THE LONG-TERM EFFECTS OF NEONATAL HYPOTHYROIDISM ON β-ENDORPHIN AND SOMATOSTATIN IN THE BRAIN

by

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A Thesis

Presented to the Faculty of Graduate Studies
in Partial Fulfillment of the Requirements for the Degree

Master of Science

Department of Physiology
Faculty of Medicine
University of Manitoba

August 1981

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ACKNOWLEDGEMENTS

My greatest appreciation and gratitude is extended to Dr. Viktor Havlicek, whose untimely death on August 5, 1981 has affected me deeply. His supervision throughout the two years of my M.Sc. program and review of my thesis manuscript has provided a valuable learning experience for me. I sincerely thank Dr. H. G. Friesen for his guidance and constructive criticism during the course of these studies and thesis preparation, as well as for provision of antisera for the radioimmunological studies.

I would like to express my gratitude to Dr. M. West, Dr. N. Kato, Dr. Y. Hoh, Dr. I. Worsely, Dr. K. Shah, Sona Pacin, Darrell Backman, Gurleen Sikand, and Gordon Blair for teaching me specific laboratory techniques and providing technical assistance at various times during this study. In addition, I thank Dr. C. Pinsky of the Dept. of Pharmacology for his review and suggestions concerning my thesis manuscript, and Ellen Elsholtz and Janet Greer for their assistance in typing.

Finally, I wish to thank in a special way, my parents whose many phone calls helped me through difficult times and to the Elsholtz family for their close friendship, interest and concern during my stay in Winnipeg.

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The financial assistance of a University of Manitoba Fellowship and Medical Research Council (Ottawa) Studentship is gratefully acknowledged.

ABSTRACT

Lactating rats receiving 0.02% propylthiouracil (PTU) in their drinking water transfer its goitrogenic effect to the offspring through their milk. This treatment induces temporary mild hypothyroidism of the pups, until weaning after which they are maintained on a normal diet and water. After exposure to the goitrogen from day 0 to 19 postnatally, the offspring are found to exhibit a high incidence of audiogenic seizures which persist into adulthood, suggesting that a permanent deficit in brain function has developed during this critical neonatal growth period. Severe neonatal hypothyroidism results in depression of the electroshock seizure threshold, indicating increased brain excitability despite its effect of diminishing amplitude of the EEG and evoked potentials. These observations are interesting in view of the fact that β-endorphin and somatostatin have been shown to produce varying degrees of epileptic seizure activity, from non-convulsive epileptiform EEG spikes to violent tonic-clonic seizures, when administered i.c.v. in low doses. In addition, both peptides have a role in regulation of neuroendocrine and behavioral processes, functions which are also extensively affected by neonatal hypothyroidism. As a result, the present series of experiments were carried out to investigate β-endorphin and somatostatin concentrations in the brains of mature PTU-treated rats (PTU rats) with and

without exposure to a seizure-producing stimulus. Learning and behavioral functions, variations in the severity of neonatal antithyroid treatment, and effects of stress were also examined. The results have indicated that both β-endorphin and somatostatin levels in the brain are permanently altered by mild neonatal hypothyroidism, β-endorphin specifically in the thalamus and somatostatin extensively affected throughout the brain. The alterations of these peptides appear to be related to the audiogenic seizure sensitivity of these rats, but not to the actual occurrence of seizures. Severe neonatal hypothyroidism (experimental cretinism) produces similar changes in brain peptide levels. Mild neonatal hypothyroidism is associated with several behavioral changes including hyperactivity, impaired learning ability, altered function of central arousal mechanisms, and reduced responsiveness to stress. In conclusion, somatostatin in particular may exert a significant role as a neurotransmitter in the pathogenesis of abnormalities observed in PTU rats. Therefore, even mild neonatal hypothyroidism during the critical growth period causes a permanent impairment of brain function which manifests itself in part by altered brain peptide content.

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List of Abbreviations

Hormones and related compounds

SRIF	somatostatin
TRH	thyrotropin releasing hormone
TSH	thyroid stimulating hormone
T_3	triiodothyronine
T_4	thyroxine
GH	growth hormone
PRL	prolactin
LPH	lipotropin
ACTH	adrenocorticotrophic hormone
LHRH	luteinizing hormone releasing hormone
LH	luteinizing hormone
FSH	follicle stimulating hormone
MSH	melanocyte stimulating hormone
cAMP	cyclic adenosine monophosphate
GABA	gamma amino-butyric acid

Chemicals and drugs

BSA	bovine serum albumin
EDTA	disodium ethylenediamine-tetraacetate
CMC	carboxymethyl-cellulose
PTU	propylthiouracil

Buffers

PB	phosphate buffer
PBS	phosphate-buffered saline

Sera

NRS	normal rabbit serum
SAR	sheep anti-rabbit gamma globulin

Terminology

CNS	central nervous system
EEG	electroencephalograph
EST	electroshock seizure threshold
MES	maximal electroshock seizure
L.I.D.	low iodine diet
ANOVA	analysis of variance
DRL	differential reinforcement of low rates
EGL	external granular layer
S.E.M.	standard error of mean

Units of measure

g	gram
mg	milligram
µg	microgram
ng	nanogram
pg	picogram
l	litre
ml	millilitre
µl	microlitre
cm	centimetre
yr	year
mo	month
wk	week
hr	hour
min	minute
sec, "	second
msec	millisecond
MW	molecular weight
M	molar
mM	millimolar
N	normal
K	MW x 10^3
K.I.U.	kallikrein inhibiting units
KW	kilowatt
KHz	kilohertz
dB	decibel
cpm	counts per minute
x g	times force of gravity

INTRODUCTION

Overview of Neonatal Hypothyroidism

I. Mechanism of Thyroid Hormone Action

Thyroid hormones, L-thyroxine (T_4) and 3,5,3'-triiodothyronine (T_3), are iodinated tyrosine derivatives which are well documented as having an essential role in normal brain development during a critical perinatal growth period in mammals (1,2). T_3 is considered to be the active form since it is the form which binds to specific nuclear receptor sites and accounts for most of the thyromimetic effects (3,4). T_4 is converted to T_3 either peripherally or within the target cell, where it combines with a cytosolic thyroxine-binding protein and is transported into the nucleus. Here the T_3 molecule binds to a core receptor (non-histone chromosomal protein) which subsequently associates with histones, becoming a 'holo' receptor, and influences transcription of specific genes. The biochemical events that follow are not well understood, but recent evidence has indicated that thyroid hormones affect at least four basic processes - activity of RNA polymerase II which assembles messenger RNA, activity of tRNA sulfurtransferase which confers codon specificity onto transfer RNA, release of nascent polypeptide chains from ribosomes, and activity of thymidine kinase and other enzymes which assemble nucleotides (5-8). Rather than stimulating the release of genetic information, it appears that thyroid

hormones act to control the rates of several critical enzymatic steps in the flow of that information from storage in DNA to expression in structural and enzymatic proteins. Thus, in neonatal hypothyroidism, the overall rate of protein synthesis is reduced, and because not all reaction rates are slowed or accelerated equally by thyroid hormones, the brain develops asynchronously.

II. Developmental Processes Affected by Neonatal Hypothyroidism

The literature on the multitude of effects of neonatal hypothyroidism on brain development is extensive, and can be summarized within seven major categories: (a) growth of neurons and brain (cell division) as a whole (9,10), (b) protein and nucleic acid synthesis (11,12), (c) development of CNS enzyme systems (13-15) (d) myelination and quantities of brain lipids (16), (e) tissue respiration (17), (f) electrical activity of the CNS (18,19), and (g) learning and behavior (20,22). All of these show a reduction or delay relative to the normal euthyroid animal, except in the case of brain excitability which increases, as evidenced by lowered electroshock seizure threshold (19).

Another important consequence of neonatal hypothyroidism is impaired development of neuroendocrine functions, in particular the hypothalamic-pituitary-thyroidal and-gonadal axes. Several hormonal systems may be adversely, and permanently

affected by early thyroid hormone deficiency (23,24). It is these consequences, together with the last two mentioned above, electrical activity and behavioral processes, which are relevant to the present area of investigation and hence, will be examined in detail.

III Development of Neuroendocrine Functions in the Hypothyroid Rat.

The rat has provided a useful model for studying the effects of neonatal hypothyroidism on CNS and neuroendocrine functions because a significant degree of brain development takes place after birth. Thus, in humans the critical period of thyroid hormone influence extends from the third trimester of fetal development to the end of the first postnatal year, whereas in the rat the corresponding period extends from about day 18 of gestation (of the 21 day gestational period) to the end of the third postnatal week (weaning age) (25).

(i) Ultrastructural Changes

When rat pups are thyroidectomized at birth by a single injection of NaI^{131}, several cytological changes are evident in the hypothalamus and pituitary after three weeks of age. Within the medial basal hypothalamus (arcuate nucleus and median eminence), a region which concentrates labelled T_3 (26), the arcuate neurons exhibit a large proportion of endo-

plasmic reticulum transformed from rough to smooth, and arranged in a "whorled body" configuration (concentric rings of cisternae) (27), abnormalities which clearly indicate a reduction in protein synthesis. In the anterior pituitary, somatotrophs are largely degranulated, and many cells are transformed into "thyroidectomy" cells characterized by dilated rough endoplasmic reticulum containing spherical electron-dense structures (thought to be secretory granules). The total number of pituitary cells undergoing mitosis is reduced, and a shift in the type of mitotic cells from predominantly somatotrophs to increased numbers of thyrotrophs is evident.

(ii) Endocrine Changes

Hypothyroidism can also be induced by administering thioureylene antithyroid drugs such as propylthiouracil (PTU) which block the organification of iodide, thereby preventing synthesis of thyroid hormones (28). PTU also acts peripherally by inhibiting the conversion of T_4 to T_3. Kikuyama et. al. (24) induced neonatal hypothyroidism by adding 0.04% PTU (w/w) to the diets of lactating rats from day 18 of gestation to postnatal day 20 of the offspring, and demonstrated endocrine changes which persisted into adulthood. On postnatal day 20, pituitary GH and PRL content was significantly lower than in normal controls. This observation correlates well both with the cytological data in rats of the same age showing decreased

numbers of somatotrophs following neonatal thyroidectomy (27) and PTU treatment (29), and with kinetic data showing a 50% loss of somatotrophs after 20 days of neonatal PTU treatment (30). Decreased pituitary PRL content persisted at 60 days of age (maturity) in both males and females, whereas in hypothyroid pups treated with thyroxine from day 1 to 20, pituitary GH and PRL levels largely returned to normal values (24). Recently, pituitary GH content was measured during the early stages of neonatal hypothyroidism. When pregnant rats were given 0.05% PTU in their drinking water beginning on gestational day 14, 2-day-old pups already had significantly reduced pituitary GH content (31). The levels stabilized until 8 days of age after which they declined further to below normal levels by 20 days of age. Furthermore, T_3 administered to 14-day-old hypothyroid rats restored the pituitary GH content to 70-80% of normal after 5 days of therapy, indicating a considerable degree of reversibility even in the late stages of the critical period. Both synthesis and release of PRL and synthesis of GH by the pituitary *in vitro* were also found to be suppressed, suggesting a direct influence of the hormone on maturation of the pituitary (24). In male rats thyroidectomized at birth, both basal PRL secretion and PRL responses to TRH and haloperidol were shown to be decreased in all periods of development tested, from 9 to 75 days of age (33).

There is controversy in the literature concerning growth patterns in hypothyroid pups. Some studies have reported maintenance of normal body weights until weaning at 21 days, whether hypothyroidism was induced by neonatal thyroidectomy (27), PTU treatment (34), or exposure to a low iodine diet (L.I.D.) (34,35). After this age, growth in both thyroidectomized and PTU-treated subjects is almost completely arrested, while L.I.D. rats continue to grow, although at a reduced rate compared to controls. In contrast, other studies have reported significantly reduced body weights before weaning, followed by a gradual but suppressed growth rate to maturity (19,24,33). Nutritional factors probably contribute to these differences, issues which will be examined further in the Discussion.

The effects of hypothyroidism on the developing gonads persist into adulthood as well. Perinatal treatment with PTU or thyroidectomy resulted in a significant reduction in absolute weights of testes and ovaries at 28 days (23,24,27), and delayed puberty (delay in vaginal opening and onset of estrous cycles) together with impaired cyclicity (lengthened estrous cycles) in females (23). When PTU treatment is stopped on day 20 in males, testis weights return to normal by 60 days (24). Male rats exhibited lower basal and LHRH-stimulated LH release before weaning age (9 and 15 days) following neonatal thyroidectomy (33). The LH values were higher than controls at 30 days, and later the same as

controls at 75 days. There are no reports of LH or FSH levels in hypothyroid females before or after maturity, although it is likely that abnormalities occur in view of the persistent impairment of cyclicity. The weights of pituitaries in female PTU-treated rats were lower both at 20 and 60 days of age (24). The thyroid glands of both sexes were hypertrophied and significantly greater in weight especially at 20 days and less so at 60 days.

(iii) Development of the Hypothalamic-Pituitary-Thyroid Axis

It has been established that during fetal development, the mammalian placenta is essentially impermeable to both thyroid hormones and TSH (36), whereas thyroid hormones are excreted in the milk of lactating mothers (37,38). The fetal rat thyroid is capable of concentrating iodide (39) and synthesizing thyroid hormones (40) after about gestational day 18 or 19. These facts indicate that fetal development in the rat is, for the most part, independent of thyroid hormones. Dussault and Labrie (41) showed that the hypothalamic pituitary-thyroid axis matures during the first 3 postnatal weeks in the rat, and the various hormonal components of the axis were found to develop as follows: Hypothalamic TRH levels, which are detectable in the fetal hypothalamus as early as day 16 of gestation (42), increase 5 to 6-fold from birth to peak between 16 and 28 days of age. Pituitary and serum TSH attain maximum levels at 10 to 12 days (5 to 6-fold increase

in pituitary TSH, 2-fold rise in serum levels), and remain elevated to the end of the third postnatal week. Levels subsequently decline to adult values by 40 days. Serum T_4 concentrations are very low in the newborn rat and decrease transiently in association with postpartum adaptation (41,43). T_4 levels and production rates increase rapidly to peak values between days 4 to 16, while T_3 levels and production rates begin to increase simultaneously, but reach their peak later at 21 to 28 days (41,44). The production rate of T_4 to T_3 ratio progressively decreases as well, due to increasing peripheral monode-iodination of T_4 to T_3 (44).

This pattern of development indicates that parallel maturation of the hypothalamus and pituitary portal vascular system is associated with an increase in activity of the thyroid gland. The increases in serum T_4 and T_3 are proportionally greater than the increment in serum TSH levels, reflecting a progressive maturation of the negative feedback control system. Krulich et. al. (45) demonstrated a progressive maturation of both pituitary sensitivity to TRH and negative feedback sensitivity to thyroid hormones during the first 2 postnatal weeks, as evidenced by increasing serum TSH response to exogenous TRH, exogenous T_3, and methimazole (thioureylene drug) blockade.

In the neonatal rat pituitary, TSH secretion is independent of the hypothalamus for at least the first postnatal week (46,47), before the establishment of an anatomical

communication between the median eminence and anterior pituitary (48,49). In addition, serum TRH levels were recently reported to be undetectable in newborn rats, but reach adult levels by 10 days of age (50). TRH antiserum administered i.p. did not affect serum TSH levels in rats from birth to 5 days, but significantly reduced serum TSH levels in 7 to 14 day old rats. As expected, exogenous TRH produced a greater pituitary TSH response in 5 day old than in adult rats, which progressively declined to day 14 in parallel with increases in serum T_4 and T_3 concentrations (51). The ability of T_3 to inhibit an increase in serum TSH 10 min. after administration of TRH was markedly reduced in the neonatal group, indicating that pituitary control of TSH release is immature in the early postnatal period. The neonatal thyroid is also considered relatively unresponsive to TSH stimulation (52). The question then arises as to the factors responsible for TSH regulation during early postnatal life in the rat. Serum TSH levels appear to be inappropriately low during this period in relation to the very low serum T_4 and T_3 levels. This may be due in part to enhanced conversion of T_4 to T_3 in the neonatal rat pituitary (53), a process which has been suggested to inhibit TSH secretion (54,55). The mechanism of control of early neonatal secretion of TSH by the pituitary remains to be elucidated, although hypothalamic somatostatin may have a role, since it is known to exert physiological control over TSH secretion (56).

Rats exposed to 0.05% PTU in the neonatal period show a marked derangement of maturation of the hypothalamic-pituitary-thyroid axis (34). Hypothalamic TRH levels during all periods of development were significantly depressed compared to controls. Pituitary TSH levels were similar in both groups until day 18, at which time levels in controls began to decline slightly whereas levels in PTU rats continued to rise sharply to a peak at 32 days. Serum TSH in PTU rats was already significantly elevated on day 1, confirming the presence of a functional pituitary-thyroid axis prior to birth. This was followed by a rapid rise at 12 days which remained high. By contrast, serum TSH levels in controls were consistently lower and relatively stable throughout development. Serum T_4 and T_3 levels were undetectable at all ages to 36 days in PTU rats, despite cessation of treatment at weaning. In rats thyroidectomized at birth, T_4 levels were observed to be very low, but detectable during development (33). Basal serum TSH levels and TSH response to TRH were elevated at all ages examined before weaning. The peak basal serum TSH level was delayed by 2 weeks in the hypothyroid group. The elevated TSH levels were suppressed after the first month of age, possibly due to increased secretion of thyroid hormones by regenerating thyroid tissue.

Rats receiving a low iodine diet from gestation to weaning generally exhibit hormonal levels intermediate to controls and PTU-treated animals (34,35) during the various

stages of development. However, low serum T_4 levels in L.I.D. rats are compensated for by increased levels of T_3 after 18 days, an effect precluded by the use of PTU. This reflects the progressive, though delayed maturation of negative feedback control of TSH synthesis and release in these animals. The fact that the development of this feedback system is greatly impaired by PTU treatment is noted by the chronically elevated serum TSH levels. This persistent elevation may be partially due to a reduction in TSH membrane binding capacity in the thyroid gland associated with chronic hypothyroidism (57). Thus, serum TSH levels would remain high without an appropriate stimulation of T_4 and T_3 to exert a negative feedback influence. In neonatally PTU-treated rats after maturity, Bakke et. al. (23) demonstrated a diminished rate of net synthesis of TSH, impaired synthesis of TSH upon PTU challenge, and slightly depressed serum T_4 and T_3 levels. These effects indicate persistent mild hypothyroidism after maturity, and were found to be dependent on neonatal rather than prenatal hypothyroidism.

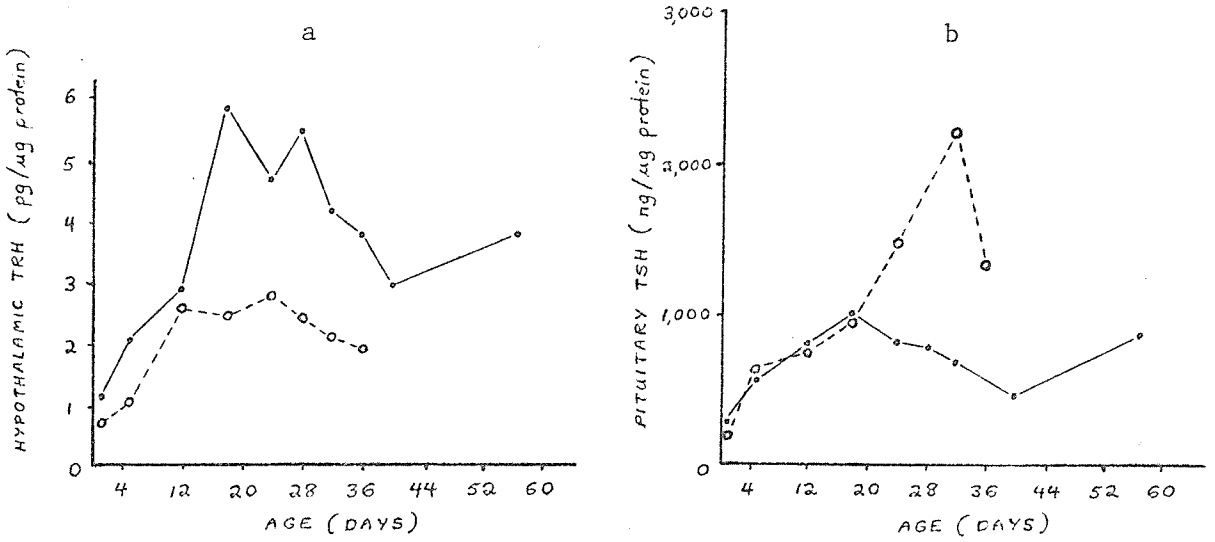
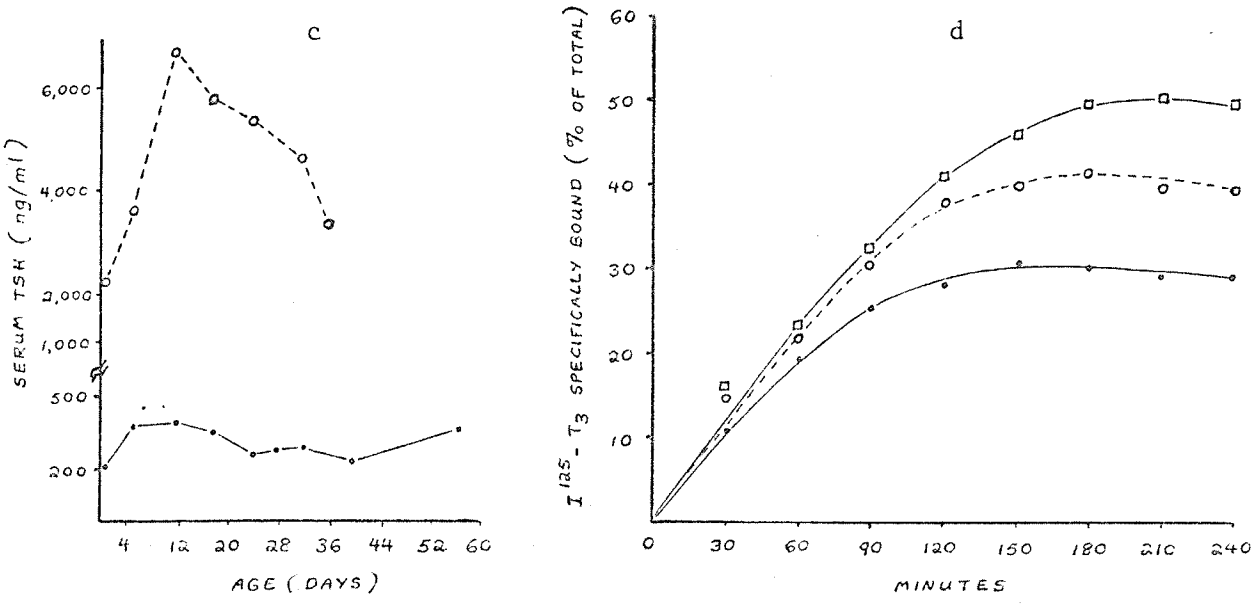


Fig. i. Effect of chronic PTU treatment (0.05% in drinking water from early gestation to post-weaning age) on development of the hypothalamic-pituitary- thyroid axis and binding of I^{125}-T_3 to specific nuclear receptor sites. a-c. Hypothalamic TRH, pituitary TSH, and serum TSH concentrations from birth to 36 days in PTU rats o-----o , and to 60 days in normal controls •———• (from Dussault and Walker (34)). d. Time course of specific binding of T_3 to brain nuclei in 2-day-old controls □———□ , 14-day-old controls •———• , and 14-day-old PTU-treated rats o-------o (from Ishiguro et. al. (63)).



(iv) Central Nervous System T_3 Receptor Ontogenesis

T_3 receptors have recently been demonstrated in late fetal whole brain with a binding capacity (concentration of receptor sites/mg. DNA) only slightly lower than in adults (58). Plasma T_3 levels are exceedingly low at this time, indicating that T_3 receptor ontogenesis is independent of circulating T_3. The receptor binding capacity doubles by 2 days of age and declines gradually over the subsequent 4 weeks. Correlating with this data, the metabolism of T_3 by rat brain tissue in vitro was shown to be elevated during the first 3 postnatal weeks, whereas it was virtually absent in adult brain (59). This pattern suggests an orderly and biologically meaningful sequence of events: first, a preparatory surge in T_3 receptor concentration and second, a rise in serum T_3 over the first 2 postnatal weeks to establish critically high levels of T_3 at specific nuclear receptor sites in order to effect the necessary maturational changes brought about by T_3.

Receptor levels in the cortex are high during the first postnatal week (60), a time period corresponding to maturation of the cortex (61). Neonatally thyroidectomized rats had significantly higher numbers of receptor sites in their cortical cell nuclei than euthyroid subjects at both 13 and 30 days of age, but binding affinity was not affected (60,62). Therefore, T_3 may have a role in regulating density of nuclear T_3 receptors during development. Neonatally PTU-treated

rats also exhibited elevated nuclear T_3 receptor concentrations in brain from 14 to 35 days of age (63). These observations imply that nuclear T_3 receptors in the brain normally mature by about 14 days of age in association with a decrease in binding capacity, and that the process is T_3-dependent. All reports to date have demonstrated alterations in brain nuclear T_3 receptor concentration without change in binding affinity during maturation or PTU treatment, except that of Dozin-van Roye and de Nayer (64) which showed a slight decrease in binding affinity with age. In either case, it appears likely that persistently elevated nuclear T_3 receptor levels occur in neonatally hypothyroid rats as a compensatory mechanism to increase the responsiveness to T_3. A signifcant reduction in the number of cytosolic thyroxine-binding sites has also been reported in both cortex and cerebellum during development from 3 to 35 days of age (65). Again, no alteration in binding affinity was observed. The extent to which thyrotrope nuclear T_3 receptor number and/or occupancy are altered in the normal and hypothyroid neonatal rat pituitary is unknown.

IV Development of Higher Central Nervous System (CNS) Functions in the Hypothyroid Rat

(i) Timetables of Maturation in the Developing Brain

The hypothalamus and median eminence are easily identifiable by 14-15 days of gestation in the fetal rat, and

neural cells begin to form by 16 days (66). The period from gestational day 18 to approximately postnatal day 19 is associated with rapid myelinogenesis, intense proliferation of dendritic and axonal processes, and synaptogenesis in most areas of the brain (61). There is also continuing proliferation of neuroblasts and glial cells in some areas such as the cerebellum. By examining the incorporation of labelled precursors into protein and RNA of brain slices in vitro, it was shown that the influence of thyroid hormones on these processes is both age- and region-dependent (61). Specifically, the differentiation and maturation of the hypothalamus was found to begin on prenatal day 18 and extend to postnatal day 12 (as indicated by its sensitivity to T_3 during this interval), at which time the hypothalamic-pituitary-thyroid axis becomes functional (41). The cerebral cortex begins its maturation at about the time of birth and continues for the first 2 postnatal weeks. The cerebellum, having the slowest rate of development, matures between postnatal days 12 to 19. These observations correspond closely to studies of neuronal differentiation timetables which showed that pyramidal cell differentiation occurs during the interval 0 - 10 days, and Purkinje cell differentiation from 6 - 21 days (67 - 70). The rates of microtubule assembly, an index of neurite growth, are greatly enhanced during these periods in normal rats, and significantly depressed by con-

current hypothyroidism (71).

Maturation of individual brain structures consists of an orderly sequence of neuronal and glial events involving phases of cell proliferation, migration and differentiation. The completion of each event sets the stage for the next in order of complexity, resulting in a series of events which are all critical and interdependent. Morphological changes which occur in the brains of hypothyroid developing rats include altered brain shape, reduced brain weight, decreased size and increased density of cortical neurons, simplification of neuronal dendrites, decreased numbers of cortical axons, delayed disappearance of the cerebellar external granular layer, and retarded myelination (22 72,73). When daily T_4 therapy is initiated on the 10th postnatal day in rats made athyrotic at birth (at which time the morphological features of the cerebral cortex are analogous to those found in the human at birth (74), growth is largely restored so that the mean body weight at 50 days is about 80% of age - matched controls (67). In addition, the characteristic cretinoid features in the young adult rats disappear so that they cannot be phenotypically distinguished from saline-injected controls (10). However, and most importantly, these apparently normal rats were shown to exhibit chemical and histological abnormalities of cortical maturation identical to those found in untreated hypothyroid animals (67). These observations point out the irreversibility of brain damage which

results from delayed replacement therapy, and suggests that even treatment initiated at birth in the human is too late to allow full recovery of normal brain function. The permanency of impairment is also evident in the cerebellum in which it was shown that 90 day old neonatally PTU-treated rats continued to exhibit malformed Purkinje cell dendrites and deficient synaptic development (75). In this brain structure, thyroid hormones have been demonstrated to act as a "time clock", signalling the termination of cell proliferation and the onset of neuronal differentiation (76). The growth and disappearance of the external granular layer (EGL) in the developing cerebellum is particularly affected. Rapid cell proliferation begins in the EGL on about postnatal day 3 or 4 (77), undoubtedly stimulated by the sharp concurrent rise in serum T_4 which occurs at this age (41). Following the peak T_4 levels attained on day 15 to 16, the gradual decline corresponds to the cessation of cell proliferation and onset of neuronal differentiation. In hypothyroid rats, the EGL was found to persist longer and continue proliferating, resulting in poorly differentiated cells after maturity.

(ii) Development of Electrocortical Activity

The genesis of cortical evoked potentials is generally regarded as the result of activation of different afferent systems (79,80). The nerve impulses which produce the

primary cortical responses are conducted through a fast pathway called the specific thalamic radiation. Those impulses that elicit the secondary repetitive slow waves following the primary responses are conducted centripetally by an extrathalamic non-specific route. The slow negative component develops early during ontogeny, whereas the fast positive component of the evoked response appears much later in life (81). In neonatally thyroidectomized rats, the evoked potential has a significantly longer latency and duration, as well as diminished amplitude of the EEG (18). In normal rats, the EEG amplitude increases during development, but even with T_4 replacement therapy in hypothyroids, the amplitude remains unchanged. Administration of T_4 for a period just sufficient to restore metabolism improved the temporal parameters of the electrocortical response however, indicating that metabolic factors contribute to this aspect of the activity. The hypothyroid subjects also showed no EEG response to visual or auditory stimulation between 15 to 24 days, which was evident in normal rats by 15 days of age and increased progressively to 24 days. By the 10th postnatal day, the threshold of the transcallosal response was significantly higher and its amplitude reduced in thyroid-deficient rats compared to controls (82).

In contrast to the characteristics of evoked cortical responses, it was found that the electroshock seizure threshold (EST) in neonatally thyroidectomized rats, measured

between postnatal days 10 to 30, is consistently and significantly lower than in controls (19). This paradoxical effect indicates higher brain excitability, and appears to be due in part to altered ionic conditions in the nerve cells. The sodium content in the brains of young hypothyroid rats was shown to be increased (83), and high intracellular sodium content is known to lower the EST (84). In addition, the pattern of maximal electroshock seizure (MES) measured at 25 days showed significantly shorter flexion and longer extension in hypothyroid rats, suggesting that the capacity to sustain maximal activity is increased. Prolonged recovery time from maximal seizures and sustained excitation from minimal seizures was also frequently observed. It is considered probable that both the EST and MES effects arise from defective maturation of cortical neurons in neonatal hypothyroidism (67) which would normally exert an inhibitory influence on seizure mechanisms, as well as from impaired development of subcortical structures of the thalamus and reticular activating system, also involved in seizure activity. The cortical cells responsible for excitatory and inhibitory processes mature at different developmental stages (85), and consequently, a deficiency of thyroid hormones during this period may permanently disturb the normal balance between excitatory and inhibitory actions.

Van Middlesworth (86) recently demonstrated that perinatally iodine-deficient rats exhibit a high incidence (89%)

of audiogenic seizures which persist into adulthood and are absent in control littermates. Relatively mild neonatal PTU treatment (0.02% PTU drinking solution drom day 0 to 19 postnatally) produced a similar effect (87), and re-exposure of the adult seizure-susceptible rats to dietary PTU was found to enhance their seizure-sensitivity. These observations are important in that they demonstrated for the first time an inherent susceptibility to epileptic-like seizures following neonatal hypothyroidism, in addition to the increased sensitivity to electrically-induced seizures. Both phenomena are indices of increased brain excitability, but the audiogenic seizure syndrome requires hair cell damage in the inner ear, indicating that some degree of peripheral nervous damage as well as damage within the CNS occurs following neonatal thyroid deficiency.

V Behavioral Consequences of Neonatal Hypothyroidism

When thyroid hormone deficiency occurs in humans perinatally, it causes a form of mental retardation associated with cretinism. The clinical signs of hypothyroidism in athyrotic cretins are usually not apparent until a few months after birth (88), and the symptoms which can develop such as enlarged tongue, coarse, thickened, and cool skin, decreased muscle tone, and bradycardia are only present in one-quarter of patients. Administration of T_4 during the early neonatal period significantly improves the intellectual

capacity of these children (88 - 91), whereas initiation of therapy after 4 months of age produces virtually no effect. Another problem is that clinical signs of thyroid deficiency or excess are difficult to assess during replacement therapy, and unless precise restoration of a chemically euthyroid state is achieved, the intellectual development of the human cretin will not be maximized. Indeed, it was shown that an excess of thyroid hormone administered chronically to either normal or hypothyroid neonatal rats results in abnormal development of the cerebral cortex (67).

Behavioral studies in rats examining both innately organized and adaptive (learned) behaviors have been used to investigate these problems. Eayrs and Lishman (20) examined both types of behavior using two forms of neonatal hypothyroidism, thyroidectomy shortly after birth, and methylthiouracil treatment from day 0 to 25 postnally. Both methods were found to produce similar behavioral deficits. The innate behaviors examined included sensitivity to cutaneous stimulation, reflex suspension, startle response, righting reflex and placing reflex. Hypothyroid and control rats were not found to differ in their sensitivity to cutaneous stimulation between days 4 to 18, as determined by the footshock threshold required to evoke the first reflex response of the hindlimb. In the reflex suspension test, performed daily from day 5 to 15, each subject's forepaws were touched against a horizontal wire, and the length of time the rat held on before releasing

its grip and falling was measured. Normally, the duration of grasping progressively increases over successive days, although hypothyroid rats exhibited a significant delay in the development of this response. In the startle response test, performed between days 9 to 24, a sharp auditory stimulus (click) was presented behind the rat's head, and the age at which the first motor response occurred (jerk of head and hindlimbs) was recorded. The first appearance of the response was delayed by approximately 1 week in hypothyroid rats. The first occurrence of the righting and placing reflexes were also delayed by about 1 week in these rats.

The adaptive behavior studied by these authors was ability to learn a maze route for food reinforcement. Hypothyroid rats took longer to run the maze and made significantly more errors on each trial. The additional time taken to reach the food was found to be due to slower movement rather than the greater number of errors made, since mean time spent per error was reduced compared to controls. The observation that hypothyroid rats were conspicuously less delayed by each error was interpreted by Eayrs and Lishman (20) as a reduced sensitivity to the environment, that is, the animal is less inhibited by encountering an unexpected situation. In turn, this characteristic seemed to perpetuate the rat's greater error frequency which persisted due to constant repetition of previously unsuccessful routes, persistence in long pathways

to the goalbox (whereas normal rats soon discover the shortest route) and barrier circling without recognition of repetition. It was concluded from these studies that neonatally hypothyroid rats are retarded in the maturation of innately organized responses, and in later life, not only perform learned tests more slowly, but show incapacity to react to their environment and so profit by experience.

In other types of learned behaviors such as a simple 4 - trial water escape response, hypothyroid rats failed to learn the response at all, both after weaning and as adults when PTU treatment was prolonged to 50 days (22). Both euthyroid and hypothyroid rats were unable to acquire a simple sensorimotor maze escape response at 18 days, whereas controls easily showed maze acquisition to criterion at 50 days while hypothyroid subjects (perinatal 0.2% dietary PTU treatment) were still unable to acquire the response (92). In both of these escape responses as well as in the maze - learning response, the deficits were permanent and not reversed by delayed T_4 replacement therapy. Eayrs (21) suggested that the impairment in capacity to learn is associated with deficiency in neuropil and a consequent reduction in the probability of interaction between neurons. Thus, the permanency of behavioral deficiency in hypothyroid animals is expected. Later Eayrs (93) attempted to correlate parameters such as axonal density, axodendritic interactions, and rate of decay of the dendritic field with distance from the cell body in cortical

neurons, with degrees of impairment in adaptive behavior. His approach met with limited success however, and it appeared in subsequent studies that the severity and timing of early thyroid deficiency were more suitable parameters to correlate with induction of learning disorders (94, 95). Decrements in maze performance were found to be related to the age at which thyroidectomy is performed (from 0 → 24 postnatal days) (21). Davenport and Dorcey (94) demonstrated dose-related deficits in maze-learning in adult rats after neonatal exposure to thiouracil (0.03 - 0.15% in the diets of lactating mothers) during the last 16 days of gestation and the first 16 postnatal days. In contrast, if 0.2% dietary PTU treatment was begun at birth, relatively minor maze-learning deficits resulted (95), indicating that the critical period of thyroid hormone influence on learning processes begins in the prenatal stage. Further testing revealed that the lower critical age boundary was approximately gestational day 18, since onset of exposure to thiouracil on that day produced significantly greater learning deficits in adults than when treatment was initiated at birth. Onset of treatment before gestational day 18 produced little additional impairment. Interestingly, this critical lower age boundary also coincides with the onset of thyroid function (40) and hypothalamic maturation (61) in the fetal rat.

One behavioral consequence of neonatal hypothyroidism which has been controversial in the literature is that of

general activity level. The early studies of Eayrs and Lishman (20) indicated overall hypoactivity and slowness of motor responses in both innate and adaptive response acquisitions. Subsequent studies of avoidance and escape learning, as well as the operant bar-press response showed an increased level of activity in hypothyroid rats compared to controls (75, 95). Hyperactivity was demonstrated through testing in an open-field activity apparatus, and this behavior was found to impair avoidance and escape learning due to its effect of reducing the subject's attention to specific cues required to learn the response. In an operant conditioning task requiring limited response rates (differential reinforcement of low rates -DRL), PTU treated rats (0.3% dietary PTU from 0 to 30 days postnatally) maintained significantly elevated response rates relative to controls (75), which is maladaptive in this learning situation. For operant bar-press responses generally, hypothyroid animals tended to maintain a fairly consistent response rate over testing sessions (96), and exhibited a significantly greater resistance to extinction. These patterns of persistence of the originally learned task are generally counterproductive and associated with maladaptive behavior (95). Both early T_4 replacement therapy (96) and environmental enrichment (97) were shown to lessen the observed learning deficits.

β-Endorphin and Somatostatin in the Brain - Proposed Relationship to the Neuroendocrine, Electrophysiological, and Behavioral Consequences of Neonatal Hypothyroidism

I. Role of β-Endorphin and Somatostatin in Neuroendocrine Functions

(i) β-Endorphin

Because of the essential role of thyroid hormones in normal brain development and the extensive endocrine consequences associated with deficiency during the critical period (23, 24), it is logical to assume that arrested differentiation of neural centres within the hypothalamus during maturation could lead to altered (perhaps permanently) function of hypophysiotropic hormones such as β-endorphin and somatostatin. For example, it was recently demonstrated that the arcuate nucleus of the hypothalamus contains all of the cell bodies of β-LPH, ACTH, and α-MSH neurons directed into the brain (98). Cell bodies are the site of peptide synthesis within neurons, and the cytological abnormalities observed in the arcuate neurons following neonatal thyroidectomy are indicative of reduced protein synthesis (27). In addition, β-LPH, β-Endorphin, and ACTH immunoreactivities were shown to coexist within the same hypothalamic neurons (99). These facts together strongly support the possibility that reduced numbers and/or synthesizing capacity of β-Endorphin-containing neurons in the hypothalamus could result

from neonatal hypothyroidism. Such a consequence would both severely restrict the supply of β-endorphin to extrahypothalamic brain regions as well as disturb the pituitary β-endorphin system with which the hypothalamic neurons are also connected.

Endogenous opiod peptides appear to have a physiological role in regulating pituitary hormone secretion, effects which are mimicked by morphine and antagonized by naloxone (100). In summary, they stimulate release of PRL, GH, ACTH, and ADH, and inhibit release of TSH, LH and FSH. Of these effects, β-endorphin specifically has been shown to stimulate secretion of GH and PRL, and was 500 to 2,000 times more potent than met-enkephalin in eliciting these responses (101). The opiod peptides have no direct effect on the pituitary, nor do they directly alter the action of hypothalamic hypophysiotropic hormones. Insteady they appear to increase serotonin metabolism and decrease dopamine metabolism in hypothalamic neurons, which in turn influences the release of hypothalamic hormones into pituitary portal vessels. Neonatal thyroidectomy was shown to inhibit brain serotonin synthesis (102), and thus, pituitary and serum levels of PRL and GH, which are lowered by neonatal hypothyroidism (24, 33) are also lowered by both reduced β-endorphin and serotonin levels. These results suggest a direct involvement of β-endorphin in at least some of the hormonal consequences of neonatal hypothyroidism.

In the adult animal, a relationship between primary hypothyroidism and β-endorphin levels in the brain has been established. Thyroidectomy was found to significantly increase β-endorphin concentrations in the hypothalamus (103), while primary hypothyroidism induced by a combined L.I.D. and 1% $NaClO_4$ drinking solution (competitor of iodide) for 3 weeks also increased hypothalamic β-endorphin content, but decreased levels in the pituitary and striatum (104). Hyperthyroidism, as expected, reduced hypothalamic β-endorphin levels. Conversely, chronic treatment with morphine sulfate in adult rats decreases pituitary TSH content (105), and acute administration decreases serum TSH levels (100) and reduces thyroid function (106).

Bakke et. al. (107) observed that neonatally morphine sulfate-treated rats had larger pituitary glands containing less TSH. Their thyroid glands were also enlarged relative to reduced body weights. As adults, their basal pituitary and serum TSH levels were usually normal, but upon PTU challenge they initially developed a much greater goiter growth compared to controls (suggesting an initial abnormal increase in TSH secretion), followed by a decrease in net TSH synthesis and diminished pituitary size. Interestingly, diminished net TSH synthesis was also observed in adults following PTU-induced neonatal hypothyroidism (23). These interrelationships between opiates (endogenous or exogenous) and the

development and adult status of the pituitary-thyroid axis suggest that β-endorphin levels in the brain may be permanently affected by neonatal hypothyroidism and/or that altered development and function of endorphin systems during the neonatal period (brain levels of β-endorphin increase progressively between postnatal days 6 to 25 (108) could contribute to the permanent endocrine consequences in the pituitary-thyroid axis and in GH and PRL regulation.

(ii) Somatostatin

Hypothalamic immunoreactive somatostatin (SRIF) is present in greatest concentration in the median eminence (109, 110), where it has been demonstrated immunohistochemically in nerve endings closely related to the capillary loops of the hypophyseal-portal venous system (111,112). In vitro release of SRIF from the median basal hypothalamus (113, 114) and from hypothalamic synaptosomes (115) has been shown to occur in response to depolarizing stimuli (113, 115), monoamines (115), and peptides including neurotensin, substance P, and GH (114). Synthetic SRIF has inhibitory effects on pituitary GH and TSH release in vitro and in vivo under physiological conditions (116 - 119), as well as on PRL release (117). The inhibitory action of SRIF occurs at the level of the somatotroph or thyrotroph (120), associated with binding to plasma membranes (121) and appears to be mediated in part by inhibition of cAMP generation (122). The best evidence that SRIF exerts a

physiological role as an inhibitor of pituitary GH and TSH release is derived from passive immunization studies in which administration of anti-SRIF serum in vivo results in elevation of basal and stimulated GH (123, 124) and TSH (56, 125) levels. These findings imply a tonic inhibitory role for SRIF in pituitary GH and TSH release.

Based on these observations, it is speculated that hypothalamic SRIF synthesis and release might be altered in situations of chronic TSH elevation such as during primary hypothyroidism in the adult animal, and during and subsequent to neonatal hypothyroidism (34). The first situation was recently examined by Berelowitz et. al. (126) who found that hypothalamic SRIF content was decreased in hypothyroid rats, an effect reversed by T_3 treatment. In vitro SRIF release from hypothyroid hypothalamic slices was decreased basally and under high-K^+ or dopamine stimulation. In addition, SRIF release from normal rat hypothalami was unaffected by TRH or TSH but was stimulated by T_3. These results suggest that T_3 exerts its negative feedback effect on pituitary TSH release via stimulation of hypothalamic SRIF release as well as by a direct pituitary effect. Thus, the elevated TSH levels seen in primary hypothyroidism may result in part from a decrease in the tonic inhibitory effect of hypothalamic SRIF (due to decreased SRIF synthesis and release). If this proposed physiological role of SRIF in regulation of the hypothalamic -

pituitary - thyroid axis is accurate, then one might anticipate reduced hypothalamic levels of SRIF in neonatal hypothyroidism as well. Alternatively, the GH deficiency observed in primary hypothyroidism (127) as well as in neonatal hypothyroidism (24, 32) could arise from or result in decreased hypothalamic SRIF levels. Evidence for this is provided by the observation that administration of anti-GH serum in the rat decreases hypothalamic SRIF content (128).

II Role of β-Endorphin and Somatostatin in Higher CNS Functions

There are two distinct neuronal systems of β-endorphin in the mammalian brain, one represented by the pituitary β-endorphin system, the other a neuronal network projecting to a number of limbic and midbrain structures (99). The brain system contains all the synthetic components of the pituitary system, including the 31 K precursor, and is also distinct from enkephalin pathways in the brain (130). Regional distribution studies have detected immunoreactive β-endorphin in highest concentration in the hypothalamus, with intermediate levels in midbrain and limbic structures and lowest concentrations in the striatum, cortex, and cerebellum (129, 130). Somatostatin is also widely distributed throughout the CNS, with highest concentrations in the hypothalamus, intermediate levels in striatum and hippocampus, and lowest concentrations in the cortex and cerebellum (109, 131). Somatostatin - containing axons and nerve terminals have been observed in the

neocortex, hippocampus, thalamus, hypothalamus, and several other brain areas (132), and within numerous distinct nuclei within the hypothalamus (133). Subcellular distribution studies of somatostatin have revealed that it is primarily localized in synaptosomes, indicating release from nerve terminals as a neurotransmitter (134). Ca^{++} - dependent release of immunoreactive somatostatin was also demonstrated in hypothalamic, amygdala (135), and cortical slices (136) under basal and high-K^{+} stimulated conditions.

Localized infusion of β-endorphin to limbic brain areas such as the hippocampus induces nonconvulsive epileptogenic and hypersynchronizing actions in the spontaneous EEG patterns of rats (137). Met-enkephalin and morphine produced a similar effect, although β-endorphin was much more potent on an equimolar basis, and the response occurred at doses too low to elicit analgesic or behavioral effects. Single-unit microiontophoretic studies have suggested a post-synaptic inhibitory role for β-endorphin in most brain areas except in pyramidal neurons of the hippocampus which show increases in discharge rates (138). This is tnought to be due to disinhibition by β-endorphin of inhibitory basket cell interneurons on the pyramidal cells, and suggests a role for β-endorphin in regulation of limbic excitability. Cortical administration of somatostatin to rats produces an early sign of activation and stereotypy which is later followed by

drowsiness (139). Intrahippocampal application induces stereotyped behavior and alterations in the sleep-waking cycle of the rat together with dissociation of the EEG from the behavior (140). Injection of somatostatin into the rat neostriatum elicits excitatory effects in low doses and disruption in central motor control with higher doses (141). Renaud et. al. (142) observed inhibition of unidentified neurons in the cerebellar cortex, and hypothalamus of urethane-anaesthetized rats after microiontophoretic application of somatostatin. However, the majority of reports which followed have indicated an excitatory role of somatostatin in the CNS, both in the presence or absence of anaesthesia, and under both in vivo and in vitro conditions. Ioffe et. al. (143) obtained excitatory responses from neurons of the sensorimotor cortex in the conscious, unanaesthetized rabbit. Subthreshold stimulation by somatostatin potentiated the excitatory action of glutamic acid and the effect was GABA-suppressible. An excitatory effect was also observed in the rat hippocampus using a technique of intracellular recording on brain slices, which showed a fast onset depolarization of the cell membrane accompanied by increased excitability (144). More recently it was demonstrated that somatostatin applied microiontophoretically to responsive neurons in the frontal and parietal neocortex, hippocampus, and striatum elicits a dose-dependent increase in firing rate in rats under two types of

anaesthetic conditions (145). Repeated exposure to somatostatin resulted in a gradual diminution of response magnitude, indicating desensitization. Somatostatin did not interfere with H^3-muscimol binding to GABA receptors or affect GABA release from synapses preloaded with H^3-GABA. This implies that somatostatin does not evoke excitatory responses through attenuation of GABA-mediated synaptic inhibition, but has a direct excitatory influence on neuronal membranes.

III Behavioral Actions of β-Endorphin and Somatostatin

Intracerebroventricular (i.c.v.) injection of β-endorphin and somatostatin leads to three types of behavioral effects which are common to both: analgesia (146,147), opiate withdrawal syndrome in low doses (characterized by stiffly arching tail or 'Straube sign', wet dog shakes and excessive grooming) (148), and akinesia at high doses (146,149). Several differences between the two peptides are also noted however. Application of β-endorphin i.c.v. precipitates epileptiform EEG activity in limbic areas which never develops into generalized motor convulsions (131,148), whereas somatostatin can induce violent tonic-clonic seizures. The effects of somatostatin were dose-dependent in that low doses (i.e. 0.1 ng.) initially led to motor incoordination which progressed to a phase of hyperactivity (excessive grooming and exploring), and eventually violent tonic-clonic

seizures followed by a post-ictal period (149). Higher doses of somatostatin (i.e. 5 - 10 ug) led to a generalized rigidity of all body muscles (akinesia) which upon mild sensory stimulation either led to violent tonic-clonic seizures or caused the rat to remain in an unconscious, post-ictal akinetic state. EEG measurements during the akinesia showed a depression with decrease in power at all frequencies. In contrast, high doses of β-endorphin administered i.c.v. (i.e. 50 ug.) which also produces akinesia, was associated with an increase in power of all frequencies (hypersynchrony), and could be interrupted by an EEG arousal response to sensory stimulation. In addition, this effect is naloxone-reversible whereas the somatostatin - induced syndrome is naloxone-insensitive. There is some contradiction in the reports of EEG responses to β-endorphin however, as it was also found that β-endorphin injected into the lateral but not the third ventricle caused a reduction in power of all EEG frequency bands (150). β-endorphin may be involved in the phenomenon of audiogenic seizures as well, since it was shown that systemic administration of other opiates such as morphine (151) and metenkephalin (152) both reduce the incidence of audiogenic seizures in a susceptible strain of mice, while naloxone alone increased the severity and incidence of audiogenic seizures in these mice (153).

These studies have indicated that both β-endorphin and somatostatin may have a physiological role in modulating

brain excitability. The evidence for an excitatory influence of somatostatin and its ability to induce epileptic seizure activity is clear, and thus it is postulated that pathological alterations in brain somatostatin metabolism may occur as a result of neonatal hypothyroidism, and may account in part for the seizure-susceptibility of these rats (86,87). The evidence for β-endorphin is less clear. In general it appears to exert a depressant action on neuronal excitability and seizure mechanisms, and the only excitatory effect observed was a net excitation in hippocampal neurons thought to be due to disinhibition, and which never led to convulsive seizure activity. Whatever the exact role of β-endorphin, as for somatostatin it is postulated that permanent alterations in brain β-endorphin neuronal systems as a result of neonatal hypothyroidism may contribute to the increased brain excitability and seizure-susceptibility seen in these rats.

One further point concerning β-endorphin and behavior deserves consideration. Several studies have provided evidence for a role of opiod peptides in general activity level, and learning and memory processes. Systemic administration of β-endorphin in rats selectively increased open-field behavior by stimulating grooming activity (154). Enkephalin produced faster running and fewer errors during maze training, whereas other potent opiate analogs or morphine either impaired performance or produced no effect (155). These results indicated that the effects of endogenous opiod peptides

on learning may be dissociated from their opiate effects. Met-enkephalin was found to facilitate memory consolidation, since prior administration attenuated CO_2 - induced amnesia of an avoidance task in rats (156,157). Leu-enkephalin, when administered after amnesia induction, facilitated retrieval (memory recall) of the learned response. The enhancement of memory was not naloxone - reversible in either case, and doses which produced facilitation had no analgesic effect. The effects were produced by systemic administration of these pentapeptides, and although no clear-cut effects of this type have been obtained with β-endorphin, another line of evidence suggests its involvement centrally in learning and memory processes. Studies have indicated a physiological role for β-endorphin in stimulation of vasopressin (ADH) release from the neurohypophysis (150,158, 159), while elevated ADH levels have been strongly implicated in enhancement of learning and memory processes (Rigter and Van Riezen, review (160)). Finally, in learning paradigms in which stress is involved, such as avoidance or escape learning, β-endorphin impairs learning due to its acute release in response to the stressful stimulus, which leads to analgesia and in turn attenuates the aversiveness of the learning situation (161). This effect was shown to be naloxone-reversible.

HYPOTHESIS AND EXPERIMENTAL OBJECTIVES

The evidence reviewed has provided strong support for the premise that a relationship exists between β-endorphin and somatostatin in the brain and the permanent deficits in CNS functions induced by neonatal hypothyroidism. Three lines of evidence in particular support such a hypothesis: (i) the neuroendocrine consequences of early thyroid deficiency, (ii) the resulting impairment in regulation of higher CNS activity in terms of altered neuronal excitability and seizure-susceptibility, and (iii) the behavioral consequences and learning disabilities associated with this neonatal disorder. Thus, it is hypothesized that impaired development and functioning of β-endorphin- and somatostatin - containing neuronal networks in the brain is one of the many consequences of thyroid hormone deficiency during the critical neonatal period of brain development. These early disruptions in peptide systems, once established, would then contribute to the permanent brain dysfunctions discussed.

The following studies have primarily employed the model of neonatal hypothyroidism developed by Van Middlesworth (87), in which relatively mild postnatal PTU treatment resulted in a high incidence of audiogenic seizures after weaning. The objectives were: (i) to determine the concentrations of β-endorphin and somatostatin in brain regions of control and neonatally PTU-treated mature rats (male and

female) using optimal brain extraction and radioimmunological techniques previously tested, (ii) to determine whether the occurrence of seizures in neonatally PTU-treated rats (PTU rats) is associated with alterations in brain β-endorphin and somatostatin levels, (iii) to compare various behavioral indices of brain dysfunction in PTU and control rats such as seizure-sensitivity, startle responsiveness, open-field activity, and maze-learning ability, (iv) to compare brain β-endorphin and somatostatin content in terms of severity of neonatal hypothyroidism, and with post-maturational exposure to PTU (which was shown to potentiate the seizure susceptibility of these rats), and (v) to examine whether stress, known to affect brain peptide levels, differentially affect β-endorphin and somatostatin levels in the brains of PTU rats compared to controls.

MATERIALS AND METHODS

I. Peptides, Chemical and Drug Preparations

Camel-β-Endorphin, Tyrosine^{1}-somatostatin, and synthetic somatostatin were purchased from Peninsula Laboratories, San Carlos, CA. Sephadex G-25 (fine) and carboxymethyl cellulose (CMC) - 23 were obtained from Pharmacia. Lactoperoxidase was from Calbiochem, La Jolla, CA., Trasylol (aprotinin) from Bayer AG, and NaI^{125} from New England Nuclear Co. Bovine serum albumin (BSA) fraction V and 6-propyl-2-thiouracil (PTU) were obtained from Sigma Chemical Co. The following chemicals were all from Fisher Scientific Co.: NaH_2PO_4, Na_2HPO_4, EDTA, NaCl, NaH_3, H_2O_2 (30%, v/v, solution), $NaClO_4$, $NaHCO_3$, sodium acetate, ammonium acetate, glacial acetic acid, HCl, and NaOH.

II. Preparation of Buffers

Phosphate-buffered saline (PBS) was made by dissolving 8.77 g. NaCl, 2.33 g. $Na_2HPO_4 \cdot 7\ H_2O$, 0.18 g. $NaH_2PO_4 \cdot H_2O$, and 0.10 g. NaH_3 in one liter of distilled water and adjusting to pH 7.4. Phosphate buffer (PB) 0.14 M was made by dissolving 37.5 g. $Na_2HPO_4 \cdot 7\ H_2O$ and 9.0 g. EDTA in one liter of distilled water. When dissolved, 5.0 g. of BSA was added and the final pH adjusted to 7.4.

III. Preparation of Camel-β-Endorphin and Somatostatin Antisera

Antisera were prepared in advance (generously supplied

by Dr. Friesen's Lab) according to the following procedure: Synthetic camel-β-endorphin (or synthetic somatostatin) was conjugated with BSA using glutaraldehyde as described by Reichlin et al. (162) for ACTH. This complex (500 µg. of peptide equivalent per rabbit) was emulsified in Freund's complete adjuvant and injected into two young rabbits at multiple intradermal sites. Booster injections were given intramuscularly every 3 - 4 weeks. Twelve days after the third immunizations, the animals were bled and sera tested for immunoreactivity. All radioimmunoassays were performed using β-endorphin antiserum coded (1 - 6) and somatostatin antiserum coded (S2 - 2).

IV. Iodination of Camel-β-endorphin and Tyrosine^{1}-Somatostatin

I^{125}-Camel-β-endorphin was prepared by a modified lactoperoxidase method of Thorell and Johansson (163). Twenty-five µl. of 0.4 M sodium acetate (pH 5.6) was added to the reaction tube containing 5 µg. camel β-endorphin dissolved in 5 µl. of 0.1 N acetic acid. One mCi (25 µl.) NaI^{125} and 25 µl. lactoperoxidase (1 µg/ml dissolved in 0.05 M phosphate buffer, pH 7.4) was added, followed by two 10 µl. aliquots (separated by a 1.5 min. interval) of 30% H_2O_2 at 1: 15,000 dilution. After an additional 1.5 min., the reaction was terminated by diluting the reactants with 0.7 ml. ice-cold PBS and 50 µl. of 5% BSA in PBS. Unreacted iodide and damaged peptide were separated from intact iodinated β-endorphin by gel filtration on a

Sephadex G-25 (fine) 1.0 x 20 cm. column equilibrated and eluted with PBS, pH 7.4. This separation step must be done at 4° C, and the column is pretreated immediately before use with 1 - 2 ml. of 5% BSA in PBS in order to minimize loss of iodinated proteins and prevent adherence of free iodide to the gel matrix. Twenty drop fractions were collected at a flow rate of 1 drop / 7-8 sec., and peaks of radioactivity were measured in units of millirems/ hr. using a Nuclear-Chicago geiger counter.

I^{125}-Tyr^{1}-somatostatin was also prepared by a modified lactoperoxidase method of Thorell and Johansson (163). Twenty-five µl. of 0.4 M sodium acetate (pH 5.6) was added to the reaction tube containing 5 µg. tyr^{1}-somatostatin dissolved in 5 µl. 0.1 N acetic acid. The same reaction steps used for β-endorphin were then followed, after which the reaction was terminated by addition of 1.0 ml. ice-cold 2 mM ammonium acetate (pH 4.6) and 50 µl. of 5% BSA in 2 mM NH_4OAc. The iodination components were separated by cation-exchange chromatography using a carboxymethyl-cellulose (CMC-23) 0.7 x 20 cm. column equilibrated and eluted with 2 mM NH_4OAc buffer. Before packing the column, the CMC must be activated by the following procedure: Weighed, dry CMC is stirred into 15 vols. of 0.5 N NaOH (i.e., 5 g/75 ml) and allowed to stand for 30 min. The supernatant is filtered and washed several times with distilled water until the effluent pH is about 8.0. Seventy-five ml. of 0.5 N HCl is then added and allowed to stand for 30 min. It

is filtered and washed until the effluent pH is 4.0. This HCl step is repeated again and washed until a neutral pH is achieved. Sodium azide (0.02%) is added, and the column matrix stored at $4^{o}C$ prior to packing. The iodinated sample was applied to the column and eluted at $4^{o}C$ by collecting 50 drop fractions at a flow rate of 1 drop/7 - 8 sec. Two drops of trasylol (10,000 K.I.U./ml) and 2 drops of 5% BSA were added to fractions 20 - 35 prior to the collection. Fractions 1 - 10 were eluted with 2 mM NH_4OAc (pH 4.6) and fractions 10 - 40 with 0.2 M NH_4OAc (pH 4.6). Each fraction was tested for specific radioactivity, measured in units of millirems/hr.

V. Double-Antibody Radioimmunoassay Procedures for β-Endorphin and Somatostatin

Synthetic camel-β-endorphin standards were prepared by diluting a 1 µg/ml stock solution in 0.14 M PB to produce the following concentrations: 100 pg/ml, 250, 500, 750, 1 ng/ml, 2.5, 5, 7.5, 10, 25, 50, 75, 100, and 250. On day 1 of the assay, standards and samples were prepared in duplicate using 12 x 75 mm. borosilicate glass tubes. To each tube was added 0.1 ml. of trasylol (5,000 K.I.U./ml), followed by 0.1 ml. of standard or sample together with 0.1 ml. of PB. This simultaneous aliquoting of sample and buffer was carried out using a Micro-Medic (model #25004) automatic pipettor. Lastly 0.1 ml. of anti-β-endorphin rabbit serum (1st antibody, 1 - 6, diluted 1: 10,000) was added. The reaction mixture was incubated

at 4°C overnight, and on day 2, 0.1 ml. of I^{125}-β-endorphin (25,000 - 30,000 cpm/0.1 ml.) was added. The incubation was resumed at 4°C overnight, after which 0.1 ml. of sheep anti-rabbit gamma globulin (SAR, 2nd antibody) was added on day 3 at a dilution of 1: 20, and 0.1 ml. of normal rabbit serum (NRS) at a dilution of 1: 350. The assay was again incubated overnight at 4°C, and on day 4, 1.0 ml. of cold PB was added to each tube, followed by centrifugation at 2,000 x g for 30 min. The supernatants were decanted and the pellets counted in an automatic Nuclear gamma counter.

Synthetic somatostatin standards were prepared as for β-endorphin, and the same 4 - day assay procedure was followed using anti-SRIF rabbit serum (diluted 1: 10,000) as first antibody and I^{125}-tyr^1-somatostatin (25,000 - 30,000 cpm/0.1 ml.) as tracer. Overnight incubations were carried out at room temperature. Alternatively, the entire radioimmunoassay procedure for somatostatin could be shortened to 2 or 3 days total incubation by reducing the intervals between addition of reagents from one day each to a few hours each. This method was found to be satisfactory only with tracers of 50% or higher specific binding at 1st antibody dilution of 1: 10,000. All standards, samples, and antibodies were stored frozen and kept on ice when thawed.

Tracer tests for β-endorphin and somatostatin were carried out using the basic assay procedures outlined, and consisted of duplicates of the following: (a) total count, containing

tracer only (25,000 - 30,000 cpm), (b) specific binding count (0 count), containing trasylol, PB, 1st antibody (1: 10,000), and tracer, (c) non-specific binding count (blank), containing trasylol, PB, NRS (1: 10,000), and tracer, (d) excess antibody count, containing trasylol, PB, 1st antibody (1: 100) and tracer, and (e) displacement count, containing trasylol, PB, 2.5 ng/ml standard, 1st antibody (1: 10,000) and tracer. Duplicates of (a), (b), and (c) counts were also prepared at the beginning of every radioimmunoassay.

VI. Preparation of Rat Brain Extracts

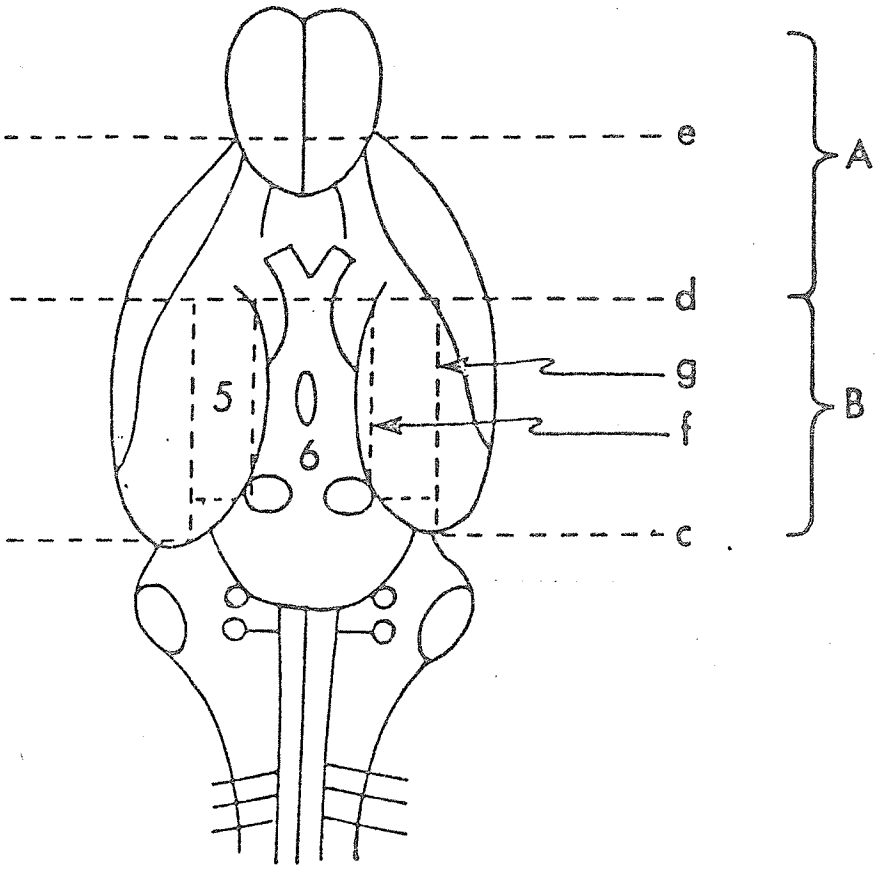
The extraction technique of Ogawa et al. (164) was used, since it had been shown to produce the greatest peptide yield compared with other commonly used procedures. Rats were weighed and individually placed in a 28 x 16.5 x 12.5 cm. clear plastic box with lid. The box was placed in a microwave oven (Philips model HN 1124) and irradiation applied at full power (2.2 KW) for 15 - 25 sec., followed by 10 - 20 sec. at half power, depending on the animal's body weight (e.g., 500 g. body wt. or greater - 25" full, 20" half; 300 - 400 g. - 20" full, 15" half). The brain was removed and dissected on ice according to Glowinsky and Iversen (165), and Konig and Klippel (166) as shown in Figure 1 (a - d). By these procedures, the following brain regions were obtained: cerebellum, hindbrain, striatum, septal nuclei, amygdala, hypothalamus, thalamus, midbrain, cortex, hippocampus, pituitary, nucleus accumbens, and pyriform

Fig. 1. Rat brain dissection procedure. a. Cross-sections c, d, and e were cut downward from the basal surface of the brain, separating cerebellum/hindbrain, mid-section, and frontal brain respectively. The olfactory bulbs, located rostrally to section e, were removed. Brain regions were coded numerically as follows:

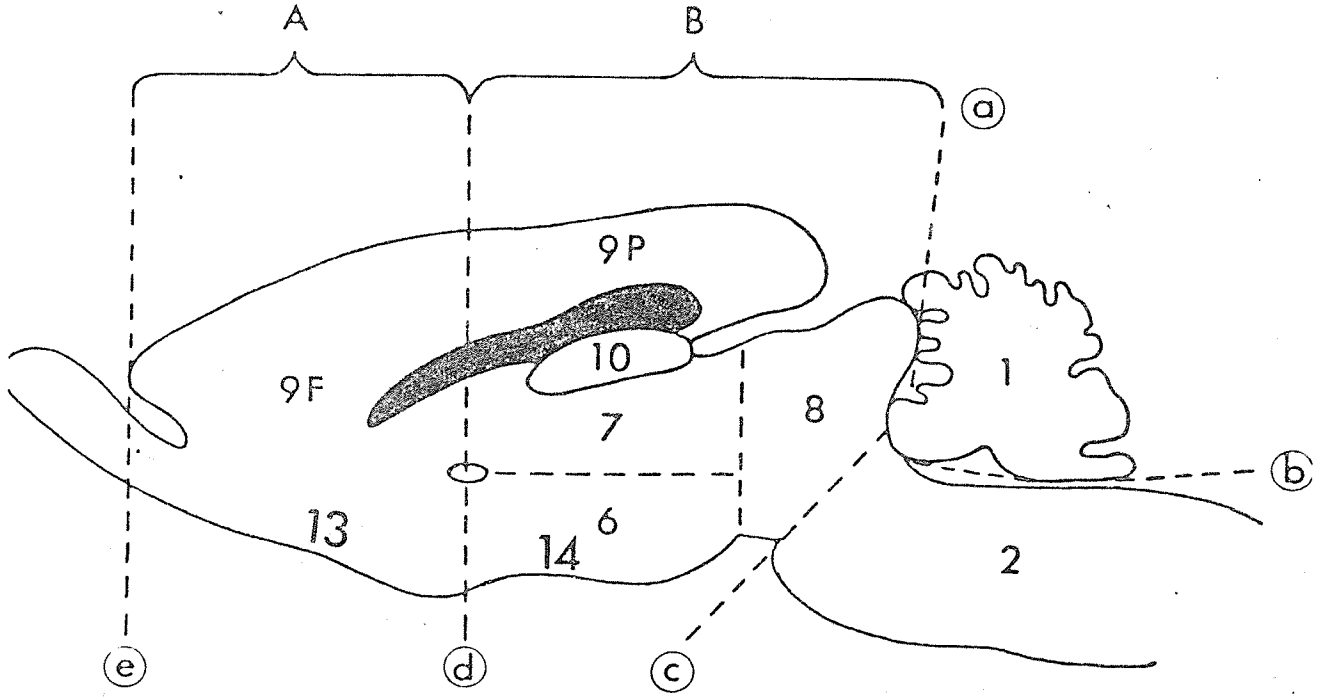
1 - cerebellum
2 - hindbrain
3 - striatum
4 - septal nuclei
5 - amygdala
6 - hypothalamus
7 - thalamus
8 - midbrain
9F - frontal cortex
9P - parietal cortex
10 - hippocampus
11 - pituitary
12 - nucleus accumbens
13 - pyriform cortex
14 - entorhinal cortex

Cuts were made at f and g to separate amygdala sections from hypothalamus. b. Lateral view of rat brain indicating location of each brain region. The frontal section of the brain was designated section A and the mid-section as section B. c. Cross-sectional view of section A as viewed from dissection cut d. d. Cross-sectional view of section B as viewed from dissection cut d. (from Glowinski and Iversen (165) and Konig and Klippel (166)).

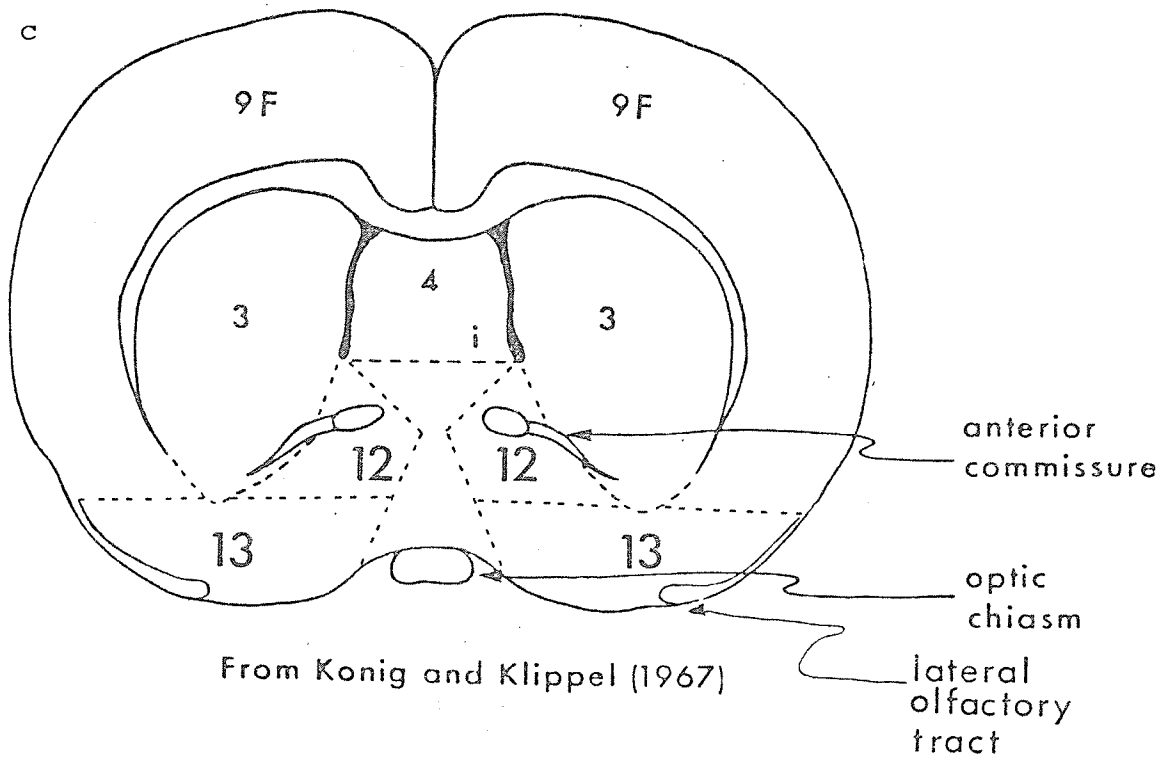
a



b

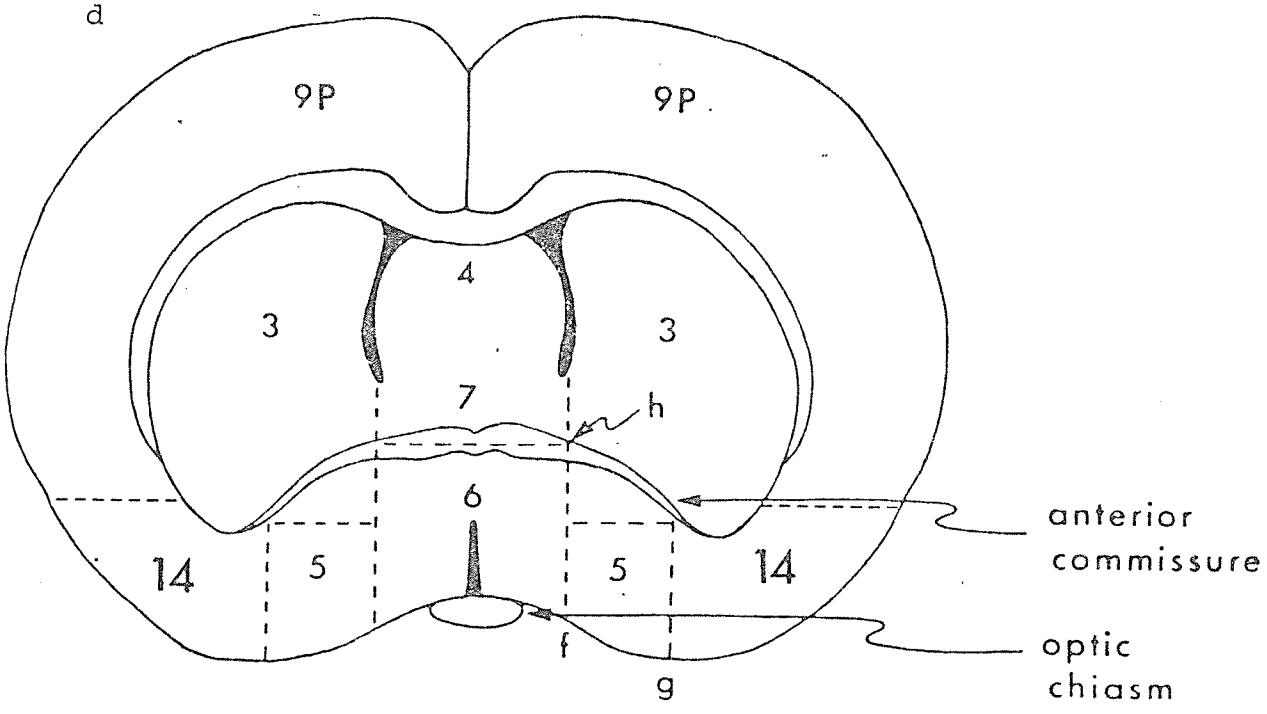


SECTION A



From Konig and Klippel (1967)

SECTION B



From Konig and Klippel (1967)

and entorhinal cortex. The weight of each brain region was determined on a Mettler HIOW balance. All samples were homogenized manually in 0.1 N acetic acid using a 1.0 ml. capacity sintered glass-lined homogenizer (model #8-417 C, Fisher Scientific Co.), or a 7.0 ml. capacity homogenizer when whole brains were being prepared. The volumes of acetic acid used depended on the weight of the brain region, as follows: septal nuclei, amygdala, hypothalamus, thalamus, pituitary and nucleus accumbens - 1.0 ml. each; cerebellum, hindbrain, striatum, midbrain, hippocampus, and pyriform and entorhinal cortex - 1.5 ml. each; cortex - 3.0 mls. and whole brain - 10.0 mls. The homogenates were centrifuged at 2,000 x g for 30 min., and the supernatants removed and kept on ice. The pellets were homogenized in the same volume of 0.1 N acetic acid used for the first extract and centrifuged as before. The second extract was then added to the first and stored frozen at -70^{o}C.

VII. Preparation of Animals, Experimental Procedures

A. Exposure of Aged PTU Rats to an Audiogenic Seizure-Inducing Stimulus

(i) Subjects - Pregnant Sprague-Dawley rats from Zivic-Miller breeders were housed individually and designated as either treatment or control condition. On the day of delivery, the experimental litters were given 0.02% PTU in tap water in place of regular drinking water. Since PTU is poorly soluble,

the drinking solution was prepared by dissolving 0.8 g. PTU and 0.6 g. $NaHCO_3$ in 100 ml. warm water and diluting the mixture to 4 liters. Blue food coloring was added to prevent errors in animal care. This treatment was continued as part of the mother's diet from postnatal day 0 to day 19 of the offspring. Each subject was tested for audiogenic seizure susceptibility at weekly intervals from weaning to adulthood as previously described (86). Seizure frequency was 100% on each occasion of testing although a slight reduction in seizure sensitivity was observed in some subjects as the rats matured. (This was noted both in terms of longer latency to onset of seizure and in more frequent occurrence of wild runs which did not progress into tonic-clonic seizures). The rats were obtained for experimentation after aging (from Dr. L. Van Middlesworth, University of Tennessee Health Sciences Center), and included 20 male and female rats, 2.5 years of age, weight range 400 - 1100 g. Nine had been PTU-treated at birth, 11 were non-treated controls.

(ii) Procedure - Each PTU rat was paired with a non-seizuring control subject on the basis of sex and body weight. The experimental subject of each pair was restrained in a cloth casing (tied around its neck) which prevented excessive movement, and placed in a glass chromatography jar, 8.5" in diameter and 18" high, with a 4" electric doorbell suspended 8" above the bottom of the jar. The rat was exposed to the ringing bell (114 dB intensity) until onset of a seizure (which

usually occurred within 20 - 30 sec.) or for a maximum of 60 sec. if no seizure developed. The rat was immediately removed from the chamber and sacrificed by microwave irradiation. The paired control subject was then restrained in the same manner,and the same procedure carried out using the specific sound stimulus duration applied to the experimental rat. Rats in this and all subsequent experiments were sacrificed between 8:30 and 11:30 a. m.

B. Maze-Learning Study in Adult PTU and Control Rats

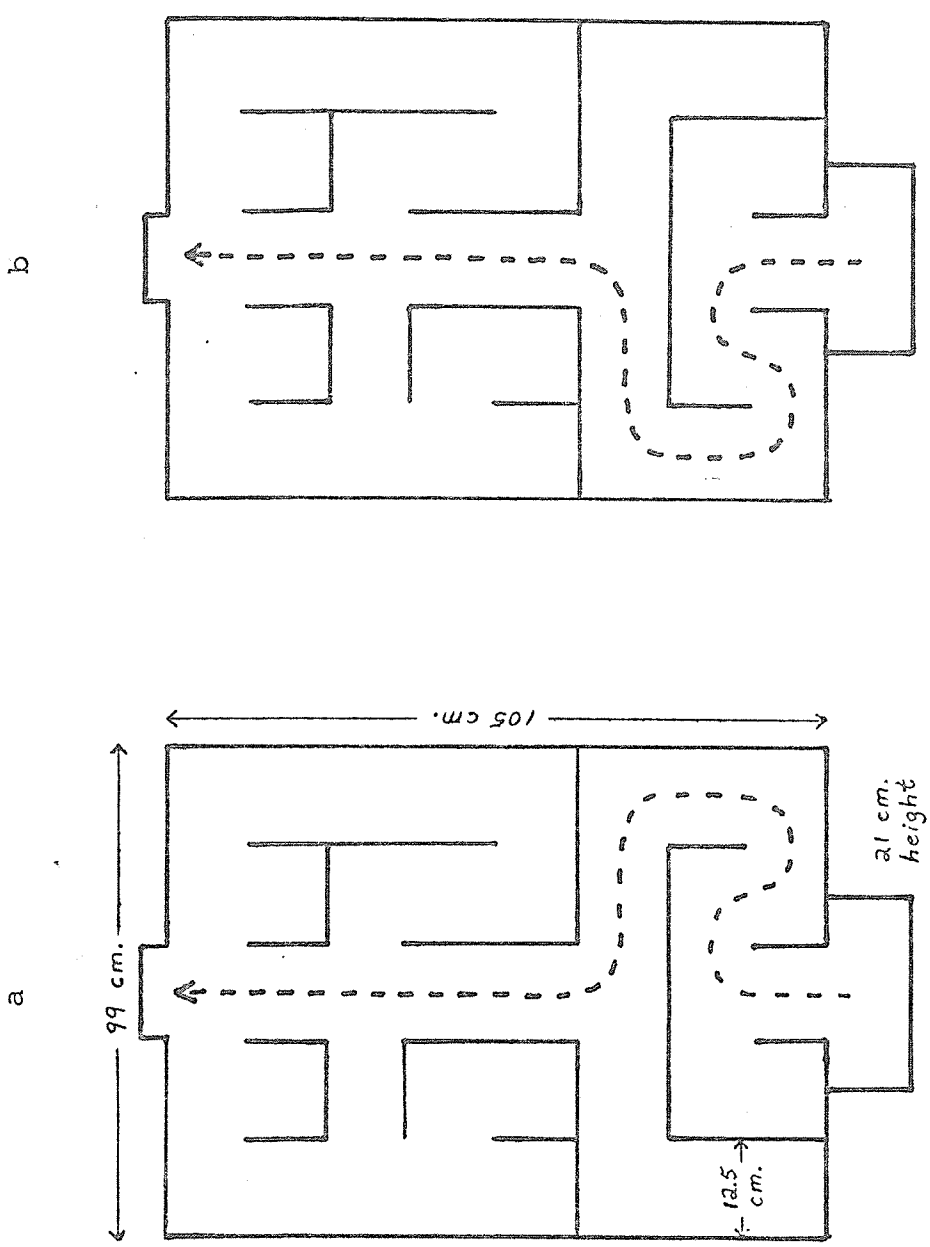
(i) Subjects - Seventeen adult (7 mos.) male and female rats obtained from litters as described above (from Dr. Van Middlesworth, St. Louis) were used. Eight were PTU-treated at birth, 9 were non-treated controls, weight range 300 - 500 g. The seizure history of these rats indicated a 90 - 100% frequency of occurrence of both wild runs and full tonic-clonic seizures on all testing occasions.

(ii) Procedure - (a) open-field activity: Each subject was tested for a 20 minute period over 3 consecutive days in an open-field apparatus. The apparatus consisted of an activity chamber, built in our laboratory, which records horizontal and vertical movements on the basis of changes in resistance measured by drinkometer-type circuitry. The base of the chamber floor (22 x 28 cm.) receives input from an oscillator which automatically senses physical movements based on disruptions

in a high-frequency electromagnetic field, and records each movement on a digital read-out. Each subject was placed in the closed chamber at the start of the 20 min. session, and after 10 min. the horizontal and vertical counts registered were recorded. At the end of the second 10 min. interval, the counts were again recorded.

(b) maze training: Each rat was housed individually and placed on a food deprivation schedule 1 week prior to the start of maze training which maintained them at approximately 80% of their free-feeding weight. The rats had free access to water at all times. On the following week, each rat began training to run a simple T-maze (as shown in Figure 2) in once daily 10-trial sessions. The rat was placed in the start box, and at the beginning of the first trial a sliding barrier was lifted which allowed the rat access to the runway and goal box. The rat's progress toward the goal box was recorded in terms of time taken to reach the food, number of errors made, and incidents of searching and grooming behavior. The trial was considered complete when the rat reached the food (4 - 5 45 mg. Noyes reward pellets per trial), or after 5 min. if the goal box was not reached before then. After this, the rat was placed back in the start box and the second trial began in the same manner. At the end of the 10th trial, the rat was taken back to its home cage and the daily ration of food was given. This training schedule continued for 2 to 3 weeks until the subject's running time

Fig. 2. Diagram of 14-pattern maze for study of learning ability in rats. Rats were placed on a 23-hr. food deprivation schedule and trained to run the maze for food reinforcement once daily in 10-trial sessions. Route a was employed for the learning phase of the study and route b for the relearning phase.


a
b
105 cm.
99 cm.
21 cm. height
12.5 cm.

and frequency of errors stabilized (i.e., until performance did not vary or fluctuate appreciably from day to day). At this point, the subject was considered trained for the learned task. On the following week, a retraining phase was introduced in which the turning direction from the start box to the runway was changed from right to left (Figure 2b), thereby requiring an alteration of the rat's established behavior pattern in order to reach the goal box. This phase also consisted of daily 10-trial sessions but continued for only 5 consecutive days. At the end of the learning study the rats were sacrificed as described previously.

C. Exposure to Loud Sound Stimulus in Habituated Control Rats

(i) Subjects - Eleven male Sprague-Dawley rats bred and raised at the University of Manitoba Faculty of Dentistry animal colony were used. At the time of the experiment the rats were 4 - 5 mos. of age and weighed 400 - 500 g.

(ii) Procedure - Rats were handled for a few minutes each and placed in plastic boxes similar to those used for microwave irradiation. This was carried out 4 times daily for 5 consecutive days, then twice daily for 3 days. Beginning on the following day, rats were paired on the basis of body weight. The first rat of each pair was placed in the chromatography jar apparatus, without restrainment, and exposed to the ringing bell for 90 sec. It was then removed and sacrificed immediately.

The second subject of the pair was sacrificed shortly after, without prior exposure to the bell.

D. Neonatal $NaClO_4$ Treatment and Exposure to PTU After Maturity

(i) Subjects - Pregnant Sprague-Dawley rats were bred and housed individually at the U. of M. Dentistry animal house. On the day of delivery each litter was randomly assigned to either $NaClO_4$ treatment or control condition. Those in the treatment group received 0.2% $NaClO_4$ in their drinking water from postnatal day 0 - 21 of the offspring. Control litters received regular tap water.

(ii) Procedure - At weaning, all rats were tested for seizure susceptibility by a 90 sec. exposure trial to a 114 dB noise. All rats subsequently received regular drinking water ad. lib. At 2 months of age, 12 $NaClO_4$-treated males and 12 control males were each assigned to one of two conditions: 0.03% PTU in drinking water or regular tap water. This procedure resulted in 4 groups of 6 subjects each: $NaClO_4$ (neonatal) + PTU (adult), $NaClO_4$ + H_2O, Control + PTU, and Control + H_2O. Body weights ranged between 260 - 475 g. The adult treatment schedule continued for 3 weeks, after which the subjects were retested for seizure-susceptibility and sacrificed 4 per day (one from each group).

E. Neonatal Treatment with 0.2% PTU

(i) Subjects - Pregnant Sprague-Dawley rats were obtained and housed as before. From day 0 to 19 after delivery, half of the litters received 0.2% PTU in the drinking water and half received regular tap water.

(ii) Procedure - On the last day of treatment, the pups were weighed and some were bled from the tail vein under light ether anaesthesia to obtain serum samples for T_4 measurements. This procedure was also carried out on subsequent 0.02% PTU and $NaClO_4$-treated litters, and in 0.02% PTU rats after maturity. A few days later, all subjects were tested for seizure susceptibility in a 90 sec. noise exposure trial. At 30 days of age, 5 control male subjects, 5 0.2% PTU males and 2 0.2% PTU females were sacrificed.

F. Hot Plate Exposure in 0.02% PTU and Control Rats

(i) Subjects - Nine adult male 0.02% PTU rats and 10 male controls (bred and raised in the U. of M. Dentistry animal house), 2.5 months of age, were used. Body weights ranged between 295 - 450 g.

(ii) Procedure - Each subject was randomly assigned to one of two groups, hot plate exposure stress or no stress (control), resulting in a total of 4 groups as follows: PTU + stress (N = 5), PTU no stress (N = 4), control + stress (N = 5),

and control no stress (N = 5). Those subjects receiving stress were exposed to 10 trials during a 30 min. period on a 55°C hot plate enclosed in a clear Plexiglas chamber. The first trial consisted of a 60 sec. exposure period followed by nine 30 sec. exposure periods, each separated by 2.5 min. rest periods. For each trial, the latency to occurrence of the first hindpaw lick or jump was recorded. Stressed subjects were sacrificed 4 per day, one from each treatment group.

G. Startle Response Test

(i) Subjects - Eleven adult female 0.02 % PTU rats and 8 female controls were used. They were 3 months of age at the start of the experiment, body weights ranged between 260 - 350 g.

(ii). Procedure - The method of Kellogg et al. (167) was used for this test. Measurements of the acoustic startle response were conducted in a double-walled sound-attenuating chamber. Rats were placed individually in a small perforated Plexiglas cage. An accelerometer mounted below the cage detected the startle response. Output from the accelerometer was fed to a Grass polygraph, amplified 500 x and exhibited on a cathode-ray tube as a change in voltage. The startle stimulus was a 10 kHz, 110 dB, 20 msec. tone that had rise and decay times of 5 msec. Background white noise was fed into the testing chamber by a Grason-Stadler white noise generator.

Presentation of five different intensities - 25, 40, 55, 70, and 85 dB was determined by Latin squares; there were five trials at each background intensity (25 trials in all). Each background noise presentation lasted for 1 minute, during which time the acoustic startle stimulus was presented once. The rat's motor response magnitude was averaged for the 5 trials of each noise level exposure.

VIII. Statistical Analysis

All statistical tests were carried out using Student's t-test (168), paired or unpaired, for single comparisons between 2 groups, and Duncan's multiple-range one-way analysis of variance (ANOVA) for multiple comparisons between groups. The levels of significance are indicated as follows:
* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

RESULTS

I. Characterization of Camel-β-Endorphin Antiserum (1 - 6) and Somatostatin Antiserum (S2 - 2)

Figure 3 shows that camel-β-endorphin and ovine β-lipotropin compete with I^{125}-camel-β-endorphin for specific binding to antiserum (1 - 6), whereas other related opiate peptides α-endorphin and met-enkephalin, as well as bombesin and naloxone do not cross-react. The proximity of the ovine β-LPH displacement curve to the β-endorphin curve indicates a 50% cross-reactivity in this assay. When serial dilutions of the antiserum were tested for specific binding, a dilution of 1:10,000 produced 40% or greater specific binding and 5% or less non-specific binding. Several other peptides and substances including γ-endorphin, leu-enkephalin, α-MSH, ACTH, vasopressin, insulin, glucagon, TRH, LHRH, myelin basic protein, GH, PRL, and morphine were also tested for cross-reactivity, none of which produced any displacement. Rat β-endorphin and rat β-LPH exhibit the same cross-reactivities as camel β-endorphin and ovine β-LPH (169). In addition to ovine β-LPH, human β-endorphin cross-reacted 30% on a molar basis.

Figure 4 shows that both synthetic somatostatin and tyr^1-somatostatin effectively and equally compete with I^{125}-tyr^1-somatostatin for specific binding to antiserum (S2 - 2). Somatostatin analogs with the substitutions ala^8, D-trp^8, and

Fig. 3. Specificity characterization of camel β-endorphin antiserum (1-6). Increasing concentrations of β-endorphin or other substance were incubated with I^{125}-camel β-endorphin tracer (25,000 - 30,000 cpm/sample) and camel β-endorphin antiserum (rabbit gamma globulin), diluted 1:10,000. Ovine β-LPH displaced the tracer 50% as effectively as camel β-endorphin, while human β-endorphin exhibited only 30% cross-reactivity (not shown). All other biologically related and unrelated peptides tested failed to cross-react in the assay.

Specificity characterization of camel β- Endorphin antiserum (1 - 6)

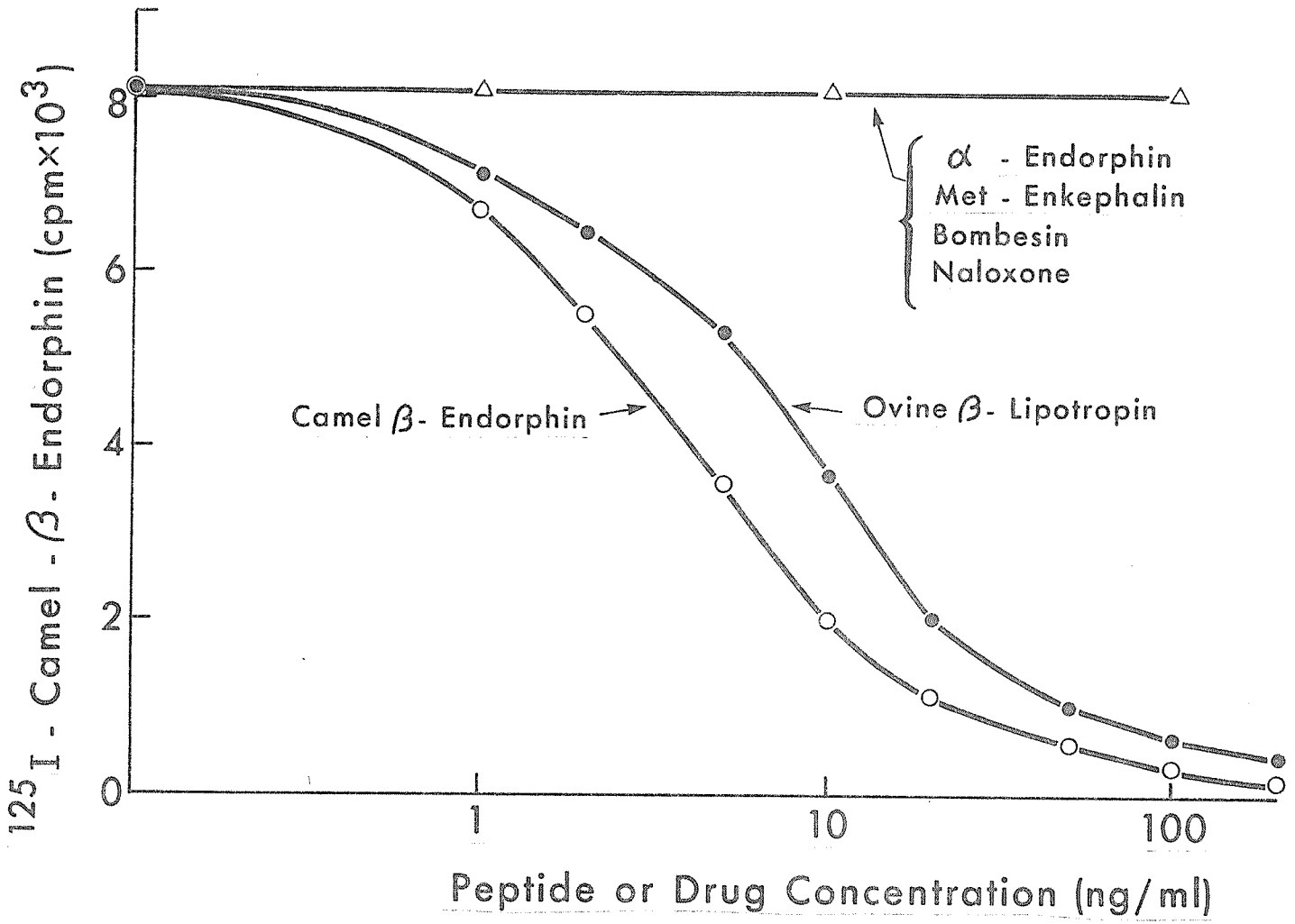
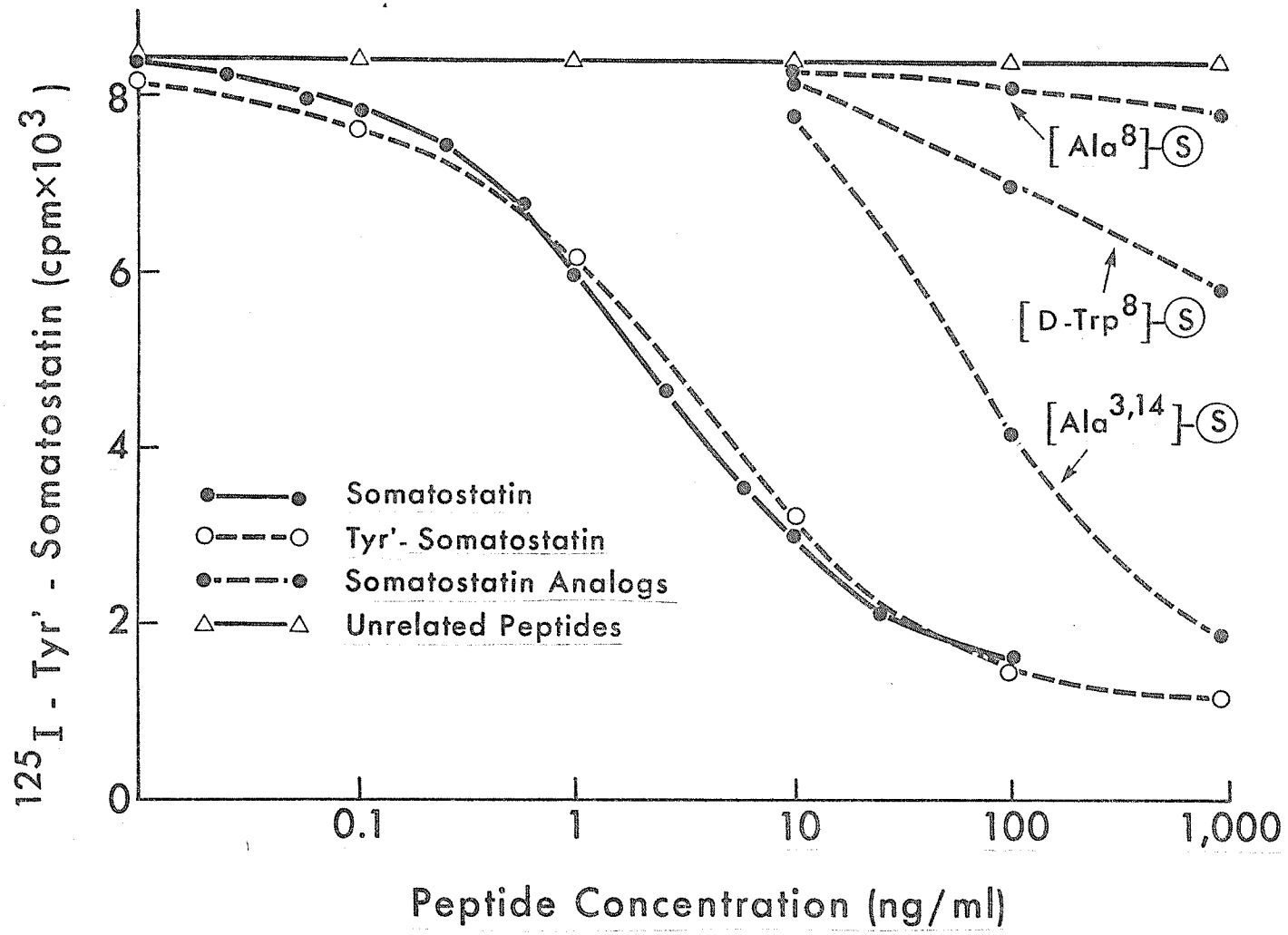


Fig. 4. Specificity characterization of somatostatin antiserum (s2-2). Increasing concentrations of somatostatin or other substance were incubated with I^{125}-tyr^{1}-somatostatin tracer (25,000 - 30,000 cpm/sample) and rabbit anti- somatostatin antiserum (1:10,000 dilution). Synthetic somatostatin, tyr^{1}-somatostatin, and I^{125}-tyr^{1}-somatostatin competed equally well for binding to the antibody. Synthetic analogs of somatostatin, $ala^{3,14}$-SRIF (a non-cyclic derivative), D-trp^{8}-SRIF, and ala^{8}-SRIF each competed for binding to varying extents at high concentrations of peptide. No other biologically related or unrelated peptides cross-reacted in the assay.

Specificity characterization of somatostatin antiserum (s2-2)



ala^{3,14} each compete to varying degrees at higher concentrations, whereas all other peptides tested, including TRH, LHRH, met-enkephalin, α-endorphin, β-endorphin, β-LPH, ACTH, porcine glucagon, vasopressin, oxytocin, secretin, PRL, GH, LH, and FSH did not cross-react. Serial dilutions of the antiserum tested for specific binding indicated that a dilution of 1: 10,000 produced 40% or greater specific binding and about 5% non-specific binding.

II. Characterization of I^{125}-Labelled Camel-β-Endorphin and Tyr^{1}-Somatostatin

Figure 5 shows that the iodination reaction mixture of β-endorphin is resolved into two peaks when eluted on a Sephadex G-25 (fine) 1.0 x 20 cm. column. The first peak contains both aggregated, damaged material (the ascending slope fractions of the peak) as well as intact iodinated camel β-endorphin (the descending slope fractions). Generally, those fractions close to the bottom of the first peak represent the best tracer. The second peak, which is approximately equal in size to the first contains free unreacted iodide. The incorporation of radioactivity into camel β-endorphin by this technique is usually 50 - 60%. A satisfactory tracer exhibits 85 - 90% specific binding and 2% non-specific binding in the presence of excess 1st antibody (1:100 dilution). At 1:10,000 dilution of the antibody specific binding is 40 - 60%, and non-specific binding is about 5%. (Note: specific binding =

$$\frac{\text{0 tube cpm} - \text{blank tube cpm}}{\text{total cpm}}$$

, 0 count - without cold peptide, blank - without cold peptide and 1st antibody).

Figure 6 shows that the tyr^1-somatostatin iodination reaction mixture is resolved into two peaks when eluted on a CMC cation-exchange 0.7 x 20 cm. column. The first peak contains free unreacted iodide, and the second peak which is eluted after increasing the molar strength of the buffer, contains mainly intact iodinated tyr^1-somatostatin. Those fractions nearest to the top of the peak usually represent the best tracer. The incorporation of radioactivity into tyr^1-somatostatin by this technique is about 60%. A satisfactory tracer exhibits binding characteristics similar to those of β-endorphin when tested at 1:100 and 1:10,000 1st antibody dilutions.

III. Characterization of Radioimmunoassays for β-Endorphin and Somatostatin

(i) Sensitivity: The sensitivity of both assays was between 0.5 and 1.0 ng/ml, with standard curves linear to about 10 ng/ml. This sensitivity range is shown in the standard curves of Figures 7 and 8 where it is expressed as ng/0.1 ml (ng/tube).

(ii) Specificity: As mentioned previously, the β-endorphin assay cross-reacts 50% with ovine β-LPH and 30% with human

Fig. 5. Elution profile of a camel β-endorphin iodination reaction mixture chromatographed on Sephadex G-25 (fine) 1.0 x 20 cm. Camel β-endorphin was iodinated by a lactoperoxidase method and eluted by gel filtration with phosphate-buffered saline (PBS pH 7.4). The first peak of radioactivity contains intact iodinated tracer in the decending slope fractions. When diluted and incubated with antibody, these fractions exhibit 40 - 50% specific binding. The second peak of radioactivity represents free iodide.

Elution profile of a camel β- Endorphin iodination reaction mixture chromatographed on sephadex G-25 (fine), 1.0×20 cm

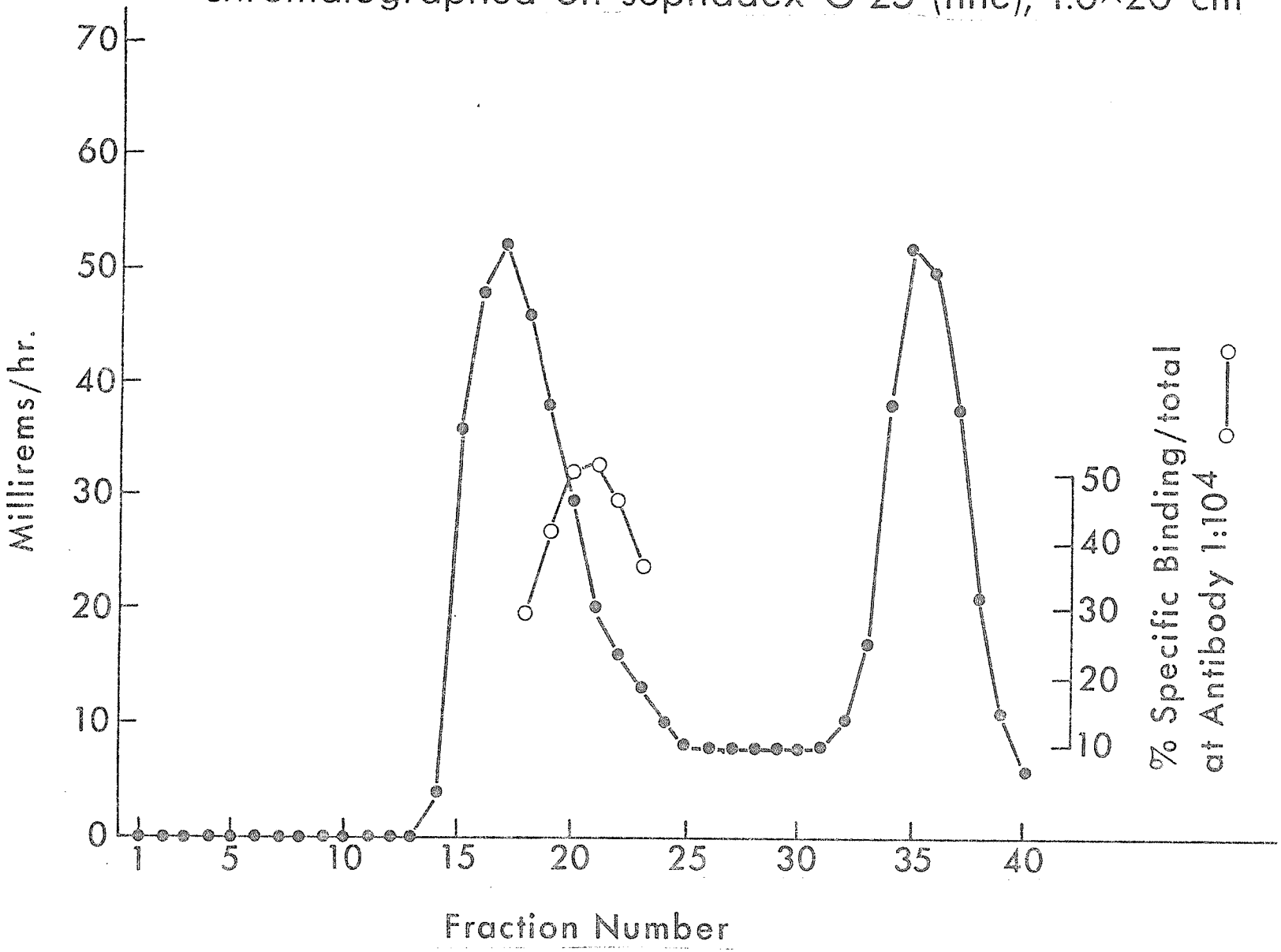
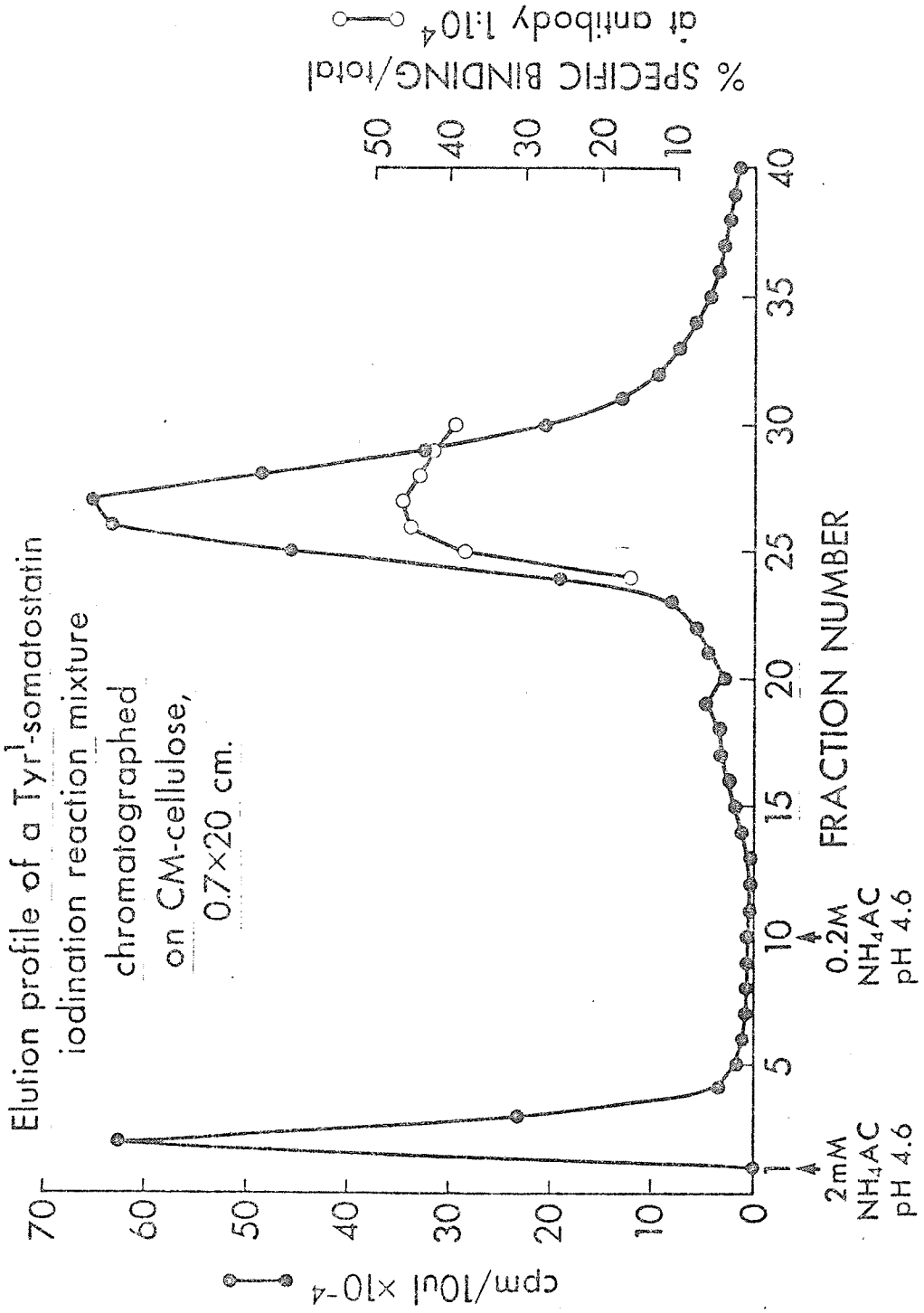


Fig. 6. Elution profile of a tyr^1-somatostatin iodination reaction mixture chromatographed on CM-cellulose 0.7 x 20 cm. Tyr^1- somatostatin was iodinated by a lactoperoxidase method and eluted by cation-exchange chromatography using 2 mM and 0.2 M NH_4OAc buffer (pH 4.6). The first peak of radioactivity is free iodide, after which the molar strength of the buffer is increased 100-fold to release intact iodinated tyr^1-somatostatin from the column. The tracer is contained in the second peak, with fractions closest to the top of the peak exhibiting 40 - 50% specific binding to the antibody.


Elution profile of a Tyr¹-somatostatin iodination reaction mixture chromatographed on CM-cellulose, 0.7×20 cm.
cpm/10µl ×10⁻⁴
% SPECIFIC BINDING/total
at antibody 1:10⁴
2mM NH₄AC pH 4.6
0.2M NH₄AC pH 4.6
FRACTION NUMBER

β-endorphin. Some cross-reactivity with the 31 K precursor and its breakdown products may also occur, but the extent to which it does is unknown. The somatostatin assay is specific at least for somatostatin-like compounds. The larger somatostatin precursor substance or breakdown products thereof may also cross-react, as is the case with the larger β-LPH molecule in the β-endorphin assay.

(iii) Reproducibility: The interassay variations of whole brain extract measurements compared to standards dissolved in PB are 12% at an average concentration of 1.5 ng/ml and 17% at 9.2 ng/ml for β-endorphin, and 7% at 1.2 ng/ml and 9% at 8.5 ng/ml for somatostatin.

(iv) Precision: The intraassay variations of whole brain extract measurements are 4.2% at 1.0 ng/ml and 5.1 % at 10.2 ng/ml for β-endorphin, and 3.9% at 1.5 ng/ml and 4.4% at 9.5 ng/ml for somatostatin.

IV. Parallelism Curves for β-Endorphin and Somatostatin

Figures 7 and 8 show that serial dilutions of whole brain extracts parallel the standard curves for β-endorphin and somatostatin. Hypothalamic, midbrain, and hindbrain extracts each produce curves identical to those of whole brain extracts.

V. β-Endorphin and Somatostatin Yield from Double Extraction

Fig. 7. Standard curve for camel β-endorphin and serial dilution curves of rat whole brain extracts. Synthetic camel β-endorphin standards were prepared in 0.14 M PB in concentrations ranging from 100 pg/ml to 100 ng/ml. The displacement curve indicated a sensitivity range of about 750 pg/ml to 10 ng/ml (.075 to 1.0 ng/tube). Serial dilutions of whole brain extracts produced curves parallel to the standard curve.

Standard curve for camel β - Endorphin and serial dilution curves of rat brain extracts

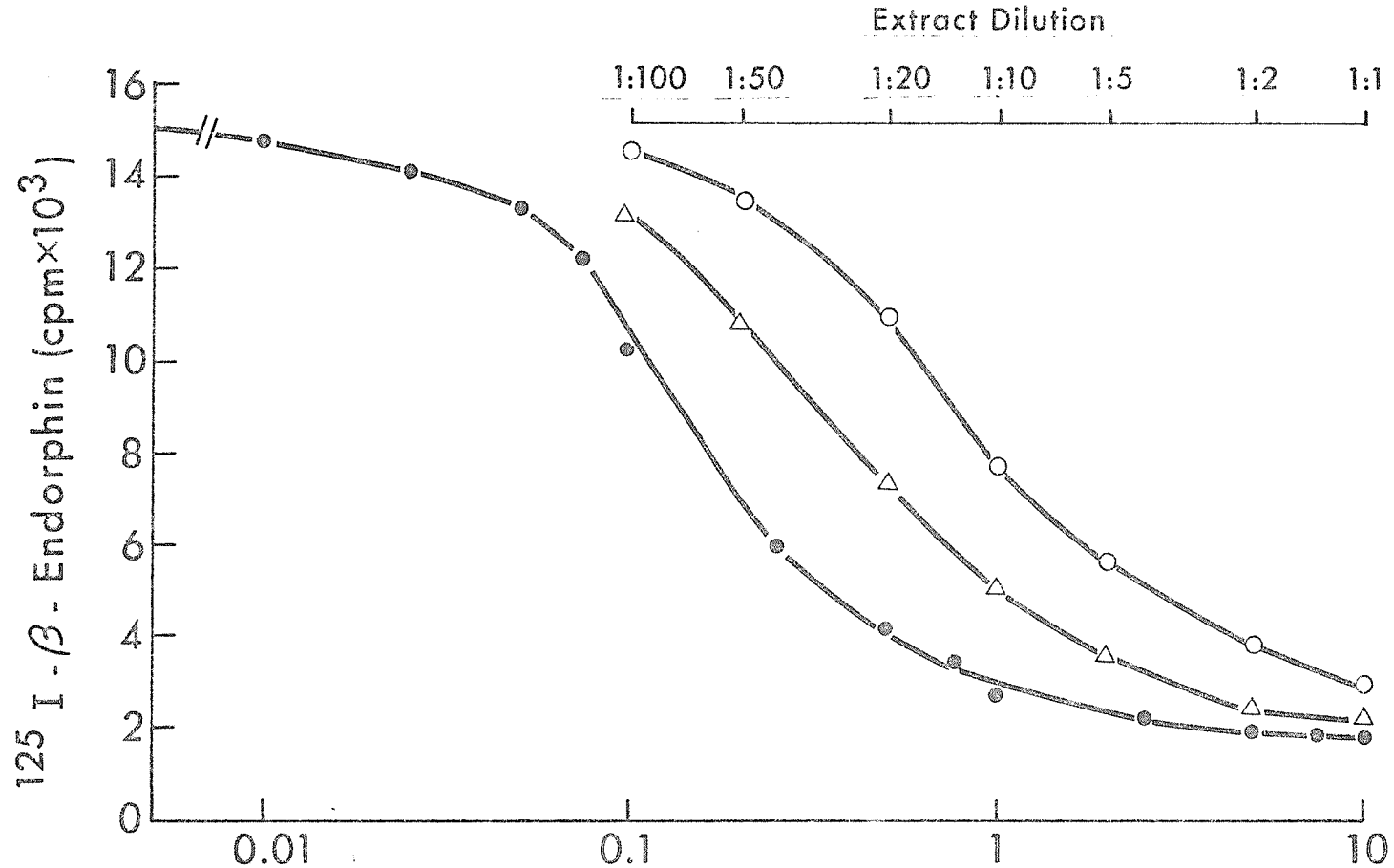
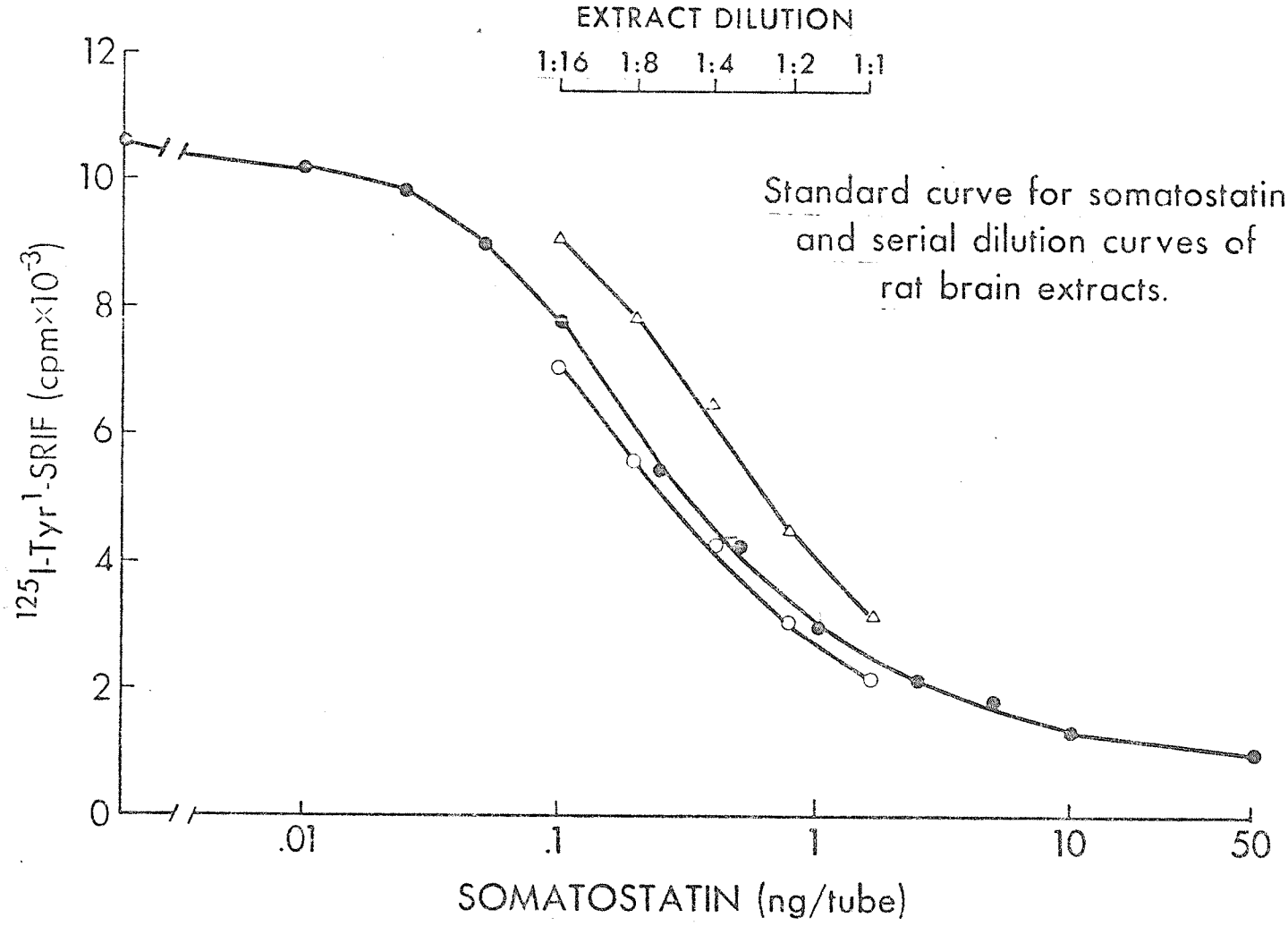


Fig. 8. Standard curve for somatostatin and serial dilution curves of rat whole brain extracts. Synthetic somatostatin standards were prepared in 0.14 M PB in concentrations ranging from 100 pg/ml to 100 ng/ml. The displacement curve indicated a sensitivity range of 500 pg/ml to 10 ng/ml (.050 to 1.0 ng/tube). Serial dilutions of whole brain extracts in 0.1 N acetic acid produced curves parallel to the standard curve.



Standard curve for somatostatin and serial dilution curves of rat brain extracts.

of Rat Brain Tissue

Table 1 lists the brain weights of 19 rats, total β-endorphin and somatostatin (ng.) in first and second extracts of whole brain and the % yield of peptide in the second extract relative to the first. The data for β-endorphin show that approximately 80% of the peptide is extracted by the first and 20% by the second extraction. These values are consistent with those obtained by Ogawa et al. (164). In contrast, the % recovery of somatostatin in the second extract was considerably greater than for β-endorphin, and varied greatly between samples. In some cases, the yield exceeded that obtained in the first extract (i.e., greater than 100%).

Table 1. β-Endorphin and Somatostatin content in First and Second Extracts of Rat Whole Brain Tissue

Sample No.	Brain Wt. (g)	Total ng. β-Endorphin 1st Ext.	2nd Ext.	% Recovery	Total ng. Somatostatin 1st Ext.	2nd Ext.	% Recovery
1	1.83	169.0	36.8	21.8	713.0	357.0	50.1
2	1.72	750.0	134.0	17.9	584.0	250.0	42.8
3	1.91	293.0	86.0	29.4	858.0	427.5	49.8
4	1.70	339.0	92.0	27.1	465.0	510.0	109.7
5	1.87	178.0	39.6	22.2	971.0	541.5	55.8
6	1.88	174.0	40.4	23.2	460.0	522.0	113.5
7	1.84	323.0	66.8	20.7	643.0	421.0	65.5
8	1.90	200.0	55.6	27.8	813.0	830.0	102.1
9	1.80	209.0	42.4	20.3	812.0	818.0	100.7
10	1.85	435.0	99.0	22.8	530.0	720.0	135.8
11	1.87	560.0	133.0	23.8	607.0	785.0	129.3
12	2.02	348.0	80.0	23.0	808.0	315.0	39.0
13	1.61	300.0	70.2	23.4	178.0	80.5	45.2
14	1.98	355.0	43.4	12.2	852.0	468.0	54.9
15	2.04	231.0	50.8	22.0	399.0	365.5	91.6
16	1.93	255.0	60.6	23.8	478.0	557.5	116.6
17	1.96	249.0	52.0	20.9	440.0	572.5	130.1
18	1.97	316.0	68.2	21.6	394.0	496.0	125.9
19	1.73	238.0	48.2	20.3	433.0	547.0	126.3
				22.3 ± 0.8 S.E.M.			88.7 ± 7.9 S.E.M.

VI. Reproducibility of Dissection Procedure

Table 2 lists the average wet weights ($\bar{X}$ ± S.E.M.) of 13 brain regions in PTU (N=13) and Control (N=28) adult rats. Cerebellar and pituitary weights were significantly lower ($p < .01$) in the PTU group, cortical weights were also reduced.

Table 2. Brain Region Weights (mg.) for Control and 0.02% PTU Rats

Brain Region	Control (N=28) $\bar{X}$ ± S.E.M.	PTU (N=13) $\bar{X}$ ± S.E.M.
Cerebellum	290.7 ± 3.7	268.4 ± 7.9 **
Hindbrain	298.8 ± 6.4	289.6 ±10.7
Striatum	198.2 ± 2.9	190.5 ±10.7
Septal Nuclei	14.4 ± 1.1	18.4 ± 2.2
Amygdala	23.8 ± 0.8	25.1 ± 1.3
Hypothalamus	58.5 ± 2.0	54.1 ± 1.7
Thalamus	36.1 ± 1.4	39.5 ± 2.8
Midbrain	139.8 ± 3.9	137.4 ± 5.0
Cortex	761.8 ±10.1	722.6 ±19.2
Hippocampus	113.5 ± 4.8	104.2 ± 6.7
Pituitary	13.3 ± 0.6	9.5 ± 0.4 **
Nucleus Accumbens	33.5 ± 2.1	33.8 ± 3.9
Pyriform and Entorhinal Cortex	90.3 ± 5.4	78.3 ± 5.6

VII. β-Endorphin and Somatostatin Levels in Rat Brain Regions Following Various Experimental Manipulations

1. Distribution of Immunoreactive (IR)-β-Endorphin and IR-SRIF in Brain Regions of Aged (Stressed) PTU and Control Rats

Of 9 PTU rats, 4 exhibited violent tonic-clonic seizures as a result of the sound stimulus exposure. In these cases, seizures occurred within 20 - 30 sec. of the stimulus

presentation. The remaining 5 did not seizure during the 60 sec. stimulus duration, nor did any of the 11 control subjects. Statistical comparison between seizuring and non-seizuring PTU rats did not reveal differences in either β-endorphin or somatostatin levels in any brain regions tested (data not shown). Figure 9 shows that the lowest concentrations of IR-β-endorphin were found in the cerebellum, striatum (basal ganglia), septal nuclei, hippocampus, and cortex. Intermediate levels occurred in the hindbrain, amygdala, thalamus, and midbrain, and highest levels were found in the hypothalamus (6 to 10-fold higher than in the thalamus). Hypothalamic IR-β-endorphin content of PTU rats was significantly lower (2.4 µg/g vs. 6.4 µg/g, $p < .01$) than in controls. A similar trend found in several other brain regions was not significant. Figure 10 shows that the lowest concentrations of IR-SRIF were found in the cerebellum, intermediate levels in the cortex, hindbrain, thalamus, midbrain, and hippocampus, and highest levels in the striatum, septal nuclei, amygdala, and hypothalamus. The IR-SRIF content of PTU rats was significantly higher in the following brain regions: striatum, amygdala, cortex ($p < .01$), cerebellum, hindbrain, midbrain, and hippocampus ($p < .05$).

2. Distribution of IR-β-Endorphin and IR-SRIF in Brain Regions of Adult PTU and Control Rats

Figure 11 shows a similar distribution pattern of

Fig. 9. IR-β-Endorphin content (ng/g wet tissue wt. $\pm$ S.E.M.) in brain regions of control stressed (N=11) and PTU stressed rats (N=9). The stressor stimulus consisted of physical restraint in a cloth casing and exposure to a 114 dB noise stimulus for 60 sec. Hypothalamic IR-β-endorphin content of PTU-treated rats was significantly lower (6.4 μg vs. 2.4 μg/g, $p \leq .01$) than in controls. No significant differences were found in any other brain regions tested.

Immunoreactive β-Endorphin in brain regions of control stressed (▨) and PTU stressed (▩) rats.

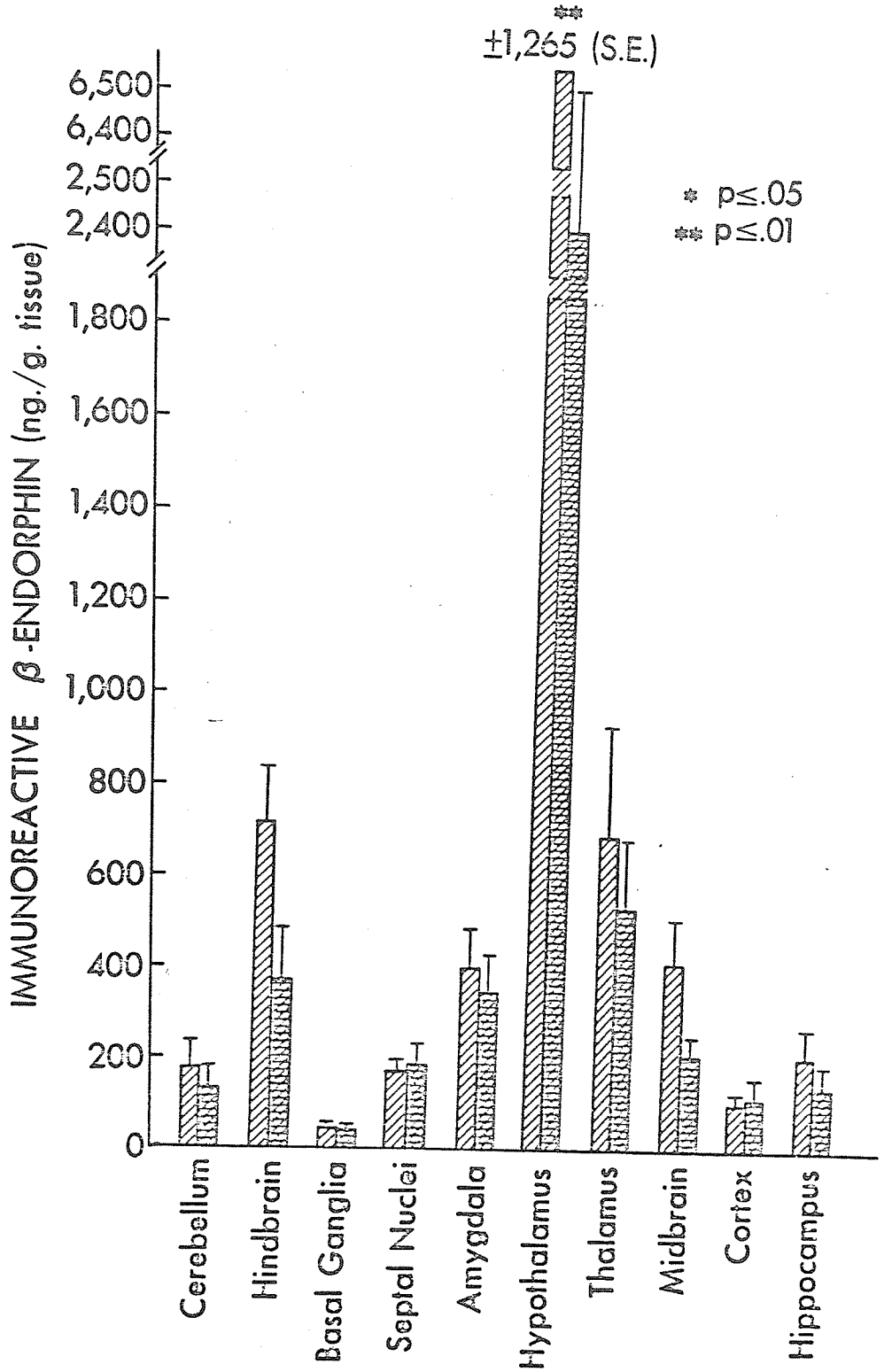
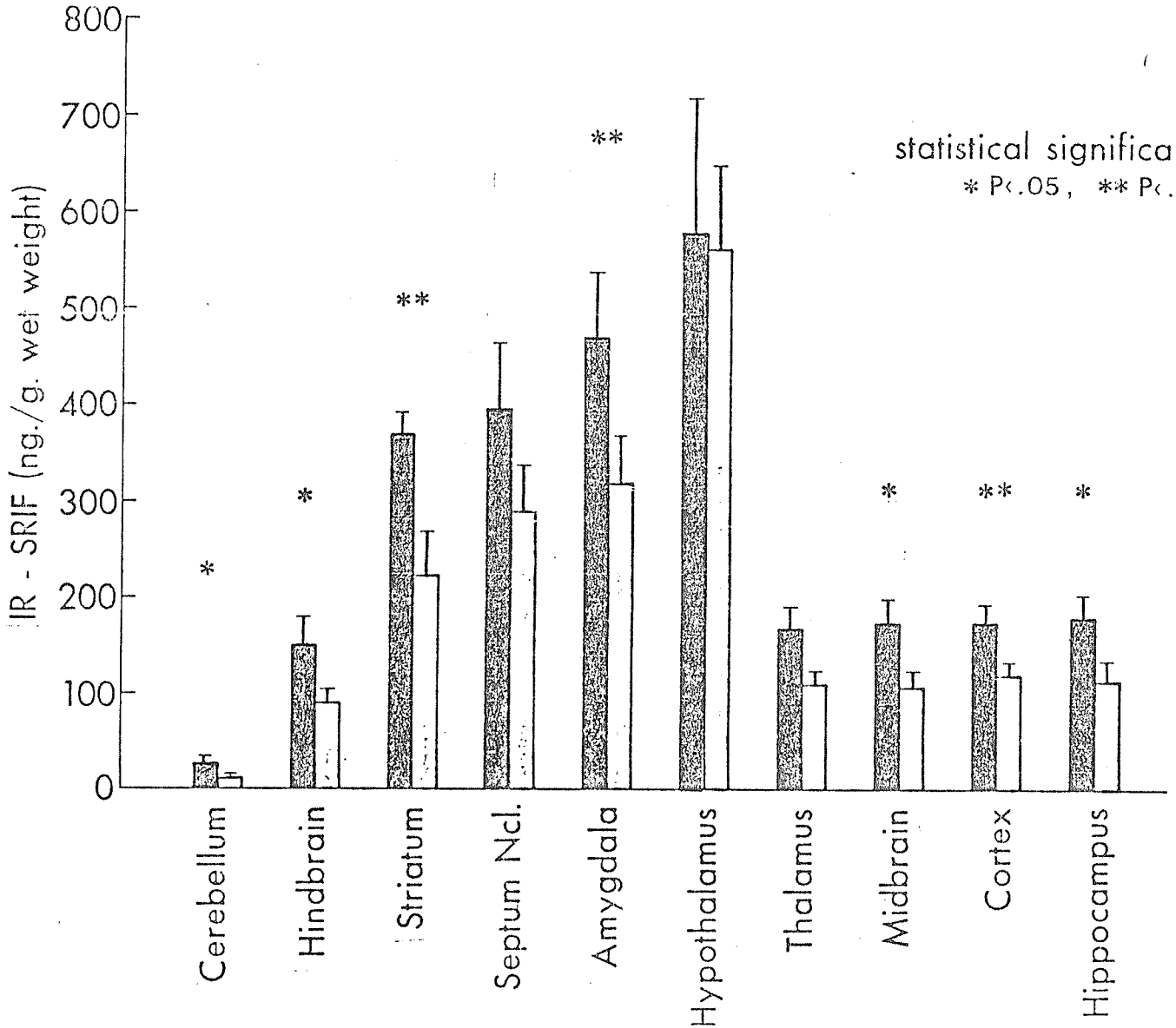


Fig. 10. IR-Somatostatin content (ng/g wet tissue wt. ± S.E.M.) in brain regions of control stressed and PTU stressed rats. Aged rats (2.5 yrs) were restrained and exposed to a loud sound stimulus (114 dB) for 60 sec. and sacrificed immediately thereafter. Significant increases in IR-SRIF content were found in PTU-treated rats in the following brain regions: striatum, amygdala, cortex ($p \leq .01$), cerebellum, midbrain, hindbrain, and hippocampus ($p \leq .05$).

Distribution of IR-SRIF in the brains of PTU-treated (▩) and control (□) aged rats.



IR-β-endorphin concentrations among different brain regions as obtained for aged, stressed rats. However, the levels were lower in most regions, except in the septal nuclei where they were increased. In addition, the nucleus accumbens, dissected from the basal forebrain (previously included with the frontal cortex), was found to have a relatively high concentration of β-endorphin. This could account for the much lower cortical β-endorphin content obtained in this second experiment relative to the first. The pituitary levels were also measured and found to be approximately 1,000-fold greater than in the hypothalamus (note: μg/g tissue). IR-β-endorphin content was significantly higher ($p < .05$) in PTU rats. A similar trend found in several other brain regions tested was not significant. Figure 12 shows a similar distribution of IR-SRIF concentrations among different brain regions as observed in aged, stressed rats. One exception was lower values found in the striatum. Nucleus accumbens and pituitary levels were high, as observed for β-endorphin. IR-SRIF content was significantly increased in the hindbrain ($p < .01$), cerebellum, striatum, hypothalamus, cortex, and hippocampus ($p < .05$) of PTU rats.

3. Comparison of Total IR-β-Endorphin and IR-SRIF in Whole Brain of Adult and Aged (Stressed) PTU and Control Rats

Figure 13 shows that total IR-β-endorphin in the brains

Fig. 11. IR-β-Endorphin content in brain regions of control non-stressed (N=9) and PTU non-stressed rats (N=8). Rats were habituated to handling during the course of a 4-week maze training period prior to sacrifice. Thalamic IR-β-endorphin content was increased in PTU-treated rats compared to controls ($p \leq .05$). Pituitary content is expressed in μg/g tissue.

Immunoreactive β-Endorphin in brain regions of control non-stressed (□) and PTU non-stressed (▤) rats.

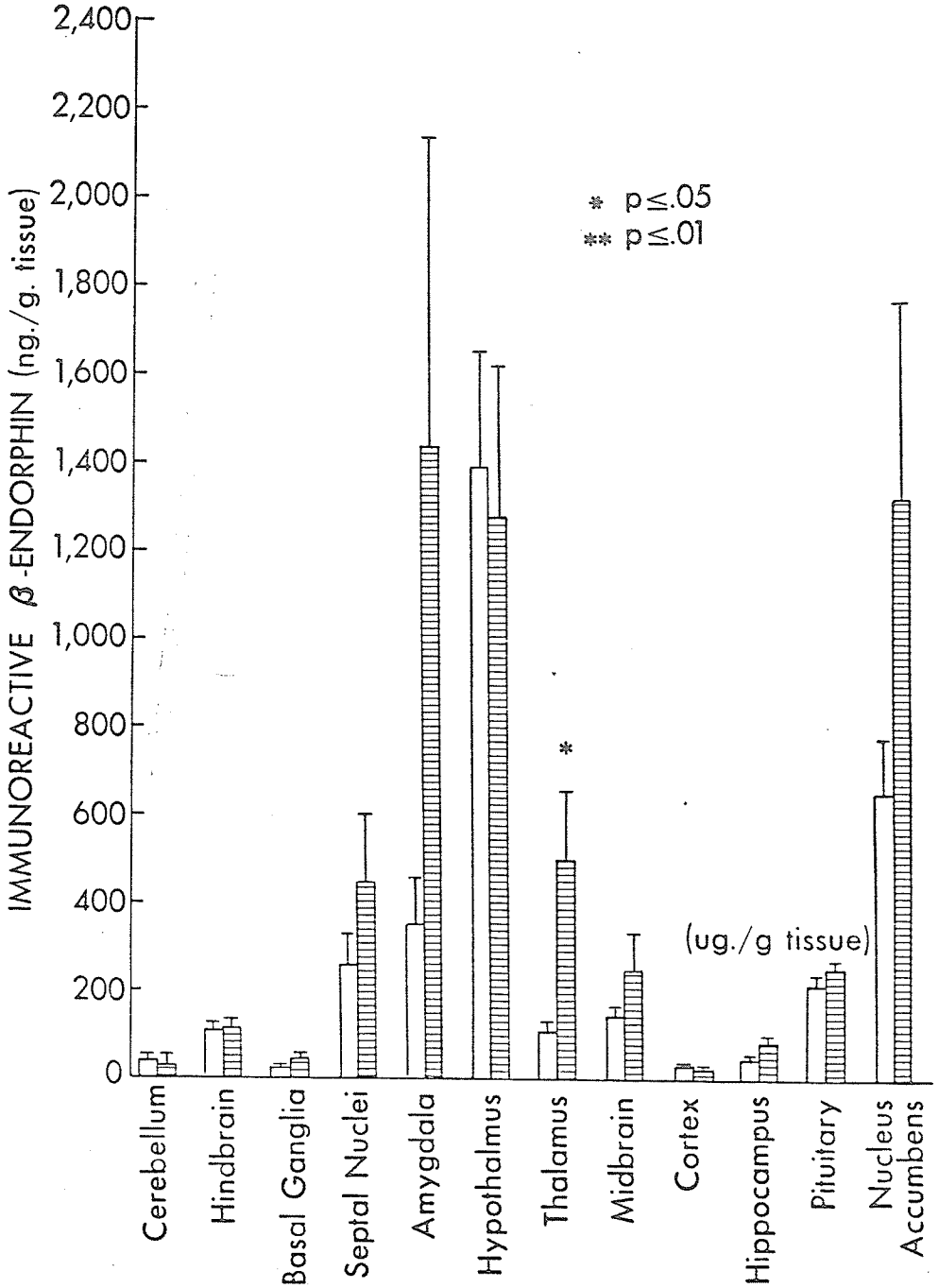
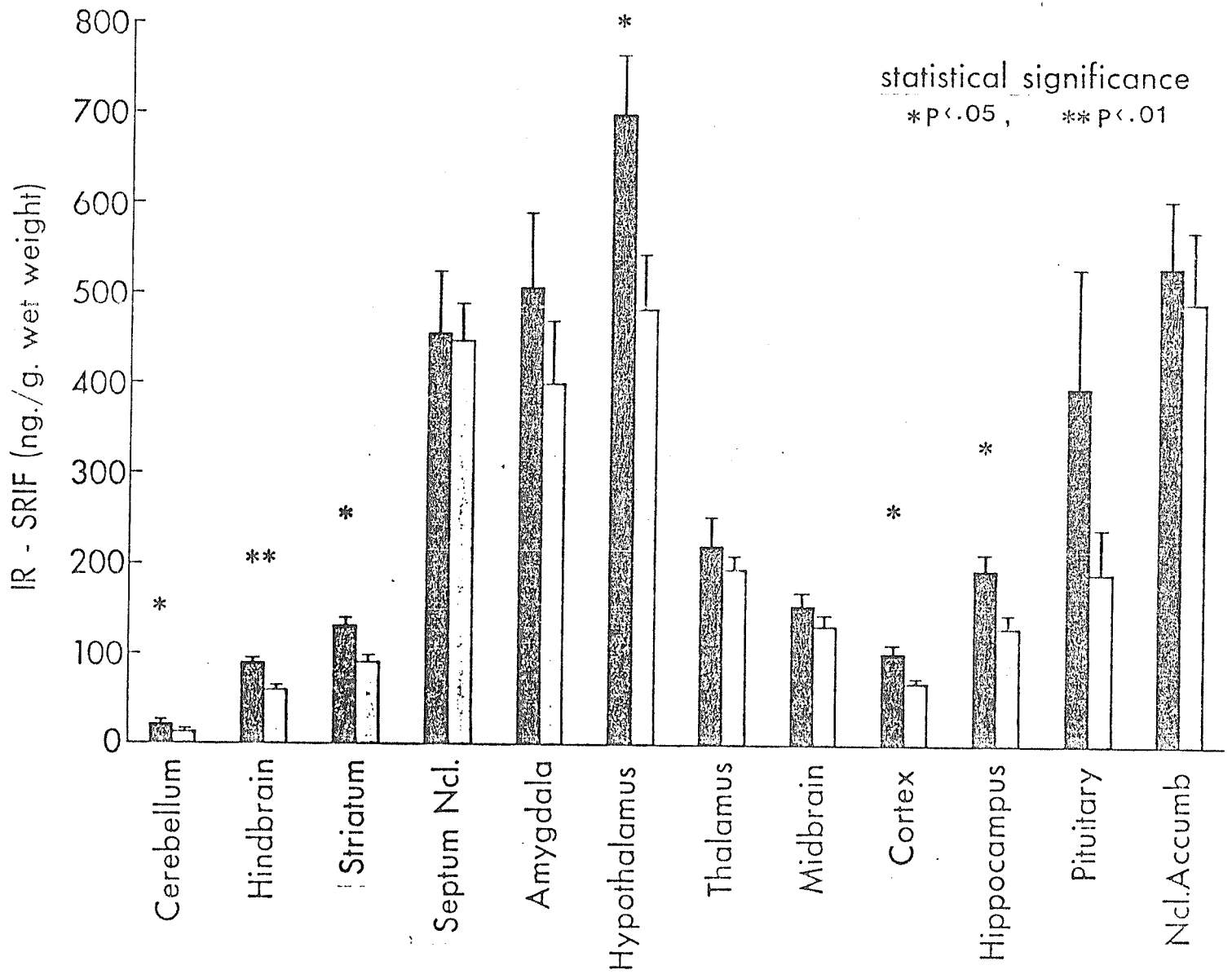


Fig. 12. IR-Somatostatin content in brain regions of control non-stressed and PTU non-stressed rats (7 mos.). IR-SRIF concentrations were significantly increased in PTU-treated rats in the following brain regions: hindbrain ($p \leq .01$), cerebellum, striatum, hypothalamus, cortex, and hippocampus ($p \leq .05$).

Distribution of IR-SRIF in the brain of PTU-treated (▩) and control (□) adult rats.



of aged, stressed control rats is significantly higher ($p < .01$) than in adult, non-stressed control rats. A similar trend found between PTU stressed and non-stressed subjects was not significant. Figure 14 shows that total IR-SRIF in the brains of adult PTU rats is significantly higher than in controls when expressed as ng./brain ($p < .05$) or ng/g wet tissue wt. ($p < .01$). In aged PTU subjects, total IR-SRIF content is significantly higher ($p < .05$) when expressed as ng/g wet tissue wt. The average ($\bar{X} \pm$ S.E.M.) wet weight of the whole brain in all PTU rats (both studies) is significantly lower ($p < .05$) than the average value of all controls, as shown in Table 3. The total IR-SRIF content in the brains of all PTU rats is significantly higher ($p < .05$, $p < .01$) when expressed as ng/brain and ng/g wet tissue wt. respectively.

Table 3. Brain Weights, Immunoreactive β-Endorphin and Immunoreactive Somatostatin Content in PTU-Treated and Control Rats

	Control (N = 19)	PTU-Treated (N = 16)
Wet Weight of Whole Brain (gm)	1.975 ± 0.032	1.868 ± 0.034 *
Total β-Endorphin Content in Whole Brain (ng/brain)	651.5 ± 161.2	479.7 ± 115.6
Total β-Endorphin Concentration in Whole Brain (ng/g wet wt.)	328.6 ± 80.2	256.5 ± 59.3
Total IR-SRIF Content in Whole Brain (ng/brain)	243.4 ± 21.2 (N = 15)	314.5 ± 22.3 * (N = 14)
Total IR-SRIF Concentration in Whole Brain (ng/g wet wt.)	122.5 ± 10.1 (N = 15)	167.8 ± 11.8 ** (N = 14)

Mean ± S.E.M.
* $p < .05$
** $p < .01$

Fig. 13. Total IR-β-endorphin content in brains of control and PTU rats. Comparison between control stressed and control non-stressed rats revealed a significant increase ($p \leq .01$) in total IR-β-endorphin of the stressed group when expressed as ng/whole brain and ng/g tissue. No other comparisons between groups showed significant changes in total peptide content.

Total Immunoreactive β-Endorphin in brains of control and PTU-treated rats.

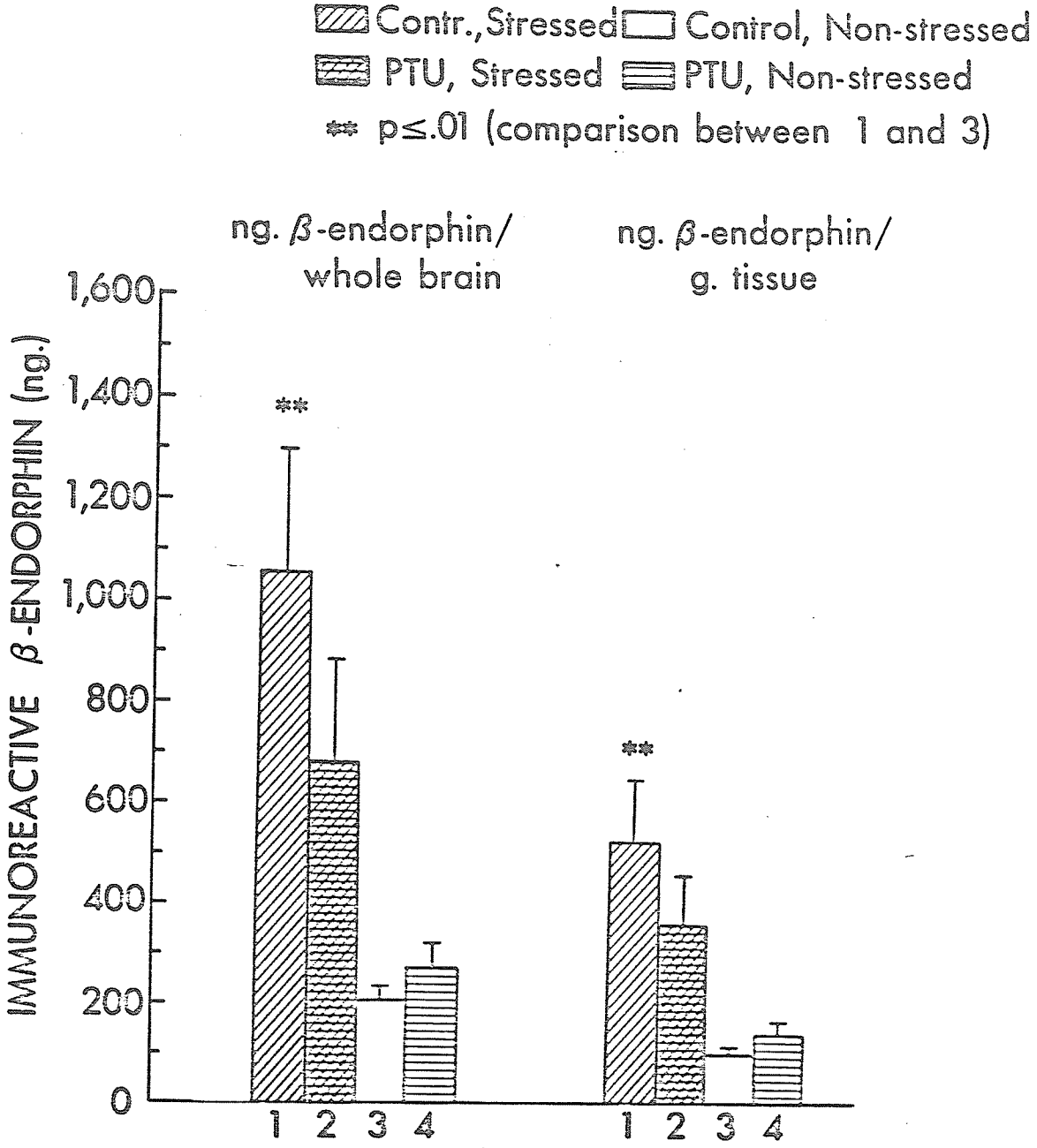
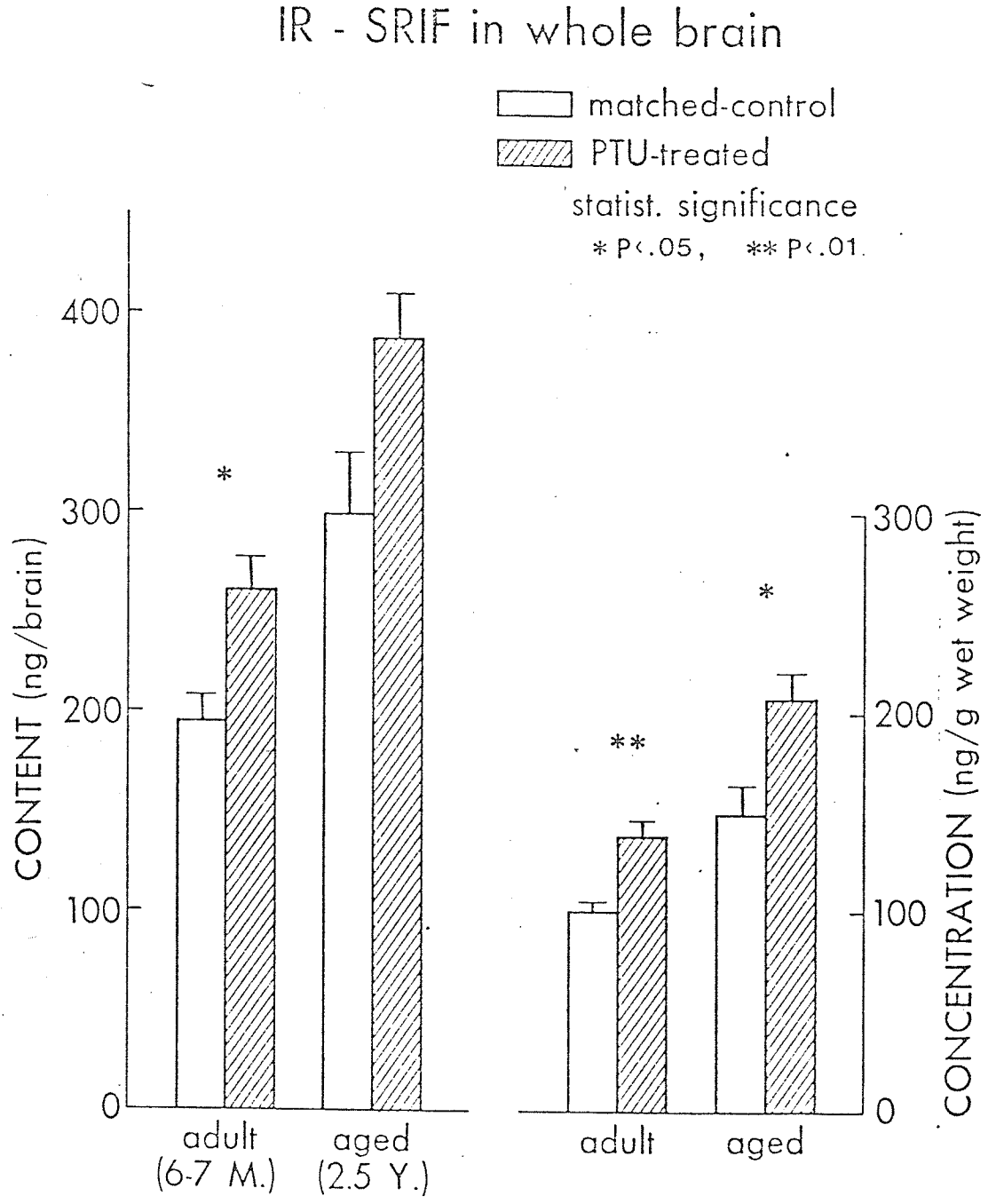


Fig. 14. Total IR-somatostatin content in brains of control and PTU rats. Comparison between control non-stressed and PTU non-stressed rats (6-7 mos.) revealed a significant increase in the PTU group when expressed as ng/whole brain ($p \leq .05$) and ng/g tissue ($p \leq .01$). Comparison between control stressed and PTU stressed rats (2.5 yrs.) showed an elevated concentration (ng/g tissue) of IR-SRIF in whole brain of the PTU group ($p \leq .05$).

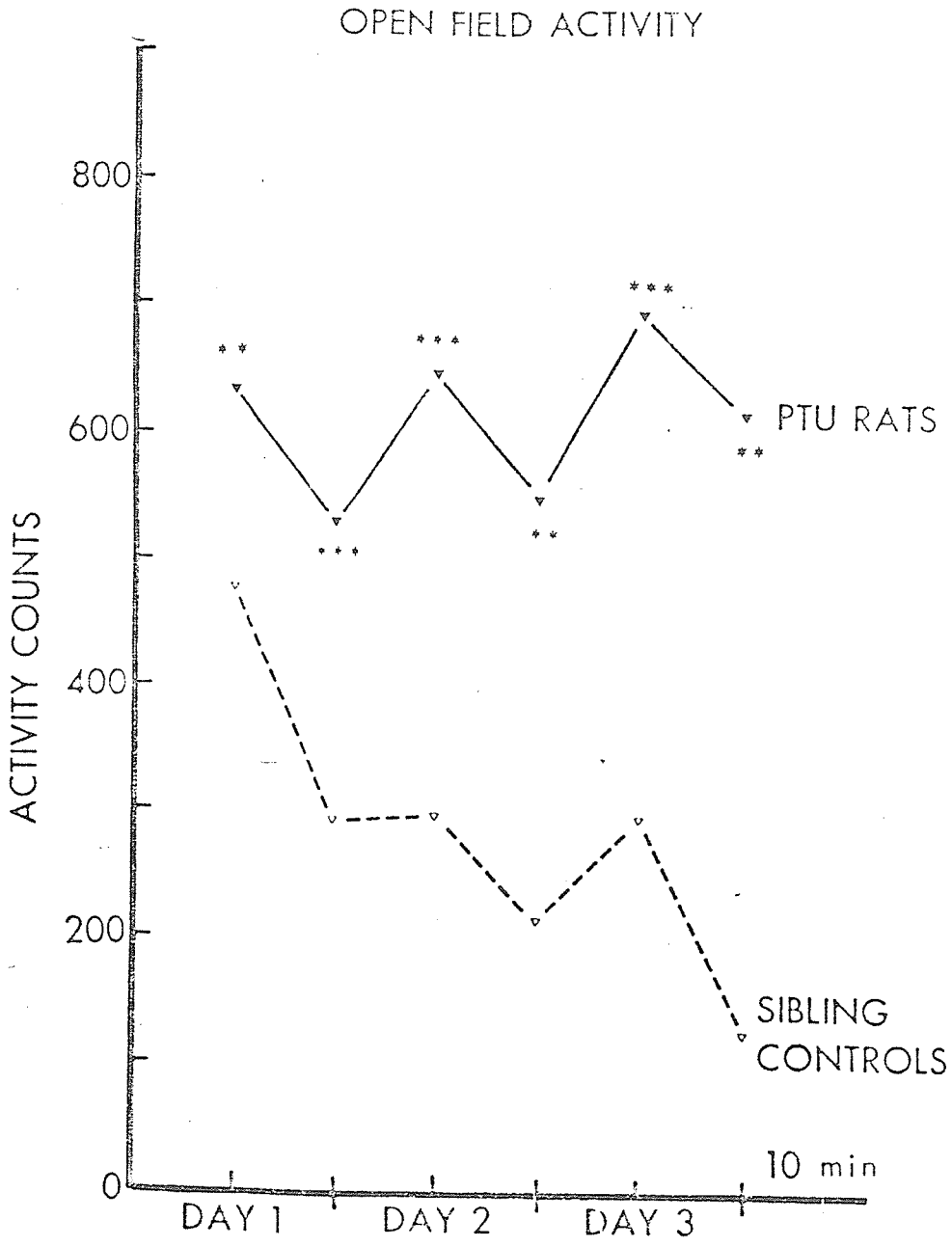

IR - SRIF in whole brain
matched-control
PTU-treated
statist. significance
* P<.05, ** P<.01
CONTENT (ng/brain)
400
300
200
100
0
*
adult (6-7 M.)
aged (2.5 Y.)
CONCENTRATION (ng/g wet weight)
300
200
100
0
**
*
adult
aged

4. Open-Field Activity and Maze-Learning Ability of Adult PTU and Control Rats

Figure 15 shows the average ($\overline{X}$ ± S.E.M.) activity counts accumulated during 2 consecutive 10 min. periods over 3 days of testing. In the control group, subjects habituated quickly to the environment during the first day of testing, as indicated by a sharp reduction in counts from the first 10 min. to the second 10 min. of confinement in the chamber. Thereafter, the activity counts dropped progressively to a low level at the end of the last day's session. In the PTU group, initial counts were significantly higher ($p < .01$) and $p < .001$) at the start of the day 1 session and continued high throughout the 3 day testing period. Some habituation occurred on each day from the first 10 min. to the second 10 min. of the session.

Figures 16 - 18 summarize the maze-learning ability of PTU and control rats. During initial training (2 week period, route as in Figure 2a), PTU rats exhibited better performance than controls, both in terms of fewer errors made and shorter running time to the goal box. Statistical comparison of both of these learning indices was significant ($p < .05$) on the last day of task acquisition (Figure 16). (Note: this day was chosen for analysis since it represented the least degree of response variability for each rat). During the relearning phase, in which the route was reversed to the mirror image of the original (Figure 2b), the performance of PTU rats was clearly

Fig. 15. Locomotor activity of PTU-treated rats (solid line, N=10) in comparison with sibling controls (broken line, N=10). Each rat was placed in the activity chamber once daily for 20 min. over 3 consecutive days. Activity counts registered were recorded after the first and second 10 min. intervals. PTU-treated rats showed persistent hyperactivity and no habituation over the 3-day testing period.


OPEN FIELD ACTIVITY
ACTIVITY COUNTS
800
600
400
200
0
PTU RATS
SIBLING CONTROLS
10 min
DAY 1
DAY 2
DAY 3

inferior to that of controls. When the average running time or number of errors of subjects on the last day of acquisition is expressed as a baseline of 100%, PTU rats were found to exhibit significantly higher error frequency ($p < .001$, Figure 17), and longer running time ($p < .05$, Figure 18) over the 5 day relearning period. On the last day of retraining, error frequency in the PTU group was still significantly higher than in controls ($p < .01$), although running time had increased to match that of controls (Figure 16).

5. IR-β-Endorphin and IR-SRIF Levels in the Brains of Habituated Control Rats Exposed to a Loud Sound Stimulus

This brief study was carried out in order to determine whether the exposure to a loud sound stimulus, as in the first experiment described, constitutes an effective stressor. Table 4 shows that β-endorphin content of the septal nuclei was significantly reduced ($p < .05$) in the bell-exposed group. No other changes were observed. Frontal and parietal cortex were dissected and assayed separately, with no regional differences in β-endorphin concentration noted between the two. In addition, pyriform and entorhinal cortex (limbic structures) were dissected out separately and the β-endorphin levels estimated for each. Concentrations were much higher than in the cortex as a whole, but again did not differ between the two. Table 5 shows no significant differences in somatostatin levels

Fig. 16. Maze-learning ability of PTU-treated vs. control rats. Average number of errors and running time were evaluated on the last day of task acquisition and the last day of retraining. Both error frequency and total running time were reduced in the PTU-treated group on the last day of task acquisition. In contrast, error frequency was significantly increased in the PTU-treated group on the last day of retraining.

Learning ability in PTU-treated vs. control rats.

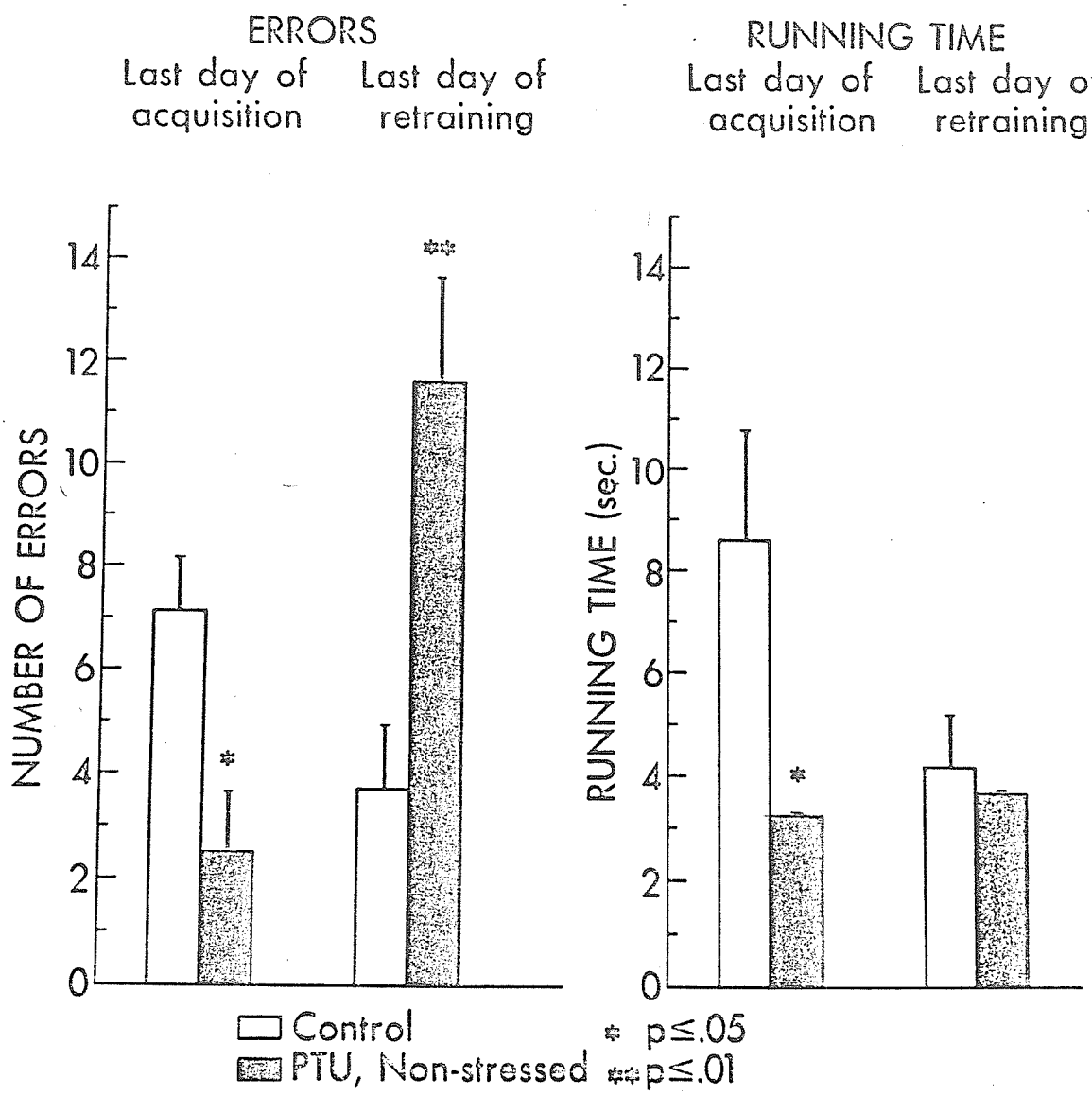


Fig. 17. Maze-learning ability during 5-day retraining phase in PTU-treated and control rats. Performance in terms of errors is expressed as a percentage of the baseline error frequency of each rat (designated as 100%, measured on the last day of task acquisition). After an initial increase in errors observed in both groups, only PTU-treated rats failed to achieve a reduction in errors over the course of 5 daily sessions.

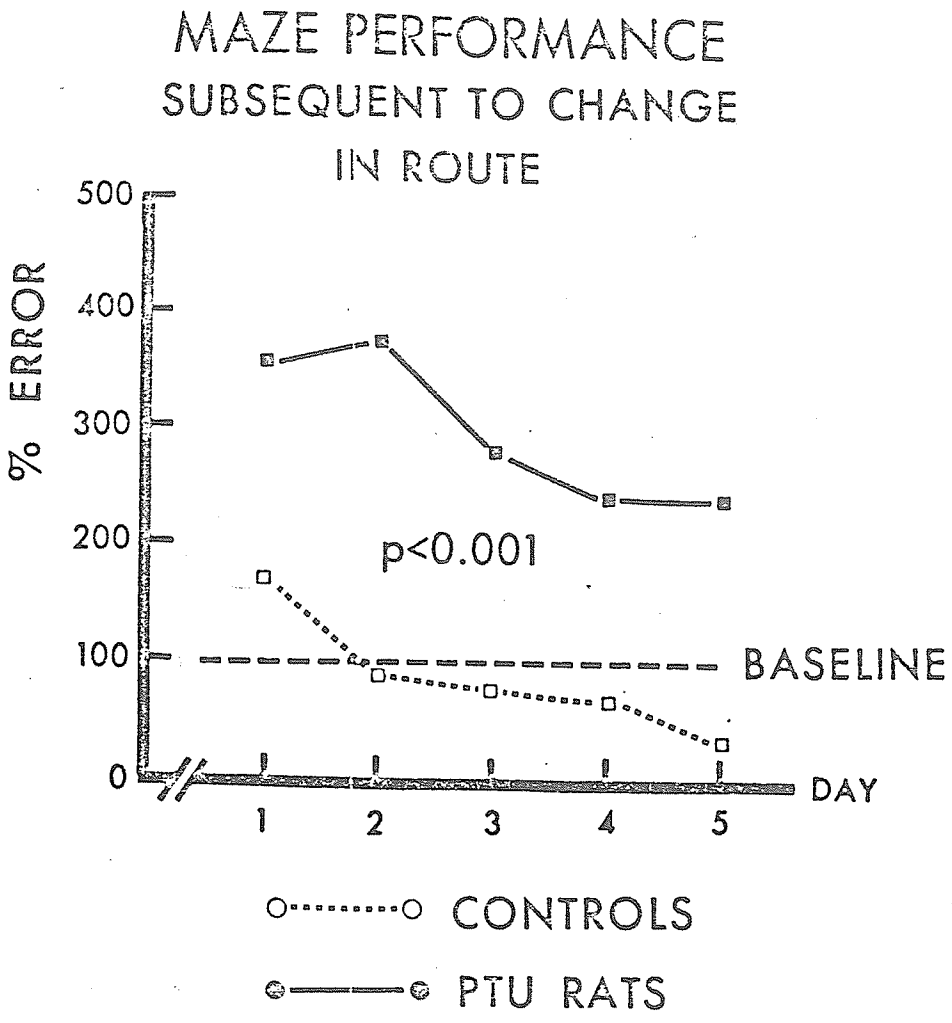
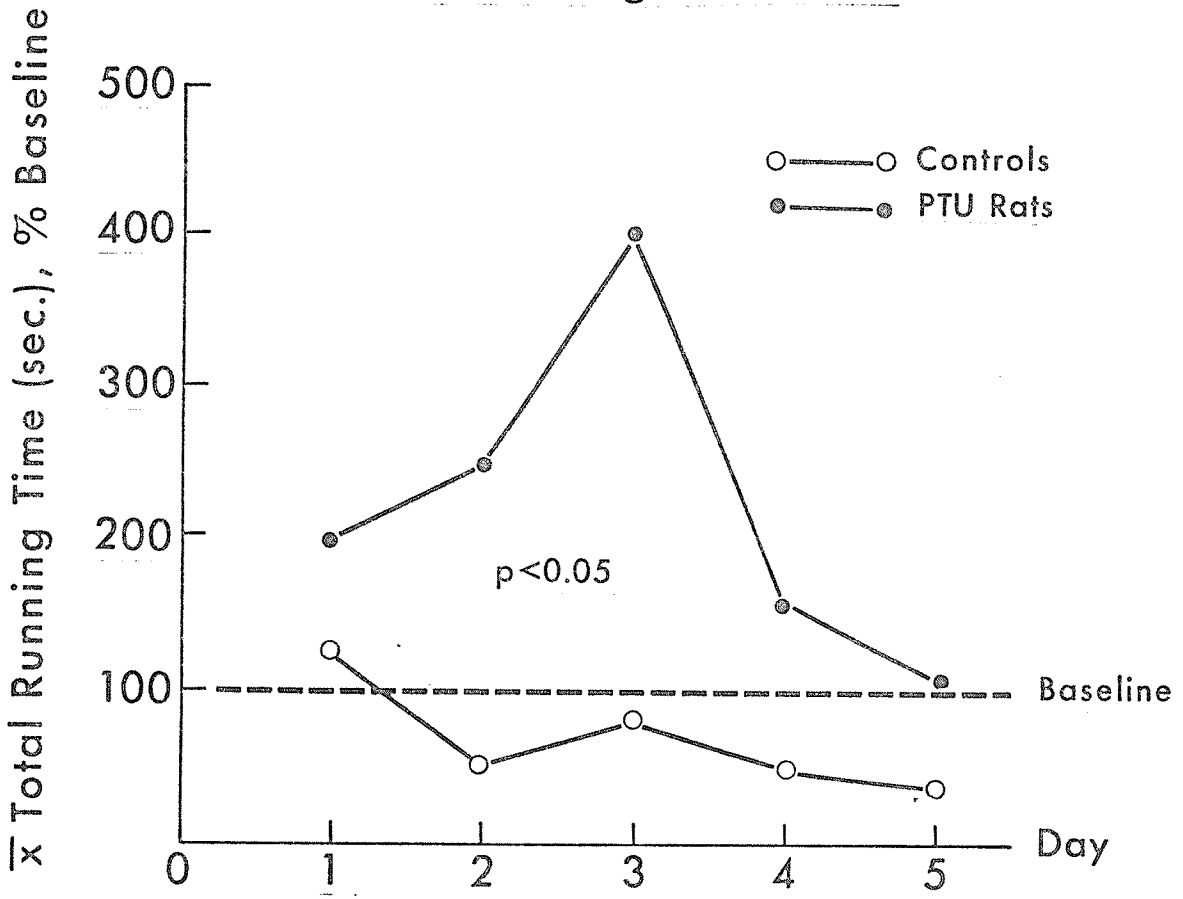

MAZE PERFORMANCE
SUBSEQUENT TO CHANGE
IN ROUTE
% ERROR
500
400
300
200
100
0
p<0.001
BASELINE
DAY
1
2
3
4
5
CONTROLS
PTU RATS

Fig. 18. Retraining performance of PTU-treated and control rats in terms of average total running time. Running time in the PTU-treated group was significantly elevated above baseline performance on the first 4 days of testing.

Maze performance subsequent to change in route



between bell-exposed and non-exposed rats in any brain areas. Frontal cortex SRIF content was found to be higher than parietal cortex levels. As for β-endorphin, pyriform and entorhinal cortical concentrations were higher than in the cortex as a whole, although no differences were found between the two.

Table 4. IR-β-Endorphin Levels in Brain Regions of Habituated Adult Control Rats Exposed to a Loud Sound Stimulus (114 dB Intensity for 90 Seconds)

Brain Region	ng/g wet wt. $\bar{X}$ ± S.E.M.	
	Control, No Bell (N = 5)	Control, Bell-Exposed (N = 6)
Cerebellum	42.9 ± 25.7	46.7 ± 25.9
Hindbrain	186.6 ± 48.3	161.3 ± 59.0
Striatum	14.4 ± 2.7	21.0 ± 6.4
Septal Nuclei	163.9 ± 14.3	101.7 ± 20.6 *
Amygdala	171.7 ± 19.9	280.5 ± 83.4
Hypothalamus	1,285.0 ±441.9	1,201.6 ± 527.4
Thalamus	114.0 ± 23.7	74.7 ± 7.0
Midbrain	132.2 ± 18.3	326.9 ± 172.3
Frontal Cortex	14.9 ± 3.1	8.6 ± 2.1
Parietal Cortex	9.1 ± 1.2	13.6 ± 3.9
Hippocampus	37.1 ± 11.7	79.4 ± 57.0
Pituitary (μg./g)	572.9 ± 61.8	552.4 ± 71.4
Nucleus Accumbens	361.9 ± 46.6	388.8 ± 105.7
Pyriform Cortex	112.2 ± 39.0	63.9 ± 20.5
Entorhinal Cortex	114.6 ± 26.7	68.4 ± 15.1

Table 5. IR-SRIF Levels in Brain Regions of Habituated Adult Control Rats Exposed to a Loud Sound Stimulus

Brain Region	ng/g wet wt. $\bar{X}$ ± S.D.	
	Control, No-Bell (N = 5)	Control, Bell-Exposed (N = 6)
Cerebellum	7.1 ± 5.5	7.3 ± 6.2
Hindbrain	83.5 ± 58.4	83.3 ± 52.8
Striatum	93.8 ± 58.0	72.5 ± 40.2
Septal Nuclei	221.7 ± 107.0	267.9 ± 110.6
Amygdala	206.7 ± 75.0	294.3 ± 59.6
Hypothalamus	421.0 ± 264.4	343.5 ± 138.4
Thalamus	64.9 ± 19.4	95.6 ± 28.4
Midbrain	97.3 ± 48.0	95.8 ± 48.6
Frontal Cortex	131.2 ± 55.9	139.0 ± 60.1
Parietal Cortex	65.4 ± 32.6	41.3 ± 20.4
Hippocampus	93.4 ± 42.1	92.2 ± 50.0
Pituitary	107.7 ± 64.4	159.7 ± 109.4
Nucleus Accumbens	341.8 ± 89.8	272.6 ± 104.4
Pyriform Cortex	262.4 ± 228.2	198.6 ± 81.3
Entorhinal Cortex	319.0 ± 39.0	205.7 ± 64.8

6. IR-β-Endorphin and IR-SRIF Levels in Brain Regions of Neonatally $NaClO_4$-Treated Rats, and with Exposure to PTU After Maturity

Figure 19 a and b shows the β-endorphin content in brain regions of Control + H_2O, ClO_4 + H_2O, Control + PTU, and ClO_4 + PTU rats (N = 6 per group). The pituitary content of β-endorphin in the ClO_4 + H_2O group was significantly elevated ($p < .01$) relative to the other 3 groups (395.5 ± 71.3 µg/g vs. 169.2 ± 32.1, 176.4 ± 23.8, and 211.1 ± 13.3). No other differences between groups were observed. β-endorphin concentrations in most brain regions were comparable to those obtained in the previous experiments, although levels were higher in the

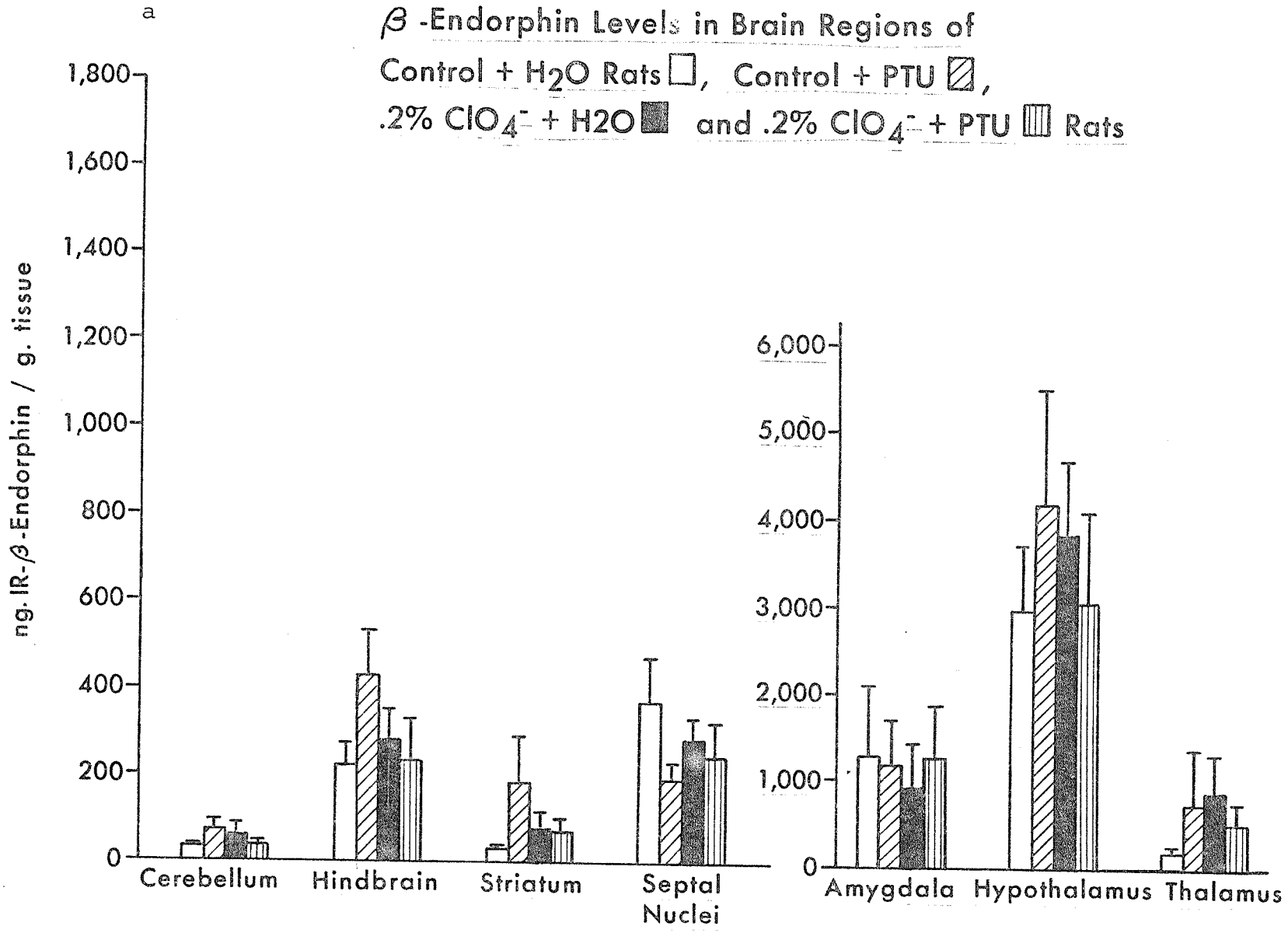
striatum and amygdala (2-fold) and pyriform and entorhinal cortex (3 to 4-fold) relatives to values reported in Table 4. Figure 20 shows IR-SRIF levels in the brains of these 4 groups of rats. Cerebellar levels were slightly increased ($p < .05$) in both groups receiving PTU, thalamic content was lower ($p < .05$) in the ClO_4 + PTU group, and cortical content was lower ($p < .05$) in the Control + PTU group. In both the pituitary and nucleus accumbens, levels were decreased in all 3 treatment groups ($p < .05$, $p < .01$) compared to the Control + H_2O group. In this experiment, IR-SRIF concentrations were generally lower (except in the pituitary) than values obtained in previous experiments, especially in the hypothalamus (3-fold decrease).

7. Development of Rat Litters Exposed to 0.02% PTU, 0.2% PTU, 1.0% $NaClO_4$ and Control Neonatal Treatments

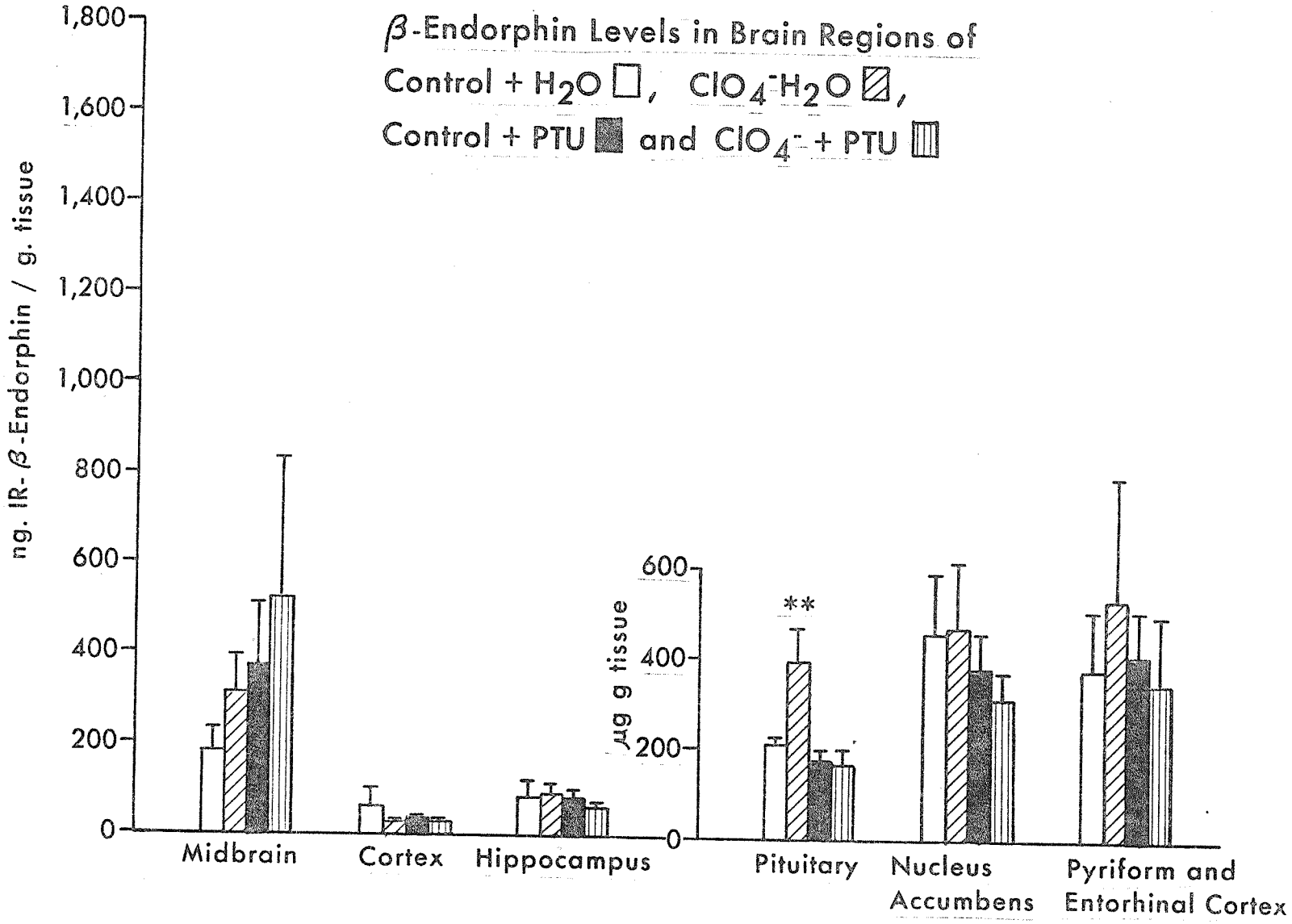
Figure 21 a - d compares the sizes and physical appearances of rat pups at 19 days of age in 4 treatment groups: control, 1.0% $NaClO_4$, 0.02% PTU, and 0.2% PTU. Control and $NaClO_4$-treated pups were very similar in size and appearance, whereas 0.02% PTU pups were smaller, with poorly developed pinnae (outer ears) which were positioned close to the head. The hair of these pups tended to be coarser, matted, and slightly yellowish in color. Growth of 0.2% PTU pups was severely depressed and physical appearance remained juvenile (rectangular head shape, poorly developed hair growth, etc.). In

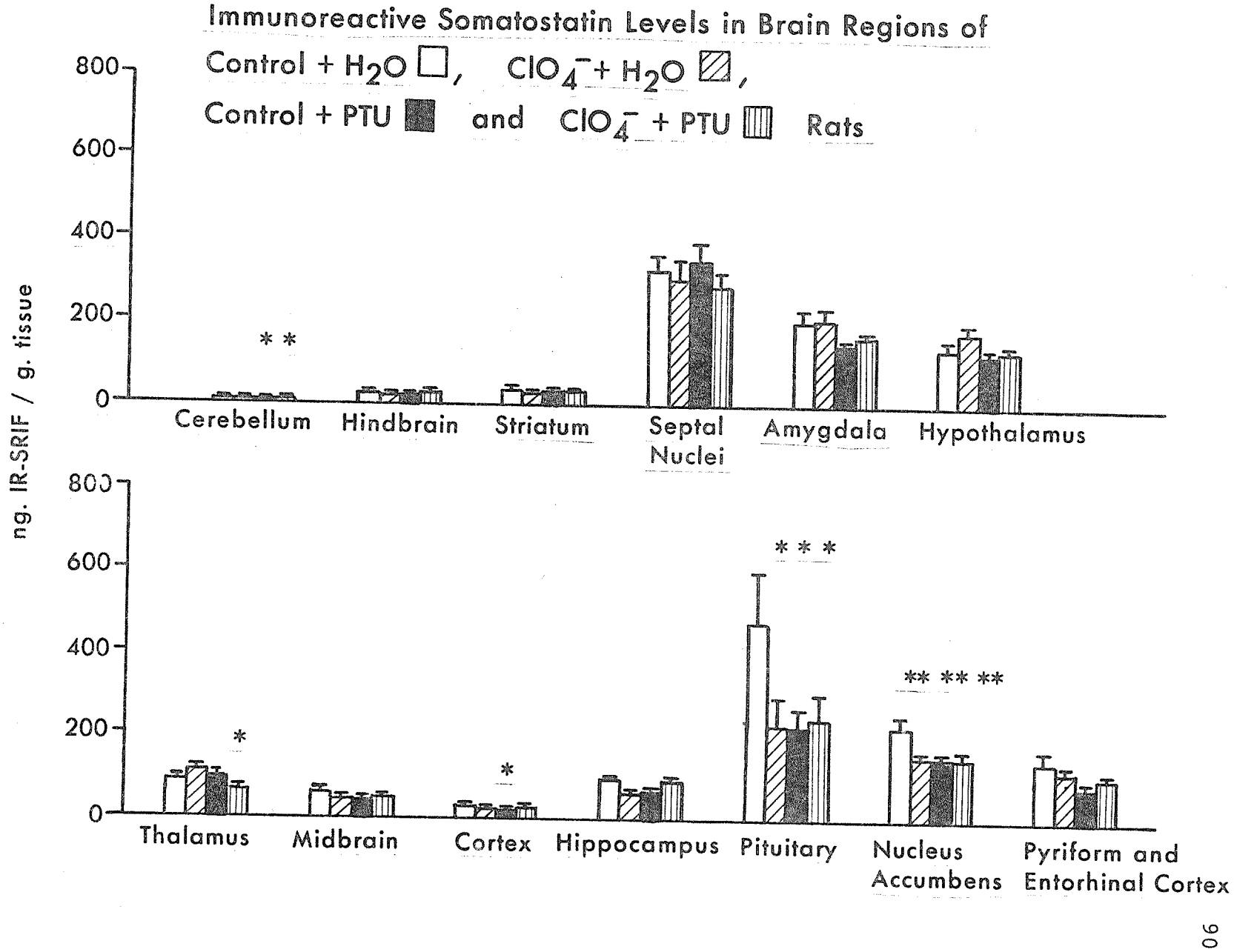
Fig. 19 a,b. IR-β-Endorphin levels in brain regions of neonatally 0.2% $NaClO_4$-treated and control rats with and without subsequent exposure to PTU after maturity (N=6 per group). The pituitary content of IR-β-endorphin in the ClO_4^- (neonatal) + H_2O (adulthood) group was significantly elevated compared to the other 3 groups. No other differences between groups were observed.

a


β -Endorphin Levels in Brain Regions of
Control + H2O Rats □, Control + PTU ▨,
.2% ClO4- + H2O ■ and .2% ClO4- + PTU ▥ Rats
ng. IR-β-Endorphin / g. tissue
1,800
1,600
1,400
1,200
1,000
800
600
400
200
0
6,000
5,000
4,000
3,000
2,000
1,000
0
Cerebellum
Hindbrain
Striatum
Septal Nuclei
Amygdala
Hypothalamus
Thalamus

b


β-Endorphin Levels in Brain Regions of
Control + H_2O □, $ClO_4^-H_2O$ ▨,
Control + PTU ■ and ClO_4^- + PTU ▥
ng. IR-β-Endorphin / g. tissue
1,800
1,600
1,400
1,200
1,000
800
600
400
200
0
μg g tissue
600
400
200
0
**
Midbrain
Cortex
Hippocampus
Pituitary
Nucleus Accumbens
Pyriform and Entorhinal Cortex


Immunoreactive Somatostatin Levels in Brain Regions of
Control + H_2O □, ClO_4^- + H_2O ▨,
Control + PTU ■ and ClO_4^- + PTU ▥ Rats
ng. IR-SRIF / g. tissue
800
600
400
200
0
* *
Cerebellum
Hindbrain
Striatum
Septal Nuclei
Amygdala
Hypothalamus
800
600
400
200
0
*
*
* * *
** ** **
Thalamus
Midbrain
Cortex
Hippocampus
Pituitary
Nucleus Accumbens
Pyriform and Entorhinal Cortex

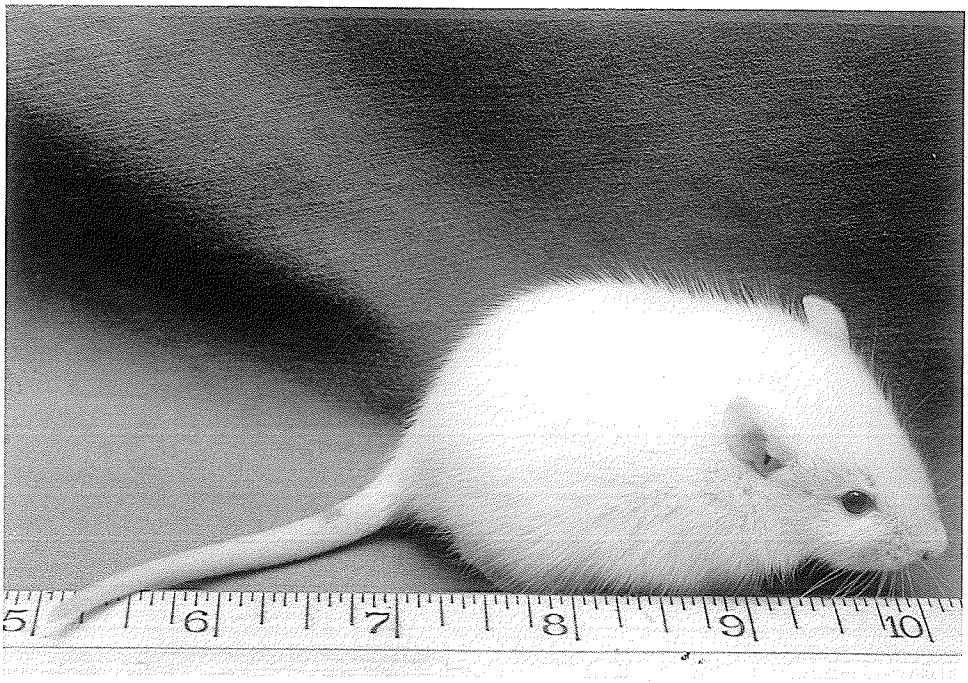
addition, they exhibited characteristic cretinoid features of swollen abdomen and enlarged neck area (goiter). Table 6 shows that the average body weight of 0.02% PTU rats at 19 days of age was significantly less ($p < .001$), about 80% that of controls. The average body weight of 0.2% PTU pups was greatly decreased ($p < .001$, about 44% of controls), and their mortality rate was high such that only 7 pups out of 3 litters survived to 19 days of age and beyond. Eye opening was delayed by 1 day in 0.02% PTU pups and 4 days in 0.2% PTU pups. No differences were observed in either body weight or day of eye-opening between control and 1.0% $NaClO_4$ pups.

T_4 levels were non-detectable at 19 days of age in all 3 treatment groups while the control value was normal, as shown in Table 7. T_3 levels were not measured at this age, although it is assumed that levels would be non-detectable in both PTU treatment groups since the drug prevents conversion of T_4 to T_3. At 4 months of age, a comparison of both serum T_4 and T_3 levels in control and 0.02% PTU rats revealed little difference between the two.

Table 8 shows the seizure-sensitivity of subjects in each of the 4 groups when tested in a 2 min. trial of exposure to the 114 dB noise stimulus. All rats were tested shortly after weaning (25 days), although 0.2% PTU rats were not yet weaned at this age. Of the subjects which reacted to the stimulus, 2 stages of seizure were distinguished, wild runs and full tonic-clonic seizures which resulted in unconsciousness.

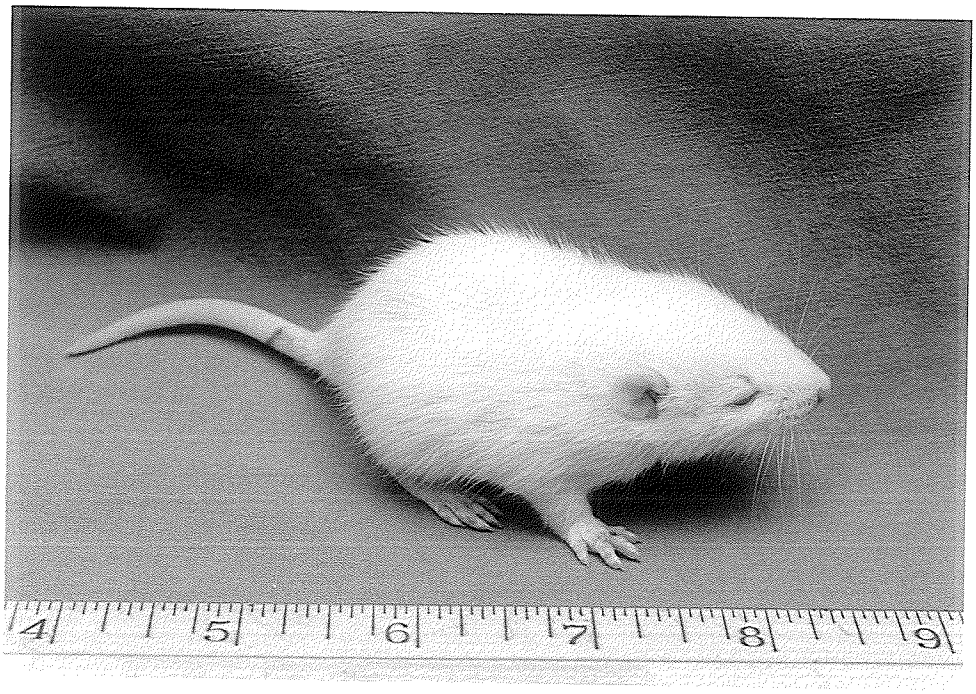
Fig. 21 a-d. Physical appearance of 19-day-old rats in 4 experimental groups: a. controls, b. 1.0% $NaClO_4$ (iodide competitor) in drinking water from 0→21 postnatal days, c. 0.02% PTU in drinking water from 0→19 postnatal days, and d. 0.2% PTU in drinking water from 0→19 postnatal days. Gross appearance of controls and $NaClO_4$ rats did not differ, whereas 0.02% PTU rats were smaller, with yellowish, matted hair and poorly developed pinnae positioned close to the head. 0.2% PTU rats were severely stunted, cretinoid in appearance, and exhibited juvenile head shape and hair growth.

a

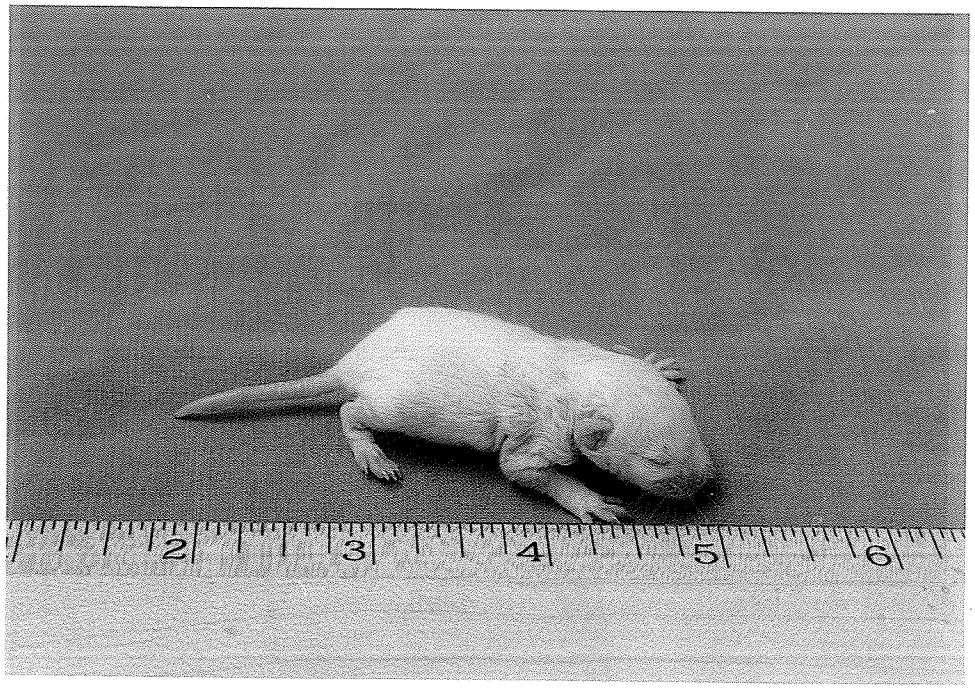


b

c



d



(All sensitive subjects initially entered the wild run stage but not all developed full tonic-clonic seizures, thus separating two levels of sensitivity). None of the control subjects reacted to the stimulus, and only 1 of 19 $NaClO_4$ subjects exhibited a wild run. Approximately 50% (10 of 21 subjects) of 0.02% PTU rats exhibited one or both stages of seizure activity, and these responses always occurred within the first 60 sec. of stimulus exposure. PTU subjects neonatally exposed to 0.2% treatment did not react, undoubtedly due to deafness since they showed no behavioral response to onset of the sound stimulus and often groomed throughout the 2 min. trial.

Table 9 shows that body weights of young adult 0.02% PTU rats (2.5 months) are somewhat lower on the average (not significant) than their control counterparts. At 30 days of age the body weights of 0.2% PTU rats are greatly decreased ($p < .001$) in comparison to controls of the same age.

Table 6. Body Weights at 19 Days and Day of Eye Opening in Rats with Different Types of Neonatal Antithyroid Treatment

No. of Litters	Treatment	Treatment Period	Body Wt. at 19 Days (g.) $\bar{X} \pm$ S.D.	Day of Eye-Opening
2	Control	-	41.6 ± 3.4	14 - 15
2	1.0% $NaClO_4$	postnatal day 0 → 21	42.6 ± 1.6	14 - 15
2	0.02% PTU	postnatal day 0 → 19	33.5 ± 2.5***	15 - 16
3	0.2% PTU	postnatal day 0 → 19	18.3 ± 4.8***	18 - 19

Table 7. Serum T_4 and T_3 Levels in Rats Following Different Types of Neonatal Antithyroid Treatment

No. of Subjects	Treatment	Age	Pooled Serum T_4 µg/dl	Pooled Serum T_3 g/dl
8	Control	19 days	6.3	-
8	1.0% $NaClO_4$	19 days	N.D.*	-
8	0.02% PTU	19 days	N.D.	-
7	0.2% PTU	19 days	N.D.	-
4	Control	4 mos.	6.4	80.0
4	0.02% PTU	4 mos.	5.9	77.0

*Non-Detectable (Assay sensitivity-2.5 µg/dl)

Table 8. Seizure Sensitivity of Post Weaning Age Rats Exposed to Neonatal Antithyroid Treatments

Treatment Group	Total N	No Reaction N	Wild Run N	Tonic-Clonic N
Control	24	24	0	0
1.0% $NaClO_4$	19	18	1	0
0.02% PTU	21	11	3	7
0.2% PTU	7	7	0	0

N = No. of subjects

Table 9. Post Weaning Body Weights of 0.02% PTU and 0.2% PTU Rats

No. of Subjects	Treatment	Age	Sex	Body Weight ($\bar{X}$ ± S.D.)
8	Control	2.5 mos.	M	364.0 ± 29.3
9	0.02% PTU	2.5 mos.	M	343.9 ± 29.0
7	Control	2.5 mos.	F	292.9 ± 14.8
11	1.02% PTU	2.5 mos.	F	257.5 ± 18.3
7	Control	7 mos.	M	495.4 ± 72.5
9	0.02% PTU	7 mos.	M	399.9 ± 28.6**
7	Control	7 mos.	F	329.6 ± 24.3
10	0.02% PTU	7 mos.	F	306.8 ± 22.2
5	Control	30 days	M	122.6 ± 4.7
7	0.2% PTU	30 days	M & F	34.6 ± 5.9***

8. Distribution of IR-β-Endorphin and IR-SRIF in Brain Regions of 30-day-old 0.2% PTU and Control Rats

Figure 22 indicates that there were no significant differences in β-endorphin concentrations found in the brains of 0.2% PTU rats compared to controls. IR-SRIF levels were significantly higher in the 0.2% PTU group in the following brain regions: striatum, septal nuclei, nucleus accumbens, pyriform and entorhinal cortex ($p < .05$), cerebellum, cortex, and hippocampus ($p < .01$), as shown in Figure 23.

9. Open-Field Activity of 0.2% PTU and Control Rats

Figure 24 shows that 0.2% PTU-treated rats exhibited hyperactivity as evidenced by both significantly higher exploratory activity counts ($p < .05$) and greater time spent in active exploratory behavior ($p < .001$) during the two 10 min. testing periods. Control rats habituated to their environment from the first to the second 10 min. period as noted by lower activity counts, whereas 0.2% PTU rats did not. Controls also spent more time grooming during the 20 min. session than hypothyroid subjects.

10. Seizure Test in Adult (2.5 mos.) 0.02% PTU Rats

The 0.02% PTU litters tested for seizure susceptibility at weaning were retested as young adults to determine possible changes in sensitivity since then. One factor believed to

Fig. 22. IR-β-Endorphin levels in brain regions of 30-day-old control (N=5) and 0.2% PTU-treated (N=7) rats. No significant changes in peptide concentration were found in the 0.2% PTU group.

Immunoreactive β-Endorphin Levels in Brain Regions of 30 Day Old Control □, and .2% PTU ▨ Rats

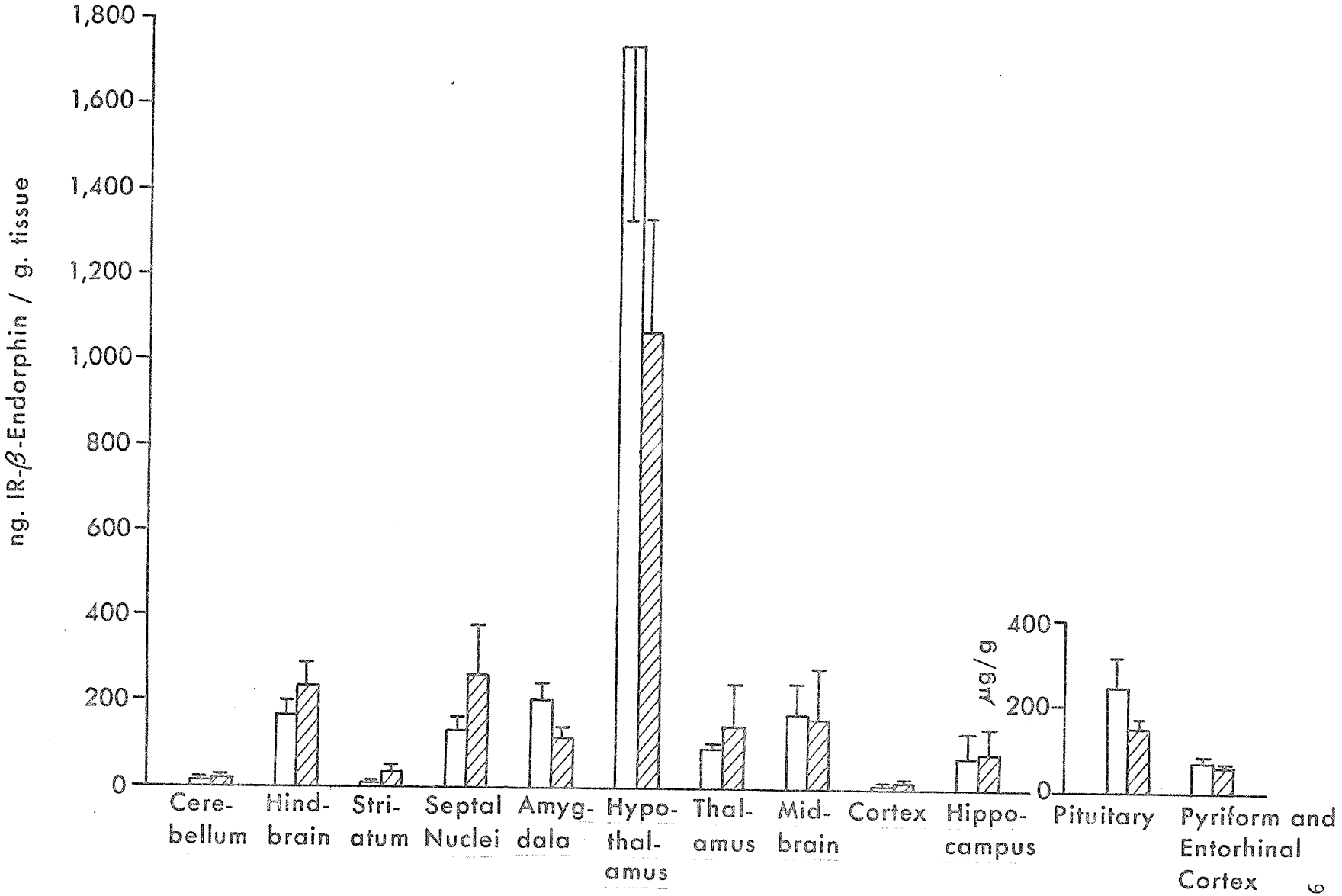


Fig. 23. IR-Somatostatin levels in brain regions of 30-day-old control and 0.2% PTU-treated rats. The 0.2% PTU group exhibited elevated IR-SRIF concentrations in striatum, septal nuclei, nucleus accumbens, pyriform and entorhinal cortex ($p \leq .05$), cerebellum, cortex, and hippocampus ($p \leq .01$).

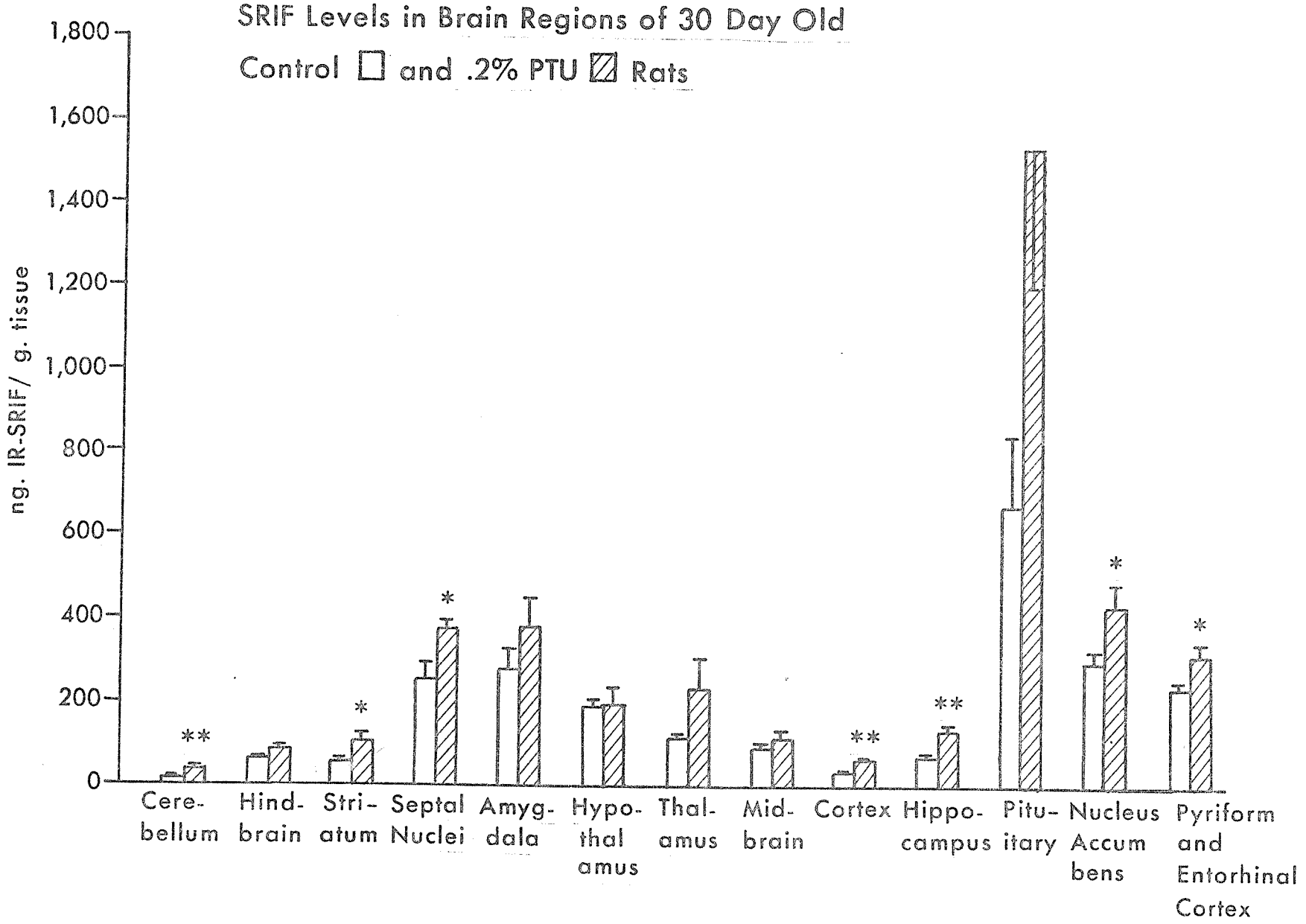
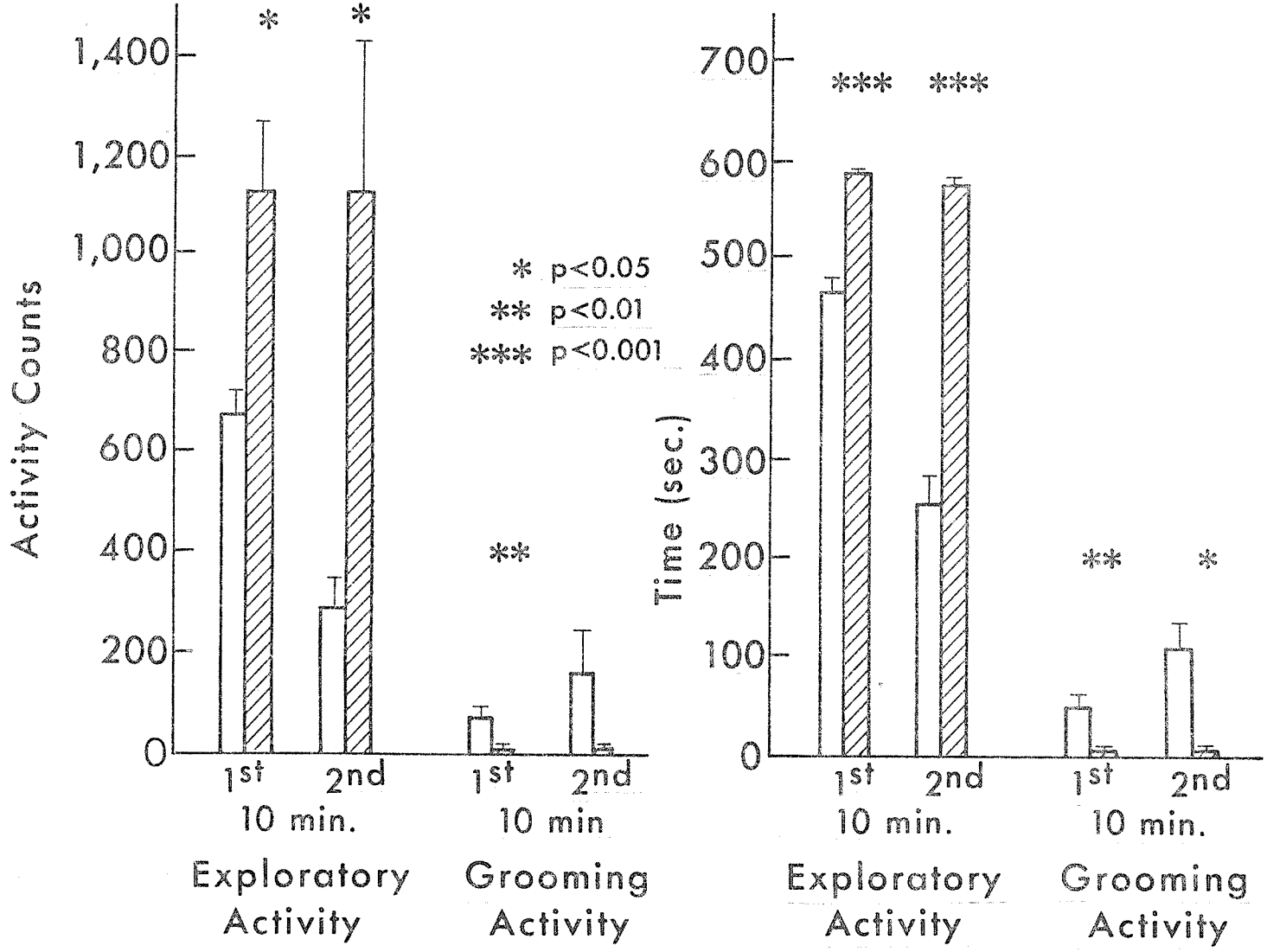

SRIF Levels in Brain Regions of 30 Day Old
Control □ and .2% PTU ▨ Rats
ng. IR-SRIF/ g. tissue
1,800
1,600
1,400
1,200
1,000
800
600
400
200
0
**
*
*
*
**
**
**
*
*
Cere-bellum
Hind-brain
Stri-atum
Septal Nuclei
Amyg-dala
Hypo-thal amus
Thal-amus
Mid-brain
Cortex
Hippo-campus
Pitu-itary
Nucleus Accum bens
Pyriform and Entorhinal Cortex

Fig. 24. Locomotor activity of control and 0.2% PTU-treated rats. Each rat was placed individually in the activity chamber for one 20 min. trial. Exploratory and grooming behavior were recorded separately after each 10 min. interval, in terms of total activity counts and total time spent in each activity. Exploratory activity counts and total time spent in exploration were elevated ($p \leq .05$, $p \leq .001$) during both 10 min. periods. Unlike controls, these rats showed no habituation from the first to the second 10 min. testing period (ie. no reduction in activity counts or total exploratory time). Grooming activity was depressed in PTU-treated subjects compared to controls.

Open - field activity in 0.2 % PTU ▨ and Control □ Rats



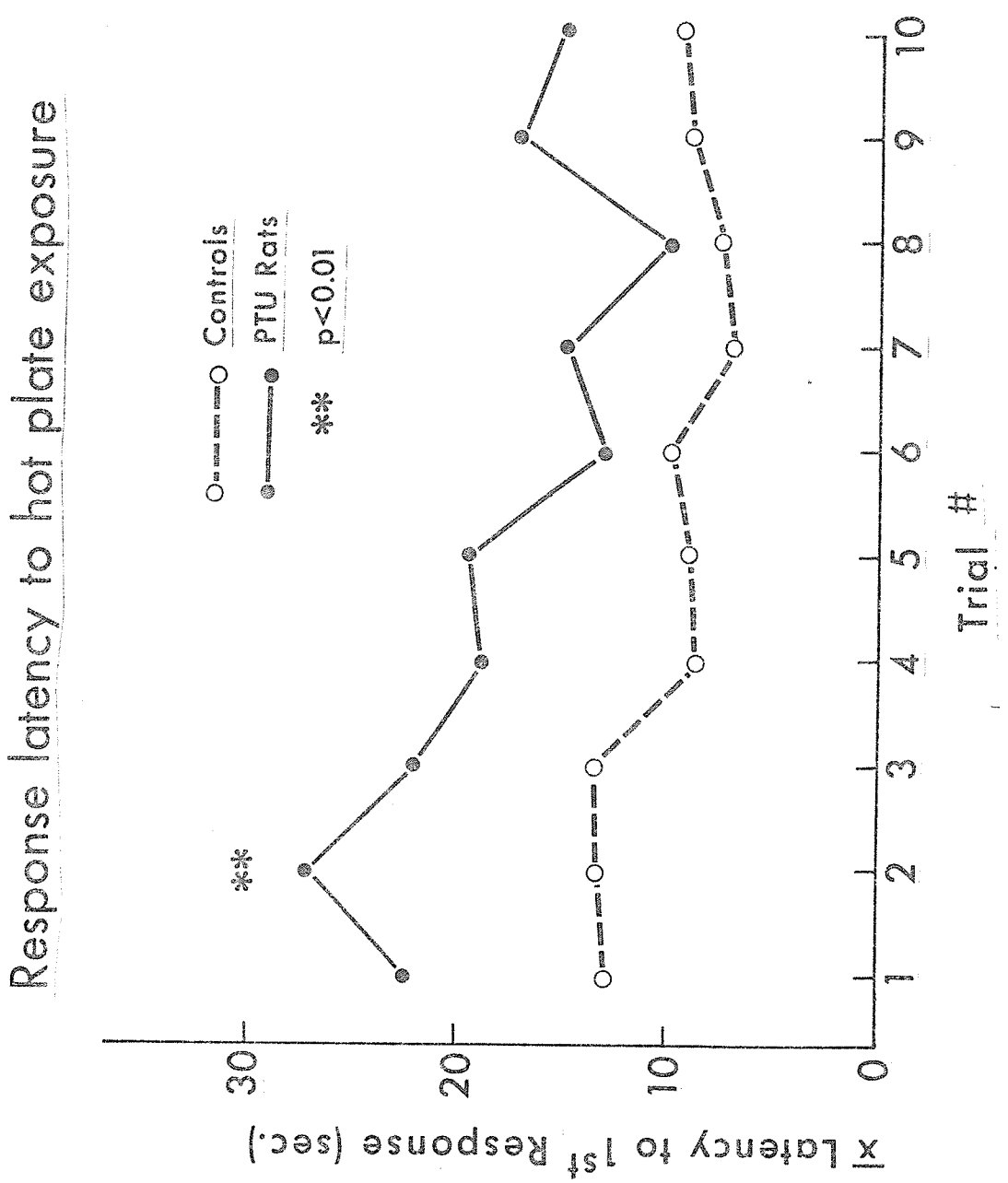
influence this was the occurrence of an unavoidable fire alarm drill in the Faculty of Dentistry animal quarters where the subjects were being housed. This took place about 2 weeks earlier (at 2 mos. of age) and lasted for 5 - 10 min., during which time it was noted that all 0.02% PTU rats experienced wild runs and violent tonic-clonic seizures. One subject died following a severe seizure episode. Of 20 subjects re-tested (9 male, 11 female), 17 experienced tonic-clonic seizures and 3 exhibited wild runs during a 2 min. test trial each. Therefore, the 50% seizure-sensitivity observed after weaning (Table 8) had increased to 100% in these young adult rats.

11. IR-β-Endorphin and IR-SRIF Levels in the Brains of PTU and Control Rats Exposed to Hot Plate Stress

Figure 25 shows that PTU rats (0.02% treatment) exhibit a longer average latency of response to the first hind-paw lick or jump over a 10-trial period of hot plate exposure. This difference was significant on the second trial only ($p < .01$). In both groups the response latency decreased with increasing number of exposure trials, the effect being more pronounced in the PTU group.

There were no significant differences in brain β-endorphin levels between control no stress, PTU no stress, control + stress, and PTU + stress groups of rats, as illustrated in Figure 26. Levels in both groups of PTU rats tended to be

Fig. 25. Behavioral characteristics of hot plate stress in control (broken line, N=5) and PTU (0.02% treatment, solid line, N=5) rats. Response latency to first hindpaw lick or jump was recorded during 10 trials of exposure (60 sec. in the first trial, 30 sec. in each of the last nine) to a 55°C hot plate. The average response latency was increased in PTU rats on all trials, with a significant increase on trial #2.


Response latency to hot plate exposure
Controls
PTU Rats
** p<0.01
**
x̄ Latency to 1st Response (sec.)
30
20
10
0
1 2 3 4 5 6 7 8 9 10
Trial #

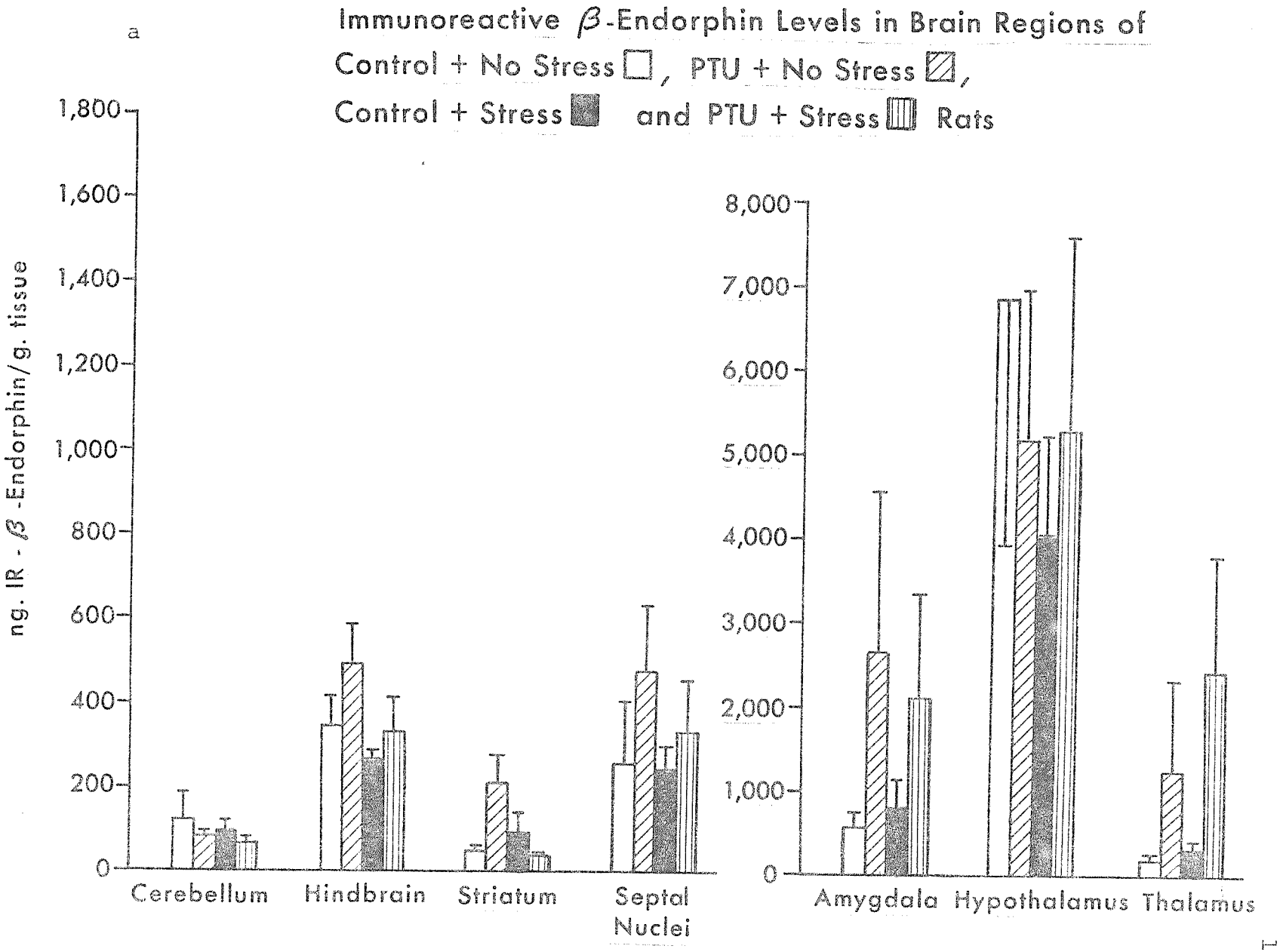
higher than control groups in the septal nuclei, amygdala, and thalamus, consistent with the results obtained for maze-trained adult PTU rats. IR-SRIF levels were also not found to differ between the groups (Figure 27) except in the pituitary, where concentrations in the control stressed group were significantly higher ($p < .05$) than in non-stressed control rats (1,082.4 ± 253.8 ng/g vs. 346.0 ± 111.5). Hypothalamic IR-SRIF levels were again unusually low in this experiment as found in the $NaClO_4$ + PTU study.

12. Startle Response Test in PTU and Control Rats

Figure 28 shows that PTU rats respond maximally to a 110 dB acoustic startle stimulus at low background white noise intensity levels whereas controls are maximally sensitive at high intensity levels. Responses of each subject at the various background white noise levels were expressed as percentages of the intensity level at which the subject's average motor response was the greatest (i.e., 100% response). This was done because individual differences in absolute magnitude of response varied greatly. The response average differed significantly between the 2 groups at all white noise intensities except 70 dB ($p < .001$ at 25 and 80 dB, $p < .01$ at 40 dB, $p < .05$ at 55 dB).

Fig. 26 a,b. IR-β-Endorphin levels in brain regions of control and PTU-treated rats, non-stressed and after exposure to hot plate stress. Stressed subjects were sacrificed 2.5 min. after the last trial of hot plate exposure. No significant differences were observed between the 4 groups in any brain region tested.

a


Immunoreactive β-Endorphin Levels in Brain Regions of Control + No Stress □, PTU + No Stress ▨, Control + Stress ■ and PTU + Stress ▥ Rats
ng. IR - β-Endorphin/g. tissue
1,800
1,600
1,400
1,200
1,000
800
600
400
200
0
8,000
7,000
6,000
5,000
4,000
3,000
2,000
1,000
0
Cerebellum
Hindbrain
Striatum
Septal Nuclei
Amygdala
Hypothalamus
Thalamus

b

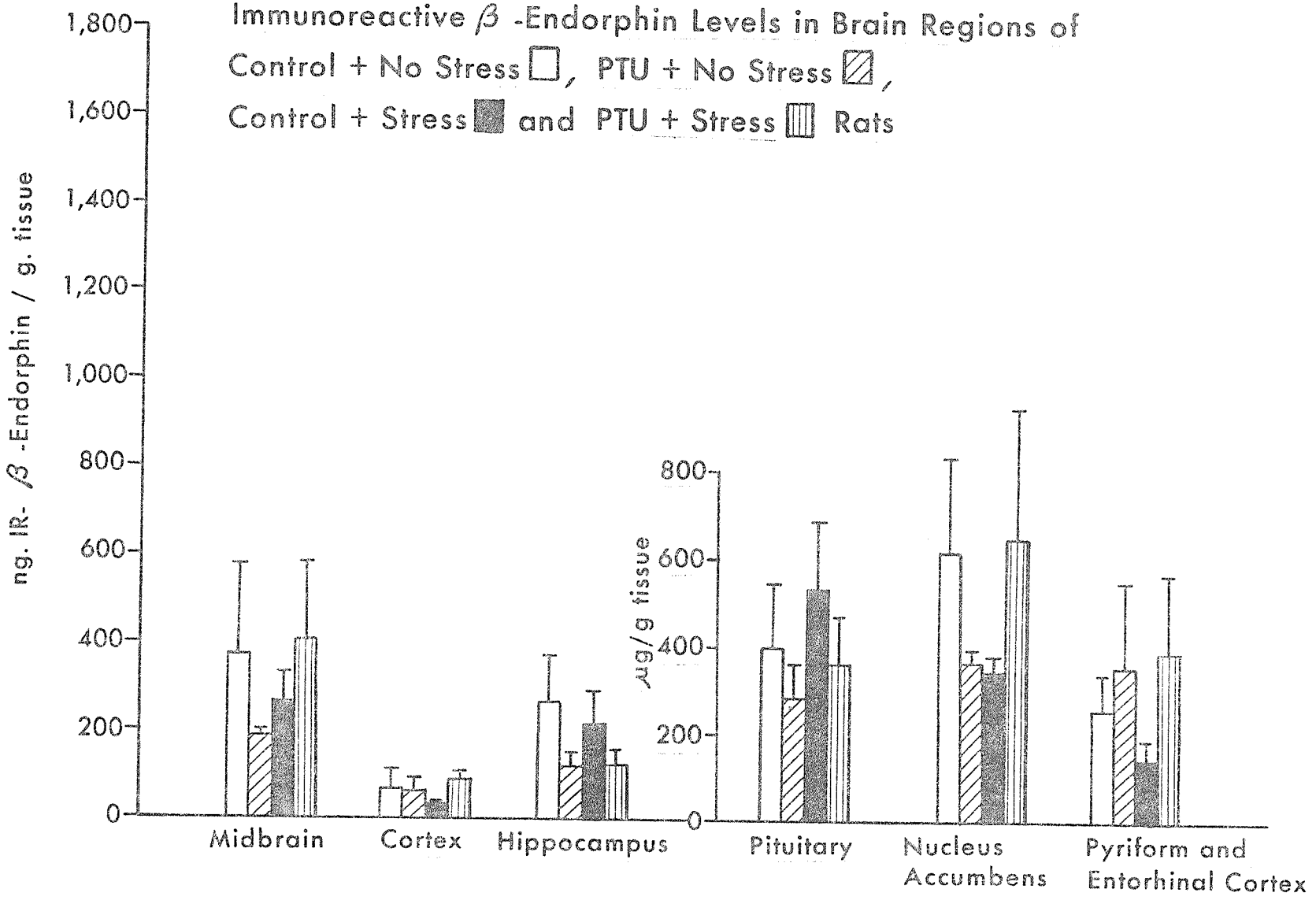

Immunoreactive β -Endorphin Levels in Brain Regions of
Control + No Stress, PTU + No Stress,
Control + Stress and PTU + Stress Rats
ng. IR- β -Endorphin / g. tissue
1,800
1,600
1,400
1,200
1,000
800
600
400
200
0
μg/g tissue
800
600
400
200
0
Midbrain
Cortex
Hippocampus
Pituitary
Nucleus Accumbens
Pyriform and Entorhinal Cortex

Fig. 27. IR-Somatostatin levels in brain regions of control and PTU-treated rats, non-stressed and after exposure to 30 min. of intermittent hot plate stress. Pituitary IR-SRIF concentrations in the control + stress group were significantly elevated ($p \leq .05$) compared to the control + no stress group. No other significant differences between the 4 groups were found.

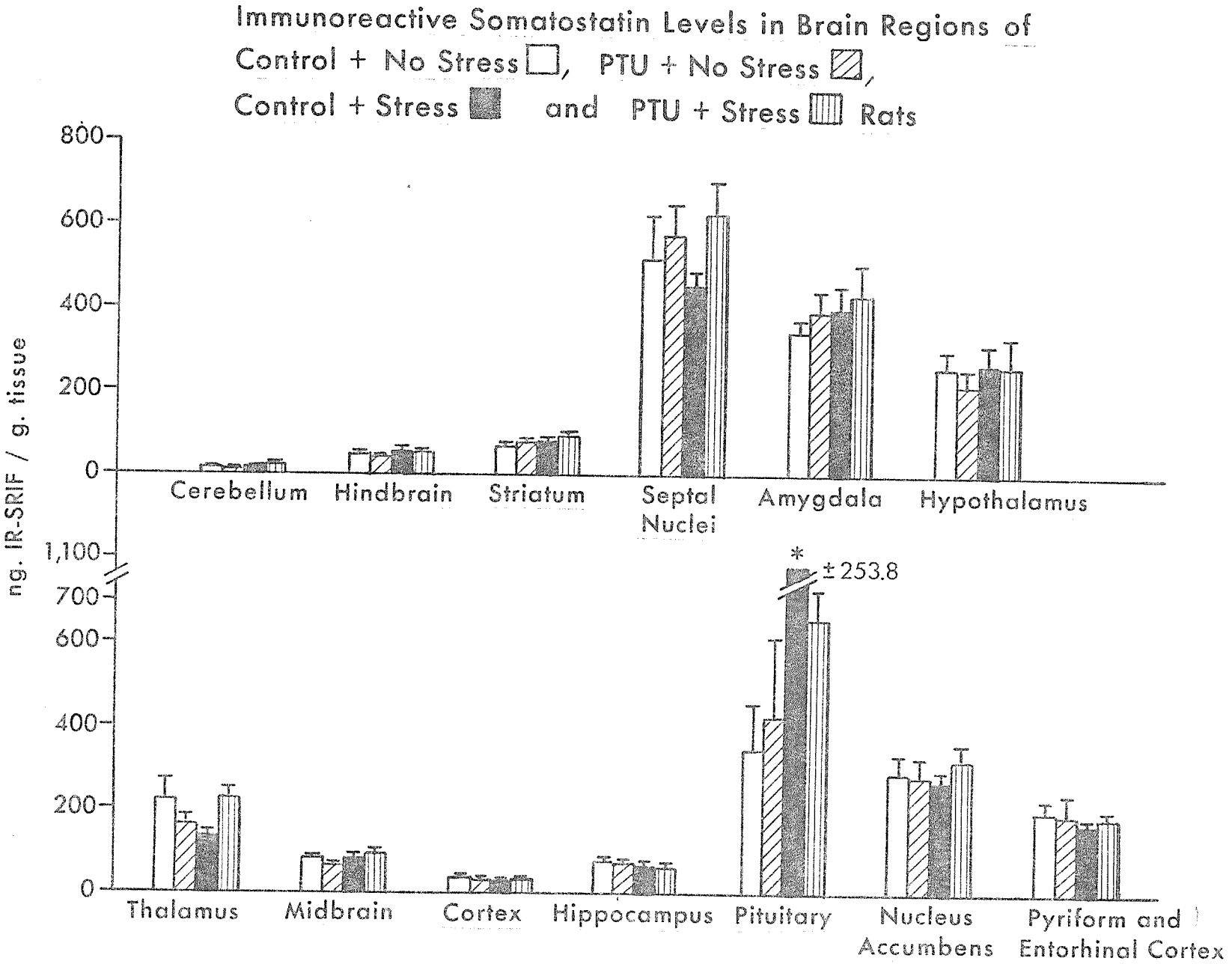
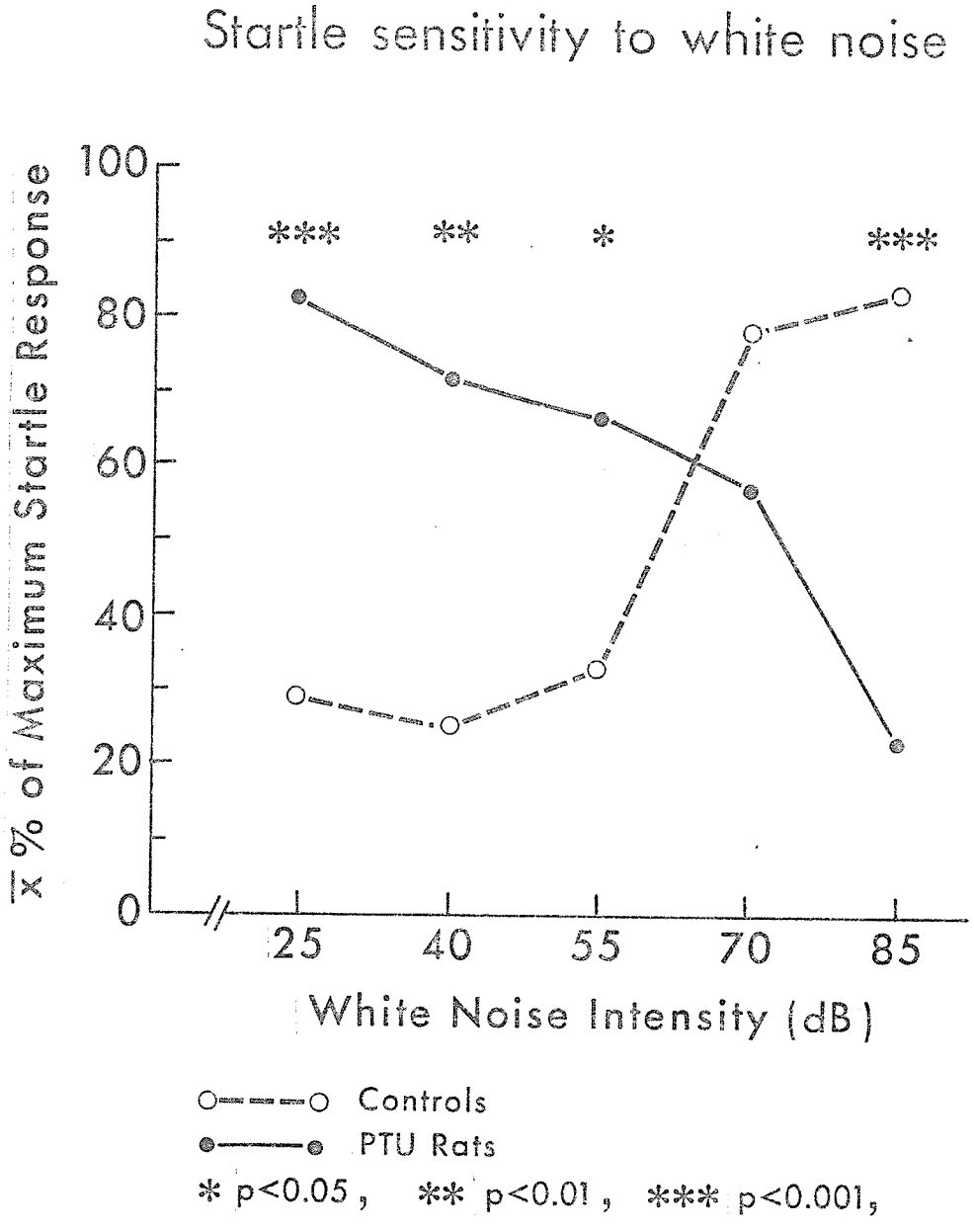

Immunoreactive Somatostatin Levels in Brain Regions of
Control + No Stress □, PTU + No Stress ▨,
Control + Stress ■ and PTU + Stress ▥ Rats
ng. IR-SRIF / g. tissue
800
600
400
200
0
1,100
700
600
400
200
0
*
± 253.8
Cerebellum
Hindbrain
Striatum
Septal Nuclei
Amygdala
Hypothalamus
Thalamus
Midbrain
Cortex
Hippocampus
Pituitary
Nucleus Accumbens
Pyriform and Entorhinal Cortex

Fig. 28. Acoustic startle response to a stimulus (110 dB click) with superimposed background white noise of varying intensity (25 to 85 dB) in PTU-treated vs. control rats. Motor response magnitude (expressed as a percentage of the maximal average motor response) was inversely proportional to the background white noise level in the PTU group and directly proportional in the control group.


Startle sensitivity to white noise
$\bar{x}$ % of Maximum Startle Response
100
80
60
40
20
0
***
**
*
***
25
40
55
70
85
White Noise Intensity (dB)
Controls
PTU Rats
* p<0.05, ** p<0.01, *** p<0.001,

DISCUSSION

I Radioimmunoassays for β-Endorphin and Somatostatin

The radioimmunoassay system for β-endorphin employed antiserum raised against synthetic camel β-endorphin. The assay can be considered homologous for rat β-endorphin, since the amino acid sequences of rat, ovine, and camel β-endorphin are identical (196). No cross-reactivity of the antiserum with biologically related or unrelated peptides was noted, except for ovine β-lipotropin which exhibited 50% cross-reactivity and human β-endorphin which cross-reacted 30% on a molar basis. Precursor forms of β-endorphin (ie. 31K molecule) as well as breakdown products may also cross-react in the assay. Ogawa et. al. (164) demonstrated that rat brain extracts of midbrain, hypothalamus, hindbrain and whole brain all produce elution profiles in which 98% of immunoreactivity co-elutes with synthetic camel β-endorphin when fractionated by gel filtration. Pituitary extracts, on the other hand, yielded a complex elution profile containing several immunoreactive species. These results indicate that immunoreactivity measurements in brain extracts closely represent β-endorphin, whereas determinations in pituitary extracts probably reflect overall content of 31 K precursor, β-LPH, β-endorphin, and opiod fragments combined. Additional evidence of the assay's specificity for β-endorphin is demonstrated by Figure 7 in which serial dilutions

of whole brain extracts produced curves parallel to the standard curve for camel β-endorphin.

The specificity of somatostatin antiserum is shown by the failure of all biologically related and unrelated peptides to displace the binding of I^{125} - tyr^{1}-somatostatin. Tyr^{1} - somatostatin displaces very similarly to synthetic somatostatin, a requirement since the added tyrosine residue is needed for iodination of this peptide molecule. Synthetic analogs of somatostatin with amino acid substitutions at various points on the cyclic structure also cross-react to varying extents. Both alanine and D-tryptamine substitions for tryptamine in position 8 lower the immunogenic potency of the peptide considerably, whereas alanine residues substituted for cysteine residues in positions 3 and 14 (an alteration which converts the cyclic form of somatostatin, created by a cys-cys disulfide bridge, to a non-cyclic form) retains greater immunogenicity of the molecule. However, the ring portion of somatostatin is necessary for its biological potency, at least in terms of effects on suppression of GH secretion (134). At present, it is impossible to rule out cross-reaction of the antiserum with some as yet unidentified substance present in the brain, as well as somatostatin precursors and breakdown products. As in the case of β-endorphin however, close parallelism of somatostatin standard curves with acidic brain extracts (as shown in Figure 8) permits the conclusion that the antiserum is specific.

II Brain Extraction Technique

Ogawa et. al. (164) demonstrated that microwave irradiation is the most effective means of sacrificing rats for extraction of β-endorphin since it appears to instantaneously stop proteolytic enzyme activity in the brain and thereby prevents degradation of β-endorphin (or other intact peptide molecules). Decapitation, on the other hand, allows considerable proteolytic degradation of peptides to occur during the time interval from killing to dissection and homogenization, thereby leading to avoidable inaccuracy in peptide measurements. 0.1 N acetic acid was found to be an optimal concentration of acid for extraction of peptides, in that higher concentrations did not improve β-endorphin yield and 0.1 N acetic acid did not interfere with the buffering capacity of the radioimmunoassay system (ie. synthetic standards prepared in 0.1 N acetic acid produced displacement curves identical to standards prepared in 0.14 M PB). Double extraction is important since it was found to increase the yield of β-endorphin uniformly by approximately 20%. Somatostatin yield is markedly increased by a second extraction, which sometimes exceeded that of the first extraction. This increased yield also varied considerably between samples and points out a technical problem in the acid extraction procedure for somatostatin. Further testing of the effect of several repetitions of each extraction on somatostatin

yield will be necessary.

III Brain β-Endorphin and Somatostatin Levels in Aged (Stressed) and Adult PTU and Control Rats

The first experiment carried out was designed to test whether a loud sound stimulus known to induce seizures in mature rats after exposure to 0.02% PTU from postnatal days 0-19, differentially influenced β-endorphin and somatostatin levels in the brain compared to non-seizuring control littermates. β-endorphin levels in most brain regions were lower in PTU rats, with a significant reduction in hypothalamic content observed. This effect occurred whether or not PTU rats actually experienced seizures during the 60 sec. stimulus presentation. Thus, it appears that altered brain content of β-endorphin (and somatostatin) is a permanent consequence of the neonatal antithyroid treatment and is not directly related to the occurrence of seizures (but perhaps to the inherent susceptibility to seizures). Unfortunately, some important aspects of this experiment have interfered with accurate interpretation of the data. Firstly, these rats were obtained in an aged condition, a factor which may itself be related to brain peptide levels, and which may have contributed to the loss of seizure sensitivity in some rats. Secondly, both the exposure to a loud sound stimulus as well as physical restraint (in particular) during the experimental procedure undoubtedly constituted severe acute stress which

was not controlled for. Gambert et. al. (170) recently showed that both β-endorphin and ACTH levels declined significantly with aging in the hypothalamus and striatum, so it is unlikely that the age of the animals contributed to elevated β-endorphin levels. No reports are known of changes in brain somatostatin levels with age. Acute forms of stress elicit a similar ACTH and adrenocortical secretory response in young and old rats (171), so it is doubtful that the age of the animals influenced their response to the stressors employed. Immobilization stress was found to produce a 10-fold elevation in plasma PRL levels, a much greater effect than found with other forms of stress (eg. cold exposure) (172). In addition, plasma GH levels were decreased significantly by this form of stress. These results suggest that both elevated hypothalamic β-endorphin and somatostatin could be involved in these physiological responses to immobilization stress.

The results of experiment 1 have shown that hypothalamic β-endorphin levels are indeed much higher than in experiment 2 in which no stress was used. The total brain content of β-endorphin was significantly higher in the control aged rats of the first experiment compared to the adult controls of experiment 2 as well. Further testing of the stress effect, using younger adult control rats, revealed that acute exposure to a loud sound stimulus generally does not affect

either brain β-endorphin or somatostatin content, indicating that the immobilization factor was the main stress component. The significance of the lowered β-endorphin content of the septal nuclei in the bell-exposed group (Table 8) is not known, but this trend was also noted following the stress procedure of experiment 1 in comparison to the no stress condition of experiment 2. It appears, whatever its significance, that this audiogenic form of stress selectively lowers β-endorphin levels in the septal nuclei. The general interpretation of experiment 1 is that PTU rats respond inadequately to severe acute stress in terms of elevated brain β-endorphin levels. West et. al. (173) showed that chronic hot plate stress significantly elevated β-endorphin levels in the hypothalamus, thalamus, and amygdala, whereas prior hypophysectomy prevented this physiological response. By an analogous mechanism, neonatal hypothyroidism in the PTU rats may have resulted in impaired mobilization of β-endorphin from the pituitary to the brain under stress, and/or reduced synthesizing capacity or numbers of β-endorphin containing neurons in the brain.

Overall β-endorphin levels were much lower in experiment 2, undoubtedly due to the absence of stress (subjects were habituated to handling during the maze-training procedure. Regional distribution patterns approximated those of previous studies (103,130,131) with highest levels in the hypothalamus, intermediate concentrations in limbic and diencephalic

structures, and low levels in cortex, striatum, and cerebellum. Experiment 2 does not provide evidence of a clear-cut relationship between brain β-endorphin levels and seizure-susceptibility in PTU rats. Given the excitatory influence (via disinhibition) of β-endorphin on hippocampal pyramidal neurons which leads to non-convulsive limbic seizures (137), one might expect elevated β-endorphin levels in the hippocampus of PTU rats. Since this was not the case, it implies that the seizure sensitivity of these rats is mainly a cortical phenomenon. Elevated thalamic β-endorphin content in the PTU rats may signify one of two things: If a direct inhibitory influence on neurons is the effect, then this may occur to produce compensatory inhibition of the thalamic portion of the reticular activating system to counteract increased cortical excitability. If indirect excitation in the thalamus is occurring however, this would contribute an added impairment of brain excitability modulation and thereby enhance seizure sensitivity. The results of experiment 2 do not appear to relate in any way to those observed during primary hypothyroidism in adulthood which demonstrated elevated β-endorphin levels in the hypothalamus (103, 104). No change in hypothalamic β-endorphin levels were found in this experiment, suggesting that long-term alterations of peptides following neonatal hypothyroidism are due to permanent CNS damage and not to short-term endocrine effects as seen in adult hypothyroidism.

Somatostatin levels do not appear to be related to the stressful nature of experiment 1 to the same extent as β-endorphin levels since the same trend in results was found in experiment 2 in which no stress was used. In both experiments, regional distribution patterns of somatostatin content in the brain closely approximate those of previous reports (109). A slight stress effect is evident however, since total brain content of somatostatin in aged-stressed rats (experiment 1) is greater than in the younger adult non-stressed subjects of experiment 2, a difference which is more likely due to stress than to age. The fact that plasma GH levels are decreased by immobilization stress (172) provides additional evidence of a relationship between brain somatostatin and stress in experiment 1.

In both experiments, somatostatin levels in most areas of the brain are permanently elevated as a result of neonatal thyroid deficiency, a consequence which probably contributes to the seizure sensitivity of these rats. Of particular interest is the observation that in both experiments cortical somatostatin levels were significantly higher, since hyperexcitability of cortical neurons is a pathological characteristic of epilepsy and somatostatin appears to have a direct excitatory influence on neuronal membranes (145). The results for hypothalamic somatostatin, which has a predominant role in neuroendocrine functions is less clear.

Experiment 1 did not indicate a change in hypothalamic somatostatin levels of PTU rats whereas experiment 2 did. Assuming #2 to be the more accurate and controlled study, elevated hypothalamic somatostatin levels could be responsible for the long-term reduction of both plasma GH (24) and TSH levels (23). In contrast however, Dussault and Walker (32) recently demonstrated reduced hypothalamic somatostatin levels together with decreased pituitary and plasma GH levels following perinatal PTU treatment (0.05%). Their interpretation was that low pituitary GH content secondarily decreases hypothalamic somatostatin content via a 'short-loop' feedback control mechanism (129), and that the resulting decrease in tonic inhibitory effect of hypothalamic somatostatin accounts for chronically elevated TSH levels, at least into early adulthood (127, 34). These differences in results and interpretations probably relate to short-term vs. long-term effects of neonatal hypothyroidism on hypothalamic somatostatin levels, since the animals in their study were tested up to 36 days of age whereas ours were examined at 7 months. In addition, their neonatal PTU treatment regimen produced a very different growth pattern of the pups (to be discussed later) which indicates differences in developmental consequences produced by different forms of treatment. Accurate periodic measurements of pituitary and serum GH and TSH levels following a specified regimen of neonatal PTU treatment from the time of weaning to early and later adulthood

would greatly assist in interpretation of these data. In particular, it is not known how long elevated TSH levels persist after neonatal PTU treatment, as past evidence has indicated a slightly diminished net synthesis and release of pituitary TSH in later adulthood (23).

IV Behavioral Studies in Adult PTU and Control Rats

Figures 15 and 24 showed that neonatal PTU treatment from day 0 to 19 at both 0.02% and 0.2% doses produced similar effects on open-field behavior. Both treatment levels resulted in hyperactivity as evidenced by significantly increased exploratory activity during 2 consecutive 10 min. sessions. The mild treatment group exhibited some habituation from the 1st to the 2nd 10 min. period, but no habituation over successive days. The severe treatment group exhibited no habituation over the course of the total 20 min. session. Grooming behavior during testing was virtually absent in this group as well, an activity common in control subjects. These results are in agreement with previous studies which have shown hyperactivity in an open-field environment as well as maladaptive hyperactivity (over-responding) in an operant DRL task (75,79). Some of these studies also employed relatively high doses of PTU treatment (ie. 0.3% dietary PTU). These persistently elevated activity counts in PTU groups of rats indicate inability to adapt to environmental conditions. I.C.V. administration of somatostatin in low doses

leads to a behavioral phase of hyperactivity (excessive exploration and grooming) (149), and thus it is possible that the elevated brain content of somatostatin in PTU rats may directly contribute to the hyperactivity seen in these animals.

Several studies have analyzed behavioral and learning deficits in children in which early diagnosis and treatment of neonatal hypothyroidism had significantly improved mental status (88-91, 174). It is possible that these clinical studies may represent analogs of the present studies of experimental "mild" neonatal hypothyroidism (using 0.2% PTU) in rats, since replacement therapy was initiated soon after birth in these children and they did not develop recognizable clinical signs of cretinism. Shortened attention span and hyperactivity were universal characteristics found in these children who had prenatal onset hypothyroidism (analogues to early postnatal hypothyroidism in the rat) (174). Other symptoms such as impaired spatial orientation, below average I.Q., and mathematical learning disability were also prevalent, and together constitute symptoms of the so-called 'minimal brain dysfunction syndrome'.

The results of our maze-learning study are in contrast to the early reports of Eayrs and Lishman (20) which showed longer running time due to slower movement, and more frequent occurrence of errors per trial. The present data on the other hand, showed that PTU rats initially acquire the

maze-running response faster, due to increased exploratory activity which led to quicker determination of the correct route to the goal box, simply by chance (ie. increased exploration increases the probability of finding the goal box). In turn, the preference of these rats to persist in established behavioral patterns allowed them to acquire performance to criterion quickly as well. Although initial frequency of errors was similar or slightly greater than in controls, by the end of training both running time and error frequency was greatly reduced in PTU rats (Figure 16). The differences in results between the two studies can be attributed mainly to the treatments used. Both began neonatal antithyroid treatment of the pups at birth, but theirs was much more severe as it involved either thyroidectomy or daily methylthiouracil injections of the pups. A more recent study which also employed relatively severe antithyroid treatment (0.2% dietary PTU beginning at birth) demonstrated minor maze-learning deficits (95). The rats appeared equally capable as controls in acquiring runway locomotion behavior, and running time and error frequency were only slightly increased. It is believed that the present data obtained following mild neonatal hypothyroidism is particularly important because the testing of animals took place long after the treatment period (6-7 months) at which time the subjects were in a euthyroid state. Previous studies employing severe

neonatal antithyroid treatment initiated testing of animals shortly after weaning or in early adulthood, at which time endocrine alterations of both the pituitary-thyroid axis as well as other hormonal systems are probably still quite prevalent. These studies did not report the thyroid status of the animals at the time of testing.

An additional consideration in the interpretation of these maze-learning studies is that the present investigation examined a retraining phase as well. It was found that upon reversal of the route, PTU rats were no longer able to perform the task adequately, exhibiting both significantly greater numbers of errors and longer running time (Figures 17 and 18). By the last day of retraining, running time had improved greatly, undoubtedly due to the general hyperactivity of PTU subjects, although error frequency was still significantly elevated. These results are interpreted as inability to adapt to changes in the environment and preference for the highly repetitive and routine response pattern initially acquired. Subjective observation of the PTU subjects during retraining also reinforced this view as they appeared upset by the presence of the new barrier which previously led to the runway, and often retraced their route from the start box to the site of the barrier (right turn) several times, eventually giving of without trying the left turn response. These subjects also often refused to run the maze, either by

remaining stationary in the start box, jumping out of the maze apparatus, or returning to their home cage (placed adjacent to the maze during testing). The striking similarity of these behavioral characteristics to the human situation is intriguing. It is well known for example, that mentally retarded individuals habitually repeat learned tasks over and over and are most easily taught in a routine environment in which daily activities are familiar and repetitious (175). Changes in environment provoke emotional outbursts, uncooperative and withdrawal behavior.

Two other forms of innate behavior in addition to open-field activity were examined in these series of studies. The first was the acoustic startle response. The startle reflex in the rat occurs in response to a brief, intense auditory stimulus and is exhibited as an abrupt contraction of the flexor muscles which yields a momentary crouching posture. If a brief auditory stimulus of relatively low magnitude (S_1) precedes the intense auditory stimulus (S_2) by an appropriate interstimulus interval (50 - 150 msec.,) then the startle response to S_2 is attenuated (176, 177). This phenomenon, known as prepulse inhibition, is highly resistant to pharmacological insults, brain lesioning, and direct interference with neurotransmitter metabolism. In contrast, a constant back-ground of white noise of moderate intensity markedly potentiates the startle response, presumable by its

activation of a central arousal mechanism (176, 177). In control rats it was found that startle responsiveness is directly proportional to the background white noise intensity up to 70 dB, beyond which further increases reduce the response magnitude (167). The present data have indicated a similar trend for control rats using the same testing procedure (Figure 28), although response magnitude was slightly higher at 85 dB than at 70 dB. This is not unexpected however, since testing of 5 dB increments of background white noise intensity between 65 to 90 dB revealed an average maximal response at 75 dB, with the decreased 85 dB response still slightly greater than the 70 dB response (177). Thus, the background range of 70 - 85 dB appears to be associated with optimal facilitation of the startle response. The PTU rats of the present study exhibited startle responsiveness which was inversely proportional to the background white noise intensity level. This shows that the characteristic potentiation of the acoustic startle reflex by background noise is absent in PTU rats, and implies a permanent impairment in the development and function of arousal mechanisms in the brain. Kellogg et. al. (167) reported that prenatal exposure to diazepam (a tranquilizer) prevents the potentiation effect of background noise when tested in the third postnatal week, resulting in a response magnitude that was similar at all noise levels from 25 - 85 dB. Their interpretation was that the drug interfered with neuronal development during the

prenatal period in such a way that the behavioral consequences of arousal are suppressed. Similarly, postnatal interference with neuronal differentiation by neonatal PTU treatment may have led to permanent suppression of arousal mechanisms in adulthood. This effect seems paradoxical in view of the hyperactivity and increased brain excitability seen in neonatally hypothyroid rats.

Another interpretation of the data relates to the auditory sensitivity of PTU rats. Van Middlesworth and Norris (87) reported that these rats have damage to the organ of Corti in the inner ear and a 40 - 60 dB loss of cochlear sensitivity at all sound frequencies tested. This partial hearing loss could account for the relative absence of motor responses at high background noise intensities (ie. 70 and 85 dB, Figure 28), an environment in which the 110 dB startle stimulus might not be detected. The development of the acoustic startle response in the rat takes place between postnatal days 12 to 21 (178), in association with concurrent maturation of auditory acuity (both peak sensitivity and range of frequencies responded to increase up to this age) (179). Eayrs and Lishman (20) had examined the development of the startle response in hypothyroid and control subjects up to weaning age and found that the first appearance of a motor response was delayed by approximately 1 week in the hypothyroid group. This observation implies delayed development of auditory receptor function during the early postnatal

period. Our results indicated that in severely hypothyroid young rats, this early receptor development must have been arrested and resulted in complete deafness, since no responsiveness to a 114 dB noise occurred at weaning age in these rats. Hence, there was also no susceptibility to audiogenic seizures noted in these rats.

The motor response latency to hot plate stress was found to be increased in PTU rats. Two interpretations of this result are possible. The rats may show delayed responding due to impaired ability to perform the response in the stressful environment, or due to reduced sensitivity to cutaneous stimulation (increased pain threshold). It is believed that the first possibility is more probable, since in an analogous situation neonatally hypothyroid rats took significantly longer to acquire a conditioned avoidance response to prevent occurrence of footshocks (96). In addition, control and hypothyroid rats were not found to differ in their sensitivity to cutaneous stimulation during postnatal development (20). At present, some degree of permanent peripheral damage to pain receptors cannot be ruled out however, since peripheral nervous damage of the hair cell receptors in the inner ear is observed in these rats.

V β-Endorphin and Somatostatin Levels in Brain Regions of Rats Following Different Degrees of Early Thyroid Deficiency, Adult PTU Treatment, and Hot Plate Stress

Van Middlesworth and Norris (87) reported that administration of 0.03% PTU in the drinking water for 3 weeks in adult rats which were exposed neonatally to 0.02% PTU treatment increased their susceptibility to audiogenic seizures after maturity. This observation suggests that inherent functional abnormalities in cortical processes of these rats may become 'masked' in adulthood (as evidenced by reduced seizure sensitivity), presumably by compensatory mechanisms, but that thyroid hormones in some way continue to be critical for adequate brain function through adulthood. Administration of 0.2% $KClO_4$ in drinking water together with a low iodine diet (L.I.D.) also produces offspring which exhibit audiogenic seizures, although at a lower percentage of incidence than following 0.02% PTU treatment (50% vs. 89%). Originally, it had been planned to duplicate this procedure in order to compare its effect on brain peptide levels of the adults to that using PTU treatment. Unfortunately, L.I.D. was no longer commercially available at the time of our study, and treatment with 0.2% $NaClO_4$ drinking solution alone proved to be inadequate. This assumption was supported by the fact that no physical differences were noted between these rats and controls during development, and none exhibited seizure

sensitivity at weaning. This was also true for a subsequent litter of pups raised on 1.0% $NaClO_4$, although at least some degree of hypothyroidism was evident at this higher dose as serum T_4 levels were non-detectable at 19 days of age (Table 11). Treatment of 0.2% $NaClO_4$ rats at 2 months of age with 0.03% PTU drinking solution for 3 weeks did not promote seizure sensitivity, and it was therefore decided to compare brain peptide levels in terms of adult PTU treatment only, in order to further distinguish between effects of neonatal vs. adult antithyroid treatment.

Adult PTU exposure was not found to affect β-endorphin levels in the brain, despite one report that treatment with 1.0% $NaClO_4$ together with L.I.D. in adult rats for 3 weeks elevated hypothalamic, and lowered striatal and pituitary β-endorphin levels (104). Elevated pituitary β -endorphin content in the $NaClO_4$ + H_2O (no adult exposure to PTU) group cannot be explained. In all 3 experimental groups of the study, $NaClO_4$ + H_2O, $NaClO_4$ + PTU, and Control + PTU, a significant decrease in somatostatin levels were observed in both the pituitary and nucleus accumbens. Slight alterations in somatostatin content were also observed in cerebellar, thalamic, and cortical tissue of particular treatment groups. None of these effects can be explained at present, and the results are viewed with caution since in this particular experiment, hypothalamic somatostatin levels, normally higher than in all other brain areas, were abnormally low and

pituitary concentrations were unusually high. Repetition of this study using either 1.0% $NaClO_4$ (preferably together with L.I.D.) or 0.02% PTU neonatal treatment would clarify these observations. Such a study would also be adequately designed to examine the combined effect of neonatal and adult antithyroid treatment (which enhances seizure susceptibility) on brain β-endorphin and somatostatin levels.

Measurements of β-endorphin levels in the brains of 0.02% PTU-treated rats showed no changes compared to controls. This lack of difference is surprising and indicates that despite the gross developmental abnormalities which occur in severely cretinous rats, β-endorphin systems in the CNS appear to remain fairly intact. It is speculated that perhaps opiate systems in the brain are phylogenetically older and their adequate functioning is essential for the survival of the animal. In contrast, a significant elevation of somatostatin content was found in several brain regions in 0.02% PTU-treated rats. These results are very similar to those obtained for each peptide in 0.02% PTU rats, and seem to indicate that the overall effect of neonatal antithyroid treatment on brain levels of these peptides is not dose-dependent. It also implies that even mild hypothyroidism in early life can induce permanent pathological changes in somatostatin-mediated functions of the brain comparable to those found in severe cretinism. One factor overlooked in this experiment was measurement of total protein content of

each brain region extract, which is known to be reduced in severely hypothyroid rats (11). Comparisons of peptide levels in terms of total protein concentrations may have revealed differences not seen when peptide concentrations are expressed in terms of wet tissue weight.

Acute hot plate stress did not reveal any stress effect in terms of altered brain β-endorphin levels in either the control or PTU group. At present, the best explanation for this relates to the temporal characteristics of the hot plate stress schedule used. Lewis et. al. (180) demonstrated that intermittent inescapable footshock over a 30-min. period produced analgesia which was antagonized by naloxone, whereas 3 minutes of continuous footshock produced analgesia of a shorter duration which was not antagonized by naloxone. These results provided evidence for the involvement of two possibly independent substrates in stress analgesia, one opiate-mediated and the other not. In our study, no analgesia was observed in either group over 10 successive exposure trials (ie. response latency decreased, indicating greater sensitivity to heat), suggesting that this stress schedule (30 sec. of exposure ever 3 min.) was inadequate to stimulate either β-endorphin-mediated or other response mechanisms. One reason for this may have been the relatively short exposure periods and the ability of subjects to lessen nociceptive sensations by jumping or licking their paws. On the other hand, footshock was unavoidable and of

uniform intensity at each presentation in the Lewis et. al. study.

No changes in somatostatin levels were observed following hot plate stress, except for elevated content in the pituitaries of the control stressed group. Problems in interpretation of the somatostatin data in both this and the $NaClO_4$ + PTU experiment have arisen. In both studies hypothalamic somatostatin levels were abnormally low, while pituitary levels tended to be unusually high. These trends are inconsistent with the first experiments carried out as well as reports in the literature, as shown in Table 10.

Table 10. Comparison of Hypothalamic and Pituitary Concentrations of SRIF Obtained from Different Experiments and from Published Reports.

Study	IR-SRIF content (ng/g tissue) Hypothalamus	Pituitary
A. Ours:		
(1) maze-trained, 7 mos.	483.1 ± 59.5	191.4 ± 45.4
(2) control habituated, 5 mos.	421.0 ± 264.4	107.7 ± 64.4
(3) 30-day-old	187.3 ± 16.2	672.8 ±176.1
(4) ClO_4 + PTU exp. (2.5 mos.)	144.6 ± 19.8	471.4 ±122.2
(5) Hot plate stress exp.	259.8 ± 44.1	346.0 ±111.5
B. Brownstein et. al. (109)	2,120 ± 80	-
C. Epelbaum et. al. (134)	1,663 ± 253	-
	IR-SRIF content (ng/mg protein)	
D. Kronheim, S. et. al. Clin. Endocrinol. 5:619 (1976)	-	10.02 ± 2.31
E. Patel, Y.C. et. al. Metabolism 27:1243 (1978)	-	4.9 ± 0.4 (posterior pituitary only)

Note: No published reports were found which expressed pituitary SRIF concentrations as ng/g tissue. All measurements listed are from control rats only (X ± S.E.M.).

It is possible that some undetected factors relating to either brain extract preparation or radioimmunoassay cross-reactivity may be involved. For example, the application of high energy microwave irradiation may tend to force the release of somatostatin from hypothalamic nerve endings into the pituitary, thus artificially redistributing the measurable immunoreactivity. Alternatively, biologically inactive forms of somatostatin-like immunoreactivity may be present in the pituitary which cross-react in our assay. Furthermore, the expected elevated somatostatin levels of PTU rats were not observed in the hot plate stress experiment. At present, it is felt that this may be due, in part, to differences in the rats used in this study (ie. PTU rats raised here as opposed to those provided by Dr. Van Middlesworth), particularly since the seizure sensitivity was lower in litters raised in our animal quarters. Problems of this nature are discussed subsequently in the section dealing with types of neonatal antithyroid treatments.

VI Problems and Considerations Related to the Topic of Investigation and Experimental Results

Age, sex, and body weight factors, as well as intra- and inter-assay variation undoubtedly have contributed to the variability in both β-endorphin and somatostatin measurements obtained in different experiments. No discernible sex-related differences in levels of either peptide

was noted, although rigorous testing of this in terms of possible cyclic variations in the estrous cycle of the rat has not been done. Lee et. al. (103) obtained lower mid-brain levels of β-endorphin in mature female rats compared to males, a difference not observed in these studies. Another factor contributing to variations in β-endorphin is the non-specific stress effects of handling prior to sacrifice. Each rat responds individually to human contact in any manner ranging from calmness to extreme emotional upset accompanied by squeaking and defense behavior. Because the pituitary contains a 1,000-fold greater concentration of β-endorphin than any brain region and appears to supply much of the brain content of this peptide (164, 173), even slight variations in the level of stress experienced could greatly affect brain concentrations in a short period of time. Factors such as these are probably responsible for the high standard deviation in β-endorphin concentrations among individuals of a group in all experiments carried out. These high standard deviations have often prevented demonstration of significance where a difference appears to exist, a problem which can only be resolved by further studies employing a larger number of subjects. Tables 11 and 12 summarize the variations in average concentrations of IR- β-Endorphin and IR-SRIF in brain regions of control rats obtained from different experiments.

Table 11. Comparison of IR- β-Endorphin Content in Brain Regions of Control Rats from Different Experiments

IR- β -Endorphin (ng/g tissue ± S.E.M.)

BRAIN REGION	Maze-trained (7 mos.)	Control Habituated (5 mos.)	30-day-old	ClO_4 + PTU Exp. (2.5 mos.)	Hot Plate Stress Exp. (2.5 mos.)
Cerebellum	37.3 ± 8.0	42.9 ± 25.7	17.2 ± 4.4	48.3 ± 7.9	120.9 ± 67.5
Hindbrain	107.0 ± 16.9	186.6 ± 48.3	168.1 ± 31.8	221.0 ± 55.2	344.9 ± 69.9
Striatum	23.6 ± 2.2	14.4 ± 2.7	11.2 ± 2.5	43.1 ± 8.5	50.5 ± 11.8
Septal Nuclei	257.9 ± 71.9	163.9 ± 14.3	133.1 ± 31.2	382.4 ± 108.6	257.0 ± 147.8
Amygdala	347.5 ± 106.8	171.7 ± 19.9	205.8 ± 36.5	1,320.0 ± 845.3	599.8 ± 157.0
Hypothalamus	1,386.4 ± 259.4	1,285.0 ± 441.9	1,745.7 ± 413.7	2,998.7 ± 810.1	6,853.4 ± 2,901.4
Thalamus	105.5 ± 23.5	114.0 ± 23.7	89.5 ± 12.0	325.8 ± 132.2	189.2 ± 88.9
Midbrain	140.9 ± 25.6	132.2 ± 18.3	176.8 ± 69.0	190.5 ± 53.8	378.1 ± 208.7
Cortex	30.7 ± 6.0	12.0 ± 2.2	7.7 ± 1.5	73.9 ± 48.0	70.7 ± 41.5
Hippocampus	52.3 ± 9.7	37.1 ± 11.7	78.9 ± 52.6	94.3 ± 36.3	267.9 ± 104.5
Pituitary (ug/g)	218.3 ± 22.5	572.9 ± 61.8	243.3 ± 70.2	212.0 ± 19.6	400.6 ± 146.0
N. Accumbens	646.9 ± 127.8	361.9 ± 46.6	-	472.9 ± 124.2	614.6 ± 213.1
Pyriform and Entorhinal Cortex	-	113.4 ± 33.2	69.6 ± 12.3	388.1 ± 116.5	256.0 ± 81.6

Table 12. Comparison of IR-SRIF Content in the Brain Regions of Control Rats from Different Experiments

BRAIN REGION	IR-SRIF (ng/g tissue ± S.E.M.)				
	Maze-trained (7 mos.)	Control Habituated (5 mos.)	30-day-old	ClO_4 + PTU Exp. (2.5 mos.)	Hot Plate Stress Exp. (2.5 mos.)
Cerebellum	15.4 ± 1.4	7.1 ± 5.5	13.5 ± 4.2	6.7 ± 1.3	15.3 ± 2.4
Hindbrain	60.7 ± 5.5	83.5 ± 58.4	62.1 ± 4.6	33.6 ± 6.6	52.2 ± 6.0
Striatum	91.7 ± 5.5	93.8 ± 58.0	53.6 ± 10.0	41.6 ± 7.9	69.0 ± 12.4
Septal Nuclei	450.2 ± 40.2	221.7 ± 107.0	257.6 ± 41.3	331.2 ± 34.6	519.8 ± 102.5
Amygdala	397.8 ± 71.0	206.7 ± 75.0	282.0 ± 49.5	208.6 ± 27.4	343.1 ± 32.1
Hypothalamus	483.1 ± 59.5	421.0 ± 264.4	187.3 ± 16.2	144.6 ± 19.8	259.8 ± 44.1
Thalamus	145.2 ± 14.9	64.9 ± 19.4	115.8 ± 5.9	94.6 ± 6.8	226.5 ± 51.6
Midbrain	131.1 ± 17.1	97.3 ± 48.0	88.2 ± 12.3	62.1 ± 11.8	81.9 ± 7.5
Cortex	70.0 ± 6.4	98.3 ± 44.1	36.1 ± 12.3	28.9 ± 3.2	38.1 ± 6.7
Hippocampus	130.9 ± 14.7	93.4 ± 42.1	71.7 ± 13.6	48.9 ± 3.7	76.9 ± 11.9
Pituitary	191.4 ± 45.4	107.7 ± 64.4	672.8 ± 176.1	471.4 ± 122.2	346.0 ± 111.5
N. Accumbens	490.8 ± 83.6	341.8 ± 89.8	298.2 ± 26.8	226.6 ± 26.0	288.7 ± 48.0
Pyriform and Entorhinal Cortex		290.7 ± 133.6	238.6 ± 14.6	141.5 ± 32.4	196.6 ± 30.0

Studies of the effects of neonatal hypothyroidism on developmental processes vary greatly with respect to two important parameters: method (and dosage) of treatment, and duration of treatment. There are 3 main techniques for experimentally inducing neonatal hypothyroidism - thyroidectomy, drug treatment and dietary iodine depletion, each having its own advantages and drawbacks. Thyroidectomy must be performed after birth as it is technically unfeasible in the fetus. The most common method, a single NaI^{131} injection, is difficult to administer in a controlled dose which can reproducibly cause complete inactivation of thyroid tissue. Regrowth of damaged tissue eventually occurs, and pups can receive thyroxine from the mother's milk during lactation as well (unless she is rendered hypothyroid also). The effects of iodine depletion or competing ClO_4^- ions are somewhat difficult to assess since enhanced intrathyroidal as well as peripheral conversion of T_4 to T_3 occurs to compensate for reduced T_4 synthesis. PTU and similar antithyroid drugs have found the most widespread use as their effects are more thorough in terms of greater goitrogenesis and inhibition of thyroid hormone synthesis and metabolism, both intrathyroidally and peripherally. The presence of these diverse forms of treatment make comparisons between individual studies difficult. It is essential that an investigator confirm that the animals were indeed hypothyroid at the time of experimentation and to what extent, inform-

ation which is often not provided. Even this is not entirely adequate however, since in our investigations serum T_4 levels were non-detectable at weaning in both 0.02% PTU and 0.2% PTU-treated rats (Table 7). The relatively minor consequences of the 0.02% PTU treatment on physical development is evidence in itself that hypothyroidism was less severe than in 0.2% - treated subjects, yet limitations in the assay's sensitivity prevented differentiation between the two.

Another problem that arises when comparing different methods of antithyroid treatment is that differences in endocrine state occur despite a comparable level of hypothyroidism. In particular, plasma insulin levels as well as glucose-induced insulin secretion are higher in PTU-treated subjects than in thyroidectomized or $KClO_4$-treated rats (181). PTU was found to stimulate pancreatic insulin secretion, an effect which was independent of either adrenal or pituitary influences. As a result, nutritional utilization may be comparatively superior in rat pups receiving PTU, thereby reducing the complications of undernutrition to developmental processes. In comparing different methods of PTU treatment, additional problems occur. Pups can receive treatment indirectly through either the food or the drinking water of the lactating mother, or directly via daily injections. A specific dosage of consumed PTU (eg. 0.2%) represents two different units of concentration in dietary

(g./dry wt.) vs. drinking water (g./vol. H_2O) routes of administration, and its presence in either form may affect the daily quantity consumed (PTU is very bitter so that taste aversion may affect consumption, and to varying extents with each subject). In turn, either reduced food or water intake could result in secondary malnutrition, which would interfere with the study of the effects of hypothyroidism. Problems of this nature may have contributed to the discrepancies in brain peptide data between rats obtained from Dr. Van Middlesworth's laboratory and those raised in our own laboratory.

The fact that different forms of neonatal antithyroid treatment do not produce comparable developmental effects is most evident when comparing body weight gain before and after weaning. Comparisons among several studies, divided into 2 groups, are summarized in Table 13. In the first group, no body weight differences were reported up to weaning at 21 days, after which growth was virtually arrested. Measurements continued up to a maximum of 43 days in these studies. In the second group, body weight differences were noted as early as 8 or 9 postnatal days of age and all reported significant differences at 18-20 days (some studies did not measure weights before this age). Body weights were persistently lower to maturity, but did gradually increase as observed in L.I.D. animals (34). The contrasts

Table 13. Comparison of Patterns in Body Weight Gain During Development in Rats Exposed to Different Forms of Neonatal Antithyroid Treatment.

Study	Antithyroid Treatment	Serum T_4 and T_3	Pre-weaning Body wt. gain	Post-weaning Body wt. gain
Group 1:				
1. Cramer and Ford, 1977 (27)	Radiothyroidectomy at birth	Not reported	No difference	Decreased at 26 days, continuing to 36 days
2. Dussault and Walker 1978 (34)	0.05% PTU drinking solution, early gestation 21 postnatal days	T_4 & T_3 non-detectable at all ages from 1 - 36 days	No difference ($PTU \bar{X}$ b.w. at 18 days = 18.3g, controls = 35.0 g)	Cessation of growth from weaning - 36 days
	L.I.D. + Deionized water, early gestation - 21 postnatal days	T_4 depressed at all ages from 1 - 57 days, T_3 highly elevated from 5 - 57 days	No difference	Lower, but gradually increasing from 24 days to adulthood.
3. Greer, et. al., 1975 (35)	0.15 dietary PTU in L.I.D. for 2 wks. premating in . Mated with chronically Iodine-deficient . L.I.D. from onset of gestation - postnatal day 43	T_4 depressed, measured only during 1st postnatal week. T_3 not reported	No difference	Virtual cessation of growth to 43 days

Table 13 (continued)

Study	Antithyroid Treatment	Serum T_4 and T_3	Pre-weaning Body wt. gain	Post-weaning Body wt. gain
Group II:				
4. Kikuyama et. al., 1974 (24)	0.04% PTU in diet from gestational day 18 - postnatal day 20	Not reported	Decreased, 24.0 g vs. 39.0 g at 20 days.	Decreased, 119.0 g. vs. 213.0 g. at 56 - 60 days
5. Sowers et. al., 1980 (33)	Radiothyroidectomy at birth	T_4 uniformly low to 30 days T_3 not reported	Decrease noted at 9 days	Persistent decrease at 30 and 75 days
6. Meisami et. al., 1970 (19)	L.I.D. from last wk. of gestation - post-natal day 6, radio-thyroidectomy on post-natal day 1.	Not reported	Decrease evident by 15 days	Persistently lower to 30 days (end of experiment)
7. Schalock et. al., 1979 (75)	0.3 % PTU in diet from day 0 - 30 post-natally + 0.001% PTU drinking solution	T_4 depressed to weaning at 30 days, normal at 60 days, then slightly de-pressed to the end of study at 120 days. T_3 not reported	Decrease evident by 8 days	Persistently lower at all ages from 30 - 126 days

Table 13 (continued)

	Study	Antithyroid Treatment	Serum T_4 and T_3	Pre-weaning Body wt. gain	Post-weaning Body wt. gain
8.	Our Study	0.02% PTU drinking solution, day 0 - 19 postnatally.	T_4 non-detectable at 18-19 days T_4 and T_3 normal in adults	Decreased at 18 days (33.5 g. vs. 41.6 g.)	Slightly lower at both 2.5 and 7 months
		0.2% PTU drinking solution, day 0 - 19 postnatally	T_4 non-detectable at 18-19 days	Decreased at 18 days (18.3 g. vs. 41.6 g.)	Much lower at 30 days (34.6 g. vs. 122.6 g.)

in these two trends seem to indicate that Group I forms of treatment were more severe, due to later cessation of growth and high mortality rate. Also, these rats were apparently not experiencing malnutrition during the pre-weaning period (although a large reduction in mean body weight at 18 days was reported as 'no difference' by Dussault and Walker (34)). The early decrements in body weights observed in Group II studies on the other hand may have resulted from some degree of concurrent malnutrition.

The question is frequently raised whether or not the developmental consequences of neonatal hypothyroidism are in fact mediated in part by undernutrition. Removal or addition of any hormone during ontogeny disturbs the remaining endocrine balance, making it difficult to demonstrate that developmental changes that follow are not mediated through effects on blood supply, nutritional state, or metabolic rate, but rather through direct intervention of the hormone with differentiation of the target cell. These questions have been studied and reviewed extensively, and although some overlap exists,the bulk of evidence has indicated that the pathology of cretinoid brains is sufficiently different from those subjected to early malnutrition to argue against the proposition that one is mediated through the other (182, 183). In general, undernutrition appears to affect predominantly the process of

cell replication - hypothyroidism that of differentiation and maturation. In addition, hypothyroid pups were shown to suckle well despite the cretinoid appearance of the body and sluggish movements, and maintained stomachs full of milk as frequently as controls.

Finally, some of the effects of thyroid hormones on growth and development are attributable to induction of GH synthesis at the pituitary level, which occurs via a direct stimulatory action on GH mRNA production (184). Recent evidence has shown that T_3 and GH act synergistically to develop enzyme systems in the brain (185). However, the role of GH is only partial, since GH administration to neonatally hypothyroid rats in the early postnatal period improved somatic growth and some indices of brain development, but did not abolish the alterations of cerebral and cerebellar differentiation and maturation (183).

SUMMARY AND FUTURE CONSIDERATIONS

The present series of experiments have provided insight into the relationship between two important brain peptides - β-endorphin and somatostatin, and the long-term effects of neonatal hypothyroidism. The main conclusions derived from these studies are:

(1) Both β-endorphin and somatostatin levels in the brain are permanently altered by neonatal thyroid deficiency, β-endorphin specifically in the thalamus and somatostatin widely affected throughout the brain.

(2) Alterations of both peptides appear to be a permanent consequence of the neonatal treatment which is related to the audiogenic seizure sensitivity of the rats, but not to the actual occurrence of seizures.

(3) PTU treatment in adulthood only (primary hypothyroidism is not associated with similar changes in brain β-endorphin and somatostatin as observed following neonatal treatment.

(4) Severe neonatal hypothyroidism (experimental cretinism) produces similar alterations in peptide levels as found in mild neonatal hypothyroidism, indicating that even mild thyroid deficiency during early development produces permanent consequences in CNS peptide-mediated processes in the absence of gross morphological cretinism.

(5) Mild neonatal hypothyroidism is associated with several behavioral changes including hyperactivity, impaired learning ability, impairment of central arousal mechanisms in the brain,

and diminished responsiveness to stress. This experimental model exhibits several similarities to clinical cases of diagnosed human cretinism in which T_4 replacement therapy was initiated soon after birth, suggesting its usefulness as an animal model for such studies.

We have investigated brain concentrations of β-endorphin and somatostatin in discrete regions of the brain under a variety of experimental conditions. However, this approach does not provide information concerning the underlying mechanisms responsible for changes that occur. For example, are the observed changes in total concentration of the peptide due to alterations in synthesis rate, degradation rate, release from nerve terminals, or all of these ? Approaches that would help to gain such information include in vitro studies of peptide release from incubated brain slices, administration of specific synthesis and degradation blockers of each peptide (not yet available), and i.c.v. injection of antisera to each peptide, which would deplete the supply of biologically active peptide. In addition, an investigation of opiate and somatostatin receptor levels in the brain following neonatal hypothyroidism could contribute a greater understanding of the biological significance of the observed changes in brain peptide levels.

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