96.730
	P_2	48.630	S_2	52.119
	P_3	10.714	S_3	42.588

P = pellet; S – supernatant

immunocomplex in cells of the pituitary, indicating the presence of TSH.
Shown under low power (X 100) in Fig. 30 , and at higher magnification
(X 400) in Fig. 31 , are representative sections of pituitary with the
thyrotrophs staining a dark brown against a nonhormonal containing, un-
stained, cell background. All controls revealed a similar appearance to
Fig. 32 , that is, no uptake of PAP complex by any cell due to the condi-
tions set by the experiment. For the pituitary, therefore, it was demon-
strated that the incubation times and concentrations of antibodies and
other substituents in this immunohistochemical staining experiment to
detect TSH activity were sufficient and satisfactory.

When amygdaloid tissue was treated in a similar manner and examined
under a high magnification (X 1000), Figs. 33 and 34 , or at a lower mag-
nification (X 400), Fig. 35 , certain cell bodies were seen to stain dark
brown, indicating the presence of TSH-like immunoreactive material. Con-
trols again provide evidence that this finding was not an artifactual ob-
servation. Pre-treatment with colchicine would perhaps have shown a more
detailed picture of the involvement of the type of cell, neurone, or glia
which was being stained. Cell bodies staining for TSH were also detected
in the hypothalamus and hippocampal regions of the brain, with a few iso-
lated bodies being also distinguished in the cerebral cortex and thalamic
areas. In the amygdaloid area, all of the individual nuclei appeared to
contain TSH-like hormone. Those which were more densely stained included
the nucleus amygdaloides corticalis, amygdaloides basilis, pars mediales,
and pars lateralis. In the hypothalamus, areas in which stained cell
bodies were prominent were the dorsomedial and ventromedial nuclei.
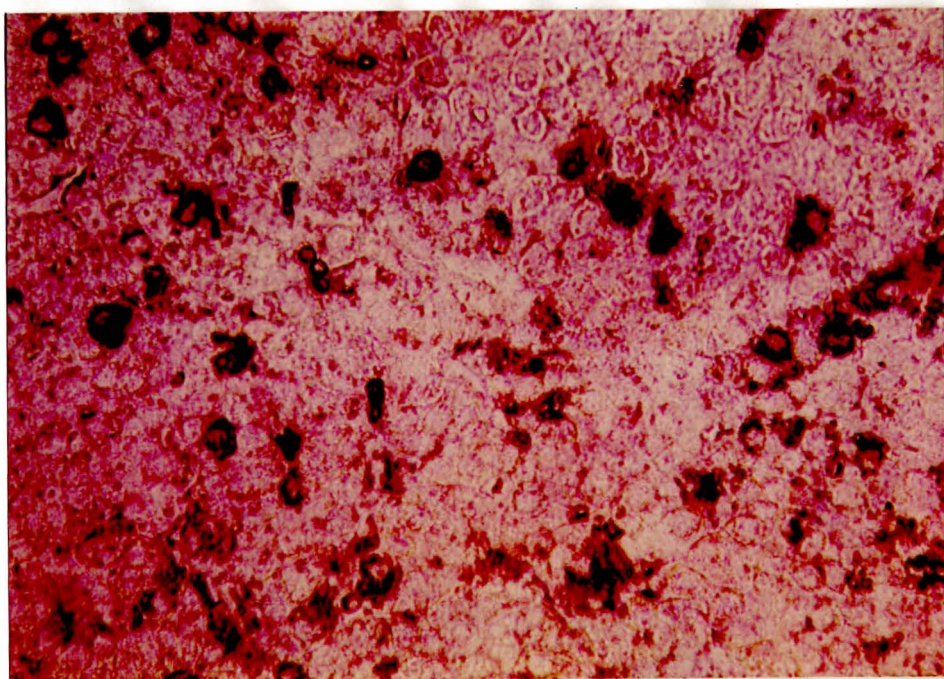
Figure 30. Immunohistochemical staining of pituitary
thyrotrophs by the PAP technique.
(Blue filter, x 100).
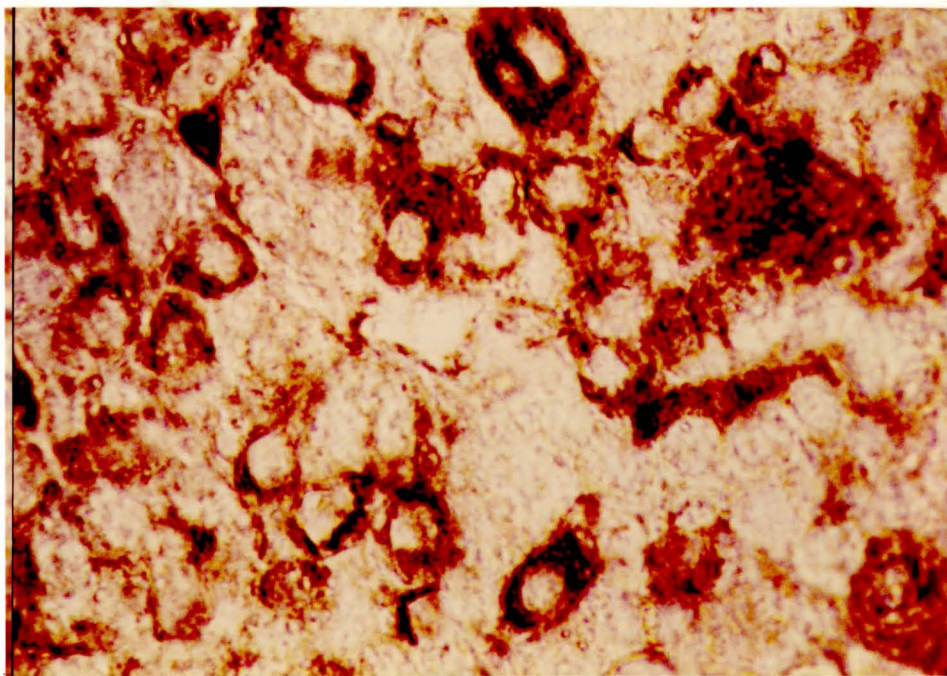
Figure 31. Immunohistochemical staining of pituitary thyrotrophs by the PAP technique (x400).
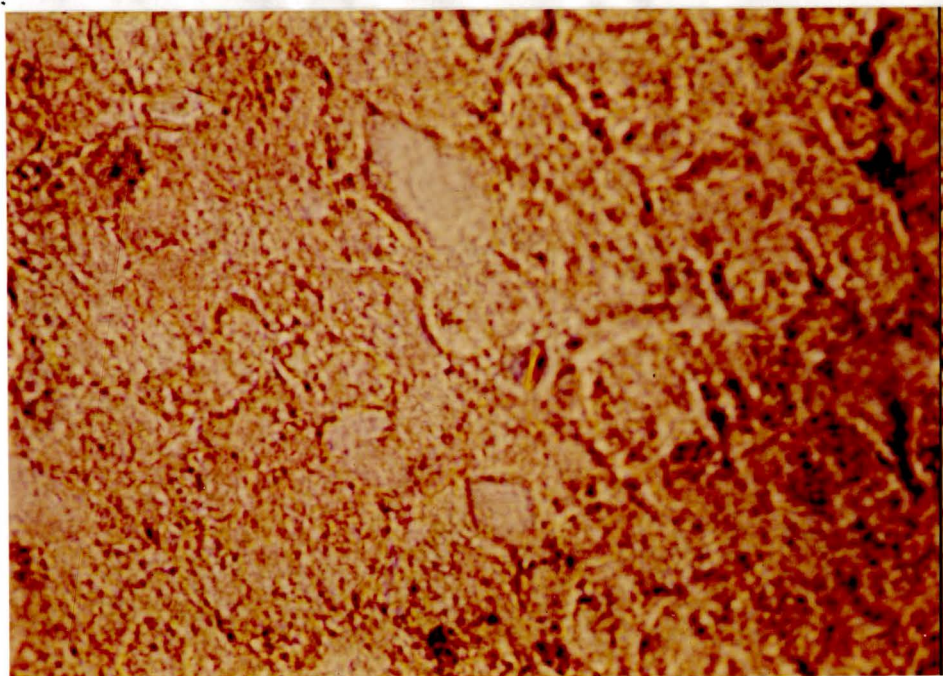
Figure 32. Control pituitary stained by the PAP technique using absorbed TSH antiserum (x 400).

Cell bodies containing
TSH

Control immunoabsorbed
1st. Ab.
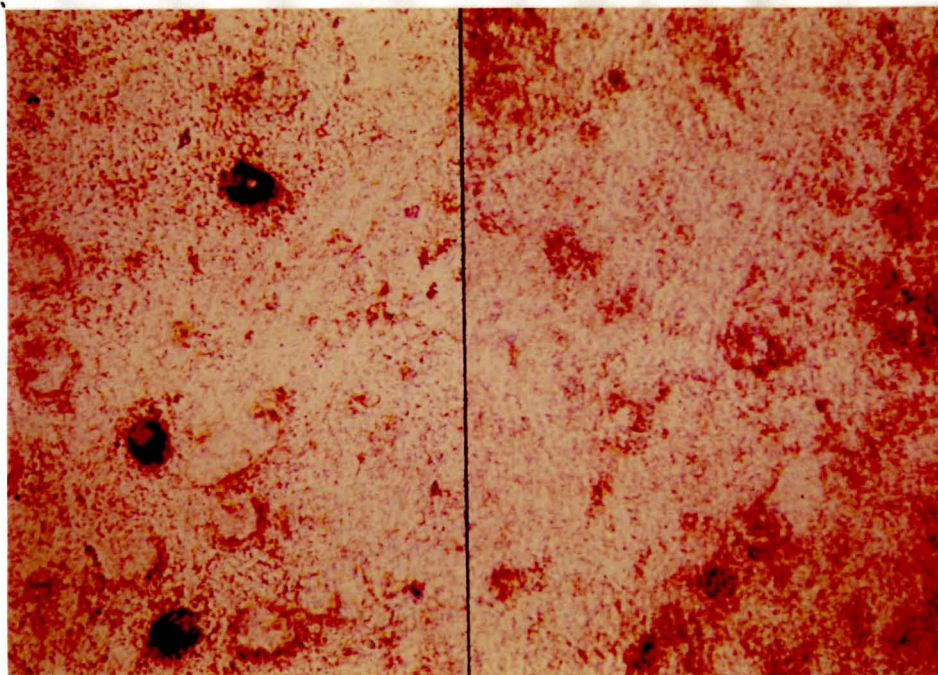
Figure 33. Immunohistochemical staining by the PAP
technique of amygdaloid tissue for TSH
(x 1000)
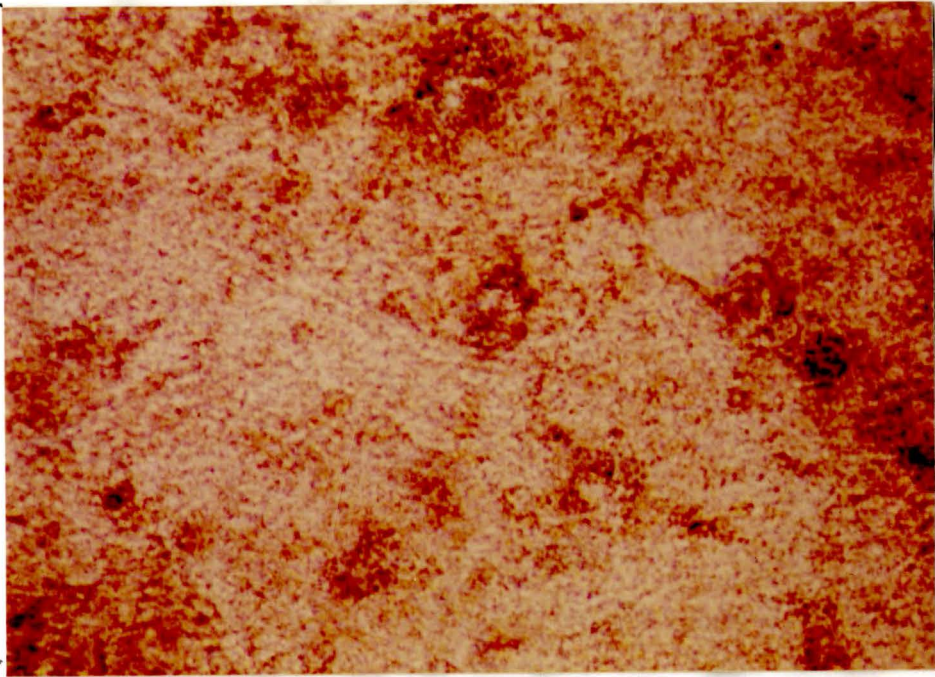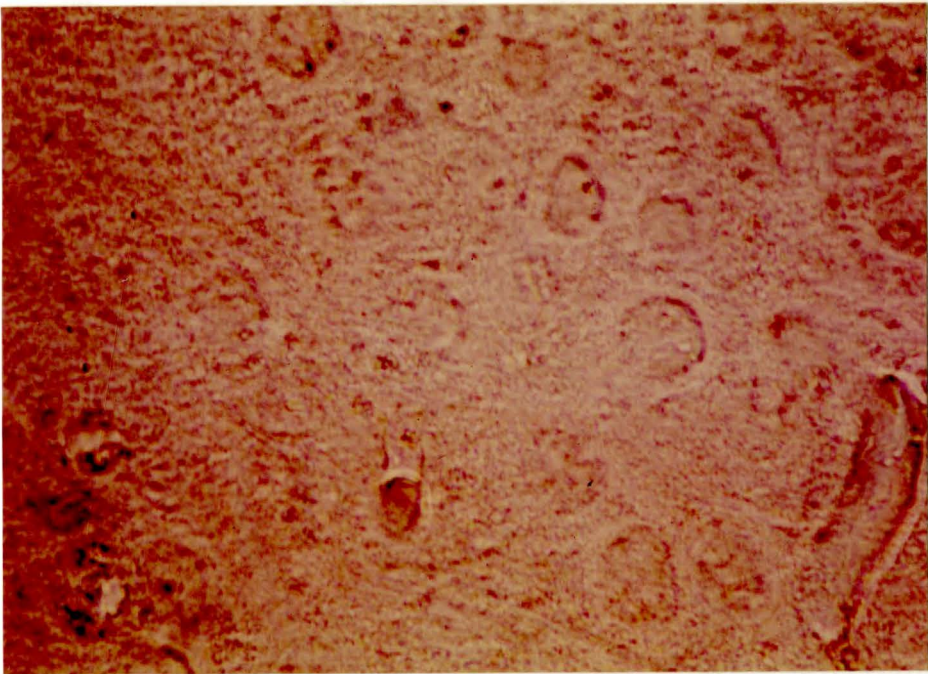
Figure 34. Immunohistochemical staining of amygdaloid tissue for TSH by the PAP technique. Controls (x 1000). A. Immunoabsorbed first antibody. B. First antibody replaced by normal rabbit serum.
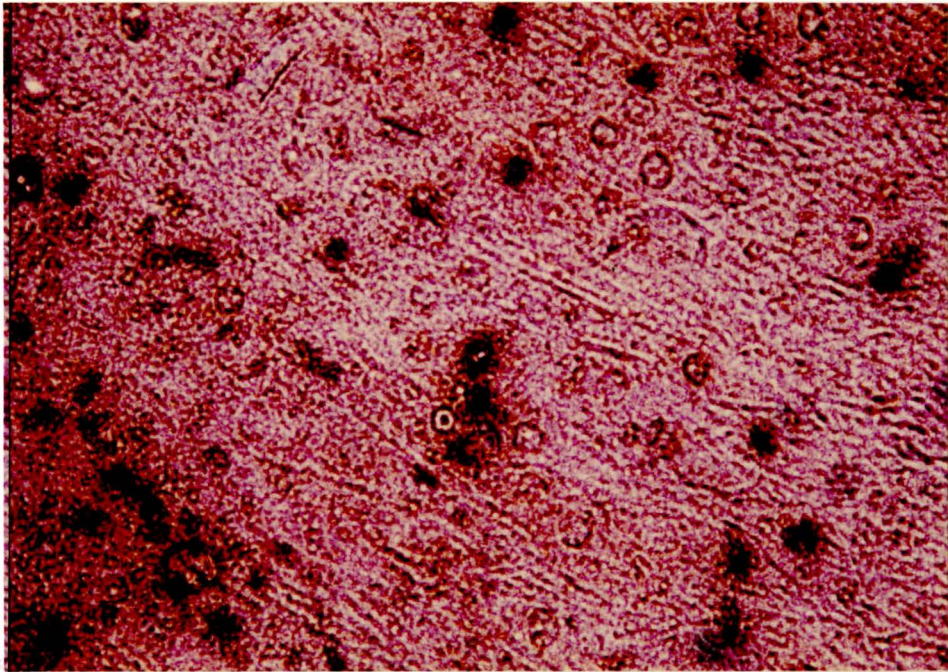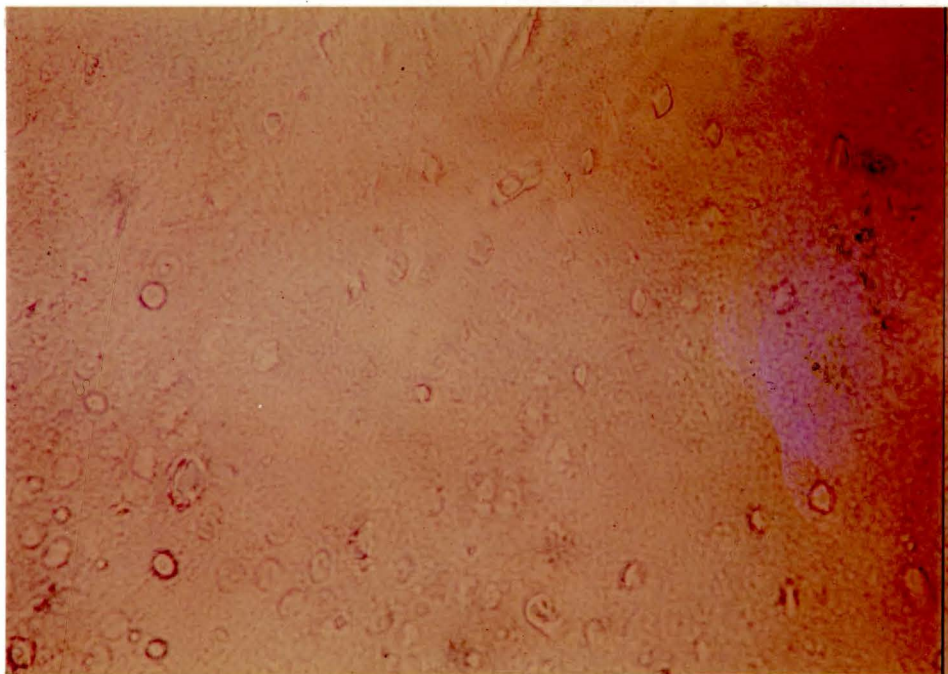
Figure 35. A. Immunohistochemical staining for TSH in
amygdaloid tissue (x400).
B. Control. First antibody replaced by normal
rabbit serum.

Similar studies with GH, not detailed in this thesis, have also detected GH-like material in cells of the amygdaloid nucleus by an indirect immunoperoxidase technique. Thus, confirming the association of hormone-like material with a particulate fraction of nervous tissue, these histochemical studies provide further evidence of a brain source for pituitary-like hormones.

I. CHARACTERIZATION OF "BRAIN HORMONES" BY GEL CHROMATOGRAPHY

a. IRGH-like Material in the Rat Brain

The elution pattern obtained when a sample of amygdaloid extract, containing immunoreactive GH-like material was placed on a Sephadex G-100 column together with a trace amount of ^{125}I labeled rat pituitary GH, is shown in Fig. 36 . The ordinates are given as the concentration of immunoreactive GH and cpm of radiolabeled pituitary growth hormone. On the abcissa, the tube number of the fractions collected is noted.

The peak of GH immunoreactivity coeluted with the trace amount of radiolabeled pituitary growth hormone, indicating molecular identity between the hormonal material found in the brain homogenate with that isolated from the pituitary. A molecular weight of approximately 46,000 D can therefore be assigned to the brain GH-like material.

b. IRTSH Material in the Rat Brain

Fig. 37 illustrates a similar experiment in which the amygdaloid extract was fractionated on a Sephadex G-100 column, together with ^{125}I
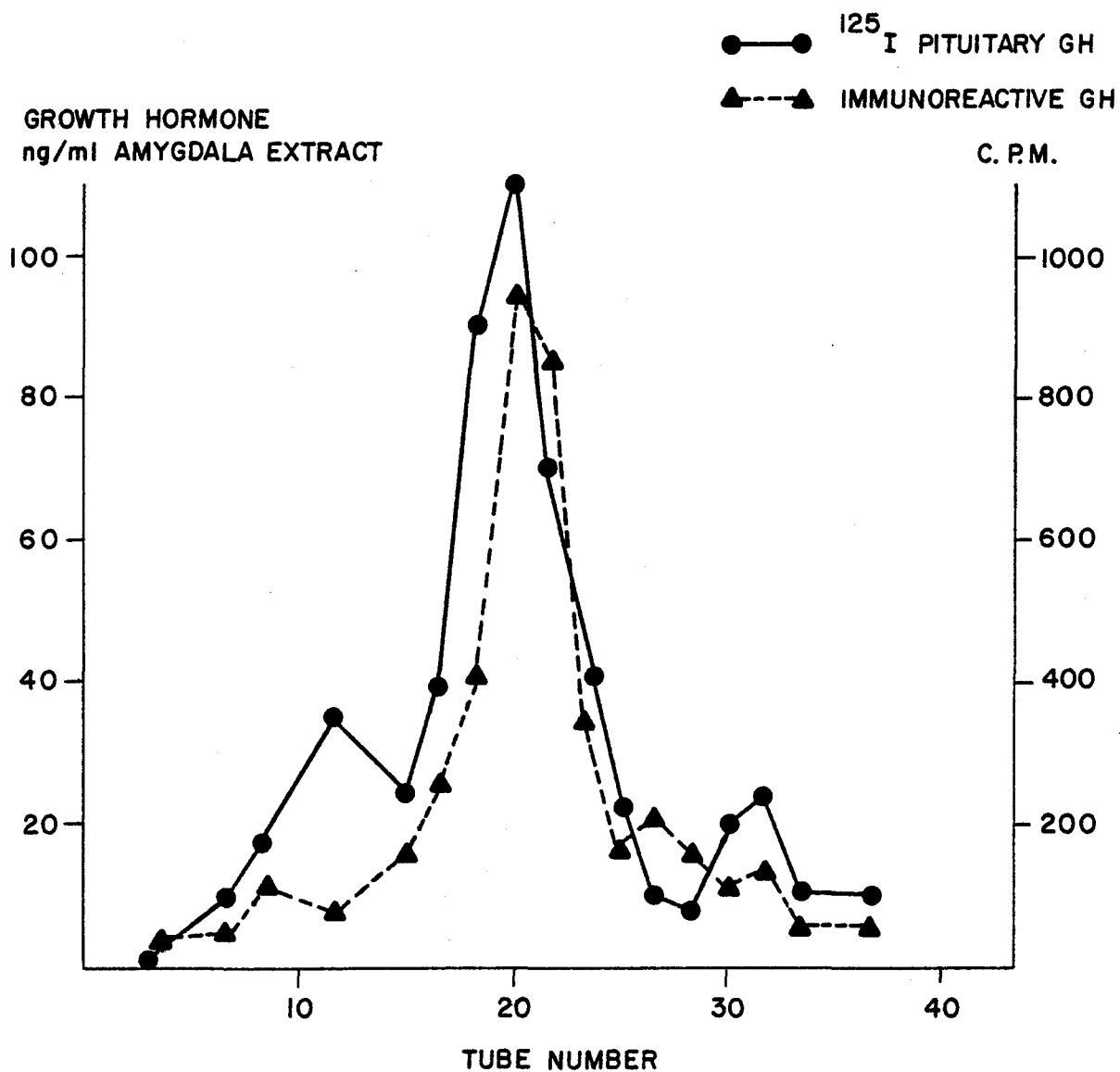
Figure 36. Elution pattern of lyophilised and
reconstituted amygdala extract IRGH
and ^{125}I rat pituitary GH on a Sephadex
G-100 column eluted with veronal buffer.

pH 8.6.
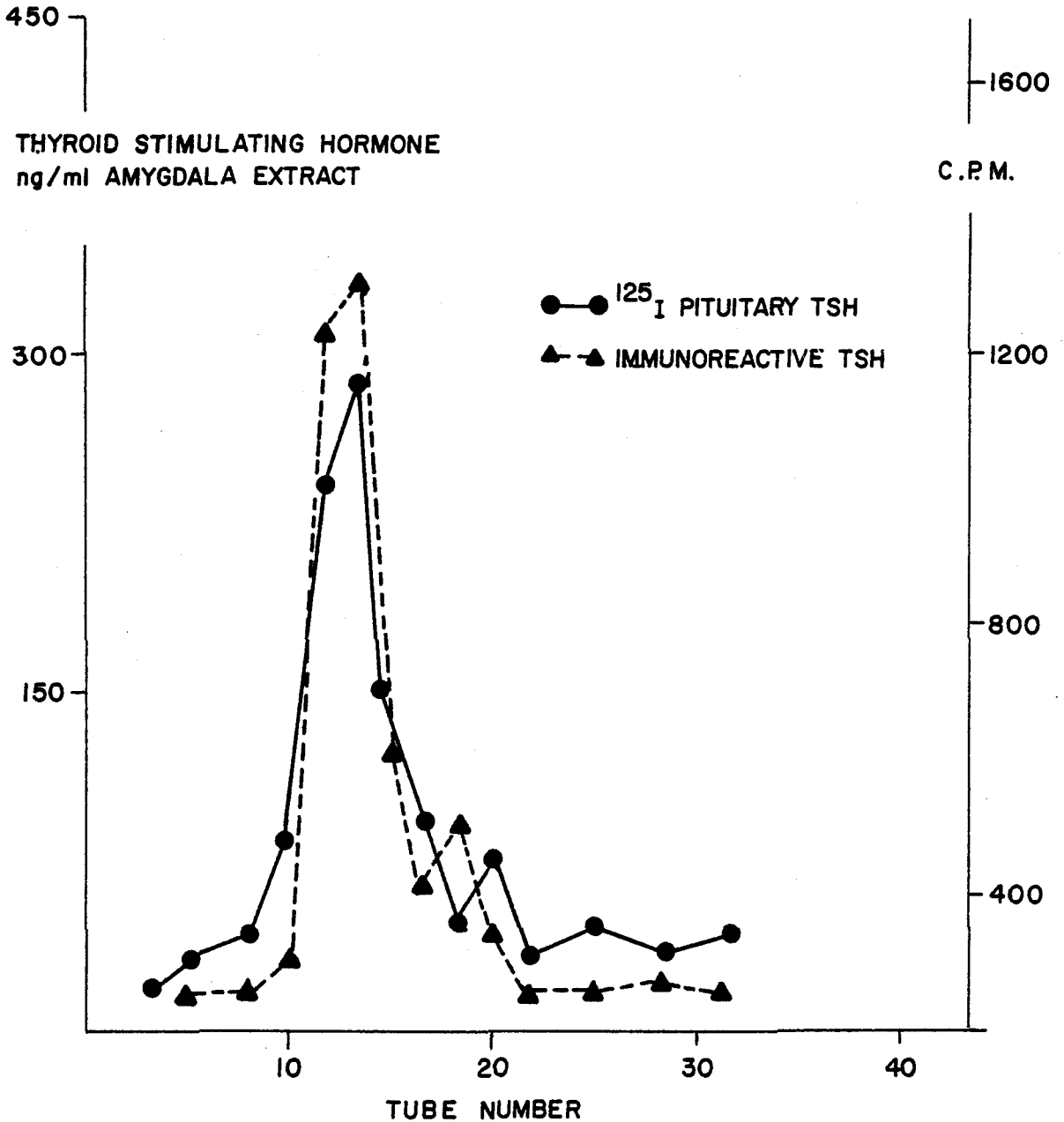
Figure 37. Elution pattern of lyophilized and reconstituted amygdala extract IRTSH and ^{125}I rat pituitary TSH on a Sephadex G-100 column eluted with 0.01 M PO$_4$ buffer pH 7.6.

rat pituitary TSH. Again, coelution of immunoreactive and labeled pituitary TSH material was observed, indicating molecular similarity between the hormones isolated from these two distinct sources. A probable molecular weight of 28,000 D can therefore be assigned to the brain TSH-like hormone. Similar results for both GH and TSH hormones were demonstrated for all brain regions examined. The existence of a prohormone or breakdown product of pituitary hormones in the brain thus seems unlikely.

    c.   <u>IR Pituitary-Like Hormones from a Cloned Mouse Neuroblastoma Line</u>

When a concentrated sample of neuroblastoma N4TGI cell extract was similarly fractionated with a trace amount of radiolabeled pituitary TSH, coelution of immunoreactive and tracer hormone was shown, as in Fig. 38 .

Similar results were obtained when non-cell line tissue extracts from amygdaloid tissue cell cultures and pituitary cell cultures were fractionated and compared. This would indicate that the immunoreactive hormonal activity detected in the <u>in vitro</u> experiments were also molecularly similar, in terms of molecular weight, to their pituitary counterparts <u>in vivo</u>.

J.   <u>IMMUNOLOGICAL CHARACTERIZATION OF BRAIN PITUITARY-LIKE HORMONES AND</u>

    <u>FURTHER IMMUNOASSAY VALIDATION</u>

Logit log plots demonstrating parallel displacement for serial dilutions of pituitary and CNS extracts are shown in Figs. 39 and 40 for GH and TSH, respectively. Although values actually fell along the same line, the hippocampal data is displaced for visual emphasis of the

IMMUNOREACTIVE TSH ●——●
ng/ml CELL EXTRACT

✗--✗ ^{125}I PITUITARY TSH
C. P.M.

TUBE NUMBER
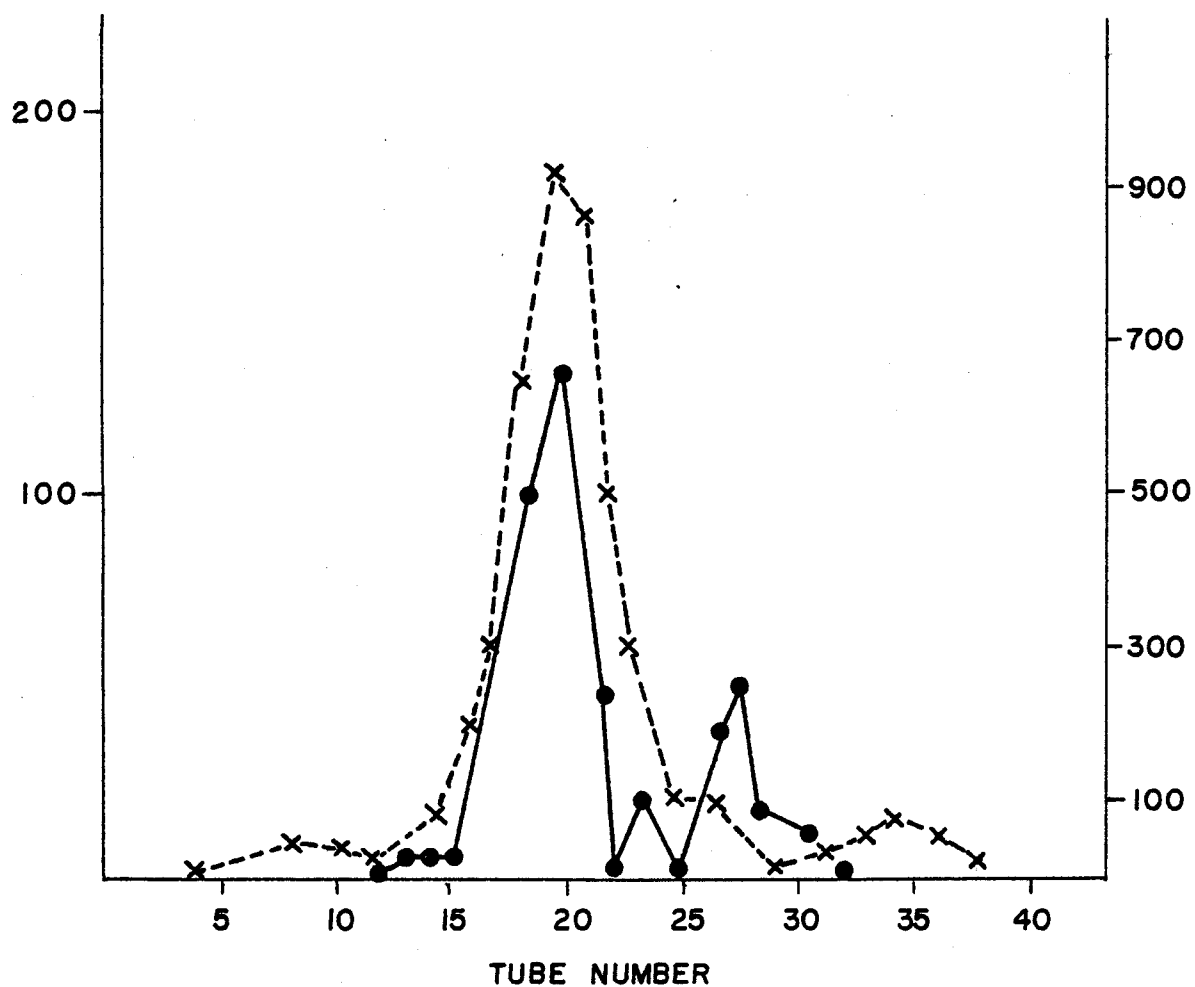
Figure 38. Elution pattern of lyophilised,
reconstituted N_4TG1 cell extract IRTSH and
^{125}I rat pituitary TSH on a Sephadex G-100
column eluted with 0.01 M PO_4 buffer pH 7.6
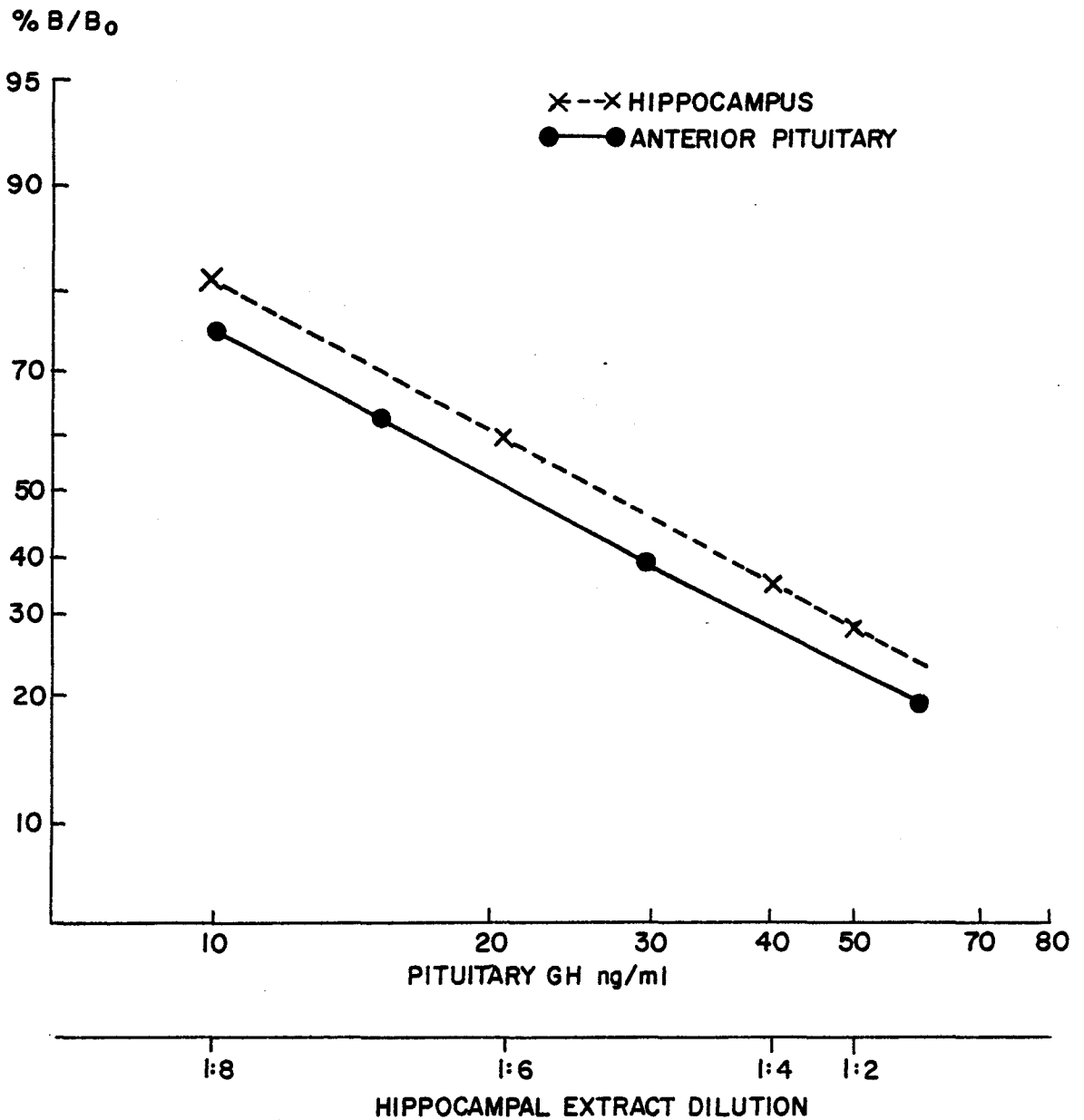
Figure 39. Logit log plot demonstrates parallel
displacement for serial dilutions of
pituitary and CNS growth hormone extracts.
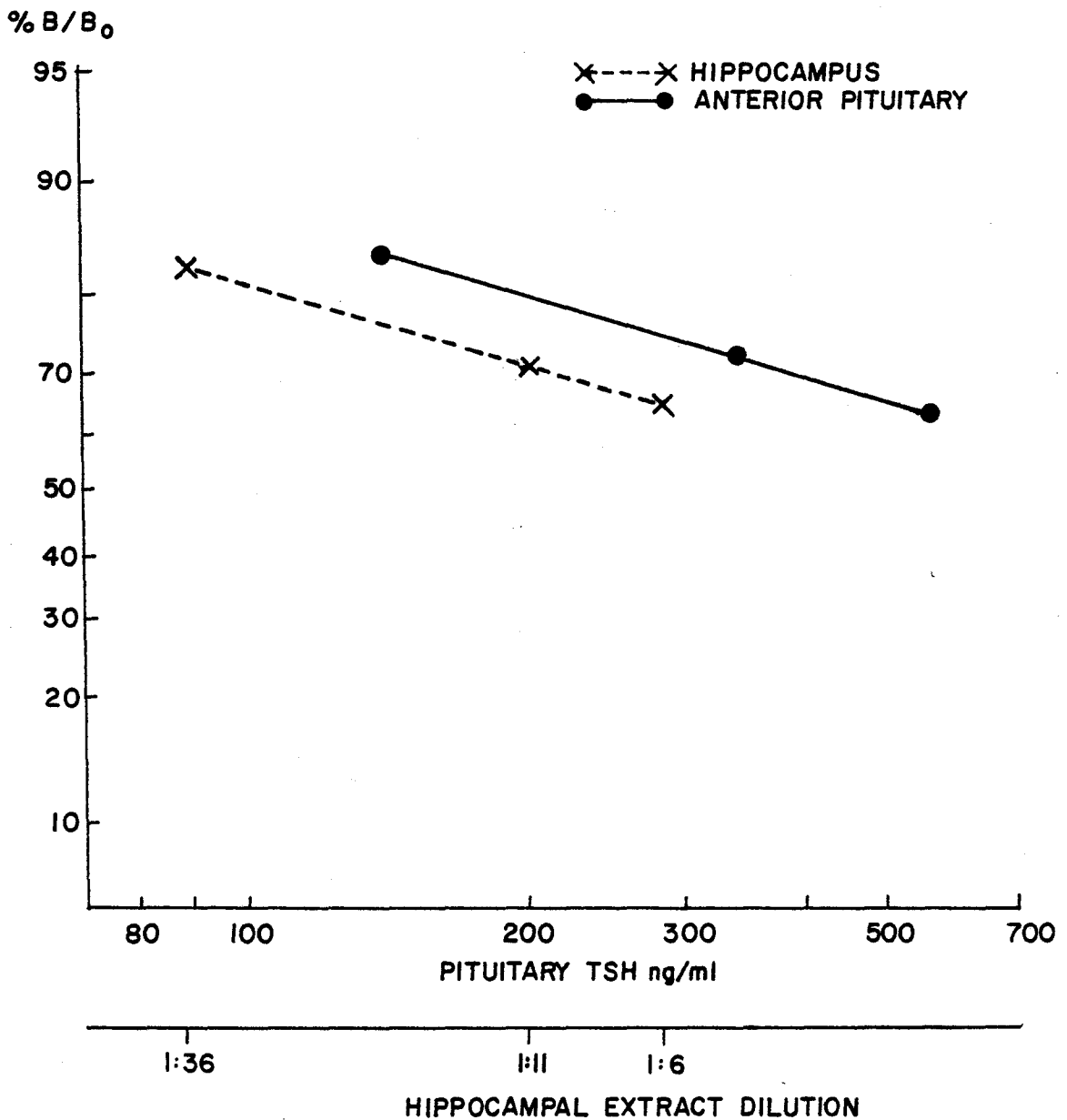
Figure 40. Logit log plot demonstrates parallel
displacement for serial dilutions of
pituitary and CNS thyroid stimulating
hormone extracts.

data's linearity. Such linearity between pituitary and brain hormones demonstrated probable immunological identity.

K. BIOLOGICAL ACTIVITY OF BRAIN PITUITARY-LIKE HORMONES AS COMPARED TO
    PITUITARY HORMONES

a. Growth Hormone Bioassay

When adult, 14-day post-hypophysectomized male and female rats received intraperitoneally injected doses of pituitary, amygdaloid extract or saline over a 5-day period, their body weights and width of the tibia epiphyseal cartilage were examined as indicators of biological activities usually attributable to growth hormone.

Table 17 shows the changes in body weights recorded over the 5-day injection period before sacrifice from two individual experiments. It is apparent that, whereas the extract from rat pituitary was able to produce an increase in body weight response, neither the amygdaloid extract nor saline-treated animals showed a rise in total body weight over the same time period. It was,however, apparent that the weight loss was substantially less in amygdaloid-treated animals when compared to that experienced by saline-treated, hypophysectomized controls. These data were not altogether surprising because, as noted before, a 100 µg growth hormone dose is usually required before a weight gain is observable. In neither experiment was sufficient immunoassayable GH given in the brain extract to the test animals.

TABLE 17

BODY WEIGHT CHANGES OVER 5-DAY

BIOLOGICAL EXPERIMENT PERIOD (gms)

Treatment	Total Quantity of IR GH (μg) Injected Over 5 Days		
	Saline (0)	RAM (73)	RAP (113)
Exp 1 ♂	− 5.26 ± 0.10	− 2.3 ± 0.21	+ 3.02 ± 0.25
Treatment	Saline (0)	RAM (90)	RAP (95)
Exp 2 ♀	− 6.17 ± 1.35	− 2.3 ± 0.95	+ 3.17 ± 0.79

The mean and SEM of the difference in body weight between Day 1 of the biological experiment and Day 5. Each point represents 5 adult animals, 14 days after hypophysectomy.

RAP = rat anterior pituitary extract

RAM = rat amygdaloid nucleus extract

However, a much lower amount of growth hormone is required to elicit a response in the tibia epiphyseal cartilage bioassay, and this was reflected in the results obtained in Table 18a. In both male and female groups of hypophysectomized animals from all four experiments, a statistically significant increase in the epiphyseal cartilage width of amygdaloid extract-treated animals over those of saline-treated controls was observable. This ability to induce cartilage growth was equivalent to the effect produced by the pituitary extract on hypophysectomized animals under similar conditions. Thus, this experiment revealed that the GH-like material present in the brain extracts was capable of eliciting a response normally attributable to, and characteristic of, pituitary growth hormone.

b. Gonadotrophic Bioactivity in Brain Extracts

From the data examining the overall weights of testes and ovaries in all three groups of treated hypophysectomized animals, Tables 18 b and c, it was apparent that the brain extracts also contained gonadotrophic bioactivity. This observation has subsequently been substantiated by our laboratory (Emanuele et al, 1980) where significant amounts of immuno-reactive luteinizing hormone have been found in certain areas of the rat brain.

c. Thyroid Stimulating Hormone Bioactivity in Brain Extracts

The thyroids of hypophysectomized animals treated with either saline, a high or low dose of rat pituitary extract, or amygdaloid tissue extract were examined biologically for structural changes.

TABLE 18

BIOASSAY OF PITUITARY AND AMYGDALA EXTRACTS

a.	DOSE/RAT μg RGH	SEX	TIBIA EPIPH. Width m.u.
	Saline-0	M	8.3 ± 0.5
	RAP-113	M	10.7 ± 0.4**
	RAM-73	M	10.3 ± 0.2**
	Saline-0	F	11.1 ± 2.0
	RAP-95	F	13.6 ± 3.2
	RAM-90	F	15.1 ± 3.2**

b.	DOSE/RAT μg RGH	SEX	TESTIS mg
	Saline-0	M	154 ± 0.7
	RAP-113	M	193 ± 4.0**
	RAM-73	M	204 ± 6.2**

c.	DOSE/RAT μg RGH	SEX	OVARIES mg
	Saline-0	F	15.2 ± 3.2
	RAP-95	F	19.1 ± 4.9*
	RAM-90	F	29.7 ± 4.5**

m.u. = 0.005 mm   ** p = < 0.01
* p = < 0.05   n = 4-5
RAP = rat anterior pituitary extract
RAM = rat amygdaloid nucleus extract

Mean and standard deviations derived from bioassay of anterior pituitary
and amygdaloid nucleus extracts in adult, 14-day, post-hypophysectomized
male and female rats. Four to five animals were included in each group,
and experimental animals received comparable amounts of immunoreactive
growth hormone in four daily divided doses administered intraperitoneally.

Details of the results observed are shown in the composite photo-
graph, Fig. 41 .

Sections of thyroid from saline-treated animals revealed the typical
follicular characteristics of hypothyroidism, that is, low cuboidal epi-
theleal cells with flattened muclei and a shrunken colloidal mass in the
follicular lumen. One of the characteristics of TSH activity, as already
discussed, is to maintain thyroid function. In both groups receiving pi-
tuitary extracts, thyroids revealed this response as demonstrated by the
appearance of tall, cuboidal epitheleal follicle cells; large, round
nuclei filling the cells, and well-maintained follicular collagen and
vacuolization of colloid.

The thyroid tissue dissected from amygdaloid extract-treated, hypo-
physectomized animals also showed these characteristics, indicating, on
the basis of biological activity, the presence of a TSH-like material.

Evaluation of this TSH-like effectiveness was also attempted using
the parameter of ^{131}I uptake into the thyroid as an activity indicator.
Table 19 shows the data obtained in terms of ^{131}I uptake into various
tissues as well as the T_4 levels of circulating serum after five different
treatments. It was apparent that only the high dose of pituitary extract,
100 µg/hypophysectomized animal, was sufficient to produce an effect com-
parable to that demonstrated in the intact saline-treated control. In
this short, four-day study, neither 2 µg of pituitary TSH nor 4.3 µg of
amygdaloid extract TSH, were sufficient to increase the labeled iodide
uptake into the thyroid or to raise circulating T_4 levels, an indirect

SALINE (0)*   LOW DOSE RAP (5)
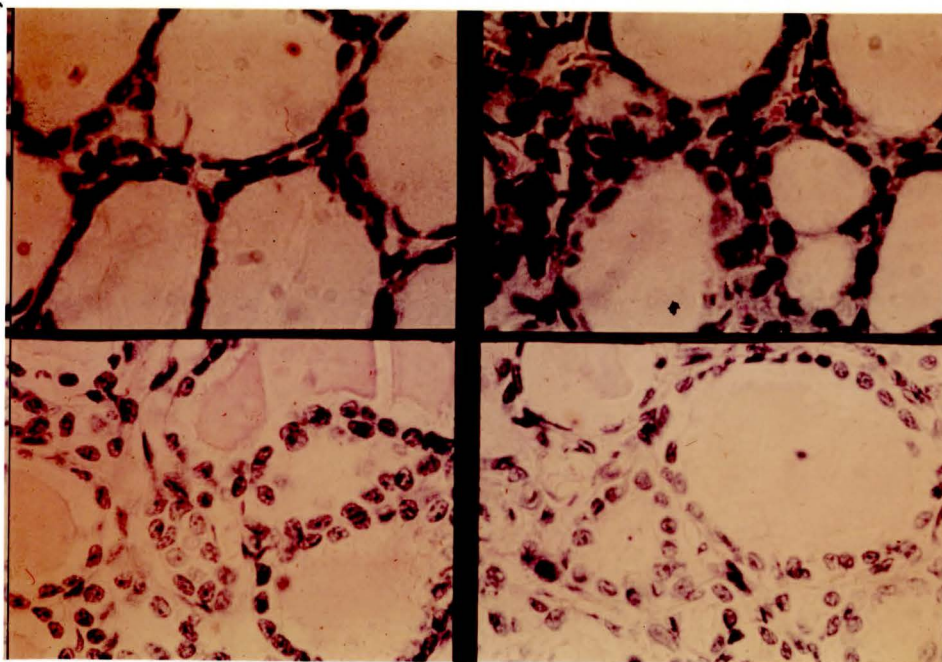
HIGH DOSE RAP (100)   RAM (4.5)

Figure 41. Histology of the thyroid gland of 14 day
           hypophysectomised animals treated with
           brain and pituitary extracts (x 400).

*( ).= µg immunoreactive TSH administered over
       a 4 day period to adult rats, 14 days
       after hypophysectomy. Note the low cuboidal
       epithelium lining of the thyroid follicles
       of saline treated animals, and the increased
       height of epithelium of pituitary **and** amygdala
       treated animals.

TABLE 19

EVALUATION OF TSH EFFECTIVENESS TO CHANGE THE

PATTERN OF ^{131}I UPTAKE INTO THE THYROID

	Total Dose TSH µg	n	(cpm ^{131}I/gm) Tissue		Serum µgm/dl
			Thyroid	Muscle	T_4
Hypophysectomized $^+$	Saline	5	4,447 ± 206	273 ± 20	0.8 ± 0.1
Hypophysectomized $^+$	100 µg RAP TSH	5	57,453 ± 129	237 ± 30	2.2 ± 0.3
Hypophysectomized $^+$	2 µg RAP TSH	5	3,000 ± 198	254 ± 16	0.9 ± 0.1
Hypophysectomized $^+$	4.3 µg RAM TSH	5	2,795 ± 216	212 ± 22	0.9 ± 0.2
Intact +	Saline	5	61,145 ± 598	189 ± 27	4.7 ± 0.3

Four groups of adult, 14-day, post-hypophysectomized rats received either saline, 100 µg pituitary extract TSH, 2 µg pituitary extract TSH, or 4.3 µg amygdaloid nucleus extract TSH over a four-day period, intraperitoneally. Eighteen hours before sacrifice, each animal received 1.5 µ curies of radioactive iodine IP.

indicator of a well-maintained thyroid. On reviewing the literature, this result was not surprising, as it was found that at least 100 μg of pituitary TSH is necessary to show an increased ^{131}I uptake into the thyroid of hypophysectomized rats (Cons et al, 1975).

L. TESTING THE INTACTNESS OF THE BLOOD-BRAIN BARRIER IN BOTH ADULT AND YOUNG ANIMALS

When ^{125}I labeled pituitary hormones were injected IV into the tail veins of adult rats and 30 minutes allowed to pass before sacrifice, levels of labeled hormone were measured in various body tissues and brain in order to observe if radiolabeled growth and thyroid stimulating hormone were capable of passing through the blood brain barrier. Results of these experiments are given in Tables 20 and 21 for GH and TSH, respectively, as cpm/mg of wet tissue. The immunoreactive hormone content of saline-injected control rat tissue was also determined, in order to compare the sites of receptor-bound circulating hormone with sites of immunologically active, extractable growth and thyroid stimulating hormones.

As shown in Table 20, the large organs of the body readily bound circulating, labeled pituitary growth hormone. The pituitary itself had a fairly high level of ^{125}I hormone/mg tissue, 11.64 ± 2.5 cpm/mg, whereas little or no labeled hormone was detected in areas of the CNS, 0.00 → 0.90 cpm/mg. By comparison, levels of immunoreactive growth hormone, as measured in tissue extracts by RIA, was highest in the CNS tissues such as amygdala, 212 ± 5.4 pg/mg tissue, and lowest in the large organs such as the kidney, 2.9 ± 0.2 pg/mg tissue.

TABLE 20

TISSUE DISTRIBUTION OF ^{125}I-RAT GROWTH HORMONE

INJECTED IV INTO ADULT RATS AND IR GH CONTENT

OF TISSUES (pg/mg wet weight)

	n	cpm/mg*	GH pg/mg wet wt.*
Kidney	5	433.84 ± 104.6	2.9 ± 0.2
Liver	5	55.56 ± 10.9	17.3 ± 2.6
Muscle	5	1.45 ± 1.0	2.2 ± 0.5
Pituitary	5	11.64 ± 2.5	1.9×10^{5} ± 0.7
Amygdala	5	0.90 ± 0.1	212 ± 5.4
Thalamus	5	0.89 ± 0.1	89 ± 3.6
Cortex	5	0.00	52 ± 5.2

n = number of experiments.

* Mean ± S. E. M.

A similar picture was shown for the effectiveness of the blood brain barrier in preventing ^{125}I labeled TSH from entering the brain via the peripheral circulation, as demonstrated in Table 21, where no radio-labeled hormone was shown to be detected in the CNS structures.

In studies with fetal material, there has been some discussion of possible leakages in the immature brain blood barrier. In order to determine whether or not this was the case, 21-day-old fetuses were given radiolabeled GH or TSH by an intraperitoneal route and the distribution of label was detected in various body tissues and brain 30 minutes after administration. Although results are only given for GH in Table 22b, similar data were obtained for TSH, revealing the total effectiveness of the 21-day-old fetal blood brain barrier to prevent the entry of circulating pituitary hormones into the brain.

Shown in Table 22a are the results of a similar experiment involving another such barrier, the fetal-placental barrier. This was also examined by injecting females in their 21st day of pregnancy with radio-labeled hormone. Dissection of the fetuses after a 30-minute delay revealed no label in any part of the fetus, demonstrating an inability of GH and TSH to cross the placental barrier.

M.   THE EFFECT OF HYPOPHYSECTOMY ON LEVELS OF BRAIN PITUITARY-LIKE

HORMONES

Animals which had been hypophysectomized for varying lengths of time were sacrificed and the levels of immunoreactive hormone present in

TABLE 21

TISSUE DISTRIBUTION OF ^{125}I RAT THYROID STIMULATING HORMONE

INJECTED IV INTO ADULT RATS AND IR TSH CONTENT

OF TISSUE (ng/mg wet weight)

	n	cpm/mg*	IR TSH ng/mg wet wt.*
Kidney	5	678.71 ± 10.5	0.040 ± 0.01
Liver	5	72.65 ± 12.3	0.075 ± 0.02
Muscle	5	18.64 ± 5.6	0.002 ± 0.01
Pituitary	5	17.33 ± 2.7	1.8×10^{5} ± 0.42
Amygdala	5	0.00	1.521 ± 0.36
Thalamus	5	0.00	0.795 ± 0.27
Cortex	5	0.00	0.651 ± 0.43

* Mean ± S. E. M.

TABLE 22

DISTRIBUTION OF ^{125}I RAT GROWTH HORMONE IN FETAL AND NEONATAL BRAIN AND OTHER TISSUES

AFTER INJECTION OF RADIOLABELED HORMONE EITHER INTRAPERITONEALLY INTO PREGNANT RATS (A)

INTO 21-DAY-OLD FETUSES (B)

OR INTO NEONATAL RATS (C)

I^{125} GH, cpm/gm Tissue

		Kidney	Pituitary	Hypothalamus	Amygdala
Rat Fetal Age (days):					
A.	21 (IP mother)	0	0	0	0
B.	21 (IP fetus)	3000	2	0	0
Rat Neonatal Age (days):					
C.	1	6676	318	0	0
	4	8759	378	0	0
	10	3764	183	0	0

the different brain regions compared to intact controls.

a. Growth Hormone: Effects of Hypophysectomy

Table 23 depicts the combined results from three separate experiments, in terms of mean hormone levels, ng/mg protein, per group of 20 animals, for the various brain regions extracted. Animals, hypophysectomized for periods of 7, 14, 28, and 48 days, were compared to intact, similar aged controls. An analysis of the data revealed that in some brain regions, namely the amygdala, hypothalamus, and thalamus, an initial fall was observed in hormonal tissue levels after one week of hypophysectomy, e.g., amygdala, $6.25 \pm 0.46 \rightarrow 1.25 \pm 0.50$ ng/mg protein. However, after 28 days of hypophysectomy, these regions were found to contain more hormone than was found in comparable intact controls, e.g. amygdala, $5.75 \pm 0.50 \rightarrow 15.00 \pm 3.10$ ng/mg protein. This is illustrated in Fig. 42 for mean and standard errors of the mean levels of growth hormone, ng/mg protein, found in amygdaloid tissue. Other areas of the brain, such as the caudate, hippocampus, and cortex, showed no fall in growth hormone levels throughout the entire 48 days of hypophysectomy examined. This kind of pattern is illustrated in Fig. 43 , showing mean and standard error of the mean levels of hormone, ng/mg protein, assayed in caudate tissue from hypophysectomized and from caudate tissue of intact rats.

b. Thyroid Stimulating Hormone: Effect of Hypophysectomy

Similar experiments were developed to determine changes in levels of immunoassayable TSH in brain tissue. Combined results are given in Table 24 for hormonal levels in hypophysectomized and intact animals,

TABLE 23

IMMUNOREACTIVE GROWTH HORMONE (ng/mg protein)*
IN EXTRACTS FROM DISCRETE BRAIN PARTS

	HYPOPHYSECTOMIZED ANIMALS				INTACT CONTROLS			
Days, Post-Hypophys.	7	14	28	48				
	A	B	C	D	A	B	C	D
AMYGDALA	1.25 ± 0.50	3.25 ± 0.50	15.0 ± 3.10	13.25 ± 1.56	6.25 ± 0.46	5.75 ± 0.88	5.75 ± 0.50	6.25 ± 0.75
HYPOTHAL-AMUS	0.57 ± 0.20	1.73 ± 0.20	7.24 ± 0.50	6.53 ± 0.90	1.99 ± 0.30	2.89 ± 0.15	3.13 ± 0.40	2.96 ± 0.25
THALAMUS	0.95 ± 0.10	1.84 ± 0.02	2.07 ± 0.60	2.01 ± 0.50	1.94 ± 0.50	1.58 ± 0.10	1.86 ± 0.10	1.75 ± 0.04
CAUDATE	1.45 ± 0.12	1.81 ± 0.15	1.75 ± 0.09	1.91 ± 0.12	1.50 ± 0.15	1.75 ± 0.24	1.66 ± 0.27	2.00 ± 0.12
HIPPO-CAMPUS	1.36 ± 0.04	1.42 ± 0.05	1.29 ± 0.20	1.28 ± 0.40	1.40 ± 0.20	1.48 ± 0.10	1.39 ± 0.94	1.25 ± 0.01
CORTEX	0.95 ± 0.03	0.72 ± 0.04	0.99 ± 0.10	1.24 ± 0.06	1.32 ± 0.40	1.26 ± 0.05	0.90 ± 0.08	1.18 ± 0.02
CEREBELLUM	0.05 ± 0.02	0.20 ± 0.04	0.03 ± 0.10	0.00	0.05 ± 0.01	0.00	0.04 ± 0.01	0.03 ± 0.01

*Mean ± S.E.M. of 3 experiments, 20 animals/group of hypophysectomized or intact controls.

AMYGDALA
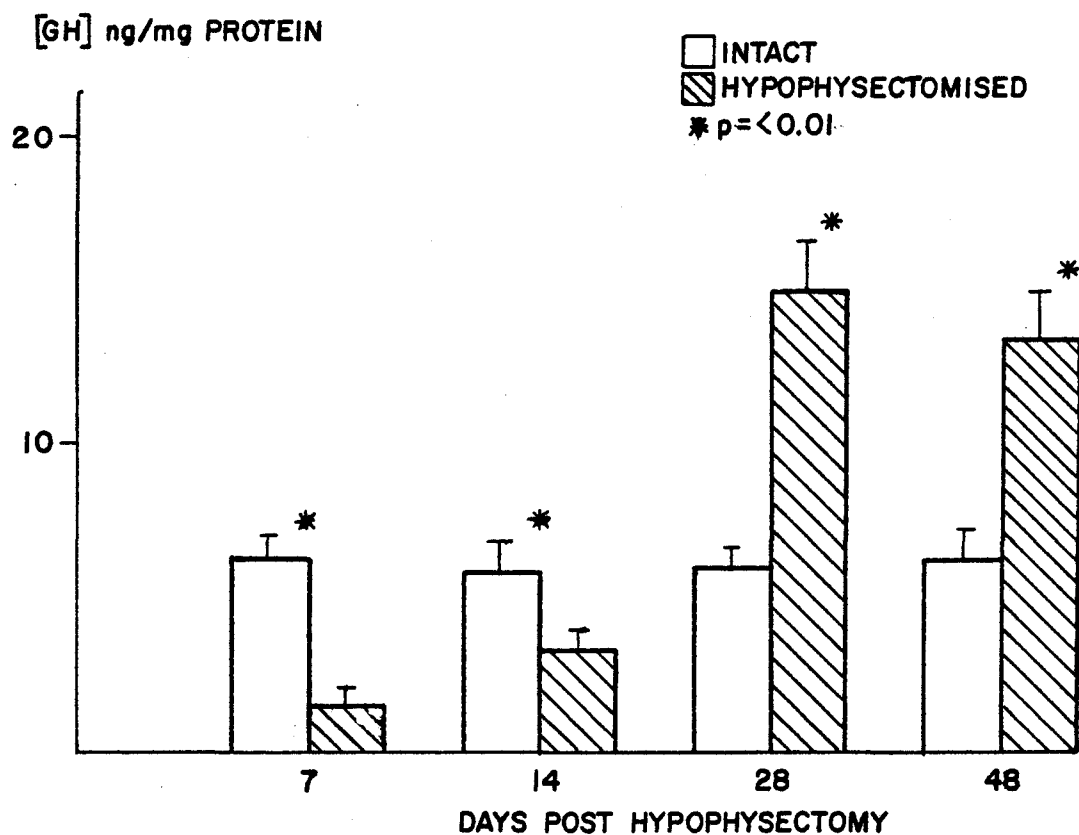
Figure 42. Immunoreactive growth hormone present in amygdaloid nucleus extracts of intact and hypophysectomised rats.

CAUDATE
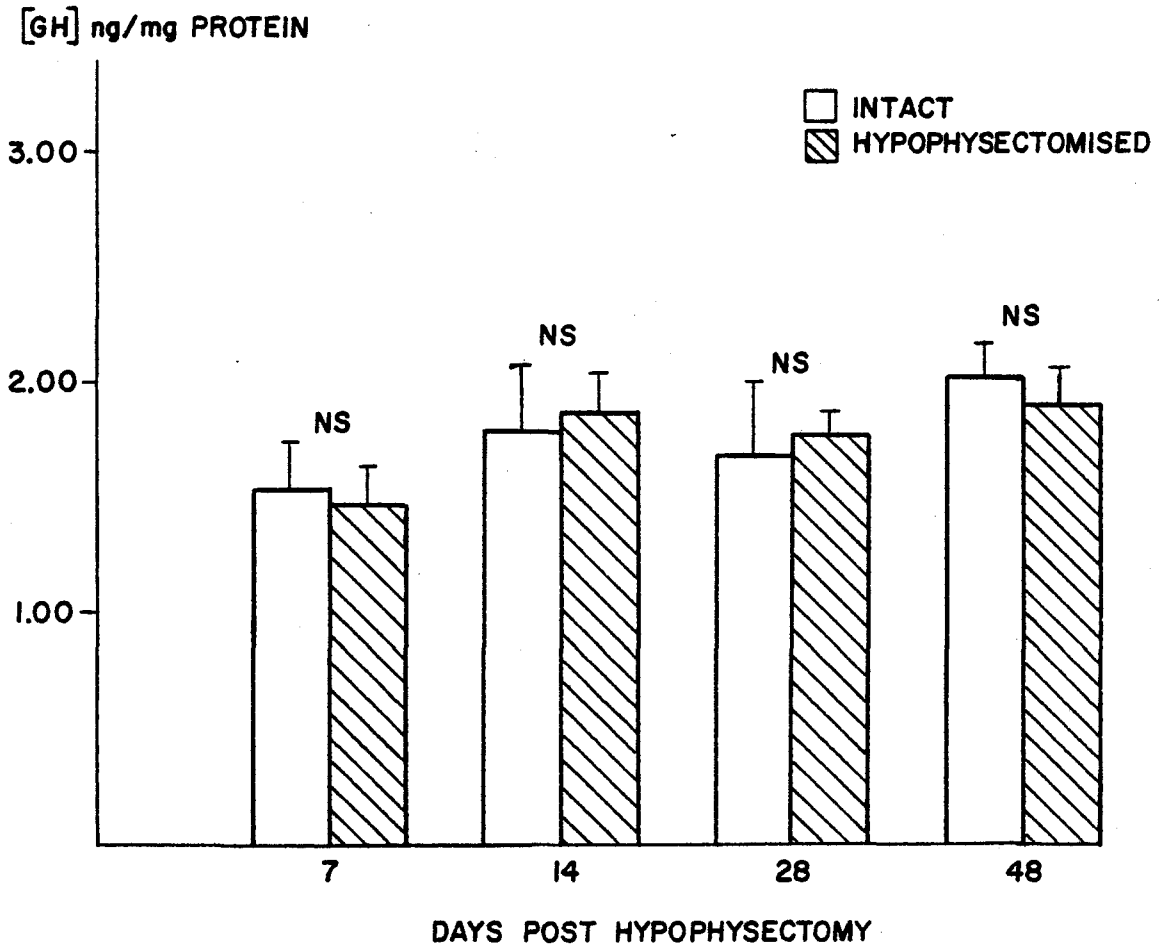
Figure 43. Immunoreactive growth hormone present in
caudate extracts of intact and hypophysecto-
mised rats.
NS = not significant.

TABLE 24

IMMUNOREACTIVE THYROID STIMULATING HORMONE (ng/mg protein)*
IN EXTRACTS FROM DISCRETE BRAIN PARTS

Days Post-Hypophys.	HYPOPHYSECTOMIZED ANIMALS				INTACT CONTROLS			
	7	14	28	48				
	A	B	C	D	A	B	C	D
AMYGDALA	18.95 ± 2.10	27.58 ± 0.30	26.79 ± 2.70	24.64 ± 1.50	21.50 ± 2.10	25.95 ± 0.50	25.79 ± 3.31	26.32 ± 2.90
HYPOTHALA-MUS	38.52 ± 0.20	31.43 ± 0.70	23.33 ± 3.40	25.95 ± 0.60	125.60 ± 1.50	143.50 ± 2.70	193.33 ± 1.66	139.60 ± 5.90
THALAMUS	16.55 ± 0.50	19.24 ± 1.50	17.32 ± 2.10	15.95 ± 1.00	17.21 ± 0.40	19.36 ± 0.80	18.30 ± 2.11	16.20 ± 1.10
CAUDATE	14.71 ± 0.80	12.92 ± 0.90	15.63 ± 0.90	14.99 ± 0.80	12.95 ± 0.20	14.83 ± 0.40	14.79 ± 0.60	15.60 ± 0.32
HIPPO-CAMPUS	12.69 ± 0.80	9.76 ± 0.20	10.30 ± 1.20	11.51 ± 0.70	12.58 ± 1.10	11.63 ± 0.70	9.62 ± 0.91	10.60 ± 1.30
CORTEX	9.51 ± 0.40	11.18 ± 0.50	10.62 ± 3.20	10.98 ± 1.20	8.79 ± 0.90	10.51 ± 0.50	11.66 ± 1.60	12.68 ± 0.63
CEREBELLUM	13.95 ± 0.30	12.69 ± 2.20	14.95 ± 1.20	15.63 ± 1.10	14.23 ± 1.50	13.65 ± 0.80	16.35 ± 0.90	17.26 ± 0.50

* Mean ± S.E.M. of 3 experiments, 20 animals/group of hypophysectomized or intact controls.

over different time periods, post-operatively.

All tissue extracts analyzed for TSH, except hypothalamic tissue, were found to show no change in immunoreactive hormone levels over the entire experimental period. However, a significant, $p = < 0.02$, fall in hypothalamic levels of TSH was noted from 7 - 28 days post-hypophysectomy. These results are illustrated for 28-day post-hypophysectomized and intact rats in Fig. 44. The data are expressed as the mean and standard error of the mean IR hormone level per group of 20 animals for three different long-term hypophysectomy experiments.

c. Validation of Hypophysectomy Procedure

The efficiency of hypophysectomy was verified not only by microscopic examination of the sella turcica, but by determination of levels of circulating TSH, T_4, GH, and % T_3 resin uptake, in intact and hypophysectomized rats. Results of these assays are given in Tables 25 and 26, and demonstrate that hypophysectomy could be considered complete for the animals used in these experiments, as levels of circulating pituitary hormones and thyroid hormones all fell to levels indicating complete loss of the pituitary.

N. TARGET ORGAN REMOVAL: EFFECT ON LEVELS OF BRAIN HORMONES

a. Growth Hormone: Effect of Removal of Target Organs

Removal of a target organ may or may not have an effect on levels of pituitary growth hormone. Lacking any one specific target organ, it
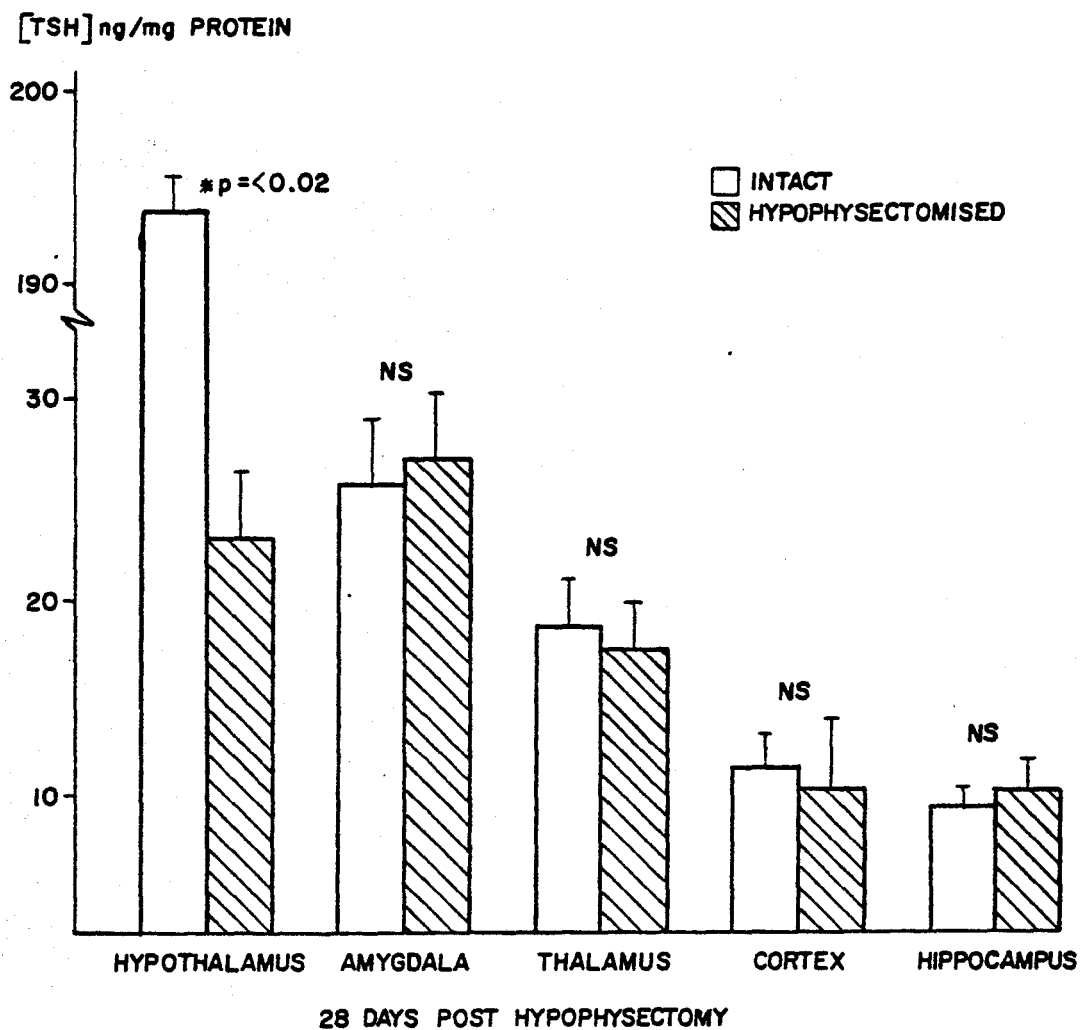
Figure 44. Immunoreactive TSH present in extracts from various brain regions of intact and 28 day hypophysectomised rats.
NS = not significant.

TABLE 25

IMMUNOREACTIVE GH AND TSH LEVELS IN SERUM OF ADULT RATS

(ng/ml)*

GROWTH HORMONE		THYROID STIMULATING HORMONE	
Intact	30 days Hypophysectomized	Intact	30 days Hypophysectomized
(n = 200)	(n = 125)	(n = 390)	(n = 120)
249.86 ± 35.32	20.28 ± 5.80	489.67 ± 52.59	59.79 ± 3.13

TABLE 26

CIRCULATING THYROID HORMONE LEVELS IN SERUM OF ADULT RATS

T_4 μg/dl		% RESIN UPTAKE T_3	
Intact (n = 40)	30-day Hypophysectomized (n = 40)	Intact (n = 40)	30-day Hypophysectomized (n = 40)
64.02 ± 0.4	47.600 ± 2.1	4.5 ± 0.3	0.63 ± 0.01

becomes more difficult to assess the possible effect of organ removal on GH levels. However, the main objective in this series of experiments was to reveal possible differences between brain and pituitary hormonal levels after one or four weeks of target organ removal with respect to any effects in sham-operated controls. The four-week data proved the most consistent throughout all three separate experiments, and results for both TSH and GH are given as the mean obtained from three experiments of 20 animals/group/experiment.

(i) Adrenalectomy

As shown in Fig. 45 , the effect of removal of the adrenal gland on levels of immunoreactive GH in serum, pituitary, hypothalamus, and amygdala was demonstrated to have no significant effect when compared to sham-operated controls. Levels of GH in other brain areas showed a similar lack of change, as given in Table 27, in terms of ng/mg protein.

(ii) Oophorectomy

When the ovaries of female adult rats were removed for four weeks, a significant drop (P = < 0.01) was observed in circulating GH, whereas pituitary levels of hormone remained unchanged (Table 28 and Fig. 46). Contrary to this observation was that shown for the amygdala and hypothalamus, which in all three experiments demonstrated a significant rise (P = < 0.01) in GH levels present in brain tissue as compared to sham-operated controls (Fig. 46). Other brain areas did not show this rise in GH following the removal of ovaries but remained unchanged four weeks postsurgery (Table 28).
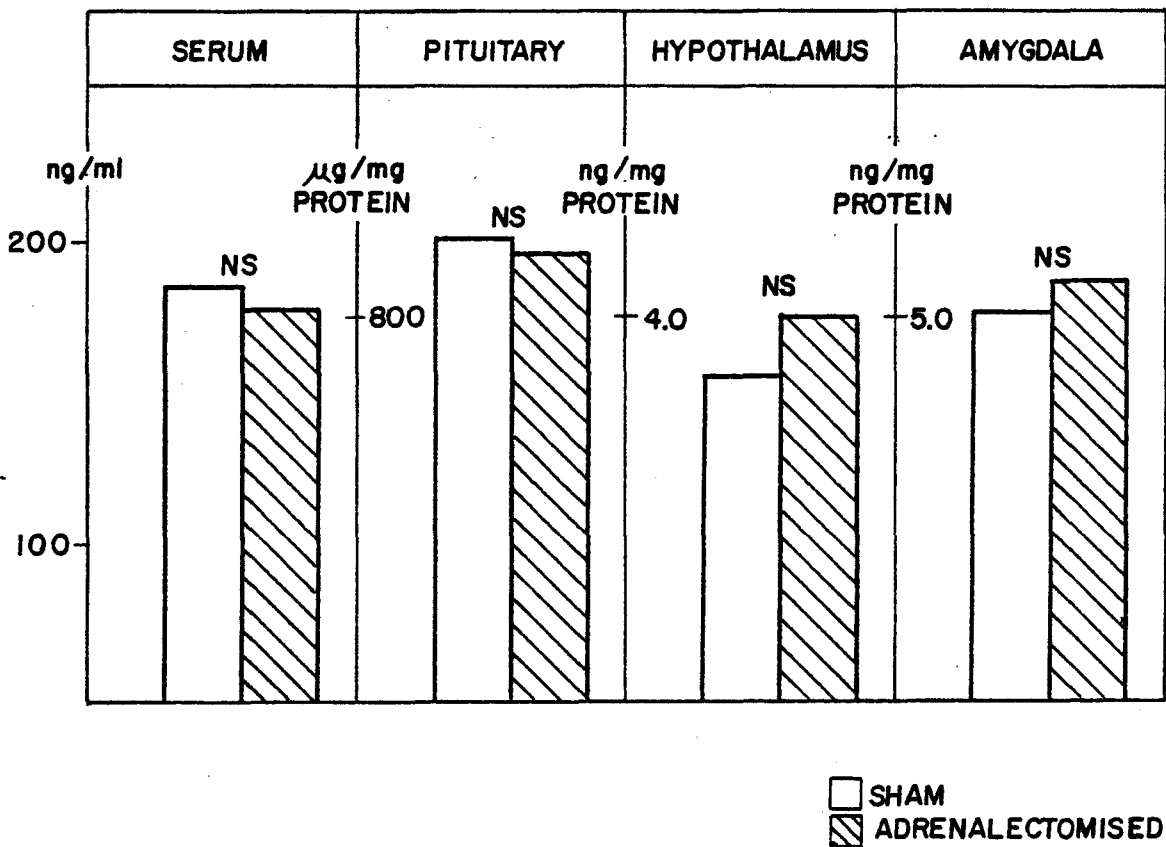
Figure 45. Effects of adrenalectomy on growth hormone
levels in serum, pituitary, and brain.
NS = not significant.

TABLE 27

EFFECT OF ADRENALECTOMY (4 WEEKS) ON BRAIN GH LEVELS

(ng/mg protein)**

	n	Sham	Adrenalectomized	Statistical Significance
AMYGDALA	60	5.1 ± 0.26	5.4 ± 1.23	NS
HYPOTHALAMUS	60	3.2 ± 1.50	4.0 ± 0.50	NS
CAUDATE	60	0.95 ± 0.26	1.10 ± 0.51	NS
THALAMUS	60	1.32 ± 0.37	1.51 ± 0.76	NS
HIPPOCAMPUS	60	1.29 ± 0.08	1.24 ± 0.10	NS
CORTEX	60	1.110 ± 0.24	0.94 ± 0.08	NS
CEREBELLUM	60	0.04 ± 0.01	0.01 ± 0.006	NS
ANTERIOR PITUITARY	60	$1.0 \times 10^6 \pm 0.51$	$0.98 \times 10^6 \pm 0.32$	NS

GH ng/ml **

| SERUM | 60 | 183 ± 33.0 | 179 ± 27.0 | NS |

n = total animals
** = mean ± S. E. M. of 3 experiments

Figure 46. Effects of oophorectomy on growth hormone
levels in serum, pituitary and brain.
NS = not significant.

TABLE 28

EFFECT OF OOPHORECTOMY (4 WEEKS) ON BRAIN GH LEVELS

(ng/mg protein)**

	n	Sham	Adrenalectomized	Statistical Significance
AMYGDALA	60	4.32 ± 1.21	9.21 ± 0.64	p = < 0.01
HYPOTHALAMUS	60	2.81 ± 0.63	5.42 ± 1.21	p = < 0.01
CAUDATE	60	2.09 ± 0.31	1.49 ± 0.52	NS
THALAMUS	60	1.31 ± 0.26	0.94 ± 0.20	NS
HIPPOCAMPUS	60	1.86 ± 0.30	1.44 ± 0.15	NS
CORTEX	60	0.81 ± 0.03	0.95 ± 0.11	NS
CEREBELLUM	60	0.11 ± 0.06	0.3 ± 0.01	NS
ANTERIOR PITUITARY	60	$8.1 \times 10^5 \pm 0.3$	$8.6 \times 10^5 \pm 0.25$	NS
GH ng/ml**				
SERUM	60	267 ± 22.6	68.90 ± 10.5	p = < 0.01

n = total animals

** = mean ± S. E. M. of 3 experiments

(iii) Orchidectomy

Unlike oophorectomy, removal of the testes did not produce any significant change in immunoreactive GH levels in serum, pituitary, or in other brain tissues (Fig. 47 , table 29).

(iv) Thyroidectomy

Four weeks after removal of the thyroid, a significant drop (P = < 0.01) was observed in both serum and pituitary immunoreactive GH levels. Levels of immunoreactive hormone in the hypothalamic and amygdaloid regions showed an opposite effect, that of a significant (P = < 0.01) increase in GH levels in operated animals, as compared to sham controls. This data is illustrated in Fig. 48 . All other brain areas showed no significant change in immunoreactive GH content when compared to sham controls four weeks after thyroidectomy, as shown in table 30.

b. Thyroid Stimulating Hormone: Effect of Removal of Target Organs

(i) Adrenalectomy

As shown in Fig. 49 , four weeks after adrenalectomy, levels of circulating immunoreactive TSH remained constant, whereas a significant fall in pituitary TSH levels was observed (P = < 0.05). Assay of hormone levels in various brain regions showed that only in the hypothalamus was any significant change brought about by removal of the adrenal glands (Table 31).

GH LEVELS 4 WEEKS AFTER ORCHIDECTOMY

Figure 47. Effects of orchidectomy on growth hormone levels in serum, pituitary, and brain. NS = not significant.

TABLE 29

EFFECT OF ORCHIDECTOMY (4 WEEKS) ON BRAIN GH LEVELS

(ng/mg protein)**

	n	Sham	Castrated	Statistical Significance
AMYGDALA	60	6.12 ± 0.35	5.80 ± 0.10	NS
HYPOTHALAMUS	60	3.60 ± 1.20	3.95 ± 0.37	NS
CAUDATE	60	1.29 ± 0.51	1.02 ± 0.81	NS
THALAMUS	60	2.10 ± 0.39	1.95 ± 0.26	NS
HIPPOCAMPUS	60	1.13 ± 0.04	1.42 ± 0.31	NS
CORTEX	60	1.72 ± 0.33	1.75 ± 0.62	NS
CEREBELLUM	60	0.04 ± 0.02	0.06 ± 0.01	NS
ANTERIOR PITUITARY	60	1.86×10^5 ± 0.40	1.72 ± 0.31	NS
GH ng/ml**				
SERUM	60	185.0 ± 22.0	177 ± 33.0	NS

n = total animals
** = mean ± S. E. M. of 3 experiments.
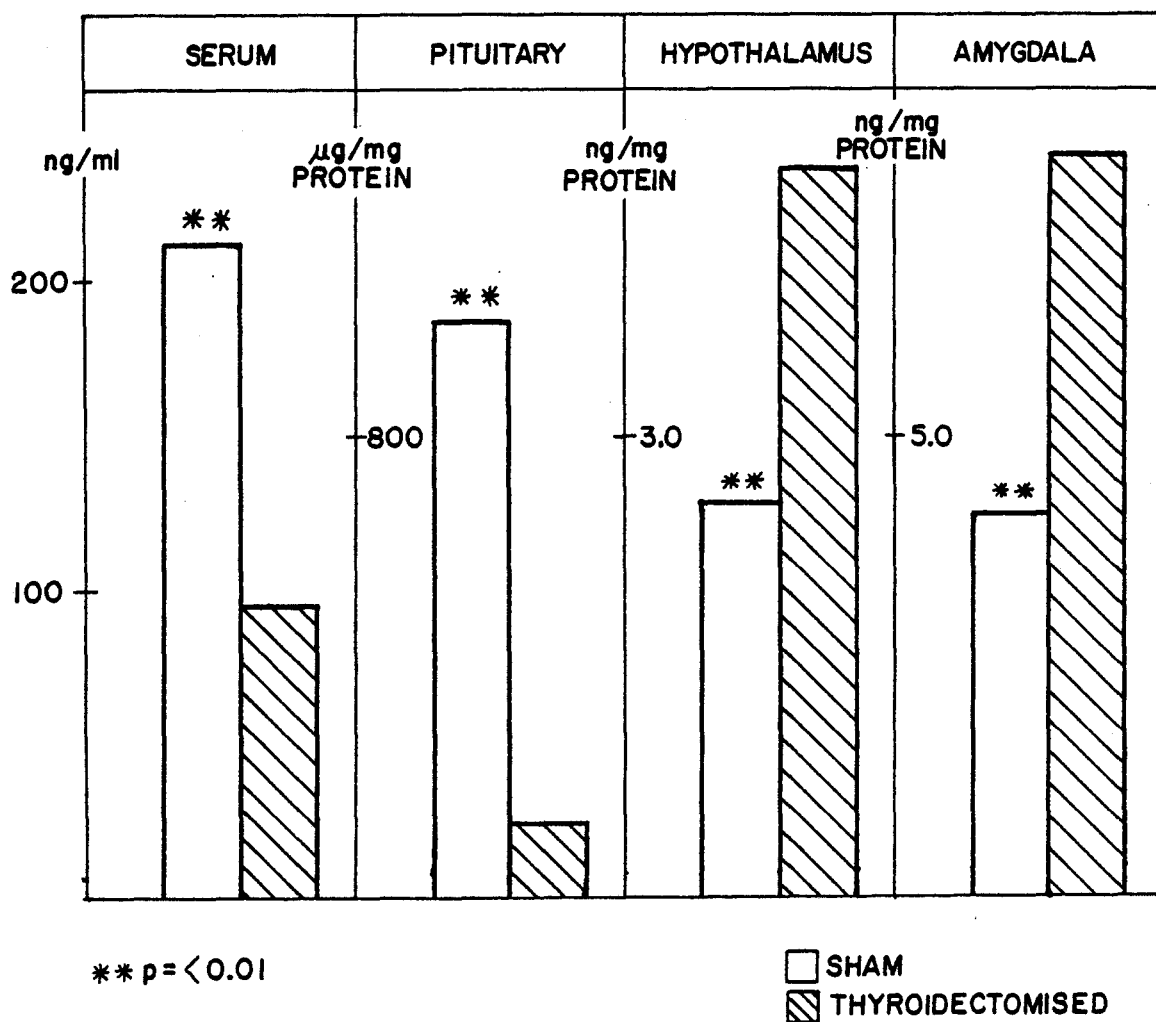
Figure 48. Effect of thyroidectomy on growth hormone
levels in serum, pituitary, and brain.

TABLE 30

EFFECTS OF THYROIDECTOMY (4 WEEKS) ON BRAIN GH LEVELS

(ng/mg protein)**

	n	Sham	Thyroidectomized	Statistical Significance
AMYGDALA	60	4.52 ± 0.63	8.21 ± 0.96	p = < 0.01
HYPOTHALAMUS	60	2.75 ± 0.25	4.82 ± 0.65	p = < 0.01
CAUDATE	60	1.35 ± 0.05	1.39 ± 0.10	NS
THALAMUS	60	1.45 ± 0.12	0.95 ± 0.31	NS
HIPPOCAMPUS	60	1.87 ± 0.31	2.10 ± 0.26	NS
CORTEX	60	1.63 ± 0.20	1.24 ± 0.41	NS
CEREBELLUM	60	0.16 ± 0.05	0.05 ± 0.01	NS
ANTERIOR PITUITARY	60	$9.5 \times 10^5 \pm 0.7$	$1.1 \times 10^5 \pm 0.4$	p = < 0.01

GH ng/ml**

	n	Sham	Thyroidectomized	Statistical Significance
SERUM	60	212 ± 25.0	93.0 ± 10.6	p = < 0.01

n = total animals

** = mean ± S. E. M. of 3 experiments
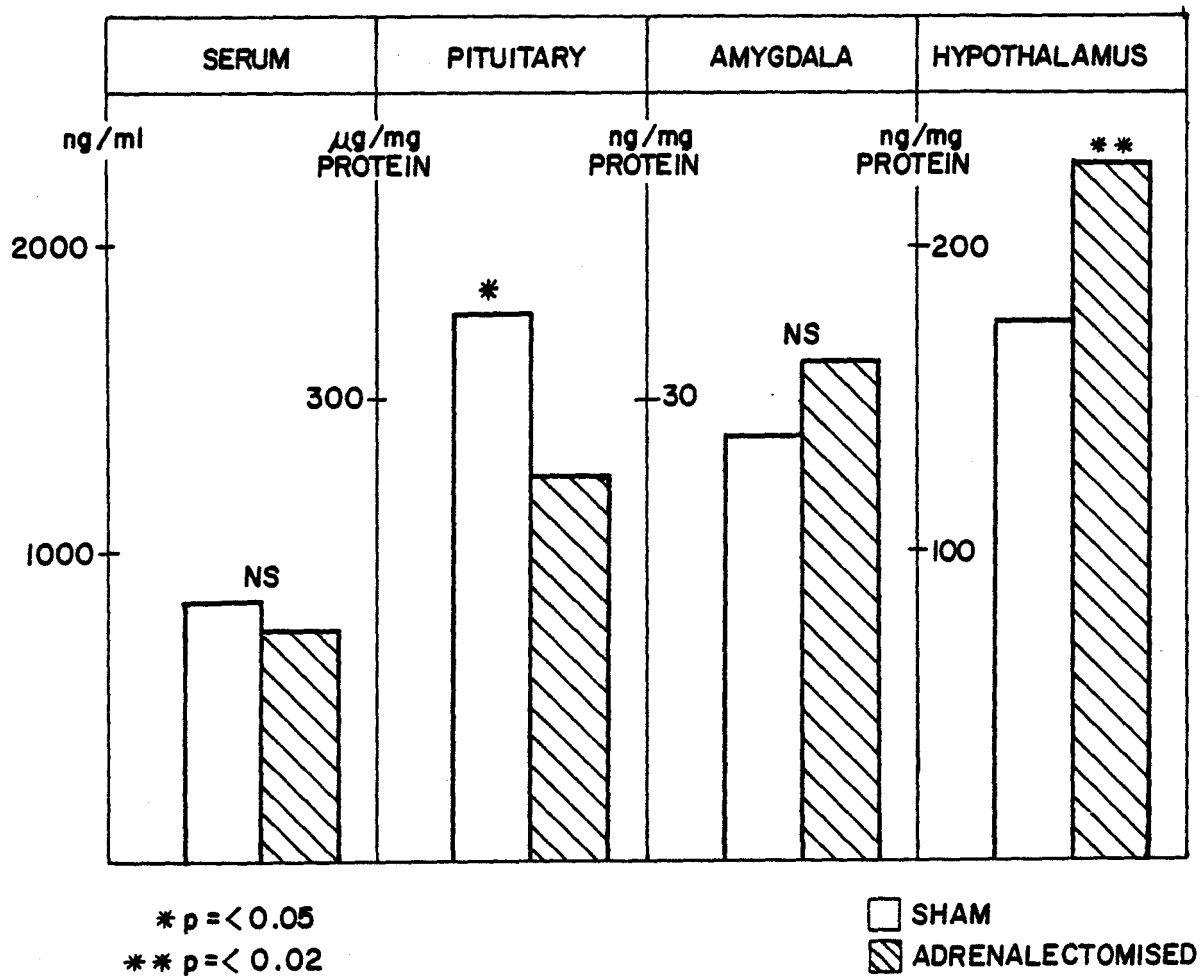
Figure 49. Effect of adrenalectomy on TSH levels in serum, pituitary and brain.
NS = not significant.

TABLE 31

EFFECT OF ADRENALECTOMY (4 WEEKS) ON BRAIN TSH LEVELS

(ng/mg protein)**

	n	Sham	Adrenalectomized	Statistical Significance
HYPOTHALAMUS	60	176.25 ± 5.31	225.65 ± 6.73	p = < .02
AMYGDALA	60	27.52 ± 2.63	32.5 ± 3.79	NS
CEREBELLUM	60	22.26 ± 6.33	20.09 ± 2.10	NS
THALAMUS	60	16.53 ± 0.59	16.75 ± 0.26	NS
CAUDATE	60	11.96 ± 0.73	12.13 ± 0.98	NS
CORTEX	60	14.25 ± 1.76	12.75 ± 0.52	NS
HIPPOCAMPUS	60	10.76 ± 0.92	9.83 ± 1.79	NS
ANTERIOR PITUITARY	60	3.6×10^5 ± 0.26	2.5×10^5 ± 0.50	p = < .05

TSH ng/ml**

	n	Sham	Adrenalectomized	
SERUM	60	826.91 ± 50.75	750.26 ± 75.63	NS

n = total animals

** = mean ± S. E. M. of 3 experiments

As shown in Fig. 49 , a significant (P = < 0.02) rise in immunoreactive

TSH levels occurred in adrenalectomized animals' hypothalamic tissue, as

compared to sham-operated controls.

(ii) Oophorectomy

Removal of the ovaries did not produce any significant change in

TSH immunoreactive hormone levels in serum, pituitary, or brain tissue,

as illustrated by the data obtained in Fig. 50 and Table 32.

(iii) Orchidectomy

Whereas removal of the testes caused a significant fall (P = < 0.05)

in immunoreactive TSH levels in serum and pituitary, a significant rise

(P = < 0.02) was shown for hypothalamic tissue four weeks postoperatively.

(Fig. 51). Other brain regions showed no significant change in TSH

levels (Table 33).

(iv) Thyroidectomy

When immunoreactive TSH was measured four weeks after thyroidectomy,

it was shown that serum levels of the immunoreactivehormone rose dramati-

cally (P = < 0.001) as compared to controls, whereas a significant fall

in pituitary levels (P = < 0.02) was observed (Fig. 52). This fall in

pituitary levels was shown in all three experiments and is contrary to

what would be expected after loss of T_4 production by thyroidectomy.

However, as noted from the literature previously, it is believed that the

turnover of TSH is so great in the pituitary, under these conditions, as

to result in measured low basal levels. Although the hypothalamus showed

TSH LEVELS 4 WEEKS AFTER OOPHORECTOMY
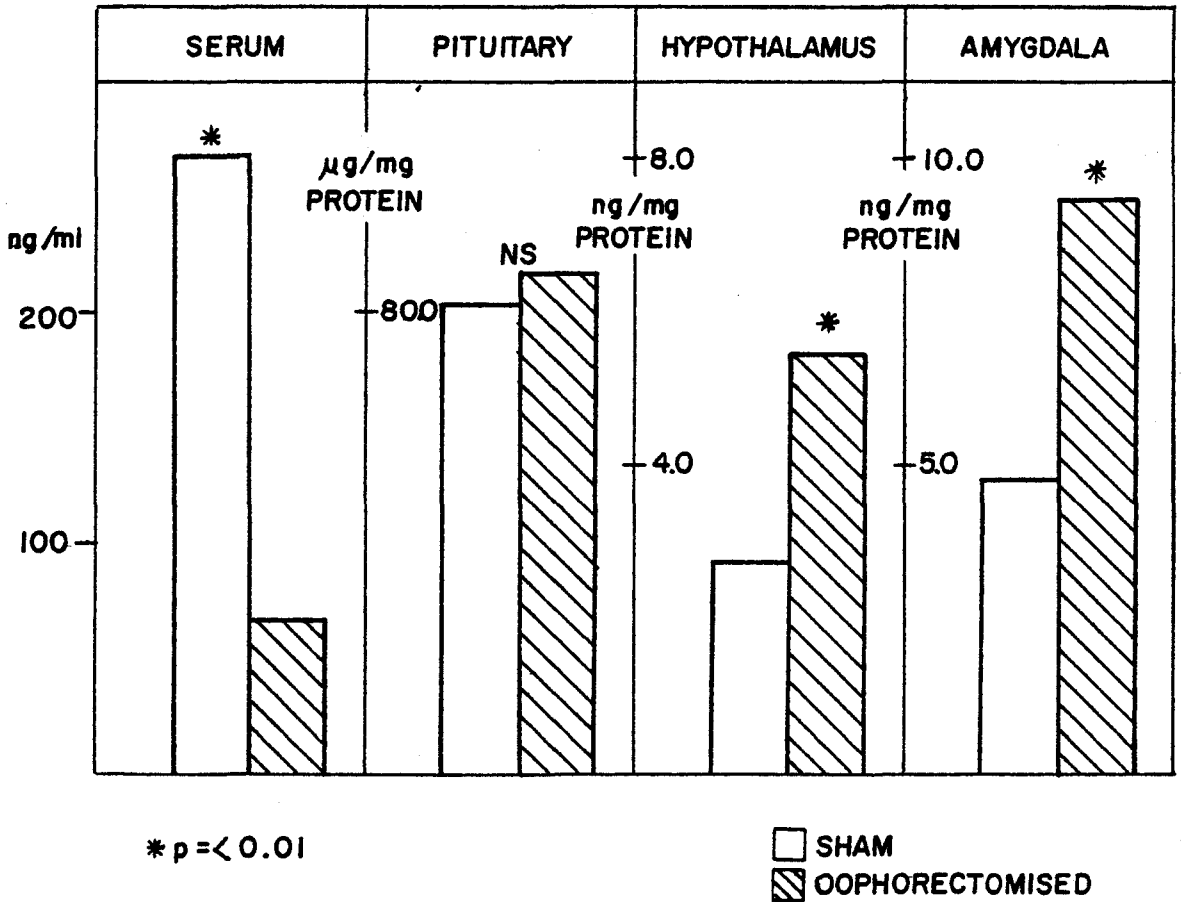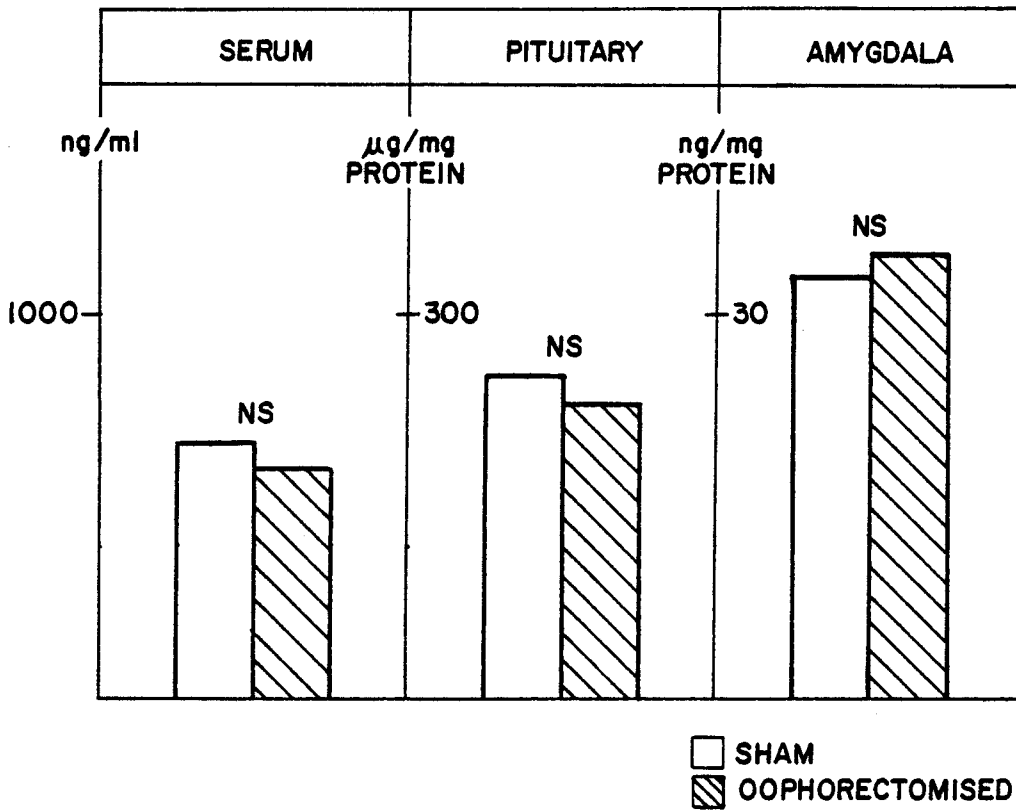
Figure 50. Effect of oophorectomy on TSH levels in serum,
pituitary and brain.
NS = not significant.

TABLE 32

EFFECTS OF OOPHORECTOMY (4 WEEKS) ON BRAIN TSH LEVELS

(ng/mg protein)**

	n	Sham	Oophorectomized	Statistical Significance
HYPOTHALAMUS	60	209.63 ± 5.11	212.90 ± 6.73	NS
AMYGDALA	60	33.96 ± 2.59	35.05 ± 3.72	NS
CEREBELLUM	60	16.22 ± 0.79	17.69 ± 0.95	NS
THALAMUS	60	18.13 ± 2.11	17.98 ± 0.63	NS
CAUDATE	60	12.93 ± 0.89	14.60 ± 0.84	NS
CORTEX	60	12.75 ± 2.30	11.98 ± 0.68	NS
HIPPOCAMPUS	60	11.38 ± 1.62	12.95 ± 0.98	NS
ANTERIOR PITUITARY	60	$2.4 \times 10^5 \pm 0.9$	$2.2 \times 10^5 \pm 0.5$	NS
		TSH ng/ml **		
SERUM	60	640 ± 120.0	602 ± 35.0	NS

n = total animals

** = mean ± S. E. M. of 3 experiments

TSH LEVELS 4 WEEKS AFTER CASTRATION

* p = < 0.05
** p = < 0.02
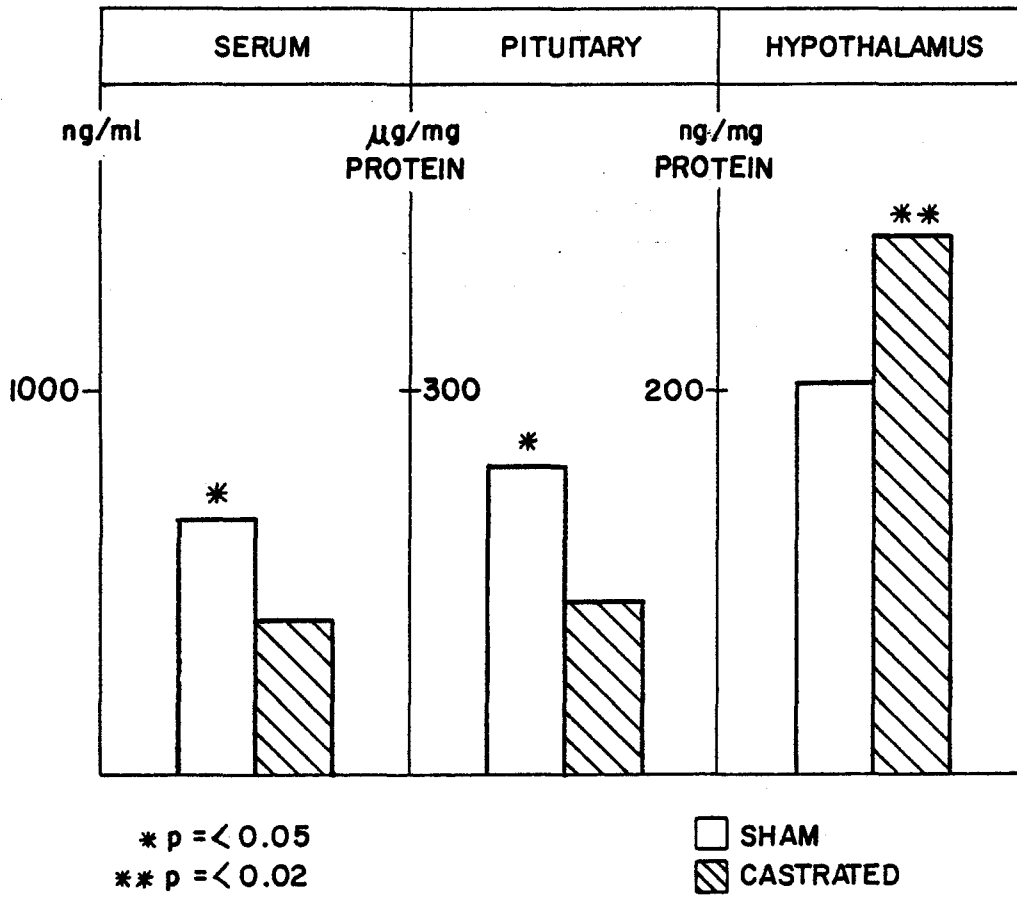
□ SHAM
▨ CASTRATED

Figure 51. Effect of orchidectomy on TSH levels in serum, pituitary and brain.

TABLE 33

EFFECTS OF ORCHIDECTOMY (4 WEEKS) ON BRAIN TSH LEVELS

(ng/mg protein)**

	n	Sham	Castration	Statistical Significance
HYPOTHALAMUS	60	205.61 ± 7.39	280.00 ± 11.65	p = < 0.02
AMYGDALA	60	26.32 ± 4.61	35.92 ± 1.95	NS
CEREBELLUM	60	13.40 ± 0.16	14.24 ± 1.36	NS
THALAMUS	60	12.46 ± 2.51	15.36 ± 0.95	NS
CAUDATE	60	16.39 ± 1.55	13.97 ± 2.60	NS
CORTEX	60	14.61 ± 0.78	12.65 ± 1.20	NS
HIPPOCAMPUS	60	12.59 ± 0.90	12.78 ± 1.10	NS
ANTERIOR PITUITARY	60	2.4×10^5 ± 0.51	1.2×10^5 ± 0.42	p = < 0.05

TSH ng/ml**

	n	Sham	Castration	Statistical Significance
SERUM	60	640.00 ± 79.6	405.00 ± 59.70	p = < 0.05

n = total animals

** = mean ± S. E. M. of 3 experiments
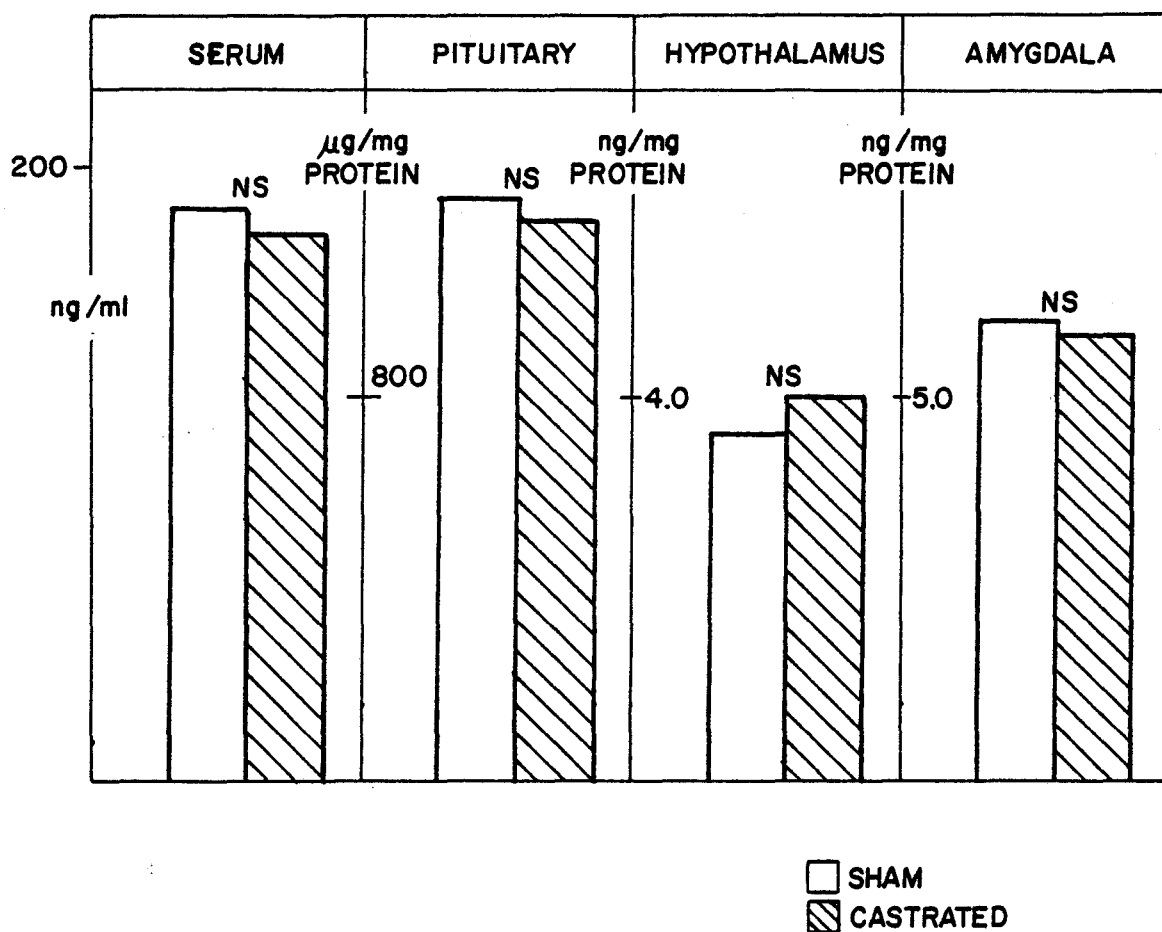
Figure 52. Effect of thyroidectomy on TSH levels in serum, pituitary and brain.
NS = not significant.

TABLE 34

EFFECTS OF THYROIDECTOMY (4 WEEKS) ON BRAIN TSH LEVELS

(ng/mg protein)**

	n	Sham	Thyroidectomized	Statistical Significance
HYPOTHALAMUS	60	158.36 ± 10.63	207.00 ± 21.35	NS
AMYGDALA	60	33.39 ± 1.65	36.89 ± 2.71	NS
CEREBELLUM	60	15.96 ± 1.30	14.63 ± 2.50	NS
THALAMUS	60	18.15 ± 0.45	17.10 ± 2.73	NS
CAUDATE	60	16.72 ± 0.87	15.96 ± 0.55	NS
CORTEX	60	14.10 ± 2.61	16.59 ± 0.71	NS
HIPPOCAMPUS	60	13.78 ± 0.60	12.11 ± 1.24	NS
ANTERIOR PITUITARY	60	3.5×10^5 ± 0.80	1.9×10^5 ± 0.52	p = < 0.02
		TSH ng/ml**		
SERUM	60	462.00 ± 59.60	2226.00 ± 102.3	p = 0.001

n = total animals

** = mean ± S. E. M. of 3 experiments

TABLE 35

SIGNIFICANCE OF CHANGES IN BRAIN GH AFTER HYPOPHYSECTOMY AND TARGET ORGAN REMOVAL (4 WEEKS)

PROCEDURE	Pituitary	Serum	Hypothalamus	Amygdala	Other Brain Regions
HYPOPHYSECTOMY	--	$p = < 0.001$	$p = < 0.01$	$p = < 0.01$	Thalamus: $p = < 0.05$ Others: NS
ADRENALECTOMY	NS	NS	NS	NS	NS
OOPHORECTOMY	NS	$p = < 0.01$	$p = < 0.01$	$p = < 0.01$	NS
ORCHIDECTOMY	NS	NS	NS	NS	NS
THYROIDECTOMY	$p = < 0.01$	$p = < 0.01$	$p = < 0.01$	$p = < 0.01$	NS

TABLE 36

SIGNIFICANCE OF CHANGES IN BRAIN TSH AFTER HYPOPHYSECTOMY AND TARGET ORGAN REMOVAL (4 WEEKS)

PROCEDURE

	Pituitary	Serum	Hypothalamus	Amygdala	Other Brain Regions
HYPOPHYSECTOMY	--	$p = < 0.01$	$p = < 0.02$	NS	NS
ADRENALECTOMY	$p = < 0.05$	NS	$p = < 0.02$	NS	NS
OOPHORECTOMY	NS	NS	NS	NS	NS
ORCHIDECTOMY	$p = < 0.05$	$p = < 0.05$	$p = < 0.02$	NS	NS
THYROIDECTOMY	$p = < 0.02$	$p = < 0.001$	NS	NS	NS
T_4 ADMINIS-TRATION	$p = < 0.002$	$p = < 0.002$	NS	NS	NS

a slight rise in TSH levels, it was not statistically significant, as was the case for all other brain areas, as shown in Table 34. Levels of circulating T_4 fell from 3.42 to 0.02 µg/dl, while the % T_3 resin uptake remained constant in thyroidectomized animals.

The significance of the changes discussed in GH and TSH after hypophysectomy and target organ removal are shown in Tables 35 and 36, respectively.

O. THYROXINE LEVELS: EFFECT OF AN INCREASE IN CIRCULATING T_4 ON SERUM AND TISSUE LEVELS OF TSH AND T_4

The effect of injecting IM, 0.4 µg T_4/gm body weight, daily for 7 days into intact adult rats on TSH levels in serum, pituitary, and amygdaloid tissue, is illustrated in Fig. 53 . Whereas approximately 200 µgm T_4 caused a dramatic, significant (P = < 0.002) fall in circulating TSH (400 → 58 ng/ml) and pituitary TSH (300 → 50 µg/mg protein), presumably by a "negative feedback" effect, no significant change in TSH levels was seen in amygdaloid or other brain tissues, including the hypothalamus. Circulating T_4's were increased from 4.70 → 17.42 µg/dl in treated animals, whereas the % T_3 resin uptake remained constant. In hypophysectomized animals, however, the effect of a similar dose of T_4 on brain levels of TSH was to cause a statistically significant (P = < 0.05) rise in the levels of IRTSH in the hypothalamus as compared to saline-injected hypophysectomized animals. T_4 levels in such animals rose from 0.8 → 24 µgm/dl, and similarly % T_3 uptake also rose from 48.5 → 70.229. Other brain regions showed no change in immunoreactive TSH levels after thyroxine treatment.
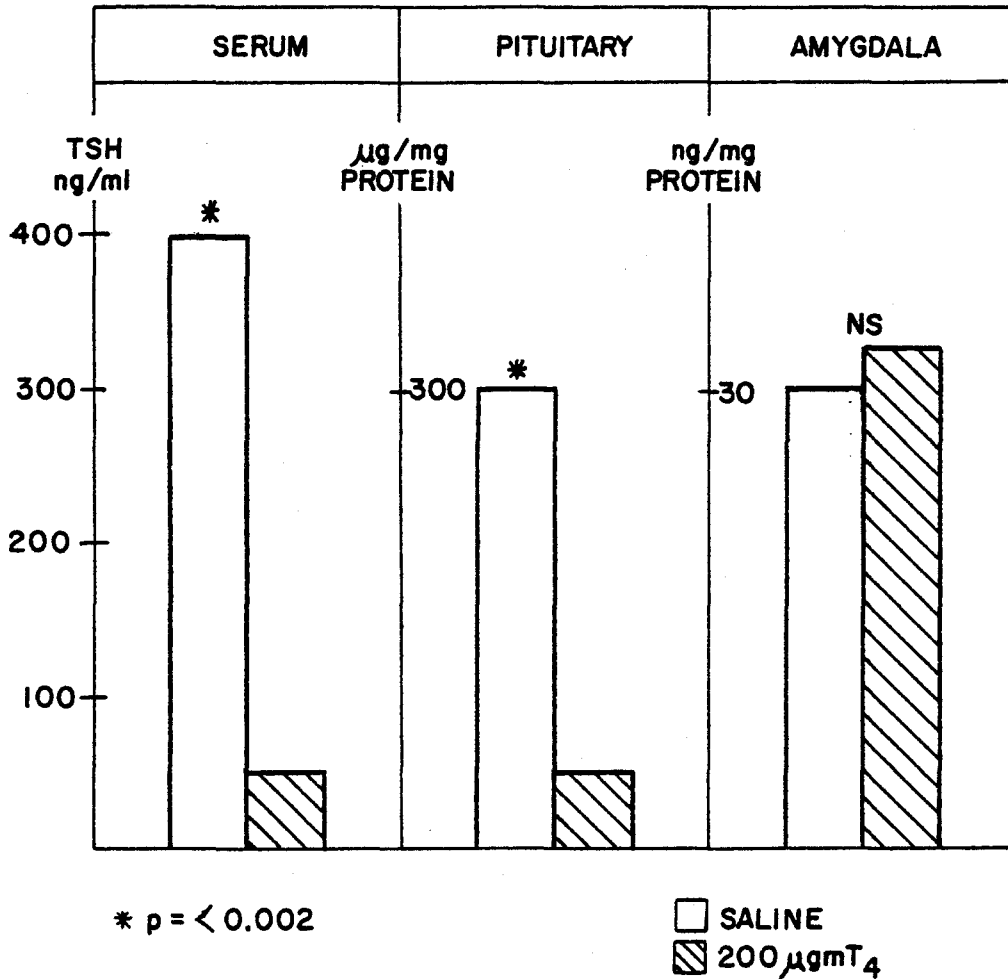
Figure 53. The effect of injecting IM. 0.4µg T_4/gm body weight daily for 7 days into intact adult rats, on TSH levels in serum, pituitary and amygdala. NS = not significant.

P. ONTOGENY OF PITUITARY AND BRAIN PITUITARY-LIKE HORMONES

In order to examine the levels of hormone at various stages of de-
velopment, rat fetuses from ten days to birth and pups from birth to
30-day-old adults were sacrificed and brain homogenates analyzed for
pituitary hormonal content by RIA.

a. Growth Hormone

(i) Growth hormone in brain and pituitary tissues

In Fig. 54 , levels of immunoreactive growth hormone, expressed as
ng hormone/mg protein, in terms of fetal and neonatal age of the animal,
are shown. This data demonstrated the appearance of immunoreactive hor-
mone at fetal day 10 and a remarkable rise in the level of detectable
hormone extracted from the hypothalamus and amygdala just prior to birth,
or day 21 of gestation in the rat. Postpartum, hormone concentration in
these brain regions fell abruptly and then rose, gradually attaining adult
levels at around 30 days of age, essentially at the time of puberty. All
other brain parts--thalamus, caudate, and cortex--showed a similar, al-
though not so pronounced, preparturitional rise over adult levels of growth
hormone. The hippocampus showed the most dramatic preparturitional in-
crease of all brain regions compared to adult levels.

Levels of pituitary growth hormone were also measured over the same
age span, as shown in Fig. 55 . Contrasting to the finding of pituitary-
like hormones in the rodent brain as early as fetal day 10, growth hor-
mone was not detectable until day 12 of fetal life in the pituitary anlage
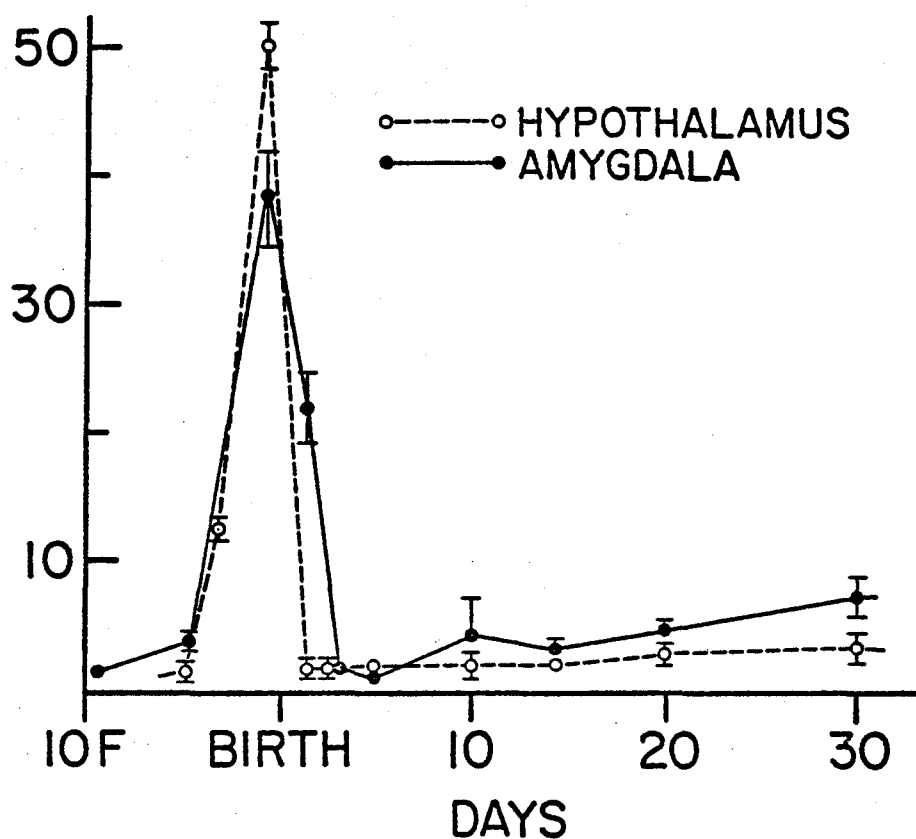
Figure 54. The ontogenetic development of growth hormone levels in the CNS from fetal to adulthood in the rat.

PITUITARY

GROWTH HORMONE
ng/mg PROTEIN

10^6

10^4

10^2

10 F   BIRTH   10   20   30

DAYS
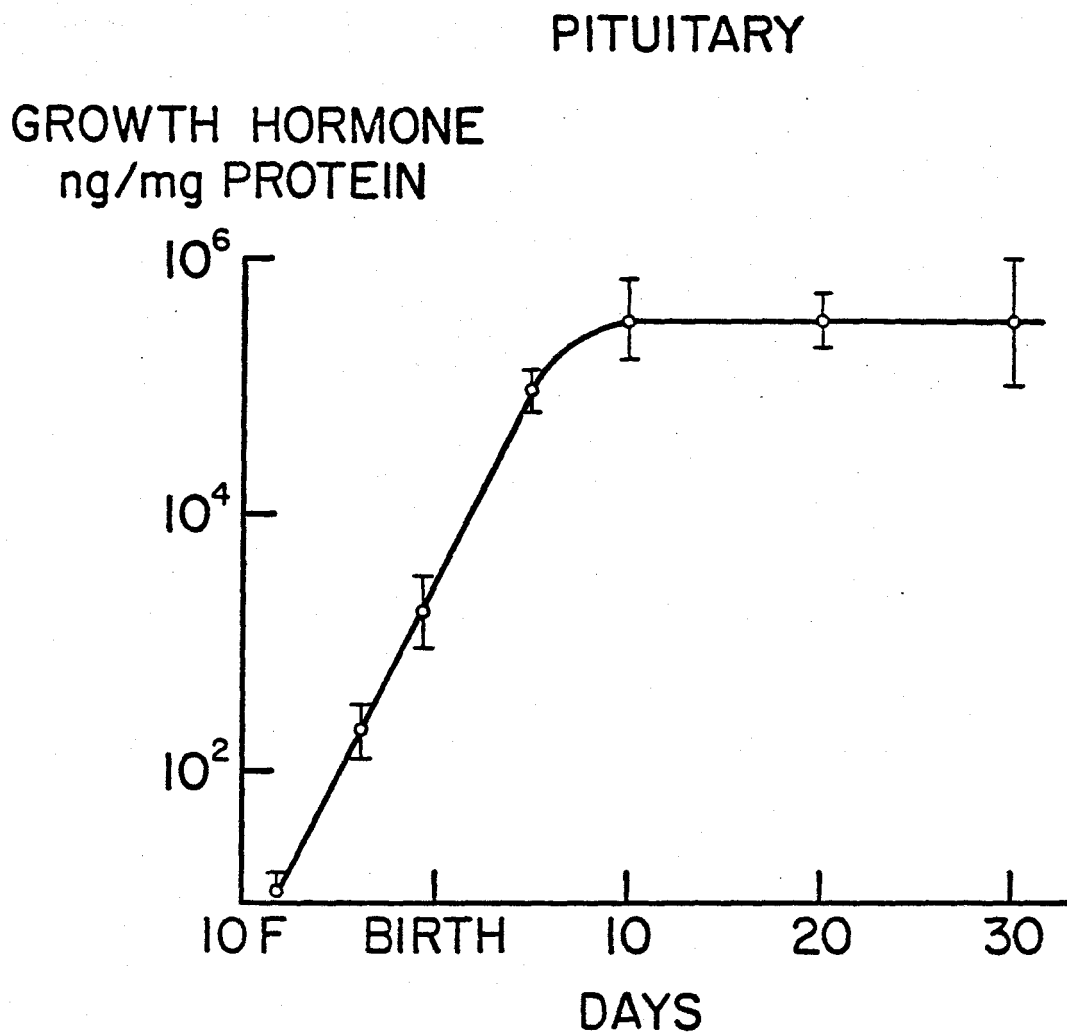
Figure 55. The ontogenetic development of growth hormone
in the pituitary from fetal to adulthood in
the rat.
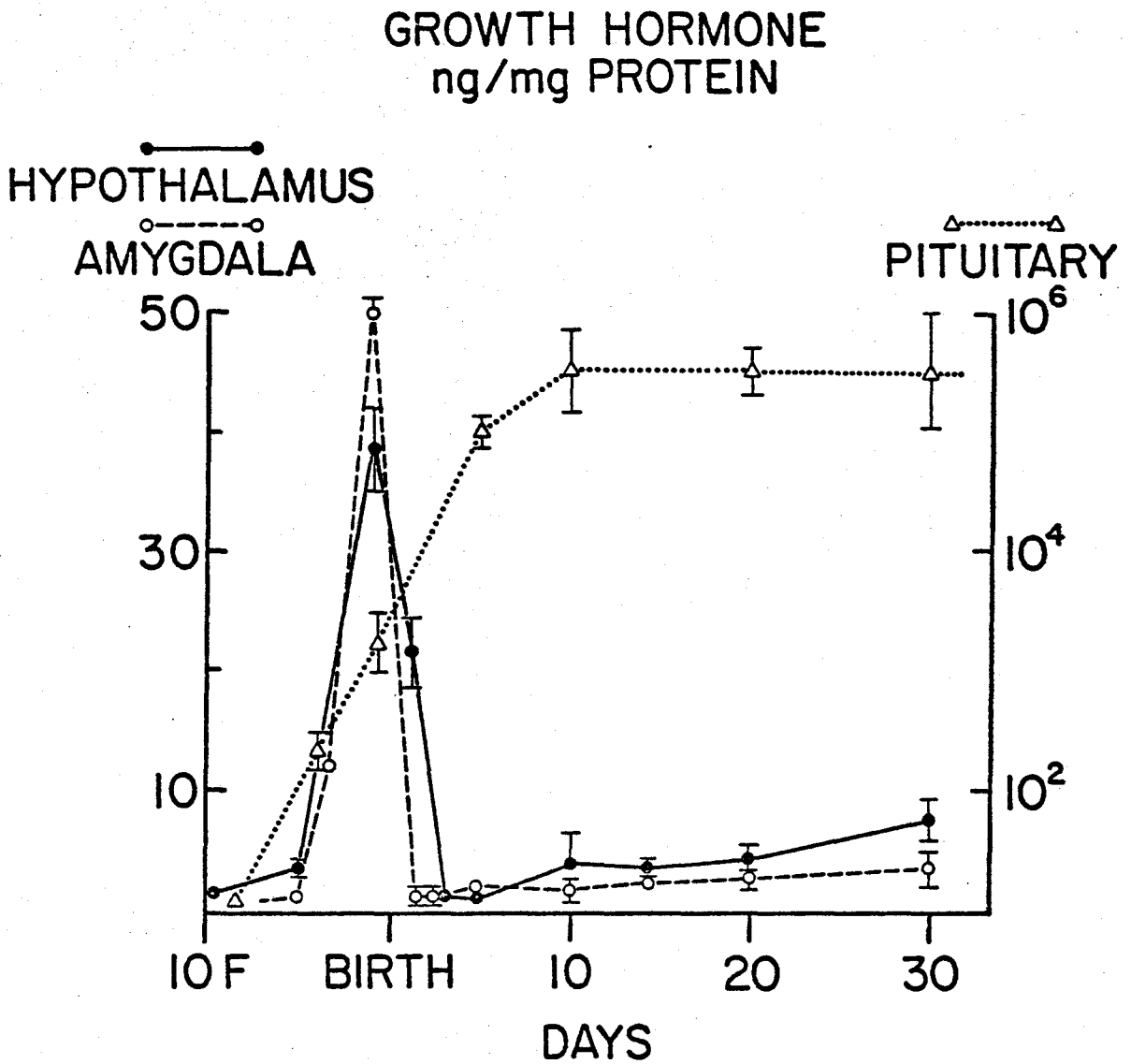
Figure 56. The ontogenetic development of growth hormone levels in the pituitary and CNS from fetal to adulthood in the rat.

and showed no preparturitional surge, but rather a gradual rise toward adult levels between 10 and 20 days after birth. These two sets of data can be superimposed, as shown in Fig. 56 , to show the relationship which exists between the hormonal levels in brain versus pituitary tissue.

(ii) Growth hormone in fetal and maternal sera and amniotic fluid

Levels of GH, in terms of ng of hormone/ml, of amniotic fluid, fetal sera, and maternal sera were also measured at day 17, 19 and 21 days gestation, and for several days postpartum in the case of sera. As shown in Fig. 57 , amniotic fluid levels continued to rise steadily until parturition, and levels of fetal serum growth hormone also rose slowly until the first day after delivery and then fell to adult levels. At day 21, immunoreactive growth hormone levels were substantially higher in fetal serum than in maternal serum, which essentially showed no change throughout pregnancy and after delivery. Levels of extracted placental immunoreactive growth hormone were also measured and remained constant and at levels approximating those of maternal serum.

b. Thyroid Stimulating Hormone

(i) Thyroid stimulating hormone in brain and pituitary tissue

A similar pattern, in terms of development, was observed when brain TSH was measured in 10-day fetal and up to 30-day old adult rat brain tissue. All brain regions containing this material showed a dramatic preparturitional rise and abrupt fall just after birth. Fig. 58 shows this phenomenon for the hypothalamus and amygdala, in terms of ng immunoreactive TSH/mg protein. When pituitary, immunoreactive TSH levels,
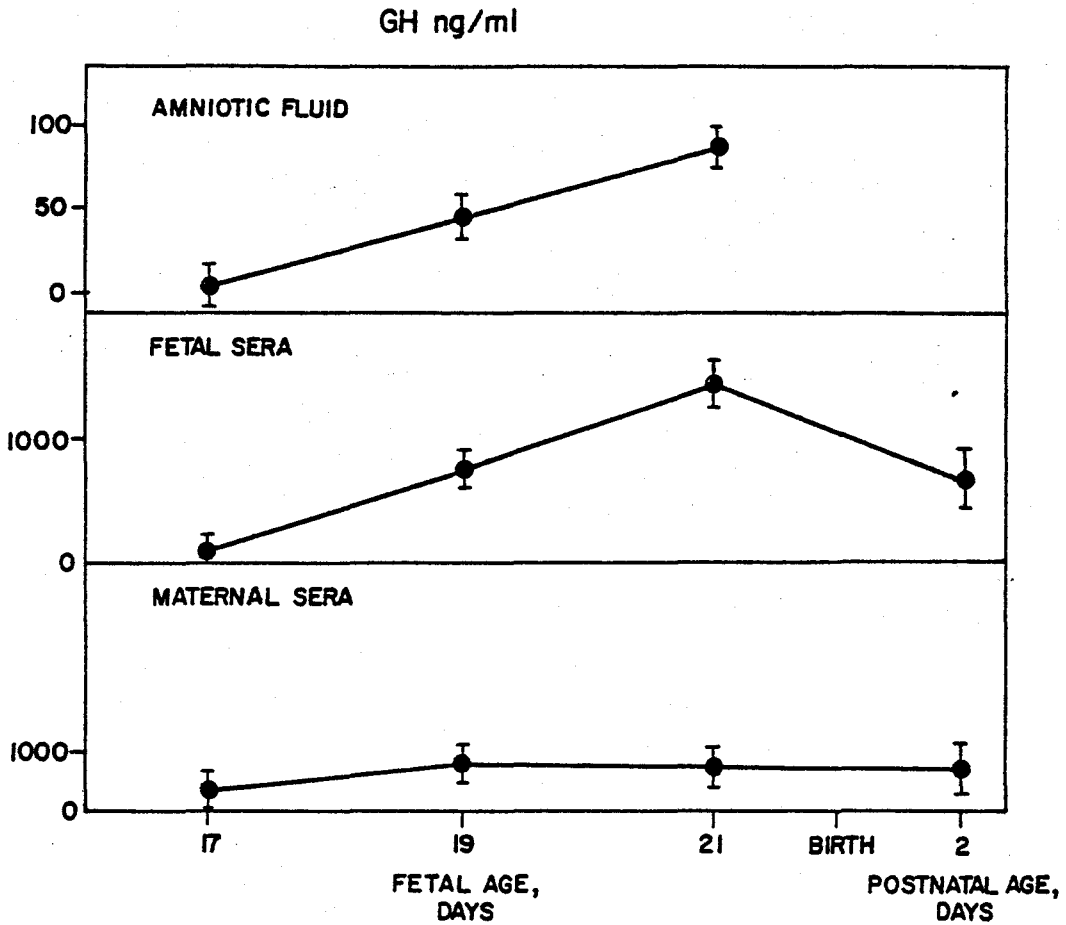
Figure 57. Levels of growth hormone (ng/ml) in amniotic fluid, fetal serum, and maternal serum from 17 days fetal to 2 days postnatal in the rat.

determined over the same time period, are superimposed on this data, as in Fig. 59 , the relationship between the development of hormonal levels in these two distinct tissues can be seen. Here again, TSH could be measured by day 10 of fetal life in the brain tissue extracts, whereas immunoassayable TSH was detectable by day 15-17 in the fetal pituitary.

(ii) Thyroid stimulating hormone in fetal and maternal sera and amniotic fluid

Unlike growth hormone, TSH amniotic fluid levels showed a fall before birth, whereas fetal serum levels remained constant and then dropped quickly after delivery. No change in maternal TSH serum levels was noted during the entire study period as shown in Fig. 60 . Placental TSH levels remained constant throughout gestation, levels of hormone corresponding to those found in maternal sera.

c. Summary of Fetal Brain Levels of Immunoreactive GH and TSH Data

In Table 37, GH and TSH levels in various regions of the 21-day fetal CNS were compared in terms of the percent rise above levels normally found in the adult rat brain. The hippocampus, hypothalamus, and amygdaloid regions showed the greatest preparturitional surge in pituitary-like hormones.

d. Immunological Characterization of Fetal Brain Hormones

When the immunological characteristics of fetal brain growth hormone were compared with those from fetal pituitary homogenates, parallel
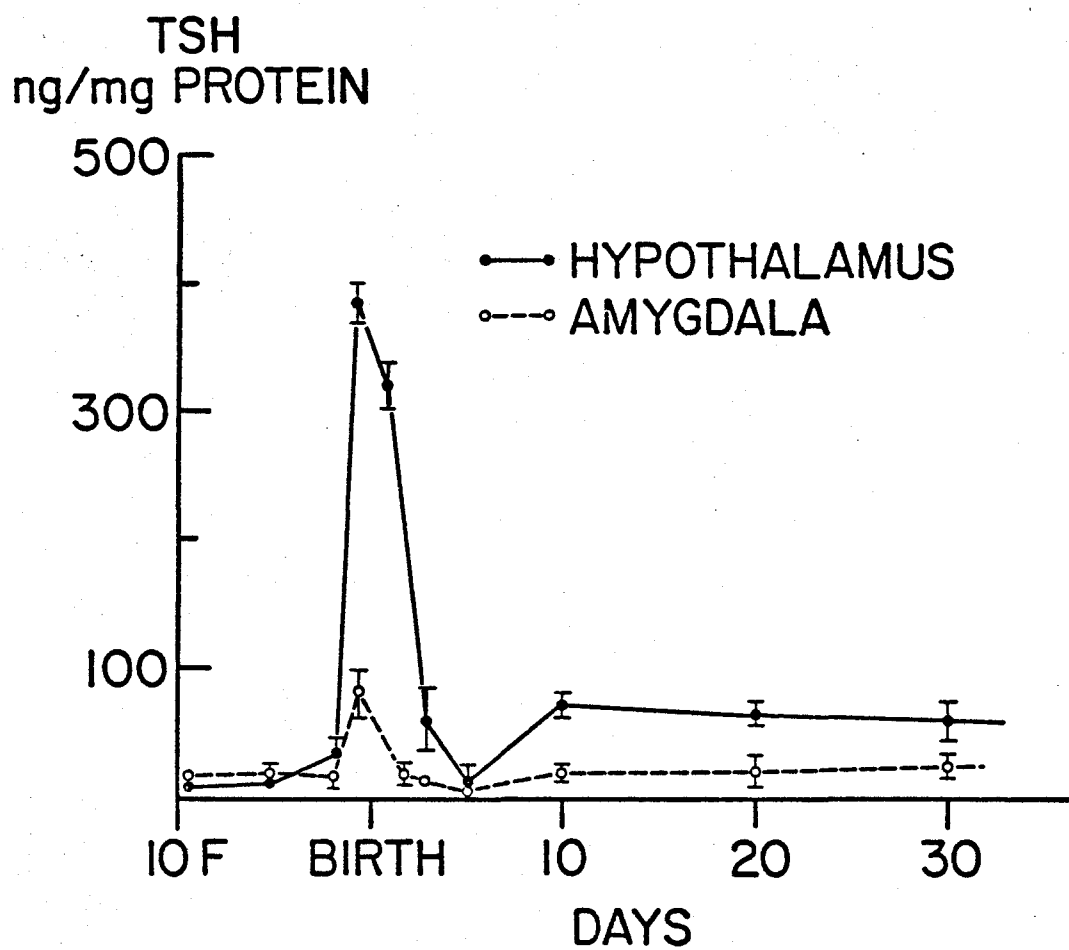
Figure 58.   The ontogenetic development of TSH levels in
             the CNS from fetal to adulthood in the rat.
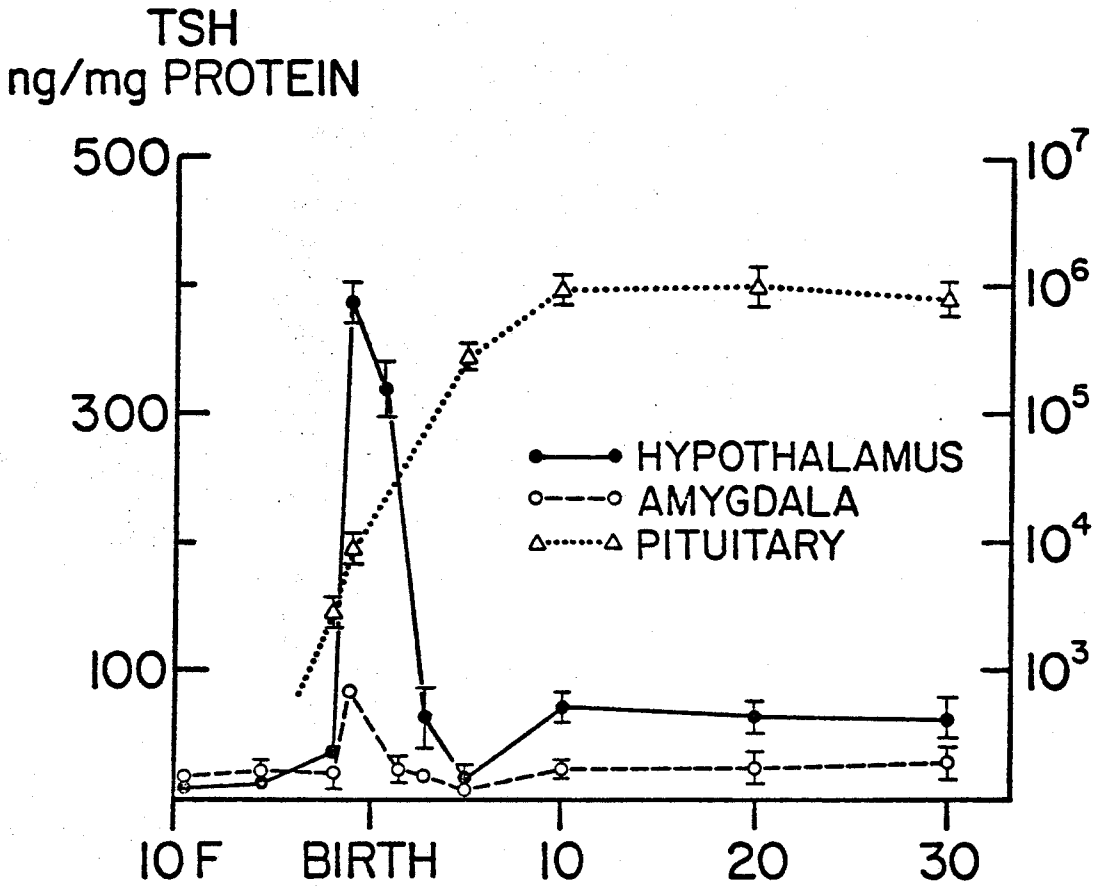
Figure 59. The ontogenetic development of TSH levels in the pituitary and CNS from fetal to adulthood in the rat
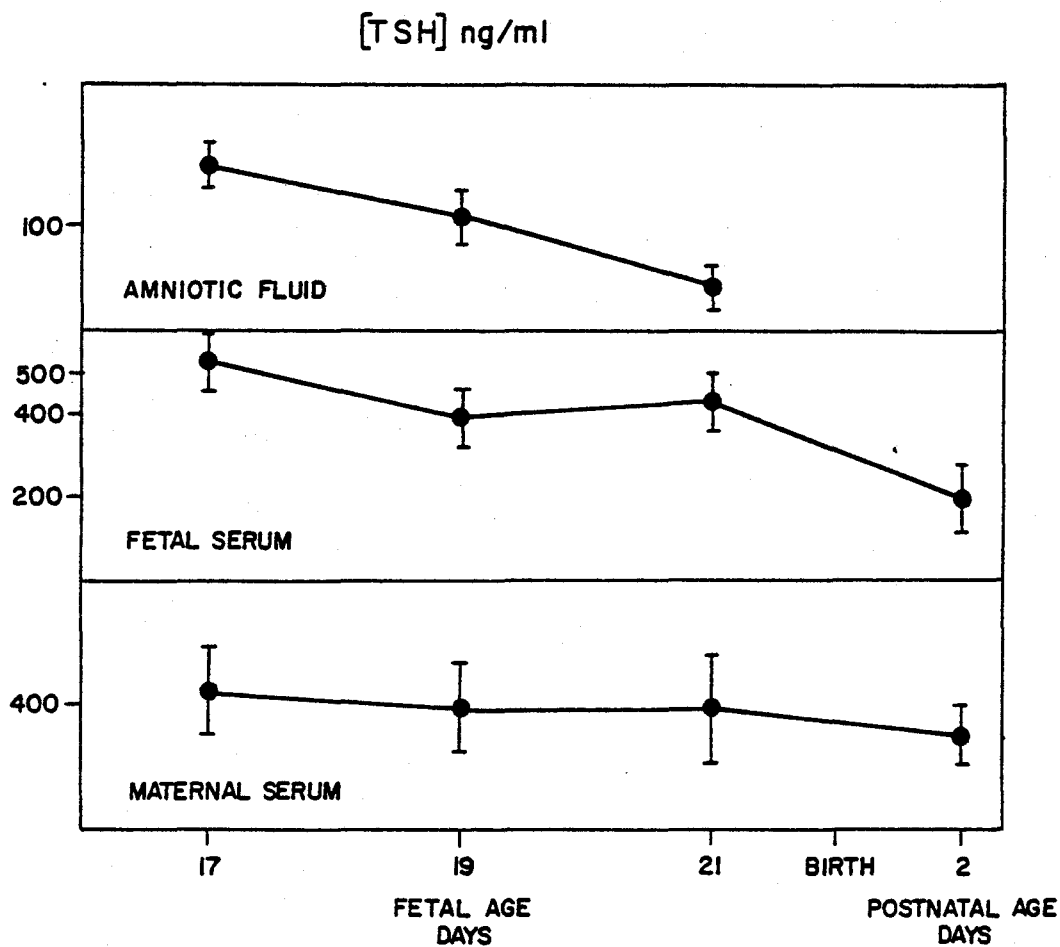
Figure 60. Levels of TSH in amniotic fluid, fetal serum,
and maternal serum from 17 days fetal to 2 days
postnatal in the rat.

TABLE 37

GH- AND TSH-LIKE IMMUNOREACTIVE PEPTIDES

IN THE 21-DAY FETAL CNS

	% ABOVE ADULT LEVELS	
	GH	TSH
Hippocampus	2000	1000
Hypothalamus	300	1000
Amygdala	600	600
Cerebellum	10	300
Cortex	800	100
Caudate	15	10

dose-response displacement curves were obtained as shown in Fig. 61 .

Fetal TSH from brain tissue showed similar parallelism with pituitary TSH. This indicated immunological similarity between the hormones from the two different sources. Although the brain curve was in fact superimposable on the pituitary curve, it had been displaced downward for visual effect.

e. Molecular Weight Determinations of Fetal Brain Hormones

The possibility of heterogenous forms of these brain peptides occurring in brain and pituitary tissue of fetuses and adult rats was also examined. As shown in Fig. 62 , using a G-100 Sephadex column, 22-day-old fetal brain extracts were co-chromatographed with ^{125}I labeled adult rat pituitary GH. It can be seen that the peak of immunoreactive GH-like material co-eluted with the radioactive marker.

In Fig. 63 , a similar column chromatography experiment demonstrated the co-elution of immunoreactive TSH-like material from fetal CNS extracts with ^{125}I TSH from adult pituitaries, suggesting molecular similarity between fetal and adult brain pituitary-like hormones.
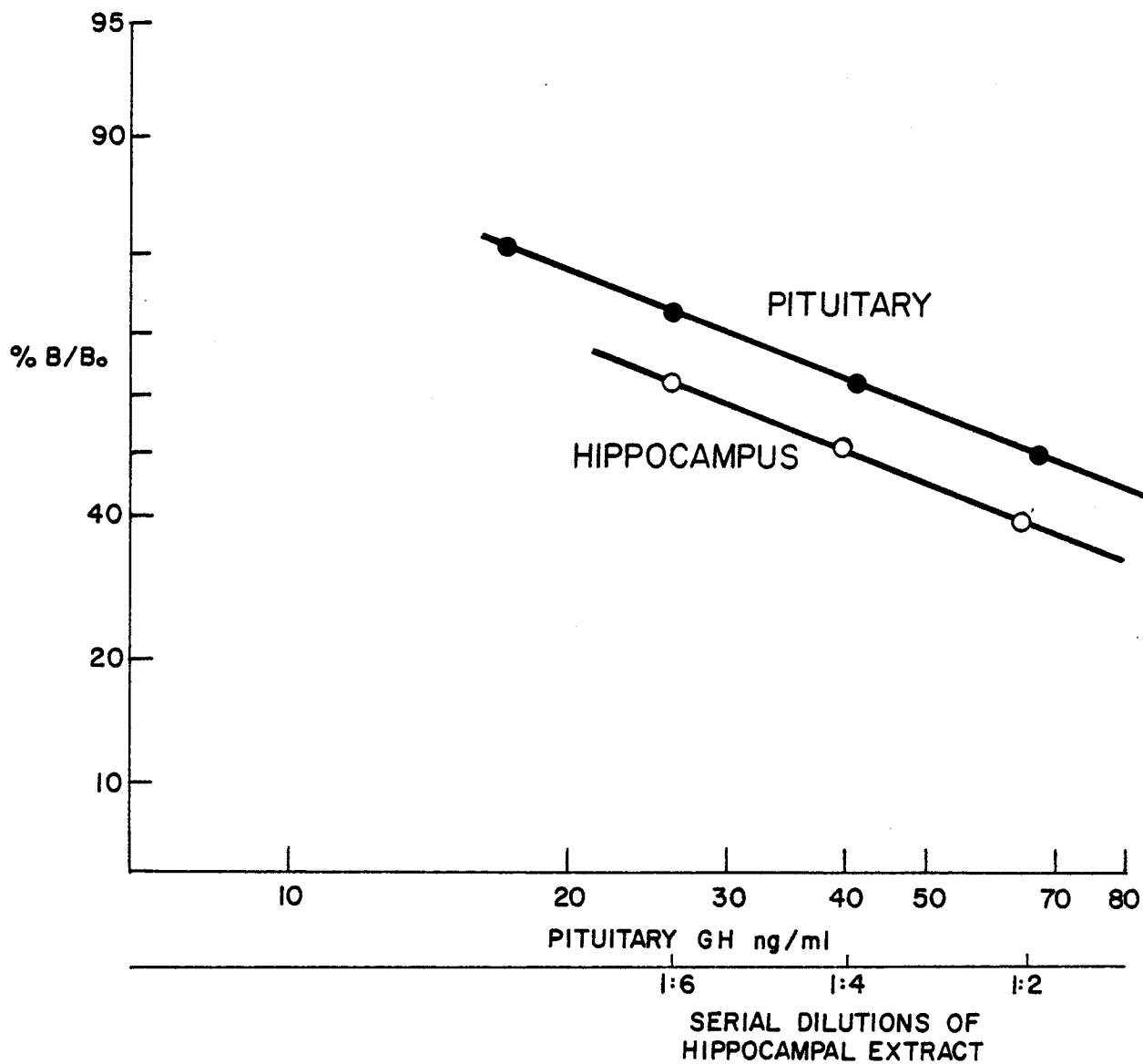
Figure 61. Logit log plot demonstrates parallelism
between pituitary and fetal CNS growth
hormone.
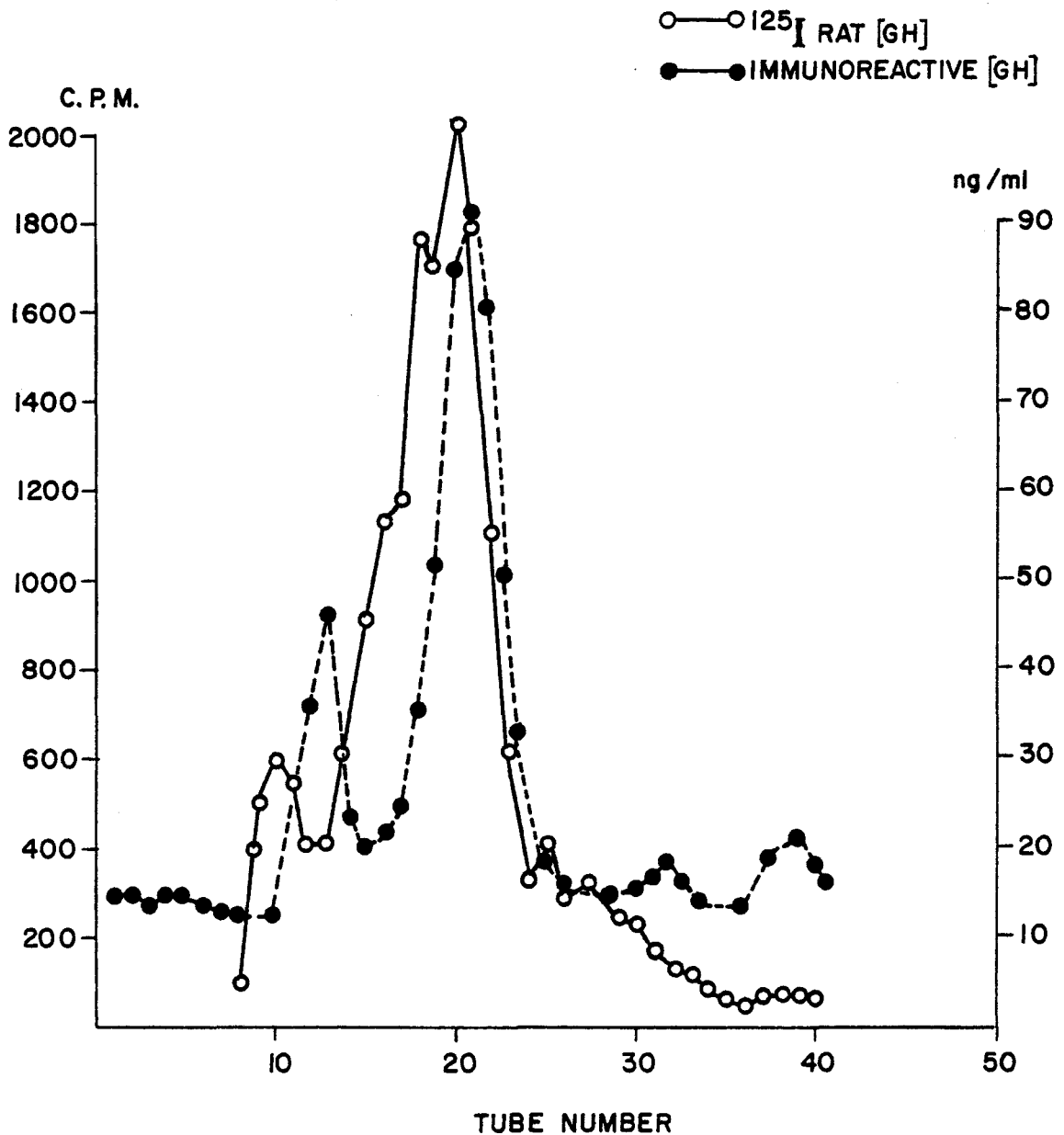
Figure 62. Elution pattern of lyophilised 21 day old fetal brain and ^{125}I adult rat pituitary growth hormone on a Sephadex G-100 column eluted with veronal buffer pH 8.6.
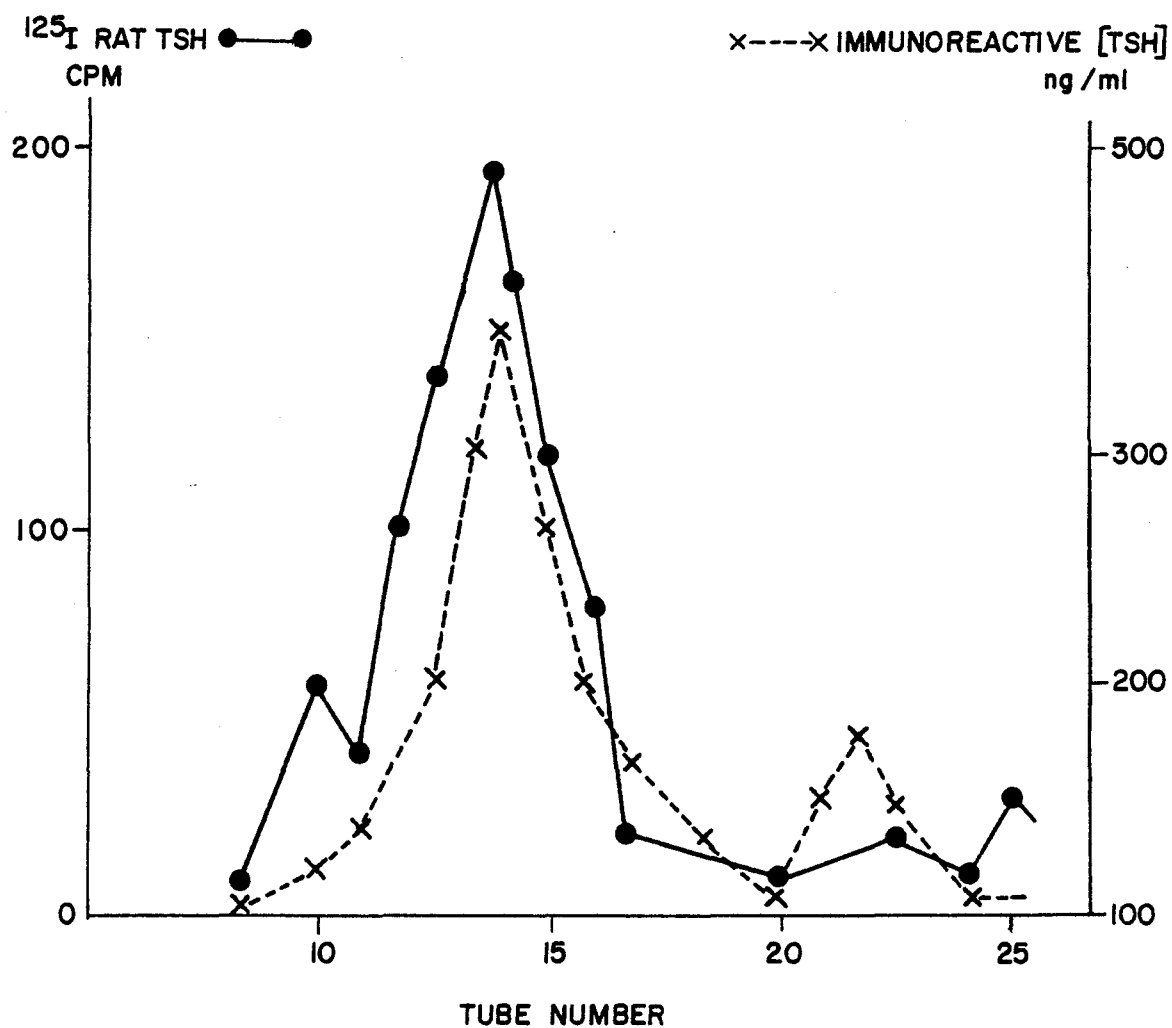
Figure 63. Elution pattern of lyophilised 21 day old fetal brain and ^{125}I adult rat pituitary TSH on a Sephadex G-100 column eluted with 0.01M PO_4 buffer pH 7.6.

6. DISCUSSION OF DATA

Initially prompted by the observation of the presence of immuno-
reactive growth hormone-like material in the media of cultured rat ner-
vous tissue reported by Pacold et al (1976), this study has attempted to
characterize, in various ways, this growth hormone-like material. In
addition, we have sought to characterize a TSH-like material, also found
in the tissue culture medium of cultured rat nervous tissue, and discov-
ered in the course of these original studies. The relevance of the data
obtained from these investigations will be discussed in the light of other
recent observations also dealing with the discovery of diverse peptides
in the central nervous system, a phenomenon only recently appreciated.
Previously, monoaminergic substances were considered the only chemical
neurotransmitters, whereas it now seems abundantly likely that peptides
of neuronal origin from cells within the CNS also have neurotransmitter
roles. In addition, new and unusual roles may be assigned which could
include effects on brain development and growth, perception of pain,
normal and disturbed behavior, and even effects on intelligence to mention
but a few possible roles for these recently discovered brain peptides.

The finding of such peptides and the speculation that they may play
a physiological role in neural function is a relatively recent sugges-
tion by de Wied (1980) and is now supported by several lines of evidence,
such as the induction of lordosis by intracerebral injection of lutein-
izing-releasing hormone in female rats (Foreman, 1977). Observations
such as these have led to the concept that many peptides, whatever

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their traditional tissue of origin, exert effects upon the brain which lead to behavioral consequences apart from their classical endocrine effects.

The main objectives of this particular study concerning the presence of two such peptides, GH and TSH, traditionally of pituitary origin, in rodent and also in primate brain tissue, is to show that these hormones are probably of CNS origin rather than diffusion contaminants from the pituitary, that their presence is largely independent of pituitary function, and finally to speculate on the possible reasons for their presence in the brain.

Initial experiments were concerned with the systematic extraction and mapping of different areas of the rodent and primate brain. Both a growth hormone-like and thyroid stimulating-like material were found, by radioimmunoassay, to be unevenly distributed throughout the central nervous system of both rat and primate. These data immediately suggested a CNS origin rather than a pituitary origin for such extractable hormone-like materials, as an overall uniform distribution of hormone concentration throughout the CNS would have been indicative of contamination from circulating pituitary hormones.

Immunoreactive growth hormone-like material was found in greatest concentrations, in terms of ng hormone/mg protein in the amygdaloid nuclear region of the rat CNS by tissue extraction experiments. The rat hypothalamus was also found to contain substantial amounts of such immunoreactive material.

Extractable immunoreactive TSH-like hormone was also detectable in both rodent and primate brain tissue. The rodent hypothalamic region was found to contain the largest amount of detectable material, and a TSH-like substance was also present in the amygdaloid nucleus and other brain regions to a lesser extent. Large differences between pituitary and brain levels of both hormones were observed, even though these two tissues underwent similar extraction procedures. Several explanations for such wide variations can be envisaged, such as the possibility of differences in the rates of peptide synthesis in each tissue, and the biological half-lives of hormones of pituitary and brain origin. Another possibility for widely variant concentrations could be that differences in tissue extractable levels of hormone are due to the types of precursors present and the subsequent post-translational processing of such precursors. De Wied (1980), for example, when discussing the two apparent pools of hypothalamic and pituitary ACTH, suggests that such processing of brain peptides may give rise to fragments of hormone which, although having biologically significant effects on the surrounding CNS milieu, are not recognizable by either immunological or biological assays. Similar arguments could be made to speculate on the apparent differences in the magnitude found between growth hormone and thyroid stimulating hormone-like concentrations in brain and pituitary pools.

Preliminary studies showed that, in the case of both immunoreactive GH- and TSH-like material in primate brains, the hypothalamus exhibited the highest concentrations of these particular CNS peptides. Although growth hormone was found in most other areas of the primate brain, the

distribution of TSH was such that large areas of the primate CNS appeared
to contain non-measurable quantities.

Further speculation on this data from primate CNS will have to
await further in-depth investigation. Its measured presence, however,
does indicate that certain pituitary-like peptides apparently are not
restricted to the rodent brain.

Mapping locations of GH- and TSH-like peptides in the rodent central
nervous system, using immunohistochemical techniques, demonstrated the
presence of TSH in various areas of the central nervous system. In the
hypothalamus, TSH was distinguishable in cell bodies of the dorsomedial
and ventromedial nuclei, and most of the nuclei of the amygdala showed
positive staining for the presence of TSH. Other areas of the CNS shown
to have extractable hormone, but which did not show positive staining by
the immunohistochemical technique, are known to be innervated by fibres
originating from cell bodies found in the hypothalamus and the amygdaloid
nuclei. Axon collaterals of hypothalamic tuberinfundibular neurons ex-
tend to brain sites quite distant from the hypothalamus, such as the
thalamus, the limbic system, other than the amygdala, and the cortex
(Martin et al, 1975), and therefore represent the means whereby hormone,
possibly produced in the cell bodies of the hypothalamus, might be axon-
ally transported to other brain regions. In preliminary studies, the
amygdala and hypothalamus also showed the largest number of positively
staining cell bodies for immunoreactive GH-like material.

At this level of investigation, it is difficult to associate any

particular cell type, either glial or neuronal, with a higher than average hormonal content, although from the immunohistochemistry data, neurons were more likely involved. However, in tissue culture studies with the neuroblastoma cell line N_4TGI, TSH-like material was found in neuronal-type cells. Despite this, the tumor origin of such cell lines cautions its use as a positive indication that normal, non-tumor neuronal cells, rather than glial or interstitial cells, are involved in hormone synthesis or storage in the CNS. It can only be concluded from our tissue culture studies, therefore, that primary mixed cell cultures of rat nervous tissue appear to release both growth hormone and thyroid stimulating hormone-like peptides into the growth media over a 30-day time period. Further studies utilizing techniques to propagate pure neuronal or glial primary cell cultures are needed to further this study.

In order to pinpoint the association of these peptides with a particular cell fraction, our ultra-centrifugation studies showed that both TSH- and GH-like immunoreactive material were isolated together with the synaptosomal-rich fraction of CNS tissue homogenates, suggesting the possibility that, like other peptides such as somatostatin, TRH and VIP, these pituitary-like hormones may be associated with pre- or post-synaptic membranes in vivo.

There exists the possibility, as shown by these same ultra-centrifugation studies, that part of the extractable pool of GH- and TSH-like material found in the hypothalamus, may be present unassociated with any specific cellular fraction. Whether the origin of this free pool of hormone is the brain or pituitary is open to speculation.

The studies which were undertaken to characterize these extractable immunoassayable peptides from rat brain tissue in a chemical, immunological, and biological manner revealed that hormones of brain origin were indistinguishable from those found in the pituitary, in terms of molecular weight, as shown by column chromatography, and in terms of immunological identity, as shown by parallelism studies. Their biological activity also demonstrated a similarity to their respective pituitary counterparts in terms of possessing the capability of eliciting a simple TSH-like response on the histology of the thyroid or, for growth hormone-like material, to increase the width of epiphyseal cartilage in the tibias of hypophysectomized animals.

Such immuno- and biological similarities between pituitary and brain hormones would suggest, without further evidence, the possibility that the presence of pituitary-like hormones in the brain is merely due to pituitary hormone contamination of these CNS tissues by circulating pituitary hormone.

Experiments designed to test this concept were carried out in this study using radioactively-labeled pituitary hormone in order to test the effectiveness of the blood-brain barrier. They show that it seems unlikely that significant quantities of pituitary TSH or GH are transported to the brain either by the general circulation or portal vessel routes. Similarly, Mezey et al (1979), showed that whole brain uptake of ^{3}H ACTH, given intravenously to adult rats, was less than 0.07% of the initial radioactive hormone dose given. In the experiments reported here, no radioactivity was found in brain tissue of either young or adult rats

after systemic injection of ^{125}I-labeled pituitary GH or TSH. This indicates that, in the particular rodent model system which was used throughout this study, the blood-brain barrier was apparently intact throughout life, eliminating any possibility of cross-contamination of brain tissue by pituitary hormones. Other indications of the bidirectional intactness of this barrier were that no increase or decrease in circulating hormone levels after experimental manipulations, such as hypophysectomy and target organ removal, produced any subsequent rise in the hormonal levels found in brain tissue. Similarly, no increase or decrease in brain tissue levels produced a concomitant increase or decrease in the concentration of circulating pituitary hormone. Experiments by Weiss et al (1975) substantiate this data in that pituitary cells implanted in the rat brain ventricles of hypophysectomized rats did not produce normalization of circulating pituitary hormone levels, even though it could be demonstrated that such implants continued to produce growth hormone and thyroid stimulating hormone. Whatever the role of the pool of brain hormones, it is unlikely to be as a reserve pool which can be drawn on in times of pituitary deficiency.

Besides the demonstration of blood-brain barrier intactness to circulating pituitary hormone, evidence of a CNS origin of brain peptides, other than GH and TSH, comes from similar experiments comparing brain tissue levels of hormone-like material before and after removal of the pituitary gland. That the concentration of ACTH, α MSH, β endorphin, and prolactin, as reported previously by Liotta et al (1978), Kobayashi et al (1978), and Toubeau et al (1979), are essentially unchanged in most

of the brain areas examined after such treatment, is consistent with the possibility of synthesis of these peptides by the brain tissue itself.·

Such variations in hormonal level that do occur after hypophysectomy in the data obtained in this study for TSH could be the result of several factors, such as changes brought about in the levels of a known regulatory factor to pituitary hormone, e.g., dopamine, by hypophysectomy. The slight decrease in TSH levels seen 28 days after hypophysectomy are most probably too late an event to be the result of tissue damage incurred by surgical intervention but could be a reflection of falling dopamine levels, which have been observed in the hypothalamus after long-term pituitary removal (Nagatsu et al, 1964). A fall in dopamine levels would have an adverse effect on the content of TSH in a normal pituitary; however, whether similar effects can be induced on all or part of the hypothalamic pool of TSH-like peptide can only be speculated upon at this time.

Another controlling factor, in terms of hypothalamic levels of TSH after hypophysectomy, could be the effect of falling TRH levels, reported by Nemeroff et al (1979) to occur in the hypothalamus, but not in other brain regions, after pituitary removal. These levels were shown by Nemeroff to be restorable by T_4 therapy. In a study for this dissertation using long-term hypophysectomized animals which received T_4 substitution treatment, levels of extractable immunoreactive TSH in the hypothalamic region were shown to be restored by such therapy. The possibility, therefore, exists of a TRH-influenced control over part of the hypothalamic TSH pool and would provide an explanation for the fall seen

in hypothalamic TSH levels and not other CNS pools of TSH after long-term hypophysectomy.

Examination of the data concerning immunoreactive GH levels in brain tissue after hypophysectomy show no change in hormonal levels for most of the brain areas examined. Exceptions were the levels of growth hormone in hypothalamic and amygdaloid tissues, which demonstrated an initial fall in hormone levels after 7 days of hypophysectomy, followed by a remarkable rise in hormone content after 28 or more days without a pituitary.

The lack of such an early fall in GH measured from tissue-cultured amygdaloid and hypothalamic tissue derived from long-term hypophysectomized animals indicates the possibility that after a certain time period of hypophysectomy, such as 7 days, a growth hormone inhibitory factor, e.g., somatostatin, may be present in sufficiently lowered amounts as to be unable to cause a normal depression of growth hormone levels in vivo. This theory is substantiated by experiments, previously quoted by Baker et al (1976), showing that somatostatin levels do fall in the hypothalamus about 10 days after hypophysectomy. The ability of somatostatin to suppress "brain" growth hormone has been shown in our study using tissue cultured normal CNS tissue. Perhaps this initial decrease in amygdaloid and hypothalamic tissue levels of growth hormone is due to somatostatin, and the increased levels after 10-14 days hypophysectomy are due to the disappearance of this inhibitory factor from the CNS milieu because of pituitary ablation. Certainly the implication that animals in such experiments were incompletely hypophysectomized (Moldow et al, 1978[a,b]) has

not been substantiated either by microscopic inspection of the empty
sella turcica, by levels of circulating T_4, TSH, and GH, which were
measured in the serum of operated animals, or by gross examination of
atrophied target glands such as the thyroid, adrenals, and gonads in
hypophysectomized rats.

Enhancement of levels of ACTH-like material in rat hypothalamus,
but not in other brain areas, 12 days after hypophysectomy has been
demonstrated by Krieger and Liotta (1979), which tends to support our
data for GH-like levels in the CNS after pituitary removal. These ex-
periments add further evidence to the possibility that these pituitary-
like peptides in the CNS are not of pituitary origin.

Other data, also corroborated by Krieger and Liotta's group, are
those obtained from the tissue culture of cells from rat central nervous
system tissues, demonstrating the presence of immunoreactive GH and TSH
in the growth medium for up to 30 days of cell culture. The ability of
cells derived from the arcuate nucleus of the hypothalamus to release
ACTH into tissue culture media for up to 17 days, has similarly been
reported (Liotta et al, 1979[a]). Using this model system of dispersed
hypothalamic cells, it has been possible to demonstrate that ^{3}H-labeled
amino acids can be incorporated into material with the characteristics of
the pituitary precursor of ACTH, giving credence to a CNS origin for this
traditionally thought of pituitary hormone (Liotta et al, 1979[b]). Evi-
dence also exists that an ACTH-like pituitary peptide other than GH and
TSH persists after long-term hypophysectomy in CNS tissues and that it
is most likely synthesized in situ within the brain.

Other experiments have sought to investigate the mechanisms of regulation of these pituitary-like peptides, compared to those regulating pituitary hormones. Such experiments have shown, for example, that concentrations of hypothalamic immunoreactive ACTH-like material are not significantly altered 24 hours or 2 weeks after adrenalectomy, after dexamethasone or corticosteroid administration, or by the stress of ether administration (Liotta et al, 1978). All of these procedures are normally associated with alteration in the levels of ACTH in the pituitary gland and serum. Alterations of levels of ACTH in the pituitary and serum due to adrenalectomy are the result of feedback mechanisms.

The secretory functions of the anterior pituitary appear to be modulated by hormonal signals originating in such peripheral target glands as the adrenals, thyroid, and gonads, and in the pituitary itself, through complicated networks of feedback mechanisms called, respectively, "long" and "short." Within each category of feedback systems, "negative" as well as "positive" mechanisms have been described. A feedback system in which the input, represented by a hormone originating in a peripheral target gland, reduces the output of pituitary hormone is called "negative" and where a feedback system, in which increased secretion of a peripheral hormone stimulates anterior pituitary function, it is generally called "positive." The unaltered hypothalamic levels of ACTH after adrenalectomy, in contrast to increased levels found in the pituitary due to a lack of negative feedback of circulating adrenal cortisone, reflect a difference in the control mechanisms of brain-situated hormone compared to pituitary hormones. Similarly, Rossier et al (1977[a]) have observed no change in

the concentrations of immunoreactive β-endorphin-like material in the brains of adrenalectomized animals. Animals subjected to the stress of foot shock showed some decrease in β-endorphin-like immunoreactivity in the hypothalamus, but not in other brain areas (Rossier et al, 1979[b]). Other studies by Havrankova et al (1979) have demonstrated that "brain insulin" concentrations are also unaffected by extreme changes in circulating hormone concentrations.

Removal of such target organs as the adrenals and testes, in our experiments, appeared to have no effect on the levels of growth hormone in either serum, pituitary, or other brain areas analyzed. Obviously, no feedback mechanisms occur in vivo involving these peripheral organs and pituitary or brain growth hormone. Oophorectomy induced a significant fall in the levels of circulating GH, but no significant difference in pituitary levels of controls or in operated animals. A significant rise in the levels of hypothalamic and amygdaloid immunoassayable GH concentrations was seen, however, after the removal of the ovaries. Other brain areas appeared unaffected by such ovarian ablation.

Thyroidectomy produced a similar picture of a fall in serum and pituitary levels of immunoassayable growth hormone and a rise in the hypothalamic and amygdaloid levels of this immunoreactive peptide. As with the gonadectomized GH content, brain regions other than hypothalamus and amygdala appeared unaffected by removal of the thyroid. What seems most striking from data derived from these experiments is the demonstration of an apparent independence between the levels of, e.g., growth hormone found in the pituitary and in serum, compared to levels found

in specific areas of the brain.

The possibility of the existence of a short loop feedback regulatory system of some kind between the hypothalamus and the anterior pituitary would be a possible explanation of the data we have described from the present studies. It is disturbing, however, that GH and TSH levels, in areas other than the hypothalamus and amygdala, are unaffected by specific target organ removal and/or trophic hormone treatments, especially if we hypothesize from the immunohistochemical data, that extractable hormone levels in one region may be derived from cell bodies in the hypothalamus and amygdaloid nuclei. The possibility, therefore, exists that in situ synthesis of peptides in these brain areas exists at a level undetectable by the immunohistochemical technique performed in these studies. Further investigations should therefore aim at improving the sensitivity of this particular technique for detecting GH and TSH in brain areas other than the hypothalamus and amygdala.

Adrenalectomy and orchidectomy in adult male rats produced similar divergent results between pituitary and serum TSH levels and those measured in the hypothalamus. Thyroidectomy resulted in a significant rise in circulating TSH and a lowering of pituitary TSH levels. No effect on brain levels of TSH-like peptides was observed after removal of the thyroid.

Immunoreactive GH and TSH of the brain, like ACTH and β endorphin, appear, therefore, to be unaffected in any particular way by the positive and negative hormonal feedback signals normally affecting the concentration of these peptides in the anterior pituitary gland and in the

circulation. This apparent dissociation was also reflected in the tissue culture studies described in this study, where T_4 added to the growth media of growing anterior pituitary or brain tissue monolayers had little or no effect on levels of immunoreactive TSH found in the culture medium of normal brain cells. TSH levels from medium of anterior pituitary cells, so treated, reflected the anticipated decline in TSH production due to a negative long feedback loop operating on pituitary TSH secretion in the presence of high levels of the thyroid hormone, T_4.

Speculation on the presence of a short loop feedback between pituitary and hypothalamic pools, similar to that postulated between pituitary and pituitary stalk TSH pools by Baake *et al* (1967), was not substantiated by data from these studies. Our data confirms, rather, the findings of others who have attempted direct implantations of TSH into the hypothalamus in order to demonstrate a feedback control mechanism on the pituitary. Levels of TSH, so implanted, apparently fail to affect the concentration of pituitary TSH by a negative short feedback loop, making TSH unique in this regard among the anterior pituitary hormones.

Further evidence for the independence of pools or *in situ* synthesis of brain and pituitary growth and thyroid stimulating hormones comes from the results of the ontogenetic studies reported in this dissertation, where brain, serum, and pituitary levels of GH and TSH were examined during gestation, in the neonatal period, and into adulthood.

In such ontogenetic studies, it was apparent from the data that a striking preparturitional surge of immunoreactive GH and TSH occurs in

the 24 hours immediately before delivery, and is followed by a fall in the first day of extrauterine life. These peptides were found to possess similar molecular amd immunological characteristics to their pituitary counterparts. The abrupt rise of a substance within the developing fetal CNS and serum just before birth is not an unusual finding. Several reports from other investigators have noted comparable patterns to those described here for GH and TSH. Some of these are shown in Table 38 , and include such identifiable substances as glycogen (Kohle et al, 1977), histamine (Pearce et al, 1969), estrogen (Dohler et al, 1975), prostaglandins (Vale et al, 1972), and receptors for serotonin and estrogen (McClusky et al, 1979), as well as angiotensin II (Baxter et al, 1980). Other data show that the levels of other substances such as somatostatin (Blasquez et al, 1974), T_4 and T_3 (Rabie et al, 1979), and TRH (Schaeffer et al, 1980) rise in the CNS in the first few days after birth, as shown in Table 39 .

Changes in hormone concentrations of TSH or GH in the fetal brain did not appear to reflect pituitary secretions as the patterns of acquisition were quite different. Growth hormone and TSH were first detected in the fetal brain at 10 days of fetal age and could not be detected in the fetal pituitary until 12-15 days of age. Furthermore, pituitary hormone concentrations did not show the dramatic preparturitional surge noted in the brain.

Radiolabeled GH and TSH given intraperitoneally to either the fetus, or pregnant rat, did not reach the fetal rat brain tissue in either case, indicating the intactness of both the fetal blood-brain barrier as well as

TABLE 38

SUBSTANCES WHOSE LEVELS RISE JUST PRIOR TO BIRTH

IN THE CNS		IN THE FETAL SERUM	
RECEPTORS FOR:			
Angiotensin II	Baxter et al, 1980	Testosterone	Weisz et al, 1980
Renin	Baxter et al, 1980	ACTH	Rose et al, 1978
Estrogen	McClusky et al, 1979	Corticoids	Rose et al, 1978
		Growth Hormone	Bassett et al, 1970
Glycogen	Kohle et al, 1977		
Spermidine	Pearce et al, 1969	LH	
Histamine	Pearce et al, 1969	FSH	Courbier et al, 1978
Substance P	Gilbert et al, 1979	TSH	
TRH in telencephalon	Schaeffer et al, 1980		
Estrogen	Dohler et al, 1975		
Vasotocin	Pavel et al, 1976	Prolactin	Riddick et al, 1979
$PGF_{2\alpha}$, PGE, PGE_2	Vale et al, 1972	Estrogen	Dohler et al, 1975
	Liggins et al, 1973		
Ketone Bodies	Haymond et al, 1979		
Membrane Phospholipids/ Cholesterol	Kapitulnik et al, 1979		
ENZYMES:			
Glucose-6-PO_4tase	Oliver et al, 1963		
Dehydrogenase	Andres et al, 1980		
Monoamine Oxidase	Snyder et al, 1979		
Fructose 1-6 Diphosphatase	Haymond et al, 1979		
Pyruvate Carboxylase	Haymond et al, 1979		
UDP-glucose-glycogen Transglucosylase Phosphoglucomutase	Kohle et al, 1977		

TABLE 39

SUBSTANCES WHOSE LEVELS RISE JUST AFTER BIRTH

C N S	DAY FIRST DETECTABLE	SOURCE
Monoamines	1 - 2	Coyle et al, 1973
Somatostatin	5	Blasquez et al, 1974
TRH in hypothalamus	6	Schaeffer et al, 1980
Androgen receptors	5	Vito et al, 1979
T_4 and T_3	2 & 12	Rabié et al, 1979
Glycogen phosphorylase	10 min.	Jacquot et al, 1964

the fetal-maternal-placental barrier, to circulating pituitary hormones at the time of the preparuritional brain surge. It is also known that the pituitary portal vessel-hypothalamic-link is not fully developed in the rat until Day 5 of life (Glydon, 1957), another indication that fetal brain hormone is not of fetal pituitary origin.

The gradual rise of levels of growth hormone and TSH in the fetal serum and their fall after birth is thought by some authors to be due to this immaturity of the pituitary-hypothalamic regulatory system. Somatostatin, for example, has failed to be detected before the fifth day of post-uterine life. The significant fall in serum levels of pituitary hormones after birth has also been postulated to be mediated by adrenergic mechanisms (Basset et al, 1970), as infusion of catecholamine and isoprenaline into fetal lambs was shown to decrease serum GH concentrations.

The dramatic rise in fetal brain levels of TSH and GH does not, however, reflect the same pattern displaced by serum and amniotic fluid and, as previously concluded with reference to pituitary levels of these hormones, the CNS concentrations are probably independent of such extra CNS pools of hormone. Any speculation on the role of this precipitous rise in fetal brain levels of immunoassayable GH and TSH should be discussed in the light of fetal morphology and physiology.

During the transition from intrauterine to extrauterine life, a number of metabolic changes and adaptions occur in fuel homeostasis. These adaptions allow the fetus to convert from a state in which it is totally dependent on maternal fuel sources, to a state in which it relies

on endogenous substrate for the maintenance of normal cellular growth. In order to maintain normal cellular metabolism and anabolic growth, the newborn must have at least three requirements: an adequate store of hepatic and brain glycogen, muscle protein and fat, the enzymatic capacity to release and utilize these substrates, and thirdly, to be able to regulate such enzyme induction, substrate mobilization, and peripheral utilization of metabolic fuels.

Unlike glycogen in the liver, the functional significance of glycogen in the brain is poorly understood, although its potential as an energy source may explain the greater resistance of young animals to hypoxia. Glycogen levels in the brain are shown by Kohle et al (1977) to rise abruptly at day 20-21 of gestation and then to decrease rapidly after birth until, 7 days postnatally, very low levels are observed. An increase in glycogen-synthesizing enzymes has also been observed to follow a similar developmental trend. Although glycogen breakdown appears to commence the day of birth, 26% of the total fetal brain glycogen store is utilized within 10 minutes of birth. This glycogenolysis is in response to accelerated cerebral glycolysis resulting from the cerebral hypoxia of vaginal delivery.

A role for fetal brain growth hormone in such adaptions to neonatal life could possibly be one in which the fetal hormone exhibits "insulin-like" properties by increasing the permeability of cells in order to feed the rapid rise in glycogenolysis. Growth-promoting hormones, in particular GH and T_4, have been shown by Levi-Montalcini et al (1968) to have complementary and synergistic effects on cell growth and the regulation of

somatic development. In the developing CNS, many more neurons are produced in fetal life than survive; in fact, cell death of up to 50% of original neurons before birth is a common feature. McClusky et al (1979) have speculated that the rapid increase in estrogen receptors and estrogen levels, reported two days prior to parturition, and their rapid disappearance after birth, could be linked to a role for estrogen in preventing cell death and stabilizing surviving neurons at this critical period of development. Perhaps a similar argument could be made for the preparturitional rise in brain growth hormone. Furthermore, it is thought that nerve cells die when they fail to contact other cells during brain development, and it is possible that growth hormone and perhaps TSH may increase nerve cell contacts by causing enlargement of cells and cell-to-cell surface contacts.

If indeed these brain hormones possess the same effects on cell metabolic activities as do their pituitary counterparts, other hormone-promoted activities, such as increased protein synthesis and m.RNA content for GH, increased mitochondrial respiration, and the increased uptake of precursors of phospholipids for possible cell wall synthesis by TSH, could perhaps contribute to the general maintenance of neuronal tissues in fetal and adult tissues.

However, in any effort to assess the functional significance of pituitary-like hormones such as GH and TSH in the brain, the possibility that the role of the hormones within the CNS differs from their currently assigned physiological role within the pituitary, should not be overlooked. For example, it is known that TRH-like activity is present in the neural

tissue of certain invertebrates, even though evidence of a role for it in the regulation of pituitary. TSH has only been found in animals higher in the evolutionary scale (Scharrer, 1978).

Another line of approach to the question of function of neuronal peptides has been the concept that "peptidergic neurons" refer not only to the neurosecretory cells of the hypothalamus producing vasopressin and hypophysiotropic hormones, but also to those neurons containing additional peptides such as Substance P, neurotensin, angiotensinogen, gastrointestinal peptides, and now more recently the hormones of the anterior pituitary such as ACTH, GH, TSH, LH, and β-lipotrophin. There is the possibility that, in the case of hypothalamic ACTH, GH, and TSH, extractable levels of hormone could be derived from two different sources. Those of CNS origin could be part of a peptidergic neural system present in addition to the classically described motor, sensory, and autonomic nervous systems, but which interrelates with such systems. Such peptides are speculated to be secreted at peptidergic terminals, and may act on a variety of effector cells, including neurons, functioning either as neurotransmitters or neuromodulators, rather than within the normal definition of a "hormone." It has been hypothesized by Barker (1977) that whereas neurotransmitters are involved in the momentary mediation of single cell-to-cell interaction, the neurohormones may be important in the sustained modulation of specific sets of target neurons. Neuromodulation implies an action of the secreted peptide in altering the effect of the classical neurotransmitter on its receptors. Possible examples of such interactions have been shown by the reported presence by Hökfelt et al (1979) of

neurons containing both peptides and classic monoaminergic neurotransmitters, and by the effects which other peptides, such as ACTH and vasopressin, have on acetylcholine, norepinephrine, and serotonin content or turnover in various brain regions (Gispen and de Wied et al, 1977). De Wied has also shown that these peptides, vasopressin and ACTH, can enhance the dephosphorylation of membrane proteins leading to a change in membrane permeability.

The reports of opioid-peptide effects on pain perception, addictive states, and psychiatric disorders have previously been reviewed, as have those reports concerning the effects which ACTH and MSH, given intraventricularly, appear to have on rat behavior. Behavioral studies suggesting a role for vasopressin in learning and memory (De Wied and Versteeg, 1979) showed that impaired learning behavior in Brattleboro rats, which lack vasopressin-synthesizing mechanisms, can be reversed by intraventricular injections of vasopressin. In normal rats, peripheral vasopressin injections appear to influence learning and memory, and in clinical trials, vasopressin may improve memory in brain-damaged human subjects. Studies with TRH demonstrate that this hypothalamic hormone can elicit behavioral excitation and anorexia in animals and may cause mood enhancement in humans (A. J. Prange et al, 1975).

In addition to such postulated behavioral effects assigned to peptide hormones in the brain, such hormones may also have an effect on endocrine regulatory processes. Experiments with intrahypothalamically injected ACTH have been reported to demonstrate inhibitory effects on pituitary ACTH release, although unphysiological doses and long periods

of treatment tend to dispute this data of Yates et al (1974). However, the possibility of such short-loop feedback mechanisms has also been alluded to in this discussion as a possible role for hypothalamic GH. It remains to be determined if such observations reflect a dual effect of these compounds on releasing factors or on the neurotransmitters that regulate such releasing factors.

The finding of GH- and TSH-like hormones in both the CNS and in the gastrointestinal tract in this study is not terribly surprising, considering the number of other peptides recently reported in such dual locations. Such peptides as somatostatin (Arimura, 1975), glucagon (Conlon et al, 1979), and VIP (Said et al, 1972) are found in the GI tract and, from this study, immunoreactive GH- and TSH-like material in extrapituitary sites such as the pancreas and small intestine. These data give further credence to one of the objectives of this dissertation, i.e., that of determining whether "brain" hormones are of CNS origin rather than the result of retrograde transport via the portal vessels from the pituitary to hypothalamus.

The finding of immunologically active, pituitary-like hormones in such extrapituitary, extra-CNS sites as the GI tract and pineal gland, leads to speculation that these peptide-containing cells can be classified as belonging to the amine-precursor uptake and decarboxylation series of cells, a concept originally developed and outlined by Anthony Pearse in 1977.

Other lines of approach to explain this apparent multisite distri-
bution of pituitary-like peptides would be that the presence of such
peptides is due primarily to incomplete gene suppression in those parti-
cular tissues, or that the detectable levels merely represent bound,
circulating, pituitary peptide to tissues with appropriate hormone recep-
tors. Removal of the pituitary as a source of circulating hormone for
up to 28 days did not appreciably lower the levels of extractable hormone
in those extrapituitary sites studied. The possibility that the observed
immunoreactive material was merely receptor-bound is therefore unlikely
in the light of the in vivo hypophysectomy experiments reported here.

In conclusion, results obtained in this study appear to add yet two
more peptides, formerly thought to have been of non-neural origin, to the
ever-growing list of brain peptides. Many of these, such as Substance P,
have been speculated to be potential neurotransmitter or neuromodulator
candidates, and represent an active field of research at the present time
seeking to unravel the parameters involved in the development and func-
tioning of the CNS.

Brain growth hormone and TSH await further investigation before
specific functions can be assigned to their presence in rodent and primate
brain. However, their apparent independence from patterns of pituitary
hormone secretion, and their probable synthesis within the CNS tissues
are tantalizing observations that await further conceptual organization.

CONCLUSIONS

The following conclusions can be drawn from the data obtained in this dissertation.

1. Immunoreactive growth hormone (GH)- and thyroid stimulating hormone (TSH)-like peptides were found to be unevenly distributed throughout the rodent and primate central nervous system (CNS) and gastrointestinal tract.

2. Highest levels of extractable GH-like material were obtained from rodent amygdaloid tissue, whereas the hypothalamus contained the largest concentration of a TSH-like peptide.

3. Both brain peptides showed a similarity to their pituitary counterparts in terms of molecular weight, immunological affinity, and biological activity.

4. Histochemical mapping of the CNS for the TSH-like peptide using a peroxidase anti-peroxidase technique showed hormonal material concentrated in neuronal cell bodies of the rodent amygdaloid nucleus and hypothalamus.

5. Ultra-centrifuged homogenates of amygdaloid and thalamic tissues showed an association between GH- and TSH-like immunoreactivity and the synaptosomal-mitochondrial pellet (SMP). In ultra-centrifuged hypothalamic tissue, the immunoreactivity was distributed evenly between the SMP and the final supernatant.

5. Tissue cultured CNS cells from intact and hypophysectomized animals released both GH- and TSH-like material into the growth medium over a 30-day period. This would indicate the CNS, rather than the pituitary, as the source of both brain hormones.

6. Contamination of CNS tissues by pituitary GH and TSH was shown to be unlikely by a demonstration of blood-brain barrier intactness throughout life, and a lack of change in brain hormone levels after hypophysectomy.

7. Target organ ablation studies showed an apparent independence between regulatory mechanisms controlling pituitary and brain hormone levels.

8. The appearance of detectable hormone in the fetus and the subsequent pattern of development differed between brain and pituitary hormone pools. A preparturitional rise in brain hormone levels was detected, followed by a fall to normal levels in the CNS after birth.

9. In conclusion, this study verified the presence in the rodent and primate brain of GH- and TSH-like materials which appear to be of CNS rather than of pituitary origin.

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<u>APPROVAL SHEET</u>.

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The final copies have been examined by the director of the
dissertation and the signature which appears below verifies
the fact that any necessary changes have been incorporated
and that the dissertation is now given final approval by the
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The dissertation is therefore accepted in partial fulfillment
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