THE ORIGIN OF LUTEINIZING HORMONE IN THE RAT BRAIN

by

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DEDICATION

To my husband, Sean, for all his emotional and financial support during my tenure as a graduate student; to my son, Brendan, for giving me so many reasons to smile and laugh; to my mother, for emotional support.

CONTENTS

LIST OF ABREVIATIONS

ACTH	adrenocorticotropic hormone
CNS	central nervous system
FSH	follicle stimulating hormone
GH	growth hormone
GMP-PNP	5'-guanyl-imidodiphosphate
hCG	human chorionic gonadotropin
hLH	human luteinizing hormone
ICC	immunocytochemistry
IR-	immunoreactive
LH	luteinizing hormone
LHRH	LH releasing hormone
ME	median eminence
POMC	pro-opiomelanocortin
Prl	prolactin
RIA	radioimmunoassay
TSH	thyroid stimulating hormone

ABSTRACT

That an LH-like substance is present in rat brain has been demonstrated using RIA, ICC, bioassay, and gel filtration chromatography. The concentration of IR-LH in hypothalamus has been shown to be markedly diminished in hypophysectomized rats, suggesting that the pituitary is the source of at least some of the LH-like substance in brain. However, intracerebroventricular administration of colchicine causes accumulation of LH in the mediobasal hypothalamus, as shown both with ICC and RIA, indicating that LH is probably also biosynthesized in neurons of the hypothalamus.

One other pituitary hormone, ACTH, has been shown to be taken up from blood in a specific fashion into the neuropil of the median eminence. Electron microscopy showed much of this ACTH (^{125}I-labelled) to be contained in nerve terminals and axons. It has been hypothesized that a similar uptake mechanism may be present for LH in the median eminence. We tested this possibility using three different experimental approaches.

In the first experiment, ^{125}I-labelled hCG (which binds to the LH receptor in gonads) was administered intravenously to male rats, the animals were sacrificed at various time points, and uptake of labelled hormone into dissected tissue areas was quantified in the gamma counter. Although ^{125}I-labelled hCG was detected in median eminence, no significant accumulation of label over time was observed in this tissue. In testis, accumulation of radiolabelled hormone over time was observed, as expected for a tissue known to contain LH/hCG receptors.

The second part of the first experiment was designed to show specificity of binding of hormone in testis and median eminence. Animals received labelled hCG either alone, or with a 500-fold excess of unlabelled hormone. Although a significant decline in uptake of labelled hormone occurred in testis when unlabelled hormone was given, the decline in median eminence was not significant. No decline was found in

neocortex, or muscle.

In an attempt to gain greater sensitivity in detecting hCG binding in tissues, a second group of experiments was completed using quantitative autoradiography. Again, animals received ^{125}I-labelled hCG, either alone or immediately preceded by unlabelled hormone (hCG or prolactin). Autoradiographic slides were prepared and silver grain densities were quantitated at the light microscope level. Although unlabelled hCG blocked the binding of labelled hormone in testis, no clear blockage of binding occurred in the median eminence. Microscopic analysis of the median eminence failed to show a concentration of silver grains over specific cytologic structures or anatomic sites. Unlabelled prolactin caused an increase in labelled hCG binding in testis; this result may be explained by studies showing changes in gonadal LH receptor number in response to alterations in serum prolactin concentration. No effect of excess prolactin treatment was seen on silver grain density in median eminence. These findings, consistent with the results seen in the previous experiment, fail to show specific binding of hCG in the median eminence.

In a final group of experiments, adenylate cyclase activity in median eminence in response to LH and hCG stimulation was examined. Ovarian luteal tissue from superovulated female rats served as a positive control for the assay. Basal values, as well as GTP and 5'-guanyl-imidodiphosphate (a GTP analogue) -stimulated values, showed adenylate cyclase activity to be present in median eminence, and to be measureable with this assay system. Addition of hCG or hLH to median eminence caused no stimulation of adenylate cyclase, although stimulation was observed in luteal tissue, as expected. These results clearly indicate that no adenylate cyclase-mediated LH/hCG receptor is present in median eminence tissue. These findings add additional evidence showing no LH/hCG receptor to be present in median eminence.

The results of all three series of experiments presented here do not support specific, receptor-mediated binding and uptake of LH/hCG in the median eminence.

Blood-borne LH or hCG does enter the neuropil of the median eminence, and therefore may account for LH measured in median eminence samples by RIA or even bioassay. However, the LH in median eminence does not appear to be actively taken up, and therefore cannot account for the LH seen in neuronal cell bodies and fibers in the brain and spinal cord. These findings, taken together with those showing increased concentration of LH in the mediobasal hypothalamus after colchicine treatment, suggest that the immunoreactive LH seen widely distributed in brain is of neuronal origin.

I. HISTORICAL REVIEW OF THE LITERATURE

A. Presence of Luteinizing Hormone in the Brain

The first report which suggested the possible presence of luteinizing hormone (LH) in the brain was made in 1964 by Craxatto et al. (11). Using the ovarian ascorbic acid depletion assay these authors found LH-like activity in extracts of human median eminence. In 1974 a second report indicating the possible presence of LH in brain was made by Petrusz who described the appearance of seemingly endogenous gonadotropins in rat brain using immunocytochemistry (ICC) (80). Experimental animals in this study were treated intravenously with human chorionic gonadotropin (hCG), while control animals received vehicle. ICC was performed on brain sections from these animals using an anti-hCG antiserum which cross-reacted with rat LH and follicle-stimulating hormone (FSH), and it was found that experimental and control sections both had extensive immunocytochemical staining, suggesting the presence of endogenous gonadotropin in these brains.

In 1981 two independent groups published more extensive studies of the LH-like substance in brain. Hostetter et al. (45) used radioimmunoassay (RIA) and ICC to demonstrate the presence of LH in the mediobasal hypothalamus, amygdala, septum, preoptic area, thalamus, hippocampus, and caudate of rat brain. The hypothalamus was found to have an immunoreactive (IR)-LH concentration of 4.7 ng/mg tissue using LH RP-1 standard, with lesser amounts in other brain areas. ICC showed LH-containing neuronal perikarya in the hypothalamic arcuate nucleus of rats treated with colchicine, while LH-containing fibers were seen widely distributed throughout the brain. Emanuele

et al. (21,22) also used RIA to show LH to be present in rat brain; this group found the hypothalamus to contain the highest level of immunoassayable LH, while other brain structures such as cerebral cortex, caudate, hippocampus, cerebellum, thalamus, and amygdala contained significant, although lesser, amounts. These investigators also showed that hypothalamic extracts possessed LH-like bioactivity using a rat Leydig cell bioassay. Both Emanuele et al. and Hostetter et al. used gel filtration chromatography to show that immunoassayable LH in brain extracts coelute with that from pituitary and with LH standard.

A group of Russian investigators has also reported the presence of immunoassayable LH in rat brain (57). These authors found that the concentration of LH in rat brain was highest in the median eminence, while the arcuate nucleus also contained a substantial amount. More recently, Emanuele et al. (23) found that extracts from extrahypothalamic brain sites show LH receptor binding activity in a testicular radioligand receptor assay, biological activity in a rat Leydig cell testosterone secretion bioassay, and a chromatographic profile similar to that of pituitary LH (23).

The LH-like substance in brain has also been studied with respect to certain physiologic parameters. The developmental pattern of brain LH has been found to be different from that of pituitary LH; in a study by Hojvat et al. (42) the pituitary LH concentration rose gradually from birth to puberty, while the hypothalamus was shown to have a surge in IR-LH concentration just prior to birth, after which the level declined by 89%, and remained at this level until 30 days of age, when it returned to the prenatal value. At one and two years of age, the concentration of the LH-like substance in brain remained steady, as did the level in serum, while pituitary LH concentration dropped by 50% (27). Immunoassayable levels of LH in the hypothalamus reportedly decrease following hypophysectomy (21), and at proestrus of the estrus cycle of female rats, when pituitary and serum levels of IR-LH are high (22).

The subcellular localization of LH was examined recently by Emanuele et al. (28). Using the technique of sucrose density separation of hypothalamic cell lysates, subcellular particle-bound LH constituted 42% of total hypothalamic LH, while the remaining 58% was contained in the cytoplasm. Of the particle-bound LH, 70% was found to be in the most synaptosomally rich fraction of the sucrose gradient. This first report suggesting a synaptosomal localization for much of the LH-like material in the hypothalamus awaits further verification.

Finally, the most recent study of brain LH by Hostetter et al. (46) utilized ICC to examine in detail the LH localization in the rat brain. Four different antisera were used independently to demonstrate LH immunoreactivity in brain sections, two of which had been generated against the entire LH molecule, and two directed against the beta subunit of LH. LH-immunopositive cell bodies were seen in the arcuate nucleus, periarcuate area, ventromedial nucleus, and retrochiasmatic area of the hypothalamus. The distribution of neuronal fibers containing IR-LH was widespread, extending into forebrain structures such as the septal area, medial preoptic area, nucleus of the diagonal band of Broca, bed nucleus of stria terminalis, and amygdala, as well as to brain stem and spinal cord structures, such as periacqueductal grey area of the midbrain, pontine reticular nuclei, nucleus of the solitary tract, dorsal motor nucleus of vagus, and substantia gelatinosa.

The studies described above provide ample evidence that a substance closely resembling or identical to pituitary LH exists in the rat brain. Although in depth chemical characterization of this LH-like substance has not yet been performed, it has been shown to have a chromatographic profile like that of pituitary LH on gel filtration chromatography, and to have immunologic and biologic activity like that of pituitary LH. Additionally, its widespread yet non-uniform distribution in the brain is suggestive of a functional role for the LH-like substance in the brain.

B. Physiological Significance of LH in the Brain

Many studies have explored the possible physiological effects of LH in brain. Most of these studies have focused on the possible role brain LH might have in governing the synthesis and secretion of LH by the pituitary. The mediobasal hypothalamus (MBH), particularly the median eminence, has been the primary site of interest for such a short feedback, or autofeedback, mechanism .

Amongst the earliest studies of LH autofeedback were those examining the effect of exogenous administration of LH on the concentration of LH in the pituitary gland and in the serum, as well as on ovarian weight and histology. Stereotaxic implantation of LH into the median eminence reduced the LH concentration in pituitary (10), plasma (38), or both (12), suggesting an inhibitory effect of LH in the median eminence on pituitary synthesis and secretion of LH. Control procedures for these studies included implantation of LH into the anterior pituitary, as well as administration of other hormones or vehicle alone into the median eminence, and generally resulted in no decreases in pituitary or plasma LH levels. Ojeda and Ramirez (71) showed that LH implantation into the median eminence caused a significant decline in ovarian weight, as well as in the percentage of animals having corpora lutea, both measures of LH stimulation of the ovaries.

The effect of LH on electrical activity in the brain was studied as early as 1959, when Kawakami and Sawyer (49) showed that intraperitoneally administered LH, or intravenously (IV) administered hCG, caused an electroencephalographic (EEG) response similar to that which follows coitus in the female rabbit. A number of additional electrophysiological recording studies have further demonstrated the ability of exogenous LH to alter neuronal activity in the hypothalamus. Single unit (50,81,84), and multiple unit (32,87) recording studies have shown changes in neuronal firing rate in response to both intravenously (32,81,84,87) and iontophoretically (50,84) administered LH. The

changes observed were mixed, however, with some studies showing primarily an increase in firing rate (32,50,87) while in another a decrease was observed (81). Sanghera et al. found that both microiontophoretic and intravenous administration of LH to ovariectomized rats lead to an increased firing rate in some neurons of the MBH, while causing a decreased rate of firing in others (84). Whatever the response of a given neuron, it was the same regardless of the route of administration of the LH. These electrophysiological studies clearly indicate that LH is capable of interacting with neurons in the central nervous system (CNS) in order to alter their rate of firing. In addition, it seems that LH in the blood is capable of reaching neurons in the hypothalamus, since electrical activity is altered even when LH is given intravenously. Information contradicting these results has been presented in two recent reports on the electrophysiological effects of LH in brain. Kesner et al. (51) observed no change in the pulsatile pattern of multiple unit activity in rhesus monkey hypothalamus following elevation of plasma LH levels by a long acting LHRH agonist. In addition, Pan et al. (78) found negligible activity of LH on firing of arcuate neurons using single unit recording from brain tissue *in vitro*.

Other approaches to demonstrating a physiological function for LH in the CNS include a study by Moguilevsky et al. (66) on the effect of LH on metabolic activity. Hypophysectomized and castrated male rats were given intraperitoneal injections of LH, and several hours later hypothalami were removed, divided into anterior, middle, and posterior portions, and assessed for oxygen uptake. The oxygen uptake was found to be depressed in the anterior hypothalamus (including preoptic area, suprachiasmatic nucleus, and paraventricular nucleus) in response to LH treatment. Kuhl and Taubert (58) examined the effect of IV administration of LH on the activity of the enzyme L-cystine arylamidase in hypothalami of female rats. This enzyme was chosen for study because it has been shown to inactivate LHRH. An increase in enzyme activity following LH

treatment was observed in brain tissue from adult rats and from immature rats treated with testosterone or estrogen. Immature animals treated with gonadal steroids but no LH did not show this increase. These studies provide a glimpse of possible biochemical modes of action of LH in the brain.

A recent study by Emanuele et al. (24) used a different approach to explore the possible physiological relevance of LH in the brain. They demonstrated the release of LH from hypothalamic tissue fragments in vitro, and found it to be stimulated by high potassium concentration, and prevented by absence of calcium ions in the incubation medium. Thus it appears that LH can be released from hypothalamic tissue by a mechanism (depolarization) similar to that causing release of neurotransmitters and other brain peptides (65) from neural tissue. The release of LH in response to high potassium concentration was limited to the ventral portion of the hypothalamus, containing the median eminence and pars tuberalis. This depolarization-induced LH release was not observed in the dorsal hypothalamus, amygdala, caudate, cerebral cortex, thalamus, hippocampus, cerebellum, or brainstem. Whether or not the depolarization-induced LH release observed in this study represents only LH released from the pars tuberalis is not entirely clear. As described previously, an immunocytochemical study has shown LH fibers apparently terminating in many areas throughout the brain, including the median eminence. It is unlikely that the LH-containing fibers of the median eminence, but not of other brain sites, would release LH in response to a depolarizing stimulus. Nor are the number of fibers in the median eminence greater than those seen in other brain areas, thus the quantity of LH released cannot account for failure to observe release of IR-LH in other brain areas. The most plausible explanation of the results of this study therefore seems to be that the LH released from ventral hypothalamic explants in response to high potassium concentration is actually from cells of the pars tuberalis of the pituitary, rather than from nerve terminals in the brain.

Results of these studies addressing the effects of LH on brain function provide evidence that LH is able to bring about physiological responses in the hypothalamus, which in some cases appear to be linked to LH release from the pituitary gland. It is unknown, however, whether or not such responses would occur under the influence of the endogenous levels of LH present in the brain. In addition, no information is provided by these studies about the possible physiological significance of the LH distributed to brain sites outside the hypothalamus as demonstrated by ICC. Thus, although these studies provide important information regarding the responsiveness of hypothalamic tissue to LH, much remains to be learned about the physiological actions of LH in the brain.

C. Other Pituitary Hormones in Brain

LH is only one example of a pituitary hormone found in brain tissue. Adrenocorticotropic hormone (ACTH) (as well as other molecules derived from the POMC precursor molecule), thyrotropin (TSH), growth hormone (GH), and prolactin (Prl) have been demonstrated in brain using methods similar to those used for LH: RIA, ICC, bioassay, and gel filtration chromatography.

1. Adrenocorticotropic Hormone

ACTH is the anterior pituitary hormone which has been studied most extensively with respect to its presence and distribution in brain. In 1977, Krieger et al. (53,54) reported on the presence of ACTH in the median eminence, mediobasal hypothalamus, and limbic areas of the rat brain using RIA and bioassay. The concentration of ACTH in the hypothalamus or median eminence was found to be about 1/100th that found in the pituitary, while other brain areas such hippocampus, amygdala, preoptic area, and septum

had lesser amounts. Moldow and Yalow (67) also used RIA to measure ACTH levels in brains of rats, rabbits, dogs, monkeys, and humans, and found that the anatomical area of distribution decreased with increasing size of the brain of the species being examined. A detailed RIA and bioassay study of ACTH and melanocyte stimulating hormone in brains of rats, cats, and monkeys was reported by Orwoll et al. in 1979 (73). In this study it was found that the ACTH concentration was highest in the hypothalamus for all species studied, with lesser amounts being detected in more peripheral brain regions. Endocrine manipulations such as hypophysectomy, dexamethasone treatment, and adrenalectomy had little effect on content of ACTH in the various brain regions examined. The use of antibodies directed against different portions of the ACTH molecule demonstrated that much of the ACTH-like immunoreactivity in brain results from the presence of the N-terminal portion of the molecule, and may belong to high molecular weight precursor molecules with little biological activity.

The anatomical distribution of IR-ACTH in the brain has been examined by a number of investigators using ICC (6,39,52,59,60,70,79,82,96). The distribution of ACTH containing neuronal cell bodies and fibers bears considerable similarity to that of LH. Cell bodies immunoreactive for ACTH are reported by all but a few authors (52,96) to be confined primarily to the arcuate and ventromedial nuclei, and periarcuate area, of the mediobasal hypothalamus. Neuronal fibers immunopositive for ACTH have been localized in the hypothalamus, septal area, amygdala, periacqueductal grey, mesencephalic reticular formation, locus coeruleus, and nucleus of the solitary tract.

Gel chromatography profiles of IR-ACTH in rat brain have been found to be similar, but not identical to those for pituitary ACTH (53,67,73).

Not only has the ACTH molecule itself been detected in brain tissue, the mRNA encoding for the POMC precursor molecule of ACTH has been demonstrated using Northern blot analysis (8) in the hypothalamus, amygdala, and cortex of rats, as well as by

the *in situ* hybridization technique in neurons of the mediobasal hypothalamus (33). Cells labelled by in situ hybridization for POMC in the hypothalamus are the same cells that stain for ACTH with ICC. However, it has been shown that the posttranslational processing of the POMC precursor molecule in hypothalamus is like that of the intermediate lobe rather than the anterior lobe of the pituitary; in the hypothalamus, desacetyl-α-MSH and endorphins are the predominant POMC products, rather than ACTH (30,34).

The anatomical distribution of ACTH in the brain led to the suggestion that ACTH may play a role in autonomic functions such as respiratory and cardiovascular regulation, as well as pain modulation (82). Ultrastructural localization of ACTH in dense-core vesicles (79) is consistent with a neurotransmitter-like mode of action of ACTH in brain, as is a study by Kapcala et al. (48) showing potassium-stimulated, calcium dependent release of ACTH and beta-endorphin from hypothalamic cells *in vitro*. Suggestive of a membrane receptor mediated action of ACTH in brain is its stimulatory effect on cAMP concentration in brain slices *in vitro* (17). Results of behavioral studies have led to the proposal that ACTH may function in memory or motivation (17).

2. Prolactin

A number of immunocytochemical studies have demonstrated the presence of a prolactin(Prl)-like substance in rodent brain (31,37,88,89). In the rat brain cell bodies immunopositive for Prl have been localized in a number of hypothalamic nuclei, including the arcuate, ventromedial, paraventricular, and supraoptic nuclei. Fibers containing IR-Prl are widespread in the hypothalamus, including the median eminence and pituitary stalk, as well as in periventricular thalamus, and in the periventricular grey area of the midbrain.

Schacter et al. (85) reported mRNA encoding for Prl in the hypothalamus as

demonstrated by the dot blot method. However, in this study only 3 of 7 samples analyzed yielded positive results, and no further studies have been reported to confirm these results.

Many studies have attempted to elucidate a possible functional role for Prl in the brain. Harlan (37) has described a facilitatory role for prolactin in the midbrain on lordosis behavior in the rat. Specific Prl binding sites have been reported in rabbit hypothalamus, thalamus, and substantia nigra which show altered binding in response to different physiological states (18,19). Other investigators have demonstrated an electrophysiological effect of Prl on neuronal activity in rabbit (9) and rat (97) brain *in vivo*, as well as on dopamine turnover in rat brain (1). Yet a more recent study found only a slight effect of Prl on firing of arcuate neurons using single unit recording from brain tissue *in vitro* (78).

3. Thyrotropin

Immunoreactive and bioactive TSH in brain tissue of rats has been reported by several authors (2,14,41,47,68). While earlier reports described the TSH-like activity in brain to be limited to the median eminence (47), or median eminence plus pituitary stalk (2), later studies showed IR-TSH to be more widespread in rat brain (present in hypothalamus, thalamus, cerebellum, limbic structures, and cerebral cortex) (14,41,68). This difference in distribution may be due to the use of more sensitive assays in the later studies. No detailed immunocytochemical studies have been published of the anatomical localization of TSH in brain. TSH has been detected also in human hypothalamus (68), as well as in monkey hypothalamus, cortex, midbrain, and hippocampus (41).

The concentration of IR-TSH in both pituitary and brain have been found to vary diurnally, but with peak levels occurring at different times in the light-dark cycle (74). The diurnal rhythm of pituitary TSH responded to inversion of the light-dark cycle,

while the brain rhythm did not. These data suggest a possible separation between brain and pituitary sources of TSH.

A variety of endocrine manipulations have been used to explore the possible physiological role of TSH in the CNS. Thyroidectomy increased the IR-TSH content in the median eminence 4 days following surgery (47), and in mediobasal hypothalamus 3 weeks postoperatively (74). However, when the entire hypothalamus was assayed, no significant change in TSH content was seen 14 days following thyroidectomy (14). Plasma levels of IR-TSH also increased following thyroidectomy, while pituitary concentration remained unchanged or decreased. The results of these studies suggest that the pituitary, via the blood, is the source of IR-TSH in brain, since median eminence and mediobasal hypothalamic levels of TSH varied in parallel with plasma levels. The failure of the hypothalamus as a whole to show any variation in IR-TSH content may be due to dilution of the TSH by a large tissue sample. Other studies, however, indicate independent brain and pituitary or plasma responses to the endocrine milieu. Treatment of rats with thyroxine for 1 to 7 days resulted in an expected decline in pituitary IR-TSH content, while median eminence/hypothalamic levels increased; plasma TSH levels either declined or remained unchanged in response to thyroxine treatment (2,14,47). Both orchidectomy and adrenalectomy caused hypothalamic levels of TSH to increase, while pituitary levels declined; serum TSH values declined following orchidectomy, and remained unchanged following adrenalectomy (44). The results of these studies indicate that hypothalamic content is not simply a reflection of pituitary or plasma content of TSH. Thus it is unlikely that the TSH detected in the hypothalamus is derived primarily from either of these sources, or that it is contained solely in cells of the pars tuberalis, which might be expected to mimic pituitary thyrotropes in function. Although it has been proposed that brain TSH serves as a feedback regulatory mechanism for the pituitary, no such role clearly emerges from the results of these studies.

IR-TSH is released from hypothalamic tissue fragments *in vitro* by depolarizing stimuli, such as high potassium concentration (60mM) and veratridine, by a calcium dependent mechanism (13,14,25). This finding indicates that IR-TSH in brain is stored in a releasable pool, which may be released in a manner similar to neurotransmitters and neuropeptides. Thyroidectomy and hypophysectomy of experimental animals caused no alteration in amounts of IR-TSH releasable from hypothalamic fragments (14), suggesting a source other than the pituitary for this releasable TSH.

Attempts at subcellular localization of TSH in brain tissue have resulted in one report of synaptosomal localization following differential and sucrose gradient centrifugation (43), and another report indicating localization in membrane bound particles similar to secretory granules (15). Both results are concordant with those from studies demonstrating release of TSH from brain tissue, and are suggestive of a neurotransmitter- or neuromodulator-like mode of action for TSH in brain.

4. Growth Hormone

Only limited attention has been given to GH in the brain. Pacold et al. (75) described IR-GH in the amygdala of rat brain as demonstrated by RIA and ICC, as well as by release of IR-GH from amygdaloid cells in culture. Hojvat et al. (40), from the same laboratory, later reported GH in rat and monkey brain measured by RIA. While IR-GH levels in rat brain were reported to be highest in the amygdala, with lesser amounts in hypothalamus, caudate nucleus, thalamus, hippocampus, cortex, and cerebellum, in the monkey brain the highest concentration of IR-GH was found in the hypothalamus, with decreasing quantities in the amygdala, pallidum, thalamus, and cortex. Results of these studies suggest that the distribution of GH in brain may be quite different from that of other pituitary-like proteins in the brain. Both Pacold et al. (75), and Hojvat et al. (40)

reported release of IR-GH from neural cells in culture. This release occurred in cultures originating from both hypophysectomized and intact animals, and continued for as long as 25 days, leading these investigators to conclude that the IR-GH in brain is of CNS, rather than pituitary, origin.

D. Origin of Pituitary-like Proteins in the Brain

Given the numerous reports of proteins in CNS tissue which immunologically, biologically, and chromatographically resemble pituitary proteins, the question arises: what is the source of these proteins? Are they actually pituitary hormones incorporated into the brain, either by a passive mechanism, or by a specific receptor mediated mechanism? Alternatively, might these pituitary-like proteins be synthesized in brain cells? A number of studies have contributed evidence concerning both possibilities for the origin of pituitary-like hormones in brain.

1. Pituitary Origin

a. Effect of Hypophysectomy on Brain Content of Pituitary-Like Hormones:

One way to address the question concerning the role of the pituitary gland as a source for pituitary-like proteins in the brain is to observe the effect of hypophysectomy on the concentration of these proteins in brain tissue. Evaluation of these studies is complicated by the presence of gonadotropins and TSH in cells of the pars tuberalis (7,35,36), some of which are undoubtedly left behind following hypophysectomy. Hormone contained within these cells, or secreted by them, might be partially or entirely responsible for detection of these hormones in brain tissue.

A number of studies have examined the effects of hypophysectomy on the brain concentrations of the anterior pituitary hormones. In a study by Bakke and Lawrence (2) no change in the TSH bioactivity measured in median eminence-pituitary stalk was observed 2 days, 9 days, 4 weeks, or 6 weeks following hypophysectomy. This result might be explained by the presence of TSH in cells of the pars tuberalis in tissue samples being assayed from both intact and hypophysectomized animals. In contrast, in 1982 Hojvat et al. (41) reported the IR-TSH content of hypothalamus to decrease by 75% following hypophysectomy, yet other brain areas in which TSH was measured showed no response to hypophysectomy. DeVito et al. (14) found that TSH content was decreased only in the dorsal hypothalamus, but not in the hypothalamus as a whole 30 days following hypophysectomy.

Krieger et al. reported that the concentration of IR-ACTH in median eminence (53) or hypothalamus (54) decreases 10 days post-hypophysectomy, while other brain areas show no change. On the other hand, Orwoll et al. (73) observed no decrease in brain IR-ACTH concentration 30 days following hypophysectomy. In an immunocytochemical study of ACTH localization in brain, Pelletier and Leclerc (79) reported the staining of cell bodies and fibers to be unchanged 2 and 8 weeks after hypophysectomy. Similarly, ICC studies of prolactin in rat brain failed to show a decrease in staining following hypophysectomy (31,88,89). In a study of IR-GH in amygdala and hypothalamus of rat brain, Hojvat et al. (40) reported an initial decline in GH concentration resulting from hypophysectomy, followed by a rebound to levels above control values 3 weeks following surgery.

The effect of hypophysectomy on levels of LH in the rat brain was examined in two studies utilizing RIA. Emanuele et al. (21) reported that the concentration of IR-LH in the hypothalamus declined significantly following hypophysectomy, although it remained detectable, and remained unchanged in other brain areas. Krivosheev et al. (57)

found that 2 months post-hypophysectomy the immunoassayable concentration of LH was decreased in the median eminence, while it was increased in the rest of the hypothalamus, as well as in thalamus, medulla, corpus callosum, and hippocampus, and remained unchanged in some brain areas.

The most consistent conclusion that can be drawn from these studies seems to be that some portion of the TSH, ACTH, GH, and LH present in the brain is most likely of pituitary origin, and that this portion resides in the hypothalamus. The pituitary hormone content of brain areas outside the hypothalamus is, in most cases, unaffected by removal of the pituitary gland, suggesting that hormone in these areas is not of pituitary origin. The brain content of Prl appears to be impervious to the loss of the pituitary as a source of Prl, although this has only been evaluated using ICC, which may not be an adequately quantitative method to detect small differences. On the whole, these studies imply that both pituitary and neuronal biosynthesis may account for the presence of pituitary-like proteins in brain.

b. Possible Routes of Transport of Hormones from Pituitary to Brain:

Pituitary hormones secreted into the blood bathing the pituitary gland might gain access to the brain after circulating through the body, or alternatively, by flowing retrogradely through the hypothalamic-pituitary portal vascular plexus. The former route of transport would result in a much lower concentration of hormone potentially coming into contact with neural tissue, and hence a slower rate of uptake. The latter possibility would result in exposure of neural tissue to much higher concentratons of pituitary hormones, and, if serving as a short-loop feedback system, would provide much greater sensitivity. Whether or not retrograde blood flow actually occurs has been explored by a number of approaches.

The experiments of Bergland and Page (3,4,76) in which they created vascular

casts of the median eminence-pituitary complex have provided some evidence that flow of blood from the adenohypophysis toward the median eminence occurs. These authors observed a continuous capillary plexus throughout the length of the neurohypophysis (median eminence, pituitary stalk, and posterior lobe) with connecting vessels to the vasculature of the anterior lobe. Examination of vascular casts from several mammalian species at the light and electron microscopic level revealed that the volume of venous drainage serving the adenohypophysis was inadequate for blood coming into this part of the gland. These results led Bergland and Page to postulate that blood from the anterior lobe might flow into the capillary plexus of the posterior lobe, and then traverse this capillary plexus in the direction of the brain, with selective vasoconstriction of various parts of the continuous neurohypophyseal capillary plexus governing the direction of blood flow toward or away from the brain. However, in a more recent study by Page (77) in which the pattern of blood flow in the median eminence-pituitary region was observed directly in female pigs using an operating microscope and dye to mark the blood, no flow from the anterior pituitary towards the median eminence was seen to occur.

The studies of Mezey et al. (63,64) have shown that substances introduced into the pituitary gland or intrasellar space may be detected in the brain in as little as 5 minutes following administration. Tritiated ACTH injected into the pituitary is found unevenly distributed in the brain, with the highest concentration being in the hypothalamus. Thirty minutes following injection, 1% of the administered dose was localized in the brain.

Further evidence in support of the notion that proteins secreted by the pituitary may traverse the portal vasculature to the median eminence is the study of Oliver et al. (72) in which several pituitary hormones were measured in hypothalamo-pituitary portal blood samples. Blood samples obtained from portal cannulae inserted in intact rats contained concentrations of LH, TSH, Prl, ACTH, α-MSH and vasopressin that were about two orders of magnitude greater than the corresponding arterial plasma levels.

Removal of the anterior pituitary or the entire gland 30 to 60 minutes prior to collection of portal blood resulted in a marked decline in levels of LH, TSH, Prl, ACTH and α-MSH.

In a study by Bergland et al. (5) it was hypothesized that if blood flowed from the pituitary directly to the brain, then the concentration of pituitary hormones in blood of the sagittal sinus (which contains venous drainage from the brain) should be higher than that of arterial blood. To test this idea blood samples were obtained simultaneously from the carotid artery, jugular vein, and sagittal sinus of intact and one hypophysectomized sheep. Indeed, IR-ACTH concentration was highest in samples from the sagittal sinus obtained from normal animals, and although somewhat reduced in hypophysectomized animals, it was still higher in sagittal sinus than in other sites. The results of this study indicate that ACTH did indeed travel from the pituitary to the brain, although the result from the hypophysectomized animal implies that some of the ACTH measured in sagittal sinus samples was actually released by neuronal cells.

One study of retrograde transport from pituitary to brain examined the biological activity of the transported substance. In this study by Dorsa et al. (20), neurotensin was injected into the pituitaries, cerebral ventricals, or blood stream of ether-anesthetized rats. Control animals received injections of saline. Because neurotensin lowers colonic temperature when given intracerebroventricularly, the colonic temperature of the animals was measured following the injections. Intracerebroventricular and pituitary injection of neurotensin resulted in a decline in colonic temperature, while intravenous injection, and saline, had no effect. These results indicate that the neurotensin introduced into the pituitary was able to undergo transport to the hypothalamus and remain biologically active.

Results of these studies demonstrate that some flow of blood from the pituitary toward the median eminence does occur, and that a small proportion of substances

introduced into the pituitary is carried to the brain. Taken together these findings suggest that retrograde blood flow may serve to transport substances from the pituitary to the median eminence, where there is potential for neuronal uptake and transport throughout the brain.

c. Specific Brain Uptake of Blood-borne Proteins:

Whether pituitary hormones reach the brain by retrograde blood flow through the portal vasculature, as many of the studies sited here suggest, or following dilution throughout the entire circulation, the question arises: would these proteins actually enter the neuropil at the median eminence (or other circumventricular organs) and be taken up into neurons? This question has been investigated for a few blood-borne proteins by van Houten and colleagues (90,91,92,93). These studies used *in vivo* autoradiography at the light and electron-microscopic level to determine the localization in brain of ^{125}I-labelled proteins introduced into the blood stream. This approach has been used to assess uptake of insulin, angiotensin-II, and ACTH. Five minutes following intravenous administration of radiolabelled insulin the label was found in neural parenchyma of all the circumventricular organs, i.e. the organum vasculosum of the lamina terminalis, the subfornical organ, the median eminence and adjacent MBH, and the area postrema and adjacent medial paravagal region (90). Specificity of these binding sites was shown by the demonstration of a significant decline in the number of autoradiographic silver grains seen in these areas when a 500-fold excess of unlabelled insulin was coinjected with the labelled insulin. A subsequent study at the electron-microscopic level of uptake of labelled insulin in the area postrema and paravagal region showed the insulin to be localized largely in the dendrites and cell bodies of neurons, particularly within various types of vacuoles (93). Angiotensin II was also taken up specifically in all the circumventricular organs and in the choroid plexus, although it was most highly

concentrated in the subfornical organ (91).

ACTH is the only anterior pituitary hormone for which specific uptake into the neuropil from blood has been demonstrated (92,95). Autoradiography showed that five minutes following injection of ^{125}I-labelled ACTH into the left cardiac ventricle either alone or together with a 1000-fold excess of unlabelled hormone, specific binding occurred in the median eminence (particularly the external zone). Although some binding was observed in all of the circumventricular organs, the median eminence was the only brain site for which specificity of binding could be demonstrated.

In a more recent study the subcellular localization of labelled ACTH bound in the median eminence was investigated using autoradiography at the electron microscope level (95). In sections from animals treated with ^{125}I-labelled ACTH, 75% of bound radioactivity was localized to nerve terminals and pre-terminal axons. In animals treated with a 1000-fold excess of unlabelled ACTH as well as labelled ACTH, 74% of the label was localized in glial cells, with only 25% in neurons, indicating that specific binding sites are localized to neurons. Inhibition of binding of labelled hormone by various unlabelled fragments of the ACTH molecule showed that the binding site in brain appears to recognize the 4-10 amino acid sequence, in distinction to the adrenal receptor, which recognizes the C-terminal portion of the molecule. It was also observed that unilateral deafferentation of the median eminence resulted in a reduction of labelling on the deafferented side, indicating a loss of binding sites due to loss of axon terminals. In an effort to determine whether or not labelled ACTH taken up into nerve terminals at the median eminence might be transported to other parts of the brain, animals were allowed to survive for 8 hours following injection of radiolabelled ACTH directly into the median eminence. Another group of animals received median eminence injection of ^{125}I-labelled wheat germ agglutinin, a substance known to be taken up by nerve terminals and transported retrogradely to the nerve cell body. Although the labelled wheat germ

agglutinin was concentrated in neurons of the supraoptic, paraventricular, arcuate, and ventromedial nuclei of the hypothalamus, there was no localization of ^{125}I-labelled ACTH seen in mediobasal hypothalamic neurons. These data imply either that there is no transport of ACTH away from the median eminence through neurons, or if such transport does occur, it is on a very different time scale than that of non-specific retrograde tracers.

It is evident from of the studies by van Houten et al. that specific uptake of some blood-borne proteins occurs in the neuropil surrounding the circumventricular organs. It has not been shown, however, that any of these proteins taken up at circumventricular sites are transported to other parts of the brain; thus it remains to be demonstrated that the uptake of blood-borne proteins can be responsible for the presence of pituitary-like proteins in various brain areas outside the circumventricular organs.

2. Brain Origin

Review of the literature has indicated that retrograde transport of proteins from pituitary to brain may occur, and that some blood-borne proteins, including one anterior pituitary hormone, are taken up and specifically bound in the neuropil of circumventricular organs, including the median eminence. In addition, studies examining the effect of hypophysectomy on brain content of pituitary-like proteins suggest that some, but not all, of the amount of pituitary-like proteins detected in brain is of pituitary origin. However, there is no indication that the pituitary is the sole or major source of the pituitary-like proteins in the brain. It therefore becomes pertinent to ask whether pituitary-like proteins are synthesized by cells within the brain.

This question has received most attention with respect to ACTH and other POMC-derived peptides. Liotta et al. demonstrated synthesis in brain of ACTH by showing incorporation of ^{35}S-labelled methionine into these peptides in hypothalamic tissue in

culture (61), and also after infusion of the labelled amino acid into the arcuate nucleus of rats (62). Gee et al. used *in situ* hybridization and ICC in adjacent brain sections to demonstrate the presence of POMC-encoding mRNA in the same hypothalamic cells as IR-ACTH (33). The mRNA encoding for the POMC molecule was also detected in hypothalamic tissue by the Northern blot and solution hybridization techniques (8).

These results corroborate the findings of earlier studies which attempted to determine the source of brain ACTH. In experiments designed to stop axoplasmic transport by treating rats with intracerebroventricular administration of colchicine, Krieger et al. found that 2 days after such treatment the concentration of IR-ACTH went up in the arcuate nucleus, while it was markedly decreased in other brain areas, including the median eminence (56). This finding is in accord with those of ICC studies which show an increase in IR-ACTH staining of neuronal cell bodies in the arcuate nucleus following colchicine treatment, coupled with a decrease in the staining of fibers throughout the brain. These findings imply that the neurons of the arcuate nucleus are the source of the ACTH distributed throughout the brain. Other evidence implicating the arcuate nucleus as the source of brain ACTH comes from the results of a study in which lesions of the arcuate nucleus were created by neonatal treatment with monosodium glutamate (55). In this study IR-ACTH was measured in a number of brain areas, and was found to be significantly decreased in all areas but one as a result of monosodium glutamate lesions; no decrease was observed in pituitary concentration of ACTH of lesioned animals.

Less attention has been given to the demonstration of biosynthesis in brain tissue of other pituitary hormones. The continued release of TSH and GH in long term cultures of hypothalamic cells has been reported (40,41). However Schorr-Toshav et al. found no mRNA encoding the alpha subunit of TSH in brain tissue, although it was detectable in the pituitary (86). Schacter et al. (85) probed mRNA gel blots prepared from rat

hypothalami with radiolabelled cDNA complementary to pituitary Prl mRNA; 3 out of 7 hypothalamic preparations contained detectable amounts of mRNA, suggesting that hypothalamic tissue does synthesize prolactin. Indirect evidence which might help to substantiate the results of Schacter et al. are ICC studies of Prl in brain in which animals were treated intracisternally with colchicine (88,89). As was the case with similar ICC studies for ACTH, colchicine treatment resulted in a markedly increased appearance of IR-Prl in neuronal cell bodies of the hypothalamus, with a concomitant decrease in staining of fibers at a distance from the cell bodies. Similar studies have not been reported for GH or TSH.

No direct demonstration of biosynthesis of LH in brain has yet been reported. However, two reports have described the effects of colchicine on the content and distribution of LH within the brain. Emanuele et al. (26) found that following intracerebroventricular injection of colchicine, the content of LH in the hypothalamus, as measured by RIA, was significantly increased to three times the level in saline-treated control animals. Other brain areas were not assayed, so no quantitative information about LH content of extrahypothalamic brain sites following colchicine treatment is available. No effect of colchicine treatment on pituitary concentration of LH was observed. In an ICC study of LH distribution in the rat CNS, Hostetter et al. (46) noted an increase in number of cells in the MBH staining for LH, as well as increased intensity of staining, following colchicine treatment; staining of fibers throughout the brain was diminished by colchicine. These two studies are suggestive of the hypothalamus as a source for the LH-like material in brain, since it would be expected that inhibition of transport along cell processes would result in accumulation of a protein at its source.

In summary, it is now well accepted that biosynthesis of the POMC precursor molecule, and consequently of its derivatives, occurs in neurons of the mediobasal hypothalamus. There is not enough evidence, however, to determine the appropriateness

of this conclusion regarding the other pituitary-like proteins in the brain: LH, TSH, Prl, and GH.

II. STATEMENT OF THE PROBLEM

Although the presence of IR-LH in neurons of the brain has been established, the source of this neuronal LH is still unknown. The LH concentration in brain tissue is a minute fraction of that in pituitary tissue; it is feasible that this protein might be transported to the brain from the pituitary, to be taken up from blood at sites deficient in a blood-brain barrier, such as the median eminence. This process might occur either by retrograde flow through the hypothalamo-hypophyseal portal vasculature, or by way of the peripheral circulation. A number of studies (3,4,5,20,64,72,76) have reported evidence in support of the notion that retrograde flow from pituitary to brain occurs. In addition, it has been shown for one anterior pituitary hormone, ACTH, that specific uptake from the bloodstream into the neuropil of the median eminence does occur (92,95).

Whether or not these phenomena apply to LH, and therefore might provide an explanation for its presence in brain, has yet to be determined. Some properties of the LH-like substance found in brain imply that it may be at least partly of pituitary origin, while others mitigate against this conclusion. In support of the hypothesis of pituitary origin, the concentration of IR-LH in the hypothalamus decreases significantly following hypophysectomy, although the concentration in other brain areas remains unchanged (21). On the other hand, intracerebroventricular administration of colchicine to rats causes an accumulation of IR-LH in the hypothalamus, whether measured by RIA (26) or observed by ICC (46). This drug interrupts axoplasmic flow and causes an accumulation of

substances within the nervous system at their site of origin; these results therefore implicate cells of the MBH as at least one source of the IR-LH found throughout the rat brain.

Uptake of LH from blood at the median eminence in a specific fashion would lead one to expect LH binding sites or receptors to be present at this site. Binding of LH to neuronal receptors might allow either for internalization and distribution of LH to a more distant site in the brain, in a manner similar to that suggested by van Houten and Posner (94) for ACTH and insulin, or alternatively for a local physiologic effect. The gonadal LH/hCG receptor mediates its effect on cellular function by stimulating the adenylate cyclase enzyme complex (83). Therefore, in an effort to identify LH receptors in the median eminence, it is of interest to examine the effect of LH on adenylate cyclase activity in this tissue.

The aims of the experiments presented in this thesis were to:

1. Examine the possibility that blood-borne LH might be taken up into the neuropil of circumventricular organs in a specific, receptor-mediated fashion. Initial experiments showed the median eminence to be the only circumventricular organ with an appreciable concentration of blood-borne LH, thus, subsequent experiments focused on this site.

2. Evaluate the possible presence of adenylate cyclase -coupled membrane receptors for LH in median eminence. The presence of such receptors in the median eminence would imply an activity of physiological significance for LH in this tissue.

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IV. MANUSCRIPT

Luteinizing Hormone in Rat Brain: Evidence Suggesting a Non-pituitary Source.

INTRODUCTION

A recent development in the field of neuroendocrinology has been the discovery of proteins in the brain which are immunologically and chemically similar to, or identical to, pituitary hormones. These proteins include immunoreactive-(IR) adrenocorticotrophic hormone (ACTH) (23,28,30,32,36), prolactin (Prl)(16,42,43), thyroid stimulating hormone (TSH)(4,29,33) and growth hormone (GH)(34). The presence of these pituitary-like hormones in the brain has been demonstrated using a variety of techniques, including radioimmunoassay (RIA), bioassay, gel filtration chromatography, and immunocytochemistry (ICC).

In 1981 Hostetter et al. (20) and Emanuele et al. (12,13) independently demonstrated the presence of immunoreactive luteinizing hormone (IR-LH) in rat brain, thus adding to the array of pituitary-like substances discovered in brain. RIA, bioassay,and ICC were used to demonstrate this protein in brain tissue, and gel filtration chromatography showed it to be of approximately the same molecular weight as pituitary LH. A more recent ICC study by Hostetter et al. (21) provided a detailed picture of the IR-LH distribution in rat brain. Cell bodies localized in the hypothalamic arcuate nucleus and periarcuate area projected to sites within the mediobasal hypothalamus as well as to other structures within the forebrain and brainstem. These findings are of interest in view of the possible physiologic action that LH may have in the mediobasal hypothalamus (10,11,13,14,19,31,38,41).

Now that many studies have demonstrated the presence of pituitary proteins in brain, a pertinent question is that of whether these proteins in brain originate through biosynthesis in neuronal cell bodies, or by transport to the brain from the pituitary. Transport of substances from the pituitary could occur through the pituitary-portal vasculature, or through the peripheral circulation, to reach the median eminence (ME).

Since the ME lacks a blood-brain barrier, protein molecules in the blood may be taken up by neurons which terminate on vessels in this area. It has been shown for POMC-derived molecules (including immunoreactive ACTH) that biosynthesis does occur in neurons of the mediobasal hypothalamus (22,24,25). For other pituitary-like hormones in brain, however, this question has not been well studied. The studies described in this report explore the possibility that the IR-LH in the brain is of pituitary origin, and is conveyed to the brain via a vascular route. Three experimental approaches were used. In the first approach, animals were treated intravenously with ^{125}I-labelled human chorionic gonadotropin (hCG), and uptake of labelled hormone into tissues was quantified directly using a gamma counter. In the second group of experiments animals were again given radiolabelled hCG intravenously, and uptake of labelled hormone was assessed using autoradiography of tissue sections. The final experiment attempted to demonstrate an LH receptor in ME that is functionally similar to the gonadal receptor by assessing gonadotropin-coupled adenylate cyclase activity in median eminence.

MATERIALS and METHODS

Animals: Male Sprague-Dawley rats weighing 200 to 220 g were obtained from Charles River suppliers. The rats were kept on a 14:10 light:dark cycle, with food and water available ad libitum. The animals were assigned randomly to treatment groups, and were used when between 200 and 250 g in weight.

Hormones: Iodination grade hCG (CR 121), and biological grade prolactin (Prl-B-5) were provided by the NIADDK. Biological grade hCG was purchased from Sigma (CG-B). hCG was chosen for use in these studies because it was found that hCG retains more of its receptor-binding activity following iodination. In addition, this hormone, which is

commonly used in studies of rat gonadotropin receptors, is more readily available than rat LH in quantities required for *in vivo* studies. The hormone was iodinated by the lactoperoxidase method by a modification of the procedure of Miyachi et al.(27). The specific activity of the hormone in terms of receptor binding capacity was determined by the self-displacement method of Catt et al.(8), using a rat testicular membrane preparation. The specific activity determined in this manner was 109.6 uCi/ug ± 38.17 (mean ± S.D.).

Experiment 1: Direct Measurement of uptake of labelled hCG

Radiolabelled and unlabelled hCG dissolved in normal saline were injected through polyvinyl jugular venous cannulae into animals anesthetized with ketamine (50 mg/kg), xylazine (10 mg/kg), and acepromazine (1 mg/kg). In the first experiment each animal received 375 uCi of labelled hCG. In subsequent experiments, each animal received 129 uCi, equivalent to approximately 1.2 ug of hCG, in a volume of 0.3 to 0.6 ml. Initially, in order to assess the time course of uptake of labelled hormone, animals were sacrificed at 15, 30, 60, and 90 minutes following hormone administration. Based on data from this first study, it was determined that 30 minutes was the optimal time to observe hormone uptake in the ME. In subsequent experiments, in order to determine the specificity of hormonal uptake, animals were given either labelled hormone alone, or labelled hormone preceded at one minute by a 500-fold excess of unlabelled hCG (Sigma). At the time of sacrifice, animals were anesthetized with pentobarbital and perfused intracardially with 100 ml of normal saline at 4°C. Following perfusion, tissue samples were taken of testis, liver, kidney, muscle, neocortex of the brain, and the hypothalamic ME. The median eminences from three animals were pooled while other tissue samples were analyzed individually. Radioactive hormone content of weighed tissue samples was determined by measurement in a gamma counter; values were converted to pg hCG taken

up per mg wet weight tissue. Data were analyzed by one-way ANOVA using Newman-Keuls test for post hoc comparisons, or by Student's t-test.

Experiment 2: Autoradiographic assessment of hCG uptake.

Labelled hCG and unlabelled hCG and Prl were administered as decribed for direct measurement of hCG uptake. Prl was included in these experiments to observe any possible non-specific inhibition of hCG binding in median eminence. Animals were sacrificed 30 minutes following injection of hormone, and perfused first with 50 ml saline, and then with 100 ml Karnovsky's fixative (2% paraformaldehyde, 2.45 % glutaraldehyde, 0.004% $CaCl_2$, buffered with 0.1M phosphate buffer, pH 7.2) at 4°C. The brain and left testis of each animal were removed and immersed in fixative at 4°C for 36-48 hours.

In preparation for autoradiographic processing, tissues were cut into 2-3 mm thick blocks and placed in 20% phosphate buffered sucrose for 24 hours at 4°C. Tissue blocks were then frozen in an isopentane slush in liquid nitrogen, and cut into 6um sections using a cryostat. Sections were mounted on gel coated glass slides, defatted by passing them through a series of alcohols, xylene, and a second series of alcohols, and stained in filtered Gill's 3X hematoxylin. Stained slides were then coated with Kodak NTB-2 photographic emulsion and stored in light-tight boxes within lead-lined pouches for 6 weeks at 4°C. Following this exposure time, the slides were developed for 6 minutes in Kodak D-170 developer at 18°C, rinsed, and fixed in Kodak Fixer for 4 minutes. Developed slides were counter-stained in Lee's stain (methylene/basic fuschsin), air dried, and mounted with cover slips.

Uptake of radiolabelled hormone was quantified by determining the density of autoradiographic grains (silver grains) over representative sections of ME, neocortex, and

testis using a Leitz microscope interfaced with an Apple personal computer and Graphics Tablet, with software written by Dr. William Woodward, Department of Neurology, Oregon Health Sciences University. For each animal, 12 unit areas through the anterior/posterior and medial/lateral extent of the median eminence, and 12 areas of parietal neocortex were quantified. In each testis, grain density was quantified in 9 to 12 interstitial areas. The data were analyzed by Kruskal-Wallis H test, followed by Mann-Whitney U tests for post hoc comparisons.

Experiment 3: Response of Adenylate Cyclase to LH and hCG in Median Eminence.

Tissue collection and preparation

Rats were anesthetized with pentobarbital and decapitated. Brains were rapidly removed and placed on a chilled surface. Coronal cuts were made through each brain just behind the optic chiasm and just in front of the mammillary bodies. From the portion of brain lying between these two cuts, a small wedge-shaped area of tissue containing the ME and a small amount of mediobasal hypothalamus was removed. These dissected median eminences, weighing an average of 1.7mg each, were pooled into a glass Dounce homogenizer, with 10 volumes of homogenization buffer (8% sucrose and 1mM EDTA in 25mM Tris-HCl, pH 7.4) at 4°C. The tissue was homogenized first with a loose-fitting pestle (10 strokes), then with a tight-fitting pestle (10 strokes). The resulting homogenate was transferred to a chilled tube, and the homogenizer was rinsed with an additional 10 ml of buffer which was then added to the original homogenate.

As a control procedure, to ascertain the validity of the adenylate cyclase assay, ovarian luteal tissue was obtained from immature female Sprague-Dawley rats which had been superovulated with hCG and pregnant mare serum gonadotropin treatment (15). Seven days following hormonal treatment, rats were sacrificed and the luteinized ovaries

removed as previously described (15). The ovaries from hormone-treated rats were pooled, weighed, minced, and homogenized as described above, with the following additional steps. After homogenization with 10 volumes of buffer, the homogenate was centrifuged at 160 x g for 2 minutes at 4°C. The resulting supernatant was saved in a chilled tube, while the pellet was rehomogenized in an additional 10 volumes of buffer. The rehomogenized pellet was then centrifuged as before, and the supernatants from the two centrifugations were pooled for use in assays. This luteal tissue homogenate was stored in aliquots at
-70°C until use in assays.

Adenylate Cyclase Assay

The adenylate cyclase assay was performed as described previously by Eyster and Stouffer (15). Each assay tube determined the adenylate cyclase activity in 20 ul of tissue homogenate (approximately 1 mg wet weight of tissue). Assay tubes contained 6 mM Na-ATP (Sigma), 1 x 10^7 cpm [α-^{32}P]ATP (ICN Pharmaceuticals), 1mM cAMP, 2 x 10^4 cpm [^{3}H]cAMP (New England Nuclear), 5 mM $MgCl_2$, 6 mM EDTA, 20 mM creatine phosphate, 0.2 mg/ml creatine kinase, and 0.02 mg/ml myokinase in 15 mM Tris-HCl buffer, pH7.5 Where appropriate the following hormonal and non-hormonal stimulators were added: GTP(50uM); 5'-guanyl-imidodiphosphate (GMP-PNP) (50 uM); hCG (CR 119, 250 uM); human LH (hLH, NIH I-1, 333 or 33 uM); prostaglandin E_2 (PGE_2, 500uM); forskolin (100 uM); and the beta-adrenergic agonist, isoproterenol (100 uM).

After all other reagents were added to tubes to a total volume of 30 ul, 20 ul of tissue homogenate was added to each tube to initiate the reaction. Tubes were incubated at 37°C for 10 minutes in a shaking water bath, after which time the enzyme reaction was stopped by adding 100 ul of Stopping Solution (40 mM ATP, 5 mM cAMP, 2% sodium

laurel sulfate), and boiling for 3 minutes.

[^{32}P]cAMP generated by adenylate cyclase in the tissue homogenate was separated from labelled substrate by sequential column chromatography on Dowex (AG50WX4 ion exchange resin, Bio-Rad) and neutral aluminum oxide (Sigma). [^{3}H]cAMP included in the incubation medium served as a recovery marker. Recovery of cAMP from columns was approximately 50%. Column fractions containing cAMP were collected into liquid scintillation cocktail (ACS, Amersham), and counted in a liquid scintillation counter for 10 minutes. Reference vials containing aliquots of labelled ATP and, labelled cAMP were included.

Protein content of aliquots of tissue homogenate was determined by the method of Bradford (6). Assay results are expressed as pmol cAMP per mg protein, per minute. Data was analyzed by one-way ANOVA for each tissue using the randomized complete block design, followed by the Scheffe test for post hoc comparisons.

RESULTS

In vivo uptake of blood-borne radiolabelled hCG

Figure 1 shows accumulation over time of ^{125}I-labelled hCG in selected tissues. Direct gamma counter quantification of labelled hormone uptake in ME showed no statistically significant accumulation over time. However, a peak appears at 30 minutes which is 54% greater than the 15 minute value, and 102% greater than the 60 minute value; this is followed by a second elevation at 90 minutes. In testis, a continual increase in radiolabelled hormone concentration is seen over time, with the 90 minute value being 168% greater than the 15 minute value ($p<0.01$, Table I). For muscle there is also a statistically significant accumulation of radioactivity in the tissue by 90 minutes ($p<0.01$),

although it is not as marked as that seen for testis. No significant accumulation of radioactivity over time is seen for liver, kidney, or neocortex.

In the second part of the first experiment uptake of labelled hormone was compared in animals receiving either radiolabelled hCG alone 30 minutes prior to sacrifice, or labelled hCG 1 minute after a 500-fold excess (by weight) of unlabelled hCG, and followed at 30 minutes by sacrifice. The thirty minute time point following hormone administration was chosen based on results of the previous experiment which indicated this to be a time of peak uptake in ME, while also being adequate to show uptake in testis. Results of this experiment (Figure 2, Table II) showed that only in testis was there a significant decline in binding of labelled hCG following pretreatment with unlabelled hormone (22%). In median eminence the decline of 16.7% did not reach statistical significance.

In Vivo Autoradiographic Analysis of Radiolabelled hCG Uptake by Testis and Median Eminence

Figure 3 depicts silver grain density in sections through ME and testis of animals treated with labelled hCG alone, labelled hCG preceded by an excess of unlabelled hCG, or labelled hCG preceded by unlabelled excess Prl. Silver grain density (Grains/um^2) in median eminence was $0.78 \pm 0.15 \times 10^{-1}$ (mean $\pm$ S.E.M.) in animals receiving labelled hormone alone, $0.62 \pm 0.10 \times 10^{-1}$ with unlabelled hCG plus labelled hormone, and $0.94 \pm 0.20 \times 10^{-1}$ when unlabelled Prl was given prior to labelled hormone. None of these groups were significantly different from one another. Silver grain density in parietal neocortex was equivalent to background. Silver grain densities were an order of magnitude greater in testis than in ME. Silver grain densities in testis were $7.89 \pm 0.41 \times 10^{-1}$ when labelled hormone was given alone, $4.25 \pm 0.61 \times 10^{-1}$ when labelled hormone was preceded by unlabelled hCG, and $17.93 \pm 1.31 \times 10^{-1}$ when unlabelled Prl preceded

hCG. Pretreatment with both unlabelled hormones caused a statistically significant change in silver grain density; a decline with hCG, and an elevation with Prl. Figures 4 and 5 show representative photomicrographs of silver grain labelling in testis and median eminence, respectively. In testis, silver grains are seen primarily over Leydig cells in the interstitium. In Figure 5, silver grains are evenly distributed in the median eminence, with only background levels extending into other brain areas. Similarly, in Figure 6, a darkfield photomicrograph, silver grains are uniformly distributed over ME.

Adenylate Cyclase Activity in Median Eminence

Table IV summarizes cAMP production by the median eminence and luteinized ovary of the rat. The basal values as well as nucleotide- and hormone-stimulated values for luteal tissue are typical of those generally obtained using this method (15). The mean basal value for ME is about three times that of corpus luteum, although the significance of this difference was not tested. The nucleotide GTP, a non-hormonal stimulator of adenylate cyclase, caused approximately a 1.8-fold increase over the basal value of cAMP production for rat luteal tissue, and a 1.54-fold increase over the basal value of ME. The non-hydrolyzable analogue of GTP, GMP-PNP, which causes a more prolonged stimulation of the enzyme, produced a 7-fold elevation of cAMP production in the luteal tissue ($p<0.01$), and a 3.2-fold elevation in ME ($p<0.01$). In accordance with expectations, in luteal tissue both hCG and hLH (given with GTP which is required for hormonal activation) caused significant elevations in cAMP production over basal values (7.5- and 4.7-fold, respectively), as well as over GTP alone ($p<0.01$). In ME, however, neither hCG nor hLH stimulated cAMP formation beyond that seen with GTP alone.

DISCUSSION

The results of this study are consistent with the conclusion that specific, receptor-mediated binding of hCG does not occur in the median eminence. In experiment 1 the results of direct quantification of uptake of blood-borne radiolabelled hCG in ME were equivocal. The concentration of radioactivity in median eminence peaked at the 30 minute time point, but this peak was not statistically significant. The overall pattern of accumulation over time of radioactivity in ME is dissimilar to that often reported for other tissues bearing LH/hCG receptors (7,17,39), with peak binding being at 60 minutes *in vivo*, and 90 to 120 minutes *in vitro*. In contrast however, Barofsky et al. (5) found that in immature female rats given ^{125}I-LH intravenously, the radioactivity content of ovary peaked at 15 to 30 minutes and then declined exponentially. The pattern in ME contrasted with the findings for testis in which a significant accumulation of radioactivity over time was seen through 90 minutes. The sequestration of hCG in testis is consistent with the many reports showing binding and internalization of LH/hCG by Leydig cells of the testis (3,17,39). A small but significant accumulation of radioactivity in the present study was also seen in muscle. This increase may correspond to retention in this tissue of iodotyrosines from degraded hCG for use in protein synthesis or energy metabolism. Liver and kidney both contained high levels of radioactivity without accumulation over time, corresponding to the high degree of blood perfusion of these tissues, as well as to their function in hormone catabolism.

In part two of the first experiment, a small, but not statistically significant, decline in radioactive labelling of ME was seen when unlabelled hCG was given to compete with binding of labelled hormone, suggesting possible low level receptor-mediated binding. The lack of statistical significance may be due to an actual lack of

specific binding of hCG in ME, to a small number of binding sites in the tissues mass assayed, or simply to the small ME sample size in this study (in actuality, 15 animals are represented, but tissue samples were pooled). In testis unlabelled hormone was found to be effective in blocking the binding of labelled hCG, indicating the receptor-mediated nature of this binding. The degree of inhibition of labelled hormone binding is not quite as marked as that seen in previous studies of gonadal uptake of labelled LH/hCG *in vivo*. One explanation for this may be that other studies have utilized immature female animals that had been treated with hCG and pregnant mare serum gonadotropin in order to increase ovarian LH receptors (7,18,37). With greater numbers of gonadal LH receptors, a greater ratio of specific to non-specific hormone binding would be obtained than with untreated mature males. In addition, in the present experiment, non-specific radioactive labelling of the tissue might have been high due to residual blood and interstitial fluid bearing labelled hormone left after perfusion. This is a problem attested to previously by Ascoli and Puett (3). Muscle and neocortex show no response to pretreatment with unlabelled hCG, consistent with the lack of LH/hCG receptor in these tissues.

A contrasting feature of parts one and two of experiment 1 is the amount of labelled hormone administered to the animals; 375 uCi in part one, and 129 uCi in part two. It is of relevance that binding of labelled hormone in ME was only slightly decreased by the lesser amount of hormone given in part two, while in testis there was a marked decline in labelled hormone binding when less was administered (compare Table I, 30 minute time point, to Table II, labelled hormone only), suggesting a dose-response relationship. This observation further supports the conclusion that, unlike the testis, the ME does not contain LH/hCG binding sites.

Experiments 1 and 2 in this study look at radioactivity in the tissues as an indication of hCG presence. It is assumed that a significant portion of the ^{125}I-labelled hCG injected into the animals remains intact until the time of tissue sampling.

Preliminary experiments in this laboratory using sephadex chromatography have shown that 15 minutes following injection, labelled hormone in serum remains intact (data not shown). Also Conn et al. (9) showed that radioactivity extracted from ovarian tissue 7 hours after injection of ^{125}I-hCG into female rats, co-chromatographed with the original labelled hormone by SDS/PAGE. Thus in the present experiments, it would be expected that the majority of labelled hormone remains intact until it is taken up by the cells, after which time it may be degraded (3,17,39).

Results of quantitative autoradiographic analysis of hCG uptake *in vivo* show no significant effect in ME of unlabelled hormone pretreatment, although a slight but non-significant decline in silver grain density was observed. As in experiment 1, there was a clear decline in binding of labelled hormone in testis when unlabelled hCG was given, demonstrating the specific receptor binding of hCG known to occur in this tissue. These results suggest that no binding of hCG to receptors occurs in ME. This conclusion is further supported by microscopic analysis of autoradiographic slides which revealed that the pattern of silver grains (figure 5) is uniform, without concentration of grains over specific cytologic structures or anatomic sites. Because nerve fibers in the zona externa of the ME terminate on blood vessels of the portal capillary plexus (26), receptors for blood-borne hormone would be localized here. If binding of blood-borne hCG in ME were specific a concentration of silver grains should be seen in zona externa, as was the case for binding of blood-borne ACTH (46), insulin (44), and angiotensin II (45) as reported by van Houten et al. However, in distinction to the present study, in van Houten's studies animals were perfused only five minutes after labelled hormone administration; this may not have allowed sufficient time for diffusion of labelled hormone into the median eminence further than the zona externa, even in the absence of specific binding. In an autoradiographic study by Tappaz et al. (40) tritiated GABA was localized to the zona externa of the ME fifteen minutes after intravenous administration. The specificity and

neuronal nature of this uptake was demonstrated in this and a subsequent study (1). Therefore, the lack of localization of silver grains over the zona externa in the present study is interpreted as further evidence that receptor binding of hCG is not occuring in the median eminence.

Although the pattern of labelled hormone uptake in response to excess unlabelled hormone is similar in experiments 1 and 2, the magnitude of difference in silver grain density between testis and median eminence in experiment 2 is not reflected in the values seen with the direct quantification method of experiment 1. The microscopic nature of the autoradiographic technique accounts for this difference since autoradiography allowed for quantification in selected histological sites, such that, in testis, interstitial areas were quantified while excluding unlabelled tubules. The approach used in experiment 1 includes all tissue elements in determining the density of radioactive labelling so that the seminiferous tubules, which make up a large proportion of testicular mass, effectively diluted the density of label seen. This effect did not occur for median eminence, because the label was uniformly distributed in the tissue.

An unexpected finding in the autoradiographic analysis of hormone binding was the response seen in testis to treatment with excess unlabelled Prl. A marked elevation in the silver grain density in testis was observed in these animals, suggesting an increase in binding of hormone to receptor, or an increase in receptor number, had occurred. An inductive effect of Prl on LH receptor number has been noted in earlier studies (2,8) and may explain this observation. However, the time period over which increased hCG binding occurs in our study (30 minutes) may not be adequate to allow for induction of increased synthesis of new receptors. It may be that binding of Prl to its receptor has a cooperative effect allowing more rapid binding of LH to its receptors. Alternatively, Prl may interact directly with the LH receptor to promote binding of LH.

The results of the autoradiographic analysis of LH uptake in median eminence

reported here are in contrast to studies by Van Houten et al. showing uptake of blood-borne ACTH (46), angiotensin II (45) and insulin (44) in ME. The studies of van Houten et al. showed uptake of labelled hormone 5 minutes after intracardiac injection, with uptake of label being markedly declined when excess unlabelled hormone was also given. Van Houten interpreted his results to indicate that specific binding of these blood-borne hormones occurred in ME.

The final experiment in this study, the assessment of adenylate cyclase activity in median eminence, provided clear evidence that no adenylate cyclase coupled LH receptor is present in this tissue. The adenylate cyclase assay worked well in luteal membranes as indicated by the expected increase in cAMP production seen with nucleotide regulators (GTP and GMP-PNP) as well as with the hormonal regulators LH and hCG. This assay also proved to be suitable for brain tissue, with results showing: 1) appreciable adenylate cyclase activity in ME; 2) an elevation in adenylate cyclase activity in response to GTP and GMP-PNP, indicating classic nucleotide regulation; 3) no gonadotropin regulation of adenylate cyclase in ME. A single assay showed increased cAMP production in both corpus luteum and ME in response to PGE_2 stimulation (data not shown). The lack of increased cAMP production in ME in response to LH or hCG indicates that either no adenylate cyclase coupled LH/hCG receptor is present, or alternatively, that receptor is present in such low density that it is not detectable above the background of other AC activity in this tissue. This finding does not exclude the possibility of a different sort of receptor-associated activity for LH in median eminence.

Three approaches have been used in this study to attempt to demonstrate specific binding of LH in ME of male rats. Such binding would be consistent with the hypothesis that blood-borne LH is the source, at least in part, of neuronal LH seen in cell bodies of the arcuate nucleus, as well as in fibers distributed in forebrain, brainstem, and spinal cord of male rats. The rather insensitive technique used in experiment 1 showed specific

binding of labelled hormone in testis, while results were unclear for ME. Using *in vivo* quantitative autoradiography in experiment 2, it became more clear that no specific binding occurred in ME, while specific binding was seen in testis. In experiment 3, assay of adenylate cyclase activity showed no stimulation of this enzyme complex by LH or hCG in ME, while such stimulation was evident in gonadal tissue. The experiments reported here have provided evidence suggesting that LH/hCG receptors are not present in median eminence of male rats. This conclusion might be further strengthened by additional studies, for example exploring *in vitro* binding of hCG to cell membranes from median eminence, or examining binding of labelled hormone to testis and ME at a time latency of greater than 30 minutes after exposure to hormone. Regardless of whatever findings might come out of further studies, the present results show that the magnitude of any specific binding of LH/hCG that might occur in the median eminence must be very small, and cannot account for the extensive distribution of LH seen in brain.

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Figure 1. Uptake of ^{125}I-labelled hCG from blood by selected tissues over time (mean±S.E.M.). **Significantly greater than all other time points for testis, $p<0.01$, ($F=10.5894$; $df=3,28$). * Significantly greater than all other time points for muscle, $p<0.01$, ($F=5.1708$; $df=3,28$).

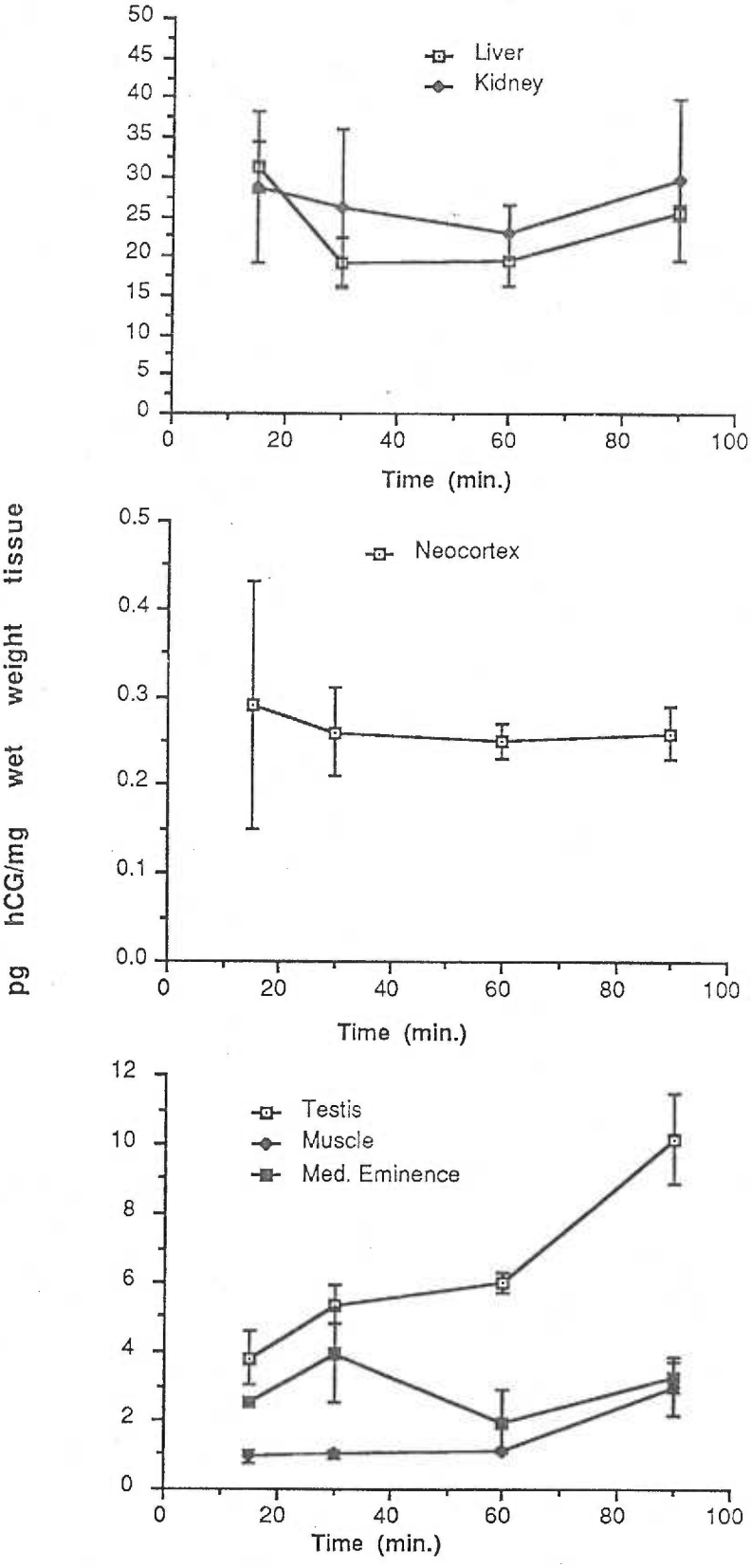

Liver
Kidney
Neocortex
Testis
Muscle
Med. Eminence
pg hCG/mg wet weight tissue
Time (min.)
Time (min.)
Time (min.)

TABLE I

Uptake of ^{125}I-labeled hCG from blood into various tissues at selected time points. Values are given in pg hCG/mg wet weight of tissue, and represent means ±S.E.M.

	Time			
Tissue	15 minutes n=5	30 minutes n=10	60 minutes n=9	90 minutes n=8
Testis	3.80±0.77	5.35±0.55	5.99±0.30	10.18±1.29[a]
Muscle	0.93±0.20	1.00±0.13	1.11±0.06	2.99±0.84[b]
Liver	31.20±3.26	19.11±3.24	19.58±3.34	25.48±0.97
Kidney	28.63±9.62	26.17±9.88	23.07±3.30	29.59±10.19
Neocortex	0.29±0.14	0.26±0.05[c]	0.25±0.02	0.26±0.03
Median Eminence[d]	2.55±0.13	3.92±1.37	1.94±0.93	3.26±0.46

a: significantly greater than all other time points for testis, $p<0.01$, ($F=10.5894$; $df=3,28$).

b: significantly greater than 60 minute time point, $p<0.01$, ($F=5.1708$; $df=3,28$).

c: n=8; d: n=2 for 15 minute time point; n=3 for all other time points. Data evaluated using one-way ANOVA for each tissue across time with post hoc comparisons using Newman-Keuls test.

Figure 2. Uptake of ^{125}I-labelled hCG by selected tissues 30 minutes after iv injection. Rats were treated with labelled hCG (*hCG) alone or with labelled hCG preceded at one minute by excess unlabelled hCG (*hCG + hCG). Values represent pg hCG/mg wet weight of tissue (mean $\pm$ S.E.M.); n=11 to 13 for each tissue except median eminence, for which n=5 (values represent tissue pooled from two to three animals). **$p<0.01$, vs labelled hCG alone, ($T=3.38$; $df=23$).

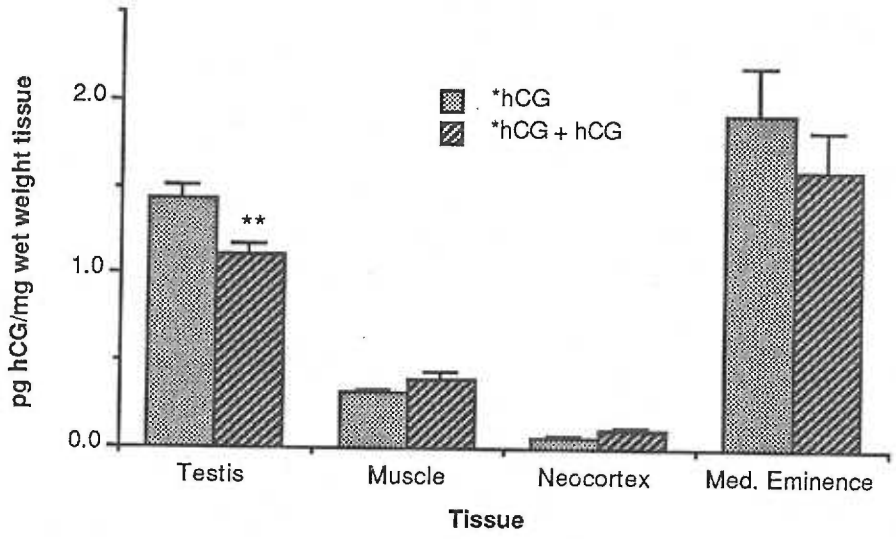

pg hCG/mg wet weight tissue
2.0
1.0
0.0
*hCG
*hCG + hCG
**
Testis
Muscle
Neocortex
Med. Eminence
Tissue

TABLE II

Uptake of ^{125}I-labelled hCG by selected tissues 30 minutes after i.v. injection. Rats were treated with labelled hCG alone or with labelled hCG preceded at one minute by excess unlabelled hCG. Values represent pg hCG/mg wet weight of tissue (mean ±S.E.M.).

Tissue	Treatment			
	n	labelled hCG only	n	labelled hCG plus unlabelled hCG
Testis	14	1.44±0.07	12	1.12±0.06[a]
Muscle	13	0.32±0.02	12	0.40±0.04
Neocortex	13	0.06±0.01	11	0.10±0.02
Median Eminence[b]	5	1.92±0.27	5	1.60±0.23

a. $p<0.01$, vs. labelled hCG alone, ($T=3.38$; $df=23$).

b. Each data point represents tissue pooled from three animals. Data analyzed by Student's t-test.

Figure 3. Silver grain density of autoradiographic slides of testis and median eminence. Animals were treated with ^{125}I-labelled hCG (*hCG)alone, or ^{125}I-labelled hCG preceded at one minute by excess unlabelled hCG (*hCG + hCG) or unlabelled prolactin (*hCG + Prl). Values represent mean $\pm$ S.E.M.
**$p<0.01$ vs. labelled hCG alone in testis.

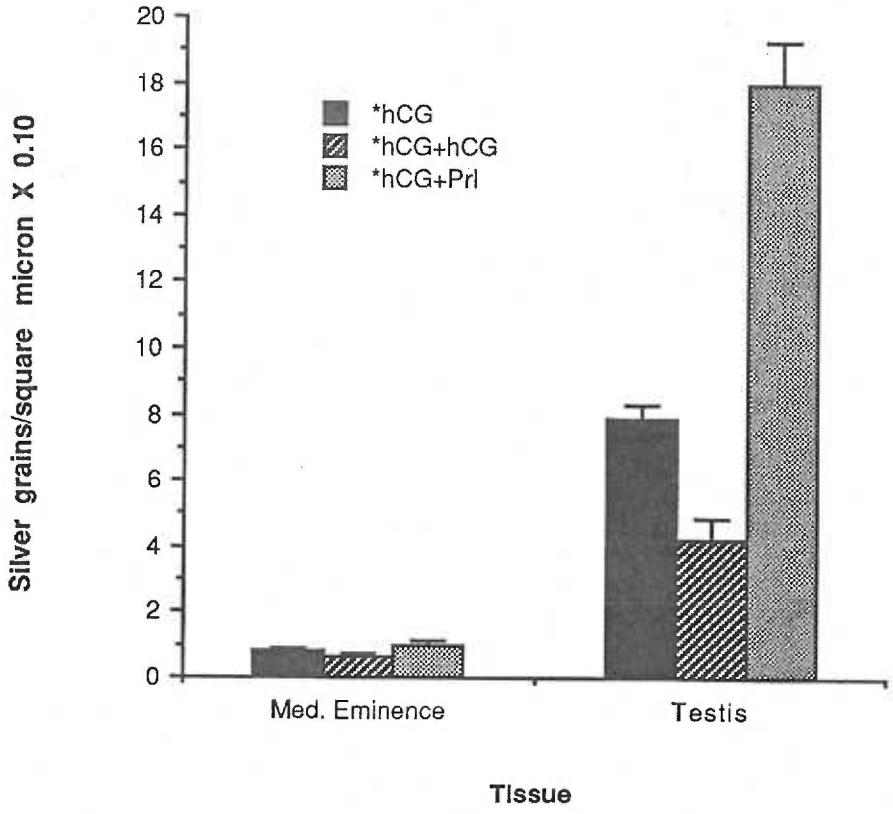

Silver grains/square micron X 0.10
20
18
16
14
12
10
8
6
4
2
0
*hCG
*hCG+hCG
*hCG+Prl
Med. Eminence
Testis
Tissue

TABLE III

Silver Grain Density of Autoradiographic Slides. Animals were treated with ^{125}I-labelled hCG alone, or labelled hCG preceded by excess unlabelled hCG or unlabelled prolactin (Prl).

(grains/um^2 x 10^{-1}; mean $\pm$ S.E.M.)

Tissue	Treatment		
	labelled hCG only	labelled hCG plus unlabelled hCG	labelled hCG plus unlabelled Prl
Testis[a]	7.89±0.41	4.25±0.61	17.93±1.31
Median Eminence	0.78±0.15	0.62±0.10	0.94±0.20[b]
Cortex	-0.01±0.04	-0.02±0.05	-0.11±0.06[b]

Data analyzed by Kruskal-Wallis H and Mann-Whitney U tests; n=5 for each group except as indicated.

a. all testis groups significantly different from one another, p=0.008

b. n=4 for these groups.

Table IV

cAMP Production in Rat Corpus Luteum and Median Eminence. Effect of hormonal and non-hormonal stimulating agents. Values are in pmol cAMP/mg protein/ minute, and represent mean ± S.E.M. of five assays for corpus luteum and three assays for median eminence.

	Tissue	
Treatment	Corpus Luteum	Median Eminence
Basal	12.52±2.60	38.80±17.08
GTP	20.92±3.83	50.83±12.41
GMP-PNP	89.20±31.80[b]**	105.47±25.90**
hCG/GTP	87.50±21.31[b]*	49.40±11.10
LH/GTP	79.92±17.21*	53.20±12.85

b: n=4 (number of assays) for these treatments.

** Significantly greater than basal value, $p<0.01$

* Significantly greater than GTP value, $p<0.01$

Data analyzed by one-way ANOVA for corpus luteum ($F=8.6063$; $df=5,15$) and ME ($F=17.9551$; $df=4,8$).

Figure 4. Autoradiographs of rat testis: a) animal treated with ^{125}I-labelled hCG only; b) animal treated with ^{125}I-labelled hCG one minute after excess unlabelled hCG; c) animal treated with labelled hCG one minute after excess unlabelled Prl. Dense accumulations of silver grains are seen over Leydig cells in the interstitium (arrows) primarily in a and c. Magnification is 314 X.

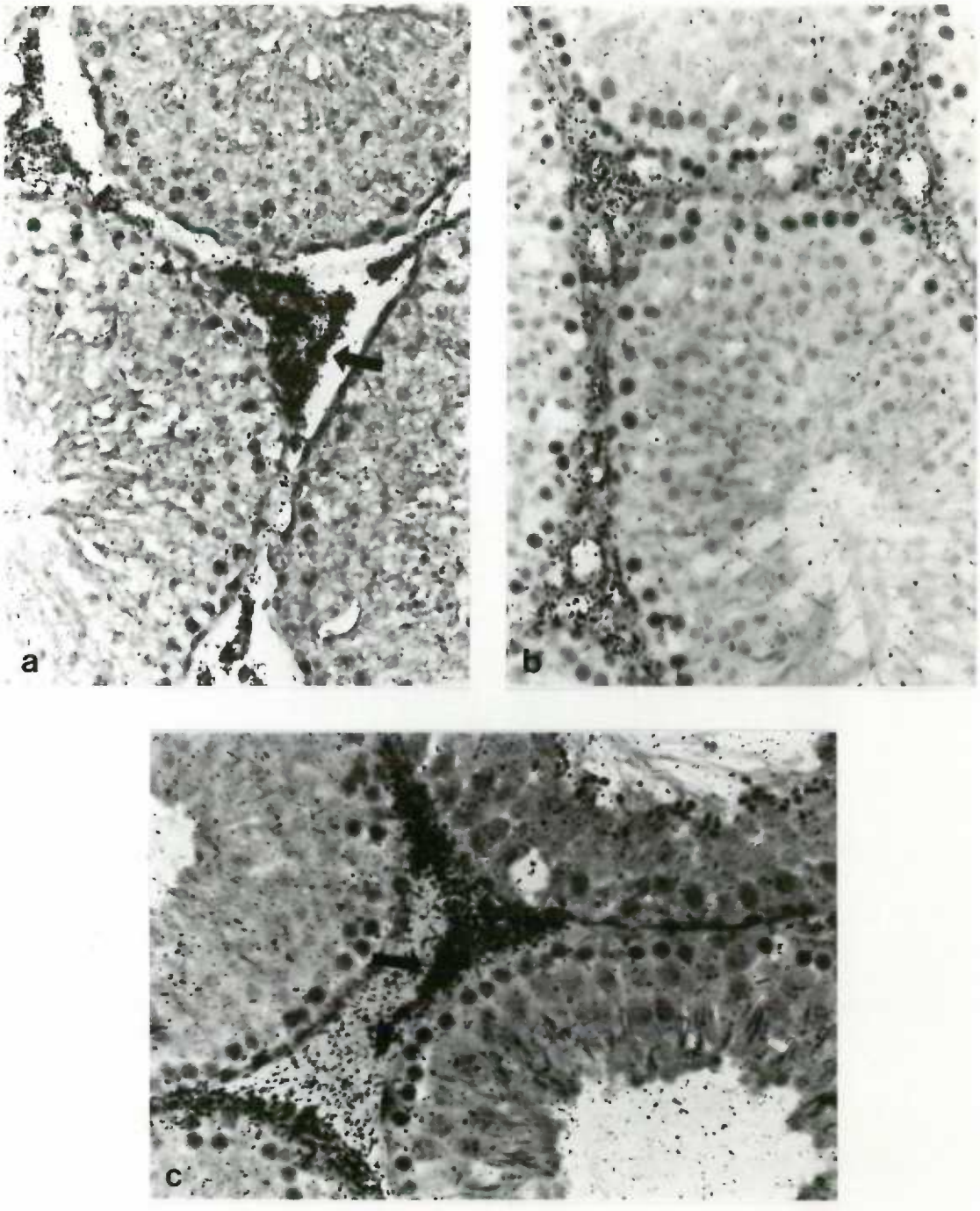
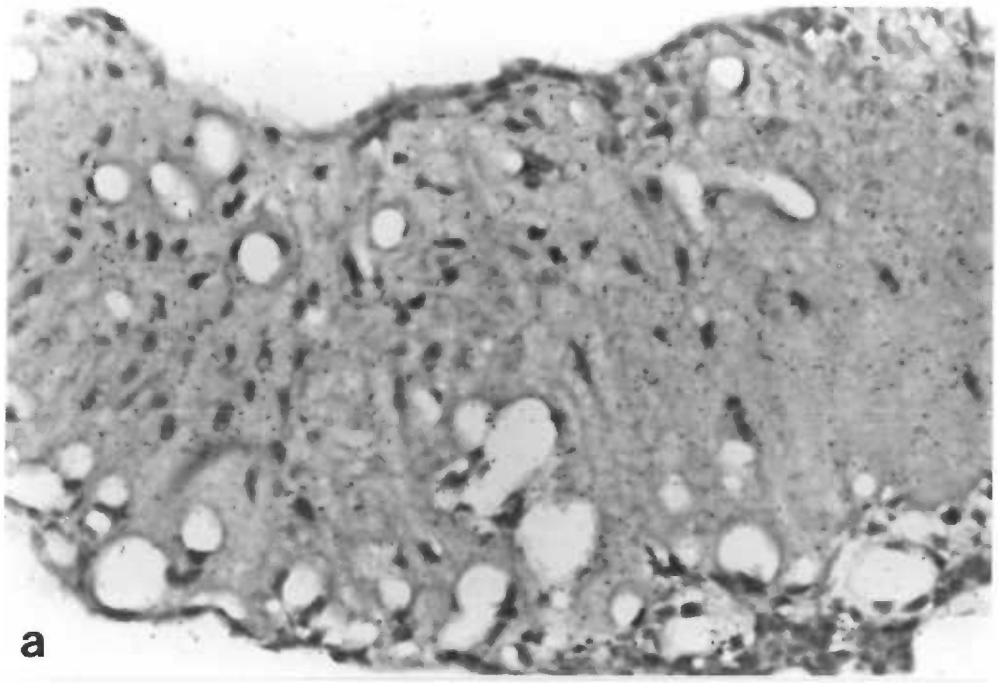
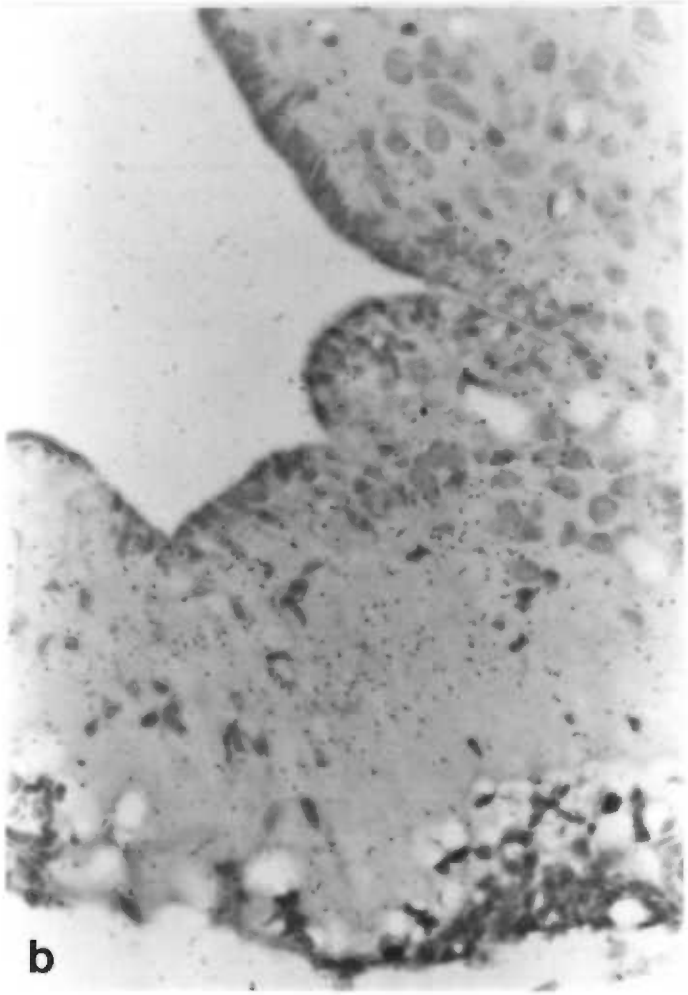

a
b
c

Figure 5. Autoradiographs of rat median eminence: a) animal treated with ^{125}I-labelled hCG only; b) animal treated with ^{125}I-labelled hCG one minute after excess unlabelled hCG. Silver grain density is approximately equivalent in both sections. No localization of silver grains over specific structures is seen. Magnification is 314 X.

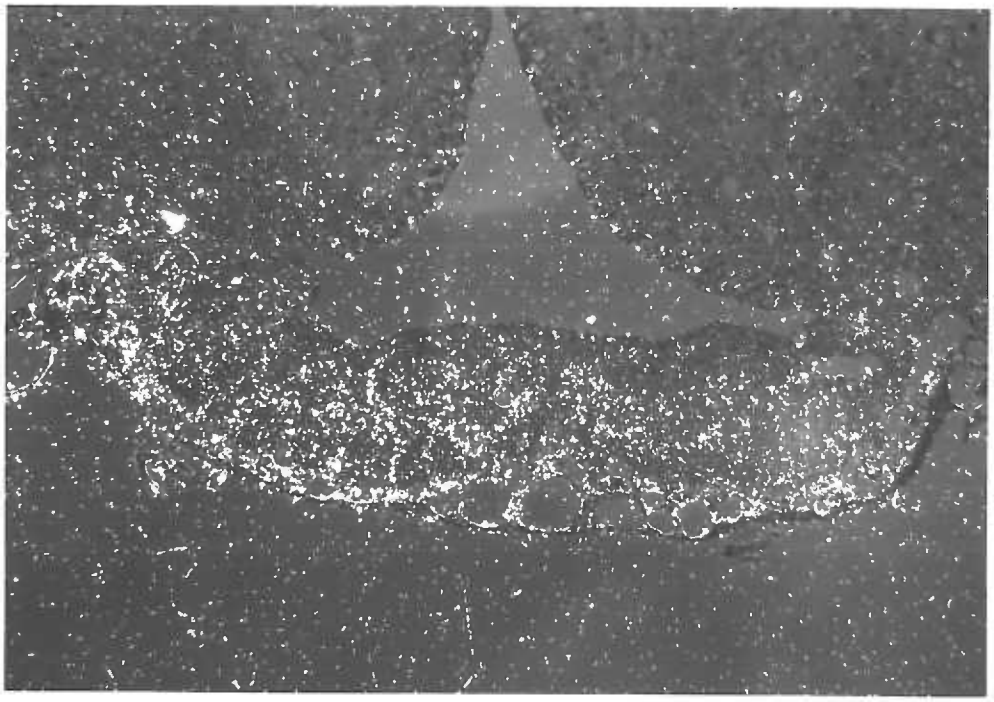


a



b

Figure 6. Darkfield Autoradiograph of radiolabelled hCG in median eminence. The animal was treated with radiolabelled hormone only. Magnification in 100 X.



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