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Roy Patrick Radcliff

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Ph.D. degree in Animal Science

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SEROTONERGIC REGULATION OF GROWTH HORMONE SECRETION IN CATTLE

By

Roy Patrick Radcliff

A DISSERTATION

Submitted to
Michigan State University
In partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Animal Science

2001

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ABSTRACT

SEROTONERGIC REGULATION OF GROWTH HORMONE SECRETION IN CATTLE

By

Roy Patrick Radcliff

Growth hormone is required for postnatal growth and galactopoiesis in ruminants. Secretion of growth hormone is controlled by hypothalamic hormones, which in turn are controlled by neurotransmitters, one of which is serotonin. Activation of serotonin receptors with quipazine induces secretion of growth hormone in cattle. However, the mechanism of quipazine-induced secretion of growth hormone is not known. My objective was to investigate the mechanism of quipazine-induced secretion of growth hormone in cattle. I hypothesized that thyrotropin releasing-hormone (TRH) mediates serotonin-induced secretion of growth hormone in cattle.

Using an in vitro perifusion culture system, I determined that quipazine did not induce secretion of growth hormone directly from anterior pituitary cells, nor were posterior pituitary cells mediating the effects of quipazine on secretion of growth hormone. Therefore, the effects of quipazine on secretion of growth hormone in vivo are not directly on the pituitary gland. With the perifusion culture system, I also determined that quipazine did not induce secretion of growth hormone releasing-hormone (GHRH) or somatostatin (SS) from bovine hypothalamic slices. These data indicate that quipazine-induced secretion of growth hormone in vivo is not mediated by GHRH or SS.

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I used a meal-fed steer model to investigate the possibility of two other hypothalamic peptides as mediators of quipazine-induced secretion of growth hormone. Quipazine induced secretion of growth hormone to similar magnitudes before versus after feeding. Pituitary adenylate cyclase-activating polypeptide (PACAP) induced secretion of growth hormone to greater magnitude before versus after feeding, indicating that PACAP probably does not mediate quipazine-induced secretion of growth hormone. Thyrotropin releasing-hormone induced secretion of growth hormone to similar magnitudes before versus after feeding, indicating that TRH might mediate quipazine induced secretion of growth hormone.

I injected meal-fed steers with 3 or 6 μg of 3,3',5-triiodo-L-thyronine (T_3) daily to increase negative feedback on the thyrotropic axis and down regulate TRH receptor expression on the pituitary gland and/or TRH synthesis and secretion from the hypothalamus. Injections of T_3 decreased endogenous secretion of 3,3',5, 5'-tetraiodo-L-thyronine without affecting basal secretion of growth hormone. Injections of T_3 did not affect GHRH- or TRH-induced secretion of growth hormone. These data indicate that injections of T_3 did not affect releasable amounts of growth hormone or TRH receptor expression on the pituitary gland. However, daily injections of T_3 inhibited but did not completely block quipazine-induced secretion of growth hormone.

In conclusion, TRH at least partially mediates serotonin-induced secretion of growth hormone in cattle.

This dissertation is dedicated to
James Edward "Uncle Ed" Norman.
A man who has always been, and will continue to be, an inspiration to me.

ACKNOWLEDGEMENTS

I would like to express my deepest and most sincere gratitude to my major professor, Dr. H. Allen Tucker. His patient guidance and strong commitment to graduate education is something I hope to pass on to the next generations of scientists. I was extremely fortunate to have such a wise and outstanding mentor throughout my entire graduate career. From him, I not only learned the lessons of science that will help me achieve my career goals, but also some lessons in life that will enable me to achieve my life goals. Thank you.

I'd also like to thank all the members of my guidance committee: Dr Donald Jump, Dr. Keith Lookingland, Dr. Dale Romsos, and Dr. Mike VanderHaar. Each member of my committee has taught me something either in the classroom or through discussions of my research and results.

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I would me amiss if I did not thank all the graduate students and friends in the Department of Animal Science. Many have helped me with experiments throughout my graduate carreer. In particular Mr. Kent Tjardes, Mr. Mike Peters,

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Mr. Tom Pilbeam and Dr. Ted Ferris. Our weekday and weekend excursions were very beneficial as stress management tools.

Finally, I would like to thank my family, especially my parents, Jack and Joan Radcliff. If every child had the love and support that I have had in life, I am sure the world would be a better place.

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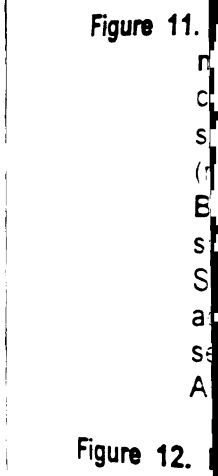

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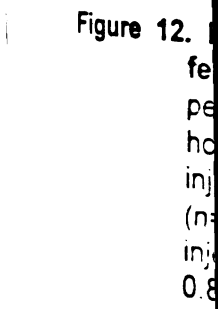

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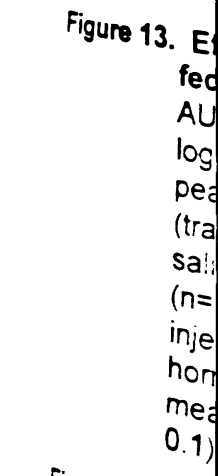

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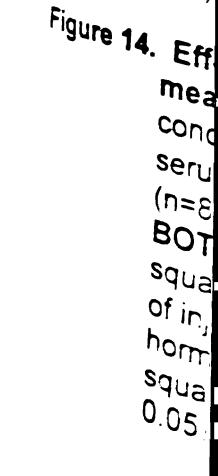

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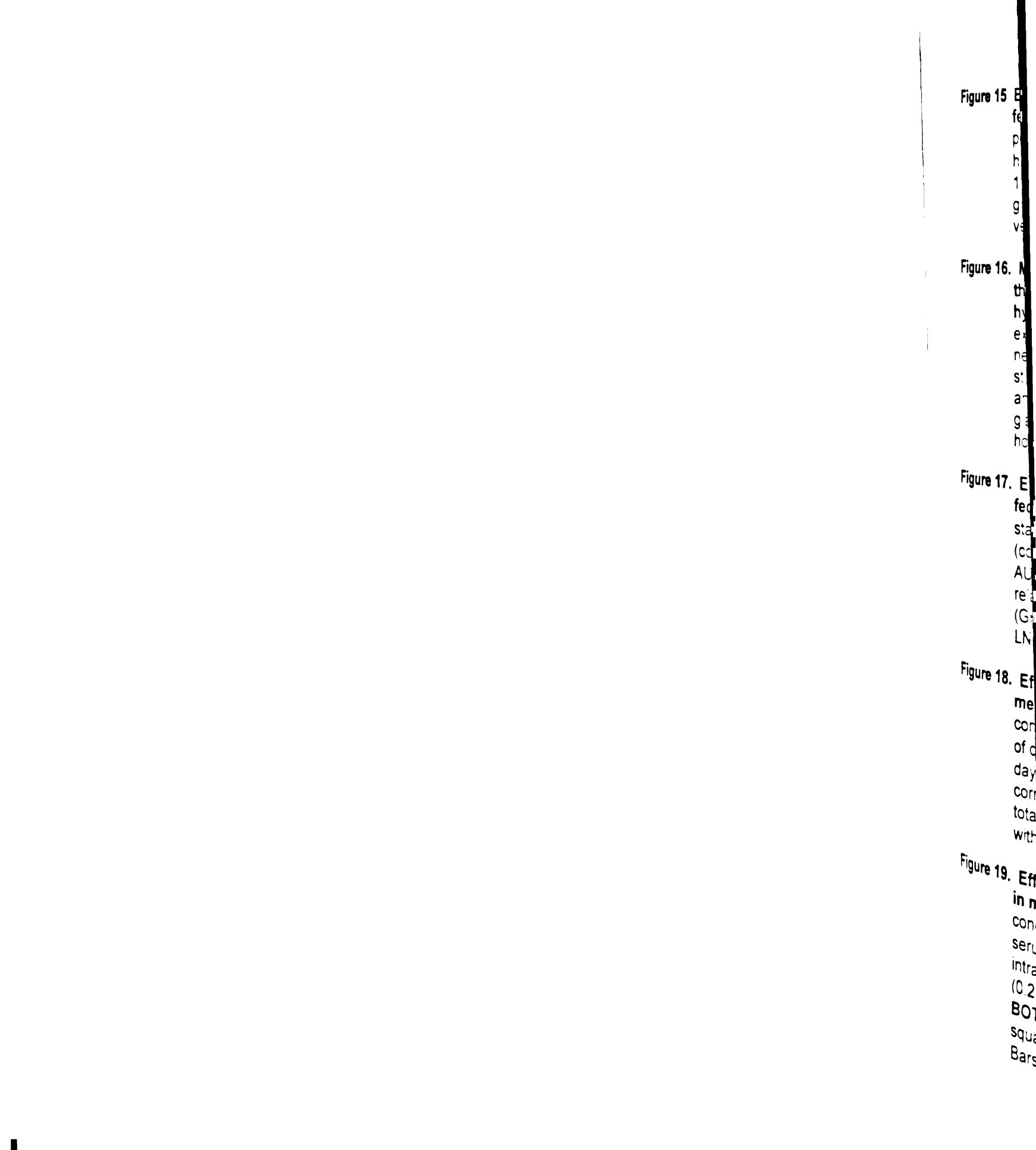

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INTRODUCTION

Animal agriculture provides nourishment to mankind through production of meat, milk and eggs. The ultimate goal of research in animal agriculture is to improve the production and production efficiency of animal agriculture. Through such research, many products have been developed that benefit all aspects of animal agriculture. For example, crops that are more nutritious have been developed to feed both man and animals. Development of products such as Lutalyse® (prostaglandin $f_{2\alpha}$) for estrus synchronization, Synovex S® (progesterone and estradiol benzoate implants) to improve growth performance in feedlot steers and Posilac® (recombinant bovine somatotropin) to improve milk production in dairy cattle all provide options to animal industry that can be used to improve production. Understanding the endogenous regulators of animal growth and development has aided scientists in developing the products and strategies that are used today to improve animal agriculture.

In cattle, growth hormone is the primary regulator of postnatal growth and milk production (Bauman et al., 1982; Bauman, 1992). Injecting cattle with exogenous growth hormone increases protein and decreases adipose deposition in the carcass (Eisemann et al., 1989; Binelli et al., 1995; Radcliff et al., 1996). Exogenous growth hormone also increases mammary development in heifers (Radcliff et al., 1996) and milk production in cows (Bauman, 1992). Thus, growth hormone is a valuable tool for improving production of meat and milk.

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Currently, exogenous growth hormone is only approved for use in lactating dairy cattle and requires a labor force to inject the cows every 2 weeks. Even if growth hormone was approved for use in growing animals the requirement for labor and pharmaceutical supplies would be cost prohibitive.

An alternative to administering exogenous growth hormone, would be to increase endogenous secretion of growth hormone. Basic research investigating the regulation of endogenous growth hormone secretion would allow development of other cost effective products or strategies to ultimately improve animal growth and development. The focus of this dissertation is the regulation of growth hormone secretion in cattle.

Secretion of growth hormone from the anterior pituitary gland seems to be controlled mainly by growth hormone-releasing hormone (GHRH), which stimulates, and somatostatin (SS), which inhibits secretion of growth hormone (Frohman et al., 1992). However, other hypothalamic peptides such as thyrotropin-releasing hormone (TRH) and pituitary adenylate cyclase-activating polypeptide (PACAP) may also play a role in regulating secretion of growth hormone in cattle (Vines et al., 1976; Vines, 1976; Vines et al., 1977; Hashizume et al., 1994). Since GHRH, SS, TRH and PACAP are all peptides, injection or implantation are the only routes of administration that will maintain their biological activity. Therefore, administration of GHRH, SS, TRH or PACAP would generate similar costs in labor and pharmaceuticals as administration of growth hormone. However, the endogenous secretion of these peptides is ultimately controlled by

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neurotransmitters that are not peptides, and these compounds may offer another less labor intensive means of manipulation.

Hypothalamic neurotransmitters such as norepinephrine and dopamine control secretion of GHRH and SS in cattle (West et al., 1997a; West et al., 1997b). Another neurotransmitter, serotonin, has also been implicated in controlling secretion of growth hormone in cattle (Gaynor et al., 1995; Gaynor et al., 1996). However, the mechanisms by which serotonin induces secretion of growth hormone is not known.

The overall goal of this dissertation was to investigate the mechanisms by which serotonin induces secretion of growth hormone in cattle. Specific objectives were to determine: 1) if activation of serotonin receptors directly induces secretion of growth hormone from bovine pituitary cells; 2) if activation of serotonin receptors affects secretion of GHRH and SS from bovine hypothalamic tissue; and 3) the roles of TRH and PACAP in mediating serotonin receptor agonist-induced secretion of growth hormone in cattle.

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REVIEW OF LITERATURE

I Characterization of Growth Hormone

Growth hormone is a 191 amino acid protein hormone that is synthesized and stored in specific cells in the anterior pituitary gland called somatotropes. Somatotropes make up approximately 50% of the cells found in the anterior pituitary gland (Thorner et al., 1998). Growth hormone plays a role in stimulation of growth as well as in a number of metabolic processes, such as hepatic IGF-I production and stimulation of hormone sensitive lipase in adipose tissue (Thorner et al., 1998). The size and amino acid sequence of growth hormone varies between species as well as within species. The majority of bovine growth hormone is synthesized as a 207 amino acid precursor and proteolytically cleaved to form the 191 amino acid protein (Woychik et al., 1982). Most variation within a species is due to alternative splicing of the mRNA. The gene coding for bovine growth hormone is approximately 1800 base pairs long. It is composed of 5 exons and 4 introns (Woychik et al., 1982). Variant forms of growth hormone and their biological activity have been reported in cattle (Hampson and Rottman, 1987; Krivi et al., 1989). However, the 191 amino acid form is the most abundant and most biologically active (Krivi et al., 1989).

Growth hormone is stored in secretory granules inside somatotropes and released in a pulsatile pattern into peripheral circulation by exocytosis (McCann and Ojeda, 1992). It is well established that secretion of growth hormone from the anterior pituitary gland is controlled by two hypothalamic neuropeptides.

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These neuropeptides are GHRH which induces, and SS which inhibits secretion of growth hormone (Thorner et al., 1998).

II Hypothalamic Regulation of Growth Hormone Secretion

A. Effects of GHRH and SS on Growth Hormone Synthesis and Secretion

Immunohistochemical experiments in cattle revealed a dense population of GHRH neuronal cell bodies located mainly in the arcuate nucleus of the hypothalamus with a smaller population located lateral and dorsal to the arcuate nucleus within and around the ventromedial nuclei (Leshin et al., 1994). The distribution of GHRH neurons in cattle is similar to that described in rats (Merchenthaler et al., 1984; Bloch et al., 1983). Terminals of GHRH neurons are located in the external zone of the median eminence (Leshin et al., 1994) juxtaposed to portal vessels. Norepinephrine induces release of GHRH in cattle (West et al., 1997a) which is transported via hypophysial portal blood to the anterior pituitary gland where it binds to specific receptors on somatotropes (Seifert et al., 1985; Velicelebi et al., 1986). Binding of GHRH to it's receptor activates the adenylate cyclase-cAMP-protein kinase A pathway via the stimulatory G_s protein (Mayo et al., 1995; Wong et al., 1995). Activation of this pathway accomplishes two things: it induces release of growth hormone from somatotropes by increasing intracellular free calcium (Holl et al., 1988) and induces transcription of growth hormone mRNA by phosphorylating c-AMP response element binding protein, which binds to DNA and induces transcription of specific genes (Barinaga et al., 1983).

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Cell bodies of SS neurons that terminate near portal vessels of the median eminence are located mainly in the periventricular nucleus of the hypothalamus of rats, sheep and cattle (Frohman et al., 1992; Leshin et al., 1994; Willoughby et al., 1995). In cattle, SS fibers and varicosities have also been reported in the arcuate nucleus (Leshin et al., 1994) indicating that SS may play a role in regulating secretion of GHRH. In fact, experiments with perifused bovine hypothalamic slices show that SS inhibits secretion of GHRH (West et al., 1997c). Dopamine induces release of SS in cattle (West et al., 1997b) and like GHRH, SS is transported to the anterior pituitary gland via portal vessels. Somatostatin inhibits GHRH-induced secretion of growth hormone in cultured bovine anterior pituitary cells in a dose dependant manner, but had no effect on basal secretion of growth hormone (Padmanabhan et al., 1987). The mRNA for five different SS receptors (SSTR1 – SSTR5) have been identified in rodent hypothalamus and anterior pituitary gland (Reisine et al., 1995; Beaudet and Tannenbaum, 1995). However, SSTR2 is thought to be the major receptor responsible for suppression of growth hormone secretion because it has greatest expression in the anterior pituitary gland of all the SS receptor subtypes. The ability of SSTR2 to bind specific SS analogs is positively correlated with a particular analog's ability to inhibit secretion of growth hormone (Reisine and Bell, 1995). Somatostatin inhibits activation of adenylate cyclase via G_i or G_o proteins thereby reducing cAMP and blocking the ability of GHRH to induce synthesis and secretion of growth hormone (Frohman et al., 1992).

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Growth hormone is secreted in a pulsatile manner in all animals studied, including cattle (Vasilatos and Wangsness, 1981; Wheaton et al., 1986; Kraicer et al., 1988). Because GHRH stimulates and SS inhibits secretion of growth hormone it is reasonable to speculate that release of growth hormone is dependant on the interaction of GHRH and SS. In fact, one hypothesis is that secretion of GHRH and SS are 180° out of phase (Tannenbaum and Ling, 1984). A pulse of growth hormone occurs when GHRH in portal blood is high and SS is low. Conversely, circulating concentrations of growth hormone are low when SS is high and GHRH is low in portal blood. There is one experiment conducted in rats that lends support to this hypothesis. However, concentrations of GHRH and SS were quantified in portal blood in one group of rats and concentrations of growth hormone in serum in another group (Plotsky and Vale, 1985). Thus, actually aligning pulses of growth hormone with peaks and troughs of GHRH and SS in the same group of animals was not done. Indeed, there is evidence that the regulation of growth hormone secretion is more complex, and this is described below.

In sheep, only 70% of growth hormone pulses occur coincident with or immediately following a pulse of GHRH. Furthermore, pulses of SS are not out of phase with GHRH pulses and do not correlate with troughs in growth hormone secretion (Frohman et al., 1990; Thomas et al., 1991). Normally, individual patterns of growth hormone secretion are random among a group of animals (Carlsson and Jansson, 1990; Wheaton et al., 1986). In calves, growth hormone secretion can be synchronized among a group of animals by restricting free

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B. Discovery

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access to feed for a fixed 2-h period each day (Moseley et al., 1988; Gaynor et al., 1993; Gaynor et al., 1995). In this meal-fed model, endogenous growth hormone secretion is pulsatile and relatively high shortly before feeding, decreases during feeding, and remains low for 2 to 3 h after feeding. Thus, the meal-fed model allows prediction as to when growth hormone will be high or low in a group of calves with reasonable accuracy. Using this model, McMahon and coworkers (2000) compared the activity of GHRH and SS neurons around the time of feeding. As concentrations of growth hormone decreased from shortly before to shortly after feeding, activity of GHRH neurons in the arcuate nucleus also decreased. However, activity of SS neurons in the periventricular nucleus also decreased during this period. These data suggest that the post-feeding suppression of growth hormone is not due to an increase in SS secretion into portal blood.

Another hypothalamic peptide, TRH, can also induce secretion of growth hormone from cultured bovine anterior pituitary cells (Smith and Convey, 1975). In fact, when activity of TRH neurons was examined in meal-fed calves, neuronal activity decreased as concentrations of growth hormone decreased from before to after feeding (Radcliff et al., 1999). These data suggested that hypothalamic regulation of growth hormone may involve more than just GHRH and SS.

B. Discovery, Characterization, and Localization of TRH

It has been recognized since the early 1950's that the hypothalamus exerts an important influence on regulation of the pituitary-thyroid axis. However,

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it was not until 1969 that TRH was isolated from ovine and porcine hypothalami and it's chemical structure determined (pyroGlu-His-Pro-NH_2) (Boler et al., 1969; Burgus et al., 1969). PreproTRH is 255 and 242 amino acids long in the rat and human, respectively (Lechan et al., 1986; Yamada et al., 1990). In the rat, five TRH peptides are proteolytically cleaved from the prepro-peptide, while the human prepro-peptide yields six TRH peptides. Regardless, the structure of TRH is conserved across species. Because TRH is a tripeptide, it was very easily synthesized. The early availability of synthetic TRH produced an initial rush of information on various aspects of it's localization and endocrinology.

The highest concentrations of TRH in the rat hypothalamus are in the median eminence (Jackson and Reichlin, 1974). Within the median eminence, however, TRH is only present in nerve fibers and terminals (Johansson and Hokfelt, 1980; Lechan and Jackson, 1982). There are no TRH neuronal cell bodies found in the median eminence. Neuronal cell bodies that stain positive for TRH are scattered throughout the hypothalamus and central nervous system (Lechan and Jackson, 1982; Hisano et al., 1986; Fliers et al., 1994). However, the majority of immunoreactive TRH neuronal cell bodies projecting to the median eminence are located in the parvocellular region of the paraventricular nucleus (Lechan and Jackson, 1982; Hisano et al., 1986; Fliers et al., 1994). Indeed, retrograde tracing of neurons with wheat germ agglutinin injected into the median eminence of rats revealed the paraventricular nucleus as the only area that had neurons that stained positive for both wheat germ agglutinin and pro-TRH (Kawano et al., 1991). Experiments utilizing ablation and deafferentation

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suggest that TRH neurons located in the paraventricular nucleus project laterally around the fornix before projecting medially through the lateral retrochiasmatic area, then caudally to terminate in the median eminence (Palkovits et al., 1982; Palkovits, 1984). Terminals in the median eminence are juxtaposed to portal vessels, which transport TRH to the anterior pituitary gland. In vitro experiments using synaptosomes isolated from sheep and rat hypothalami demonstrated that dopamine induces release of TRH (Bennett et al., 1975; Schaeffer et al., 1977). Histamine and serotonin have also been shown to induce release of TRH from cultured rat medial basal hypothalamus (Charli et al., 1978; Chen and Ramirez, 1981).

Thyrotropin-releasing hormone induces secretion of thyroid-stimulating hormone and prolactin from the anterior pituitary gland of humans (Bowers et al., 1971; Jacobs et al., 1973), rats (Mueller et al., 1973; Blake, 1974), sheep (Debeljuk et al., 1973; Fell et al., 1973), goats (McMurtry and Malven, 1974) and cattle (Schams, 1972; Convey et al., 1973; Vines et al., 1976). In addition, TRH induces secretion of growth hormone in pre- and post-pubertal cattle (Vines et al., 1976; Vines et al., 1977), pregnant cattle (Vines et al., 1977) and lactating cattle (Convey et al., 1973; Tucker et al., 1975; Bourne et al., 1977). Infusion of TRH for 6-h increased secretion of growth hormone for the entire infusion period (Tucker et al., 1975). Even though concentrations of growth hormone declined slightly during the infusion period, growth hormone was still three times greater than saline-infused controls at the end of the infusion period, and administration of a second heterologous stimulus further increased concentrations of growth

hormone. In fact, when TRH and GHRH are simultaneously administered to cattle, there is a synergistic increase in secretion of growth hormone (Hodate et al., 1985). These data indicate that GHRH and TRH may induce the secretion of growth hormone via different mechanisms, and combined with the fact that TRH induces secretion of growth hormone during various physiological states in cattle suggests that TRH may play a role in the normal hypothalamic regulation of growth hormone secretion in cattle.

C. Characterization and Signal Transduction Mechanism of TRH Receptors

The first evidence that TRH binds to a specific receptor was that tritium labeled TRH would competitively bind to plasma membranes isolated from both rat thyrotropic tumor cells (Grant et al., 1972) and bovine anterior pituitary cells (Labrie et al., 1972). Receptors for TRH on intact cells were first identified on a clonal rat pituitary cell line that secretes growth hormone and prolactin (GH_3) using tritium labeled TRH (Gourdji et al., 1973; Hinkle and Tashjian, 1973). Since then, TRH receptor binding has been demonstrated in the anterior pituitary gland, retina and throughout the central nervous system in rodents, ruminants, carnivores and fowl (Burt and Taylor, 1982; Taylor and Burt, 1982; Sharif et al., 1991). A recent report describes the presence of TRH receptor mRNA in somatotropes from the rat (Konaka et al., 1997). Using in situ hybridization, Konaka and coworkers found that 61% of the anterior pituitary cells stained positive for TRH receptor mRNA. Staining for growth hormone and prolactin in these same cells revealed that 62% and 31% of the cells that stained positive for TRH receptor mRNA were somatotropes and lactotropes, respectively. The

presence of TRH receptors on somatotropes and lactotropes may explain why TRH induces release of both growth hormone and prolactin in cattle (Vines et al., 1976; Vines, 1976; Vines et al., 1977).

The receptor for TRH has been cloned from mouse (Straub et al., 1990), rat (de la Pena et al., 1992; Sellar et al., 1993) and human tissue (Duthie et al., 1993). Comparison of the receptors from the rat, mouse and human show very high homology except for the COOH terminal tail (Duthie et al., 1993). The cloned receptors from mouse, rat and human tissues are all functional, and the differences in the COOH end have not produced any observable differences in function. The human gene encoding the TRH receptor has three exons and two introns with upstream promoter sequences for the glucocorticoid response element, activator protein-1 sites, cyclic-AMP response elements and the pituitary specific Pit-1 factor, indicating that TRH receptor expression is regulated by many different signals (Matre et al., 1999). The TRH receptor has seven transmembrane elements (Sellar et al., 1993) coupled to a G-protein (G_q or G_{11}).

Binding of TRH to the receptor causes activation of phospholipase Cβ1 and hydrolysis of phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-trisphosphate and diacylglycerol (Aragay et al., 1992; Hsieh and Martin, 1992; Svoboda et al., 1996). Inositol 1,4,5-trisphosphate induces release of intracellular calcium stores, which depolarize the cell and open voltage-gated calcium channels. The increase in intracellular calcium (from intracellular and extracellular sources) induces secretion of thyroid-stimulating hormone, prolactin or growth hormone depending on the cell type (Albert and Tashjian, 1984).

Diacylglycerol also serves as a second messenger. Diacylglycerol activates protein kinase C, which in turn phosphorylates many intracellular proteins (Kahn et al., 1998) that regulate a number of cellular functions including ion pumps, that either repolarize or maintain depolarization of secretory cells as well as transcription and translation of protein hormones.

D. Discovery, Characterization, and Localization of PACAP

Pituitary adenylate cyclase-activating polypeptide is another hypothalamic peptide that induces secretion of growth hormone from cultured bovine anterior pituitary cells (Hashizume et al., 1994). Pituitary adenylate cyclase-activating polypeptide is a 38 amino acid peptide that was originally isolated from ovine hypothalamic tissue (Miyata et al., 1989). The N-terminal portion, amino acids 1 – 28, of the peptide has 68% homology with porcine vasoactive intestinal peptide (VIP). The C-terminal portion, amino acids 29 – 38, has no homology with any known peptide. The name comes from the peptide's ability to increase cyclic-AMP in rat pituitary cells. There are two isoforms of PACAP. The more abundant form is the 38 amino acid peptide (PACAP-38); the second is a truncated form which is made up of the 27 N-terminal amino acids (PACAP-27) (Miyata et al., 1990). Immunohistochemical experiments with ovine hypothalamic tissue have shown that PACAP neuronal cell bodies are located in the paraventricular and supraoptic nuclei. Stained fibers were located throughout the hypothalamus with dense staining in the median eminence juxtaposed to portal vessels (Köves et al., 1990). As determined by radioimmunoassay, the greatest concentration of PACAP-38 was in the hypothalamus. However, PACAP-38 was also measured

in extracts from other regions of the brain and several peripheral organs. The shorter PACAP-27 was also measured in rat hypothalamic tissue but was found in much lesser amounts (Arimura et al., 1991). In fact, PACAP-38 was the major form measured in hypophysial portal blood, which contained significantly greater amounts of PACAP-38 than peripheral blood (Dow et al., 1994). Thus, the greatest source of PACAP is the hypothalamus.

In vitro, PACAP-38 induces secretion of growth hormone from bovine (Hashizume et al., 1994), porcine (Martínez-Fuentes et al., 1998b; Martínez-Fuentes et al., 1998a) and rat anterior pituitary cells (Velkeniers et al., 1994). Hashizume and coworkers (1994) also demonstrated that the stimulatory effects of PACAP-38 on growth hormone secretion from bovine anterior pituitary cells was blunted when treated with SS and PACAP simultaneously. Pituitary adenylate cyclase-activating polypeptide also increased growth hormone mRNA accumulation in porcine (Martínez-Fuentes et al., 1998b) and rat anterior pituitary cells in culture (Velkeniers et al., 1994). Experiments testing the effects of PACAP in vivo are limited. Exogenous PACAP induces secretion of growth hormone in male and lactating female rats (Rawlings and Hezareh, 1996). In sheep, however, infusion of 1 nmol/min of PACAP into the carotid artery for 10 min failed to induce secretion of growth hormone (Sawangjaroen and Curlewis, 1994). The inability of PACAP to induce secretion of growth hormone in ewes may be due to the small dose that was infused, or alternatively PACAP may not play a role in regulating secretion of growth hormone in sheep.

E. Characterization and Signal Transduction Mechanism of PACAP Receptors

Specific binding of PACAP was first demonstrated with ^{125}I labeled PACAP-27 in bovine brain membranes (Ohtaki et al., 1990). Unlabeled PACAP-27 and PACAP-38 displaced ^{125}I-PACAP-27 while VIP did not. After purification, the receptor showed a higher affinity for PACAP-38 than PACAP-27 (Ohtaki et al., 1993). To date, three distinct PACAP/VIP receptors (PVR1, PVR2 and PVR3) have been cloned. All three receptors have seven transmembrane domains and are linked to G-proteins. The three PACAP/VIP receptors can be divided into two types.

Type I receptors bind PACAP-38 and PACAP-27 with greater affinity than VIP. The PVR1 is a type I receptor that has been cloned from rat (Hosoya et al., 1993; Pisegna and Wank, 1993; Spengler et al., 1993), human (Ogi et al., 1993), and bovine (Miyamoto et al., 1994) tissue, and activates both adenylate cyclase and phospholipase C pathways. In the rat, six splice variants of PVR1 have been cloned, a short form called PVR1*s* and five variants with inserts in the putative third intracellular loop of the receptor (Spengler et al., 1993; Journot et al., 1995). There are two distinct 28 amino acid inserts (PVR1*hip* and PVR1*hop1*), a 27 amino acid insert that is identical to *hop1* except that it lacks a serine residue (PVR1*hop2*), and two combination inserts (PVR1*hiphop1* and PVR1*hiphop2*). All six variants activate adenylate cyclase and phospholipase C except PVR1*hip*, which only activates adenylate cyclase (Rawlings and Hezareh, 1996).

The PVR2 and PVR3 receptors are Type II receptors which bind PACAP-38, PACAP-27 and VIP with approximately equal affinity (Rawlings, 1994). The PVR2 receptor has been cloned from rat (Ishihara et al., 1992) and human tissue (Sreedharan et al., 1993; Couvineau et al., 1994; Gagnon et al., 1994). It probably corresponds to the classic VIP receptor. In cells transfected with the PVR2, both PACAP and VIP activate adenylate cyclase with equal potency (Ishihara et al., 1992; Sreedharan et al., 1993; Couvineau et al., 1994; Gagnon et al., 1994; Ciccarelli et al., 1994). The PVR3 receptor has been cloned from rat (Usdin et al., 1994; Lutz et al., 1993), mouse (Inagaki et al., 1994) and human tissue (Svoboda et al., 1994; Adamou et al., 1995). The PVR3 activates adenylate cyclase but not phospholipase C (Rawlings and Hezareh, 1996).

All three PVR receptors are found in the anterior pituitary gland. The PVR1 is the most abundant, PVR2 expression is lowest and PVR3 expression is intermediate (Rawlings and Hezareh, 1996). In culture, 89% of rat anterior pituitary cells that stained positive for growth hormone also bound biotinylated PACAP-38 (Vigh et al., 1993). Addition of a membrane permeable cAMP antagonist blocked the PACAP-induced influx of extracellular calcium (Canny et al., 1992; Rawlings et al., 1993). In addition, PACAP-38 failed to mobilize inositol 1,4,5-trisphosphate sensitive calcium stores (Rawlings and Hezareh, 1996) indicating that the phospholipase C pathway was not activated. These data suggest that PACAP receptors on somatotropes are Type II receptors (PVR2 and(or) PVR3) because they activate the adenylate cyclase- cAMP second messenger pathway, not the phospholipase C pathway. If the actions of PACAP-

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38 on somatotropes were mediated by Type I receptors then the phospholipase C pathway should have also been activated.

III Serotonergic Receptor Regulation of Growth Hormone Secretion

A. Characterization of Serotonergic Innervation of the Hypothalamus

Serotonin belongs to the biogenic amine family of neurotransmitters that are synthesized from amino acid precursors. Serotonin is also referred to as 5-hydroxytryptamine (5-HT). Serotonin is derived from the amino acid tryptophan by two enzymatic reactions. The first reaction, which is the rate limiting reaction, is catalyzed by tryptophan hydroxylase which puts a hydroxyl group in the 5 position on the indole ring of tryptophan to make 5-hydroxytryptophan. The second reaction, catalyzed by the enzyme 5-hydroxytryptophan decarboxylase, produces serotonin.

The serotonergic system in the brain originates from the midline of the brain stem and projects rostrally throughout the cortex, hippocampus and olfactory bulb, and caudally to the spinal cord. Serotonin has been implicated in sleep, aggression, sexual activity, appetite, learning, memory, depression, obsessive compulsive disorder, anorexia, bulimia, sudden infant death syndrome, Alzheimer's disease and schizophrenia. It is the single largest brain neurotransmitter system known and can be characterized as a "giant" neuronal system (Azmitia and Whitaker-Azmitia, 1991). Several experiments using autoradiography have demonstrated the dense serotonergic innervation of the hypothalamus (Conrad et al., 1974; Bobillier et al., 1976; Bobillier et al., 1979;

Azmitia and Segal, 1978). The serotonergic fibers within the hypothalamus originate from cell bodies in the raphe nuclei with their axons projecting to the hypothalamus through the medial forebrain bundle, ventricular tract, and arcuate tract (Azmitia and Segal, 1978). Additionally, cell bodies that accumulate labeled serotonin (Beaudet and Descarries, 1979) and stain positive for serotonin (Frankfurt et al., 1981) have been identified within the dorsomedial nucleus of the hypothalamus. However, their function has yet to be identified. Early retrograde tracing experiments using either the fluorescent dye true blue or fluorescent microspheres inserted into the paraventricular nucleus revealed labeled cell bodies in the dorsal raphe and median raphe nuclei as well as the medial lemniscus (Sawchenko et al., 1983; Petrov et al., 1992). In contrast, anterograde tracing experiments from the dorsal and medial raphe nuclei revealed very few or no labeled terminals in the paraventricular nucleus (Azmitia and Segal, 1978; Moore et al., 1978; Vertes and Martin, 1988; Vertes, 1991; Vertes et al., 1999). A more recent report by Larsen and coworkers (1996) combined retrograde and anterograde tracings to identify the origin of serotonergic terminals in the paraventricular nucleus. Injection of the tracer, cholera toxin subunit B, into the medial portion of the paraventricular nucleus or into the third ventricle resulted in labeled serotonergic neurons in the dorsal and medial raphe nuclei. In addition, injection of the anterograde tracer lectin PHA-L into either the dorsal or medial raphe nuclei resulted in labeled terminals in close proximity to the ependymal lining of the third ventricle with very few fibers and terminals labeled in the paraventricular nucleus. These data indicate that earlier retrograde tracing

experiments that attempted to identify neurons projecting to the paraventricular nucleus may have actually traced neurons projecting to the ependymal lining and was the result of the tracer leaking into the supraependymal layer. However, injection of cholera toxin subunit B into the lateral portion of the paraventricular nucleus resulted in labeling of neurons in raphe magnus and no labeling in the dorsal and medial raphe nuclei. Additionally, injection of lectin PHA-L into the raphe magnus resulted in anterograde labeling of a dense network of fibers and terminals throughout the paraventricular nucleus (Larsen et al., 1996). Thus, it appears that different nuclei in the hypothalamus receive serotonergic input from specific raphe nuclei. Regardless of the exact origin, the dense serotonergic innervation of the hypothalamus could influence the secretion of several hypothalamic hormones such as corticotrophin releasing-hormone, TRH, PACAP, GHRH and gonadotropin releasing-hormone. Specificity of serotonin's action may be a function of it's origin as well as it's receptor subtype present on the postsynaptic cell.

B. Characterization, Localization and Signal Transduction Mechanism of Serotonin Receptors

There are 14 known serotonin receptor subtypes which can be classified into seven separate subfamilies (5-HT_1 – 5-HT_7). Unless noted in this review each receptor has it's own gene. Due to the vast number of serotonin receptors, only those that are located in the hypothalamus are discussed in detail. In the 5-HT_1 family, there are five subtypes (5-HT_{1A}, 5-HT_{1B},5-HT_{1D}, 5-HT_{1E} and 5-HT_{1F}) all of which have seven transmembrane domains and inhibit adenylate cyclase

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via G-proteins (Barnes and Sharp, 1999). Of the five 5-HT_1 subtypes only 5-HT_{1E} has been reported in the hypothalamus.

Currently, there are no reports of radioligand binding specifically to 5-HT_{1E} receptors. However, in the human and monkey, 5-HT_{1E} mRNA has been isolated from entorhinal cortex and the caudate putamen with lesser amounts found in the amygdala and hypothalamus (Bruinvels et al., 1994b; Bruinvels et al., 1994a). Little is known about the physiological role of the 5-HT_{1E} receptor. However, when it is expressed in either murine Ltk-cells or human embryonic kidney cell line 293, treatment with serotonin inhibits forskolin-stimulated adenylate cyclase (Levy et al., 1992; McAllister et al., 1992), indicating that activation of 5-HT_{1E} receptors inhibits the adenylate cyclase second messenger system.

The 5-HT_2 receptor subfamily is made up of three receptor subtypes (5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C}). All three receptors have seven transmembrane domains coupled to phospholipase C, and they mobilize intracellular calcium (Barnes and Sharp, 1999). Of the 5-HT_2 receptors, 5-HT_{2B} is the only one located in the hypothalamus. However, I have also included some discussion on 5-HT_{2C} because of it's abundance in the choroid plexis which lines the third ventricle.

The 5-HT_{2B} receptor is restricted to a few locations. In the rat immunoreactivity appears only in the cerebellum, lateral septum, and dorsal hypothalamus (Duxon et al., 1997a). There are few data available on the physiological role of 5-HT_{2B} receptors. One report indicates that a 5-HT_{2B} agonist has anxiolytic effects (Duxon et al., 1997b).

Autoradiographic experiments have produced a detailed map of the 5-HT_{2C} receptor distribution in the brain. Firstly, very large amounts of the 5-HT_{2C} receptor are located in the choroid plexis. Binding sites are also widely distributed throughout the cortex, limbic system, and basal ganglia (Barnes and Sharp, 1999). Several behavioral and physiological responses have been attributed to 5-HT_{2C} receptor activation. These include hypolocomotion, hypophagia, anxiety, penile erections and hyperthermia (as reviewed by Koek et al., 1992). Recent reports indicate that 5-HT_{2C} receptors may inhibit secretion of noradrenaline and dopamine (Millan et al., 1998; Di Matteo et al., 1998). Administration of cyproheptadine (5-HT_2 receptor antagonist) to steers inhibits secretion of growth hormone (Gaynor et al., 1995)

The third serotonin receptor subfamily (5-HT_3) contains only one receptor subtype (5-HT_{3A}). However a splice variant (5-HT_{3AS}) has been reported (Hope et al., 1993). The 5-HT_3 receptor subfamily differs from the other serotonin receptors in that it is a ligand gated ion channel with homology to other ligand gated ion channels such as nicotinic, $GABA_A$ and glycine receptors (Barnes and Sharp, 1999). However, it is not present in the hypothalamus.

The fourth serotonin receptor family (5-HT_4) contains four receptor subtypes (5-$HT_{4(a)}$, 5-$HT_{4(b)}$, 5-$HT_{4(c)}$, and 5-$HT_{4(d)}$). Unlike the previously discussed subfamilies, 5-HT_4 receptors all appear to come from a single gene and are all splice variants with identical amino acid sequences up to Leu^{358} (Blondel et al., 1998). All four splice variants have seven transmembrane domains and activate adenylate cyclase (Gerald et al., 1995; Claeysen et al.,

1996; Van den Wyngaert et al., 1997), but none of the four variants have been identified in the hypothalamus.

Very little is understood about the fifth subfamily of serotonin receptors (5-ht_5). To date there is no direct evidence for the existence of a native 5-ht_5 receptor; therefore, it is still denoted with lower case letters. Initially the 5-ht_5 receptor was identified by screening mouse cDNA libraries and was named 5-ht_{5A} (Plassat et al., 1992). Later the 5-ht_{5B} receptor was identified by the same group (Matthes et al., 1993). The 5-ht_{5A} and 5-ht_{5B} receptors have since been identified from rat (Erlander et al., 1993; Wisden et al., 1993) and the 5-ht_{5A} from human (Rees et al., 1994) cDNA libraries.

The 5-ht_6 receptor was initially identified from it's cDNA sequence by two different groups (Monsma et al., 1993; Ruat et al., 1993a) and thus far is the only serotonin receptor in the 5-ht_6 subfamily. Both Northern blot analysis and in situ hybridization revealed that 5-ht_6 mRNA is mostly confined to the central nervous system (Monsma et al., 1993; Ruat et al., 1993a). The 5-ht_6 receptor has seven transmembrane regions and experiments using artificial expression systems indicate that it activates adenylate cyclase (Monsma et al., 1993; Ruat et al., 1993a; Boess et al., 1997). With the recent discovery of specific 5-ht_6 receptor ligands (Sleight et al., 1998) and demonstration of their ability to bind in the rat and porcine striatum (Boess et al., 1998), the functional roles of the 5-ht_6 receptor may soon be identified.

The last subfamily of serotonin receptors (5-HT_7) are encoded by a single gene but splice variants produce four receptors (5-$HT_{7(a)}$, 5-$HT_{7(b)}$, 5-$HT_{7(c)}$, and

5-$HT_{7(d)}$) (Heidmann et al., 1997; Jasper et al., 1997). The 5-HT_7 receptor has seven transmembrane domains and activates adenylate cyclase in both artificial expression systems and native tissues (Plassat et al., 1993; Ruat et al., 1993b; Heidmann et al., 1997; Heidmann et al., 1998). The 5-HT_7 receptor is expressed at relatively high amounts in the thalamus, hypothalamus and hippocampus (To et al., 1995; Stowe and Barnes, 1998). However, large tissue-specific expression of the different splice variants is not apparent. Artificial expression of the 5-HT_7 receptor has demonstrated it's ability to activate adenylate cyclase and increase intracellular calcium concentrations without inducing any phospholipase C activity (Baker et al., 1998), indicating that 5-HT_7 may play a role in neuronal activation.

The large number of different serotonin receptors discovered in the past decade in addition to the specific distribution of each of the receptor subtypes, lends support to the many functions that serotonin is believed to have in the central nervous system. Due to the complexity of the serotonergic system, it will be several more years before we understand all the physiological actions of serotonin.

C. Serotonergic Receptor Regulation of Growth Hormone Secretion

It is well established that the major control of growth hormone secretion is by two hypothalamic peptides, GHRH and SS. However, there is considerable evidence that GHRH and SS are not the only regulators of growth hormone secretion. Serotonin is one of several substances from the hypothalamus that may play a role in regulation of growth hormone secretion. However, reports

concerning the role of serotonin are conflicting, and the mechanism of action has yet to be delineated. For example, the serotonin receptor agonist, quipazine, which activates several different serotonin receptors, has been reported to inhibit secretion of growth hormone in chickens (Hall et al., 1983). In contrast, the same serotonin receptor agonist stimulates secretion of growth hormone in cattle. Additionally, the 5-HT_2/5-HT_{2C} receptor antagonist cyproheptadine inhibits secretion of growth hormone in cattle (Sartin et al., 1987; Gaynor et al., 1995; Gaynor et al., 1996). But, when bovine anterior pituitary cells in culture were treated with quipazine, there was no effect on secretion of growth hormone (Gaynor et al., 1996). In sheep, the 5-HT_{1A} receptor agonist buspirone inhibited secretion of growth hormone when administered either intravenously or intracerebroventricularly while cyproheptadine administration via either route had no effect (Spencer et al., 1991). In the rat, the nonselective serotonin receptor agonists quipazine and MK212 (6-chloro-2(piperazinyl)-pyrazine) and serotonin itself stimulate secretion of growth hormone (Vijayan et al., 1978; Arnold and Fernstrom, 1981; Willoughby et al., 1982; Willoughby et al., 1987; Chapman et al., 1993). However, when cultured rat anterior pituitary cells were treated with serotonin there was no effect on growth hormone secretion (Balsa et al., 1998). One investigator measured the amounts of serotonin and it's metabolite, 5-hydroxyindoleacetic acid (5-HIAA), in four different rat models that are commonly used to investigate growth hormone secretion. The ratio of 5-HIAA to serotonin (5-HIAA/5-HT) can be used as an index of neuronal activity. In all four rat models, serotonergic neuronal activity was positively correlated with

concentrations of growth hormone in serum (Smythe et al., 1982). These data suggest that the effects of serotonin on growth hormone secretion are species specific and mediated in the hypothalamus, not in the anterior pituitary gland.

There have been reports that in the rat, serotonin's action on growth hormone secretion is mediated via GHRH (Murakami et al., 1986), TRH (Chen and Ramirez, 1981), and PACAP (Yamauchi et al., 1996), all of which are possible mediators. But research to support any one of these hypothalamic hormones as the mediator of serotonin-induced growth hormone secretion in cattle is lacking. It has also been reported that there is an interaction of serotonergic and α_2-adrenergic systems with regards to growth hormone secretion. In rats, an intact serotonergic system had to be present in the hypothalamus for α_2-adrenergic receptor agonists to stimulate growth hormone secretion (Conway et al., 1990). Conversely, in cattle pre-treatment with an α_2-adrenergic receptor antagonist suppressed serotonin receptor agonist-induced secretion of growth hormone (Gaynor et al., 1996). One thing is clear from all of these data, we are not sure how serotonin induces secretion of growth hormone in vivo in cattle or rats.

CHAPTER 1

Serotonin Induced Secretion of Growth Hormone

INTRODUCTION

Serotonin has been implicated in the regulation of growth hormone secretion. However, the effects of serotonin seem to be species specific. Serotonin inhibits secretion of growth hormone in chickens and sheep (Hall et al., 1983; Spencer et al., 1991) but stimulates secretion of growth hormone in cattle and rats (Sartin et al., 1987; Gaynor et al., 1995; Gaynor et al., 1996; Smythe et al., 1975; Vijayan et al., 1978; Willoughby et al., 1982; Willoughby et al., 1987). The exact mechanisms by which serotonin exerts it's effects are not known. Serotonin does not induce secretion of growth hormone directly from cultured bovine or rat anterior pituitary cells (Gaynor et al., 1996; Balsa et al., 1998). However, Balsa and coworkers (1998) reported that serotonin induced secretion of growth hormone from dispersed rat anterior pituitary cells co-cultured with posterior pituitary cells in medium supplemented with corticosterone. Even though the increase in secretion of growth hormone was significant, the magnitude of response was quite small (approximately 3.9 versus 4.5 ng per plate for control and treated cells respectively, when incubated for 180 min). It is therefore possible that serotonin acts directly on the posterior pituitary gland to induce secretion of growth hormone from the anterior pituitary gland. In fact, Balsa and coworkers speculated that α melanocyte-stimulating hormone (αMSH) from the posterior pituitary cells was mediating the effects of serotonin on secretion of growth hormone from anterior pituitary cells. However, they could not confirm αMSH as the mediator. Thus, Balsa and coworkers were unable to

explain how serotonin caused secretion of growth hormone from anterior pituitary cells co-cultured with posterior pituitary cells.

Gaynor and coworkers (1996) tested the effects of quipazine on secretion of growth hormone from bovine anterior pituitary cells. However, the anterior pituitary cells were not co-cultured with posterior pituitary cells. Therefore, I proposed the mechanism of action depicted in Figure 1 and hypothesized that activation of serotonergic receptors would induce secretion of growth hormone from bovine anterior pituitary cells co-cultured with posterior pituitary cells. My objective was to determine if quipazine, which induces secretion of growth hormone in cattle (Sartin et al., 1987; Gaynor et al., 1996), would induce release of growth hormone from bovine anterior pituitary cells co-cultured with posterior pituitary cells.

MATERIALS AND METHODS

Perifusion of Bovine Anterior and Posterior Pituitary Cells

Two bovine pituitary glands were collected at a local abattoir, placed in ice cold calcium, magnesium free-Hank's balanced salt solution (pH 7.4, CMF-HBSS; Life Technologies Inc., Grand Island, NY) that had been sterilized by filtration with a 0.22 μm Sterivex®-GS filter (Millipore®, Bedford, MA) and transported back to the laboratory. Working inside a biological safety hood, using aseptic technique, each pituitary gland was dissected to separate anterior pituitary tissue from posterior pituitary tissue. In separate vials, anterior and

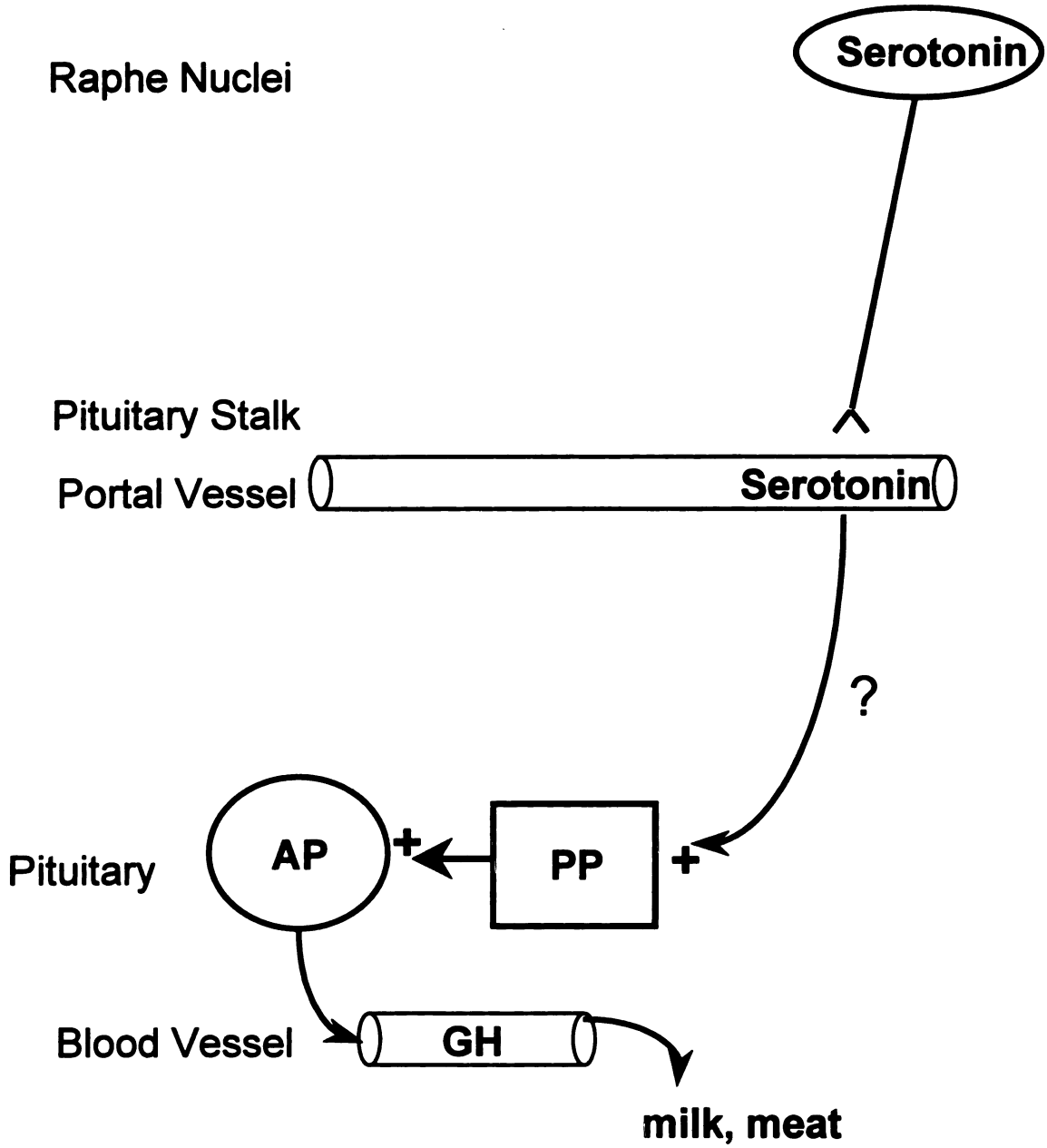


Figure 1. Proposed model for direct action of serotonin on the bovine pituitary gland. Serotonergic neurons (oval) project axons to the pituitary stalk with terminals juxtaposed to portal vessels which transport it to the pituitary gland. Serotonin acts at the posterior pituitary gland (PP) to induce secretion of growth hormone (GH) from the anterior pituitary gland (AP).

posterior pituitary tissues were individually enzymatically dispersed using techniques described previously for anterior pituitary tissue (Padmanabhan et al., 1987). Briefly, tissue was cut into approximately 1 mm cubes. The cubes were then placed in CMF-HBSS containing collagenase (3 mg/ml) and incubated in a shaking water bath at 37° C for 45 min. Cells were triturated after 15, 30 and 45 min of incubation. Collagenase solution and cells were then poured through sterile gauze into 50 ml conical tubes and centrifuged to pellet cells. Supernatant was removed, and cells resuspended and rinsed with CMF-HBSS three times.

Harvested anterior and posterior pituitary cells from each pituitary gland were suspended in separate vials with Dulbecco's modified Eagle's medium (DMEM; Life Technologies Inc.) with low glucose (1,000 mg/L) containing 1% newborn calf serum (Life Technologies Inc.), 25 m*M* N-2-hydroxyethylpiperazine-N'-2 ethanesulfonic acid (HEPES; Sigma Chemical Co., St. Louis, MO), penicillin (10 U/ml; Life Technologies Inc.), streptomycin (10 μg/ml; Life Technologies Inc.) and fungizone (0.25 μg/L; Life Technologies Inc.). Medium was also supplemented with 10^{-6} *M* hydrocortisone (H4001; Sigma Chemical Co. St. Louis, MO), a concentration similar to concentration of corticosterone used by Balsa and coworkers (1998). Concentration of cells was 10^6 cells/ml. After dispersion and resuspension, cell viability of both anterior and posterior pituitary cells was greater than 90% as determined by trypan blue exclusion.

The perifusion system of Gaynor and coworkers (1996) was modified for use in this experiment. Briefly, barrels of 3-ml syringes (Becton-Dickinson and Co.) were used as perifusion chambers. Rubber stoppers were placed on the

tips of syringes and a layer of sterile glass wool was inserted. Syringes were packed by gravity to a volume of 0.5 ml with sterile Cytodex I beads, which had been previously swollen in 0.9% sodium chloride solution. Cells from each pituitary gland were dispensed into chambers as a mixture of anterior (1 ml) and posterior (0.25 ml) pituitary cells or just anterior pituitary cells (1 ml). Chambers were then filled to 3 ml with DMEM and placed in a humidified atmosphere of 95% O_2-5% CO_2 at 37°C for 24 h to allow cells to adhere to the Cytodex I beads.

On the following day chambers were connected to peristaltic pumps, and the cells were perifused at 37°C with DMEM gassed with 95% O_2-5% CO_2 at a flow rate of 0.15 ml/min for 180 min. After this initial flushing period, chambers were ready for experimental protocols. Experimental treatments were dissolved in medium and sterilized through a 0.22 μm filter into separate vials that were connected to the peristaltic pump via a three-way stopcock. The stopcock allowed for easy switching from medium to medium containing treatments and back to medium. During experiments, 3-ml fractions of medium effluent were collected into 12 X 75 mm polypropylene tubes inside the incubator. Fractions were stored at -20°C until assayed. Radioimmunoassay was used to quantify concentrations of growth hormone in medium effluent as described previously (Gaynor et al., 1995).

Experimental Protocol

Six chambers containing 10^6 anterior pituitary cells only (three chambers per pituitary gland), and six chambers containing a mixture of anterior (10^6 cells) and posterior (0.25 X 10^6 cells) pituitary cells (three chambers per pituitary gland)

were assigned to one of two treatments in the first replicate (Table 1) and perifused with DMEM for 180 min. Medium effluent (3 ml) was collected at 20-min intervals, and all samples were assayed for growth hormone. Pre-treatment samples were collected at 20, 40 and 60 min. Treatments were either control medium or 10^{-6} *M* quipazine dissolved in medium. This dose of quipazine was the middle dose used by Gaynor and coworkers (1996) and was similar to the dose of serotonin used by Balsa and coworkers (1998). Cells were perifused with treatments from 61 to 80 min. Post-treatment samples were collected from 81 to 180 min. Cells were then perifused with medium for 120 min to allow them to recover from treatments imposed in the first replicate. Using the same cells, treatments were reversed and the experiment replicated. At the end of the second replicate, cells were perifused with DMEM containing 60 m*M* potassium chloride (KCl) from 181 to 200 min, then medium from 201 to 240 min. In viable secretory cells, KCl causes depolarization and an influx of extracellular calcium which induces secretion. These samples were assayed for growth hormone to determine somatotrope viability. Data from the first and second replicate were pooled and aligned by time of treatment for statistical analysis.

Statistical Analysis

Areas under the response curves (AUC) of growth hormone were calculated from 61 to 120 min using the trapezoidal rule and were subjected to analysis of variance using Mixed Models procedures of Statistical Analysis System (SAS;SAS, 1999). To account for differences in basal release between chambers, pre-treatment growth hormone values at 60 min were used as a

covariate in the statistical analysis. Cow, replicate, treatment and covariate were included in the model. Potassium chloride-stimulated release was calculated as the average concentration of growth hormone in medium samples collected from 200 to 240 min. Comparison between average baseline concentrations (average concentration from 20 to 60 min) and KCl-stimulated concentrations were conducted using Student's paired t-test (Gill, 1978).

Table 1. Treatment assignments for Experiment 1.

Chamber	Cow	Replicate 1	Replicate 2
1 (AP[a] cells only)	1	Control	Quipazine
2 (AP cells only)	2	Control	Quipazine
3 (AP cells only)	1	Control	Quipazine
4 (AP cells only)	2	Quipazine	Control
5 (AP cells only)	1	Quipazine	Control
6 (AP cells only)	2	Quipazine	Control
7 (AP and PP[b] cells)	1	Control	Quipazine
8 (AP and PP cells)	2	Control	Quipazine
9 (AP and PP cells)	1	Control	Quipazine
10 (AP and PP cells)	2	Quipazine	Control
11 (AP and PP cells)	1	Quipazine	Control
12 (AP and PP cells)	2	Quipazine	Control

[a] Anterior pituitary gland.
[b] Posterior pituitary gland.

RESULTS

Effect of Quipazine on Secretion of Growth Hormone from Dispersed Pituitary Cells

Profiles of concentrations of growth hormone in medium effluent are shown in Figure 2 (top panel). Perifusion of anterior pituitary cells with medium containing 10^{-6} *M* quipazine did not alter AUC for growth hormone when

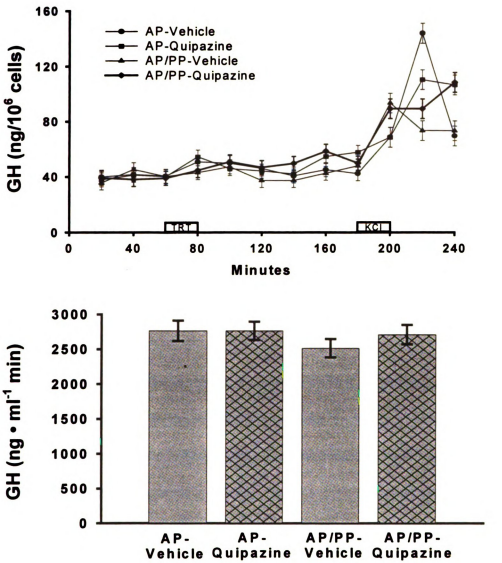


Figure 2. Effect of quipazine on secretion of growth hormone from dispersed bovine pituitary cells. TOP PANEL; Data presented are least squares mean concentrations (± pooled standard error of mean; SEM) of growth hormone in medium effluent from dispersed anterior pituitary gland cells perifused with vehicle medium (AP-vehicle; n=6) or quipazine (AP-Quipazine; n=6), and the combination of anterior and posterior pituitary gland cells perifused with vehicle medium (AP/PP-vehicle; n=6) or quipazine (AP/PP-Quipazine; n=6). Treatments were imposed from 61 to 80 min (indicated by box on X axis). All chambers were exposed to 60 m*M* KCl from 181 to 200 min (indicated by box on X axis) to test somatotrope viability. **BOTTOM PANEL;** Statistical analysis was performed on least squares mean AUC (± pooled SEM) for growth hormone calculated from 60 to 120 min and adjusted for pre-treatment concentrations using concentration of growth hormone at 60 min as a covariate. Least squares means were not different from each other.

compared with perifusion of anterior pituitary cells with medium alone ($P = 0.99$; Figure 2, bottom panel). Similarly, perifusion of a combination of anterior and posterior pituitary cells with medium containing 10^{-6} *M* quipazine did not alter AUC for growth hormone compared with anterior and posterior pituitary cells perifused with medium alone ($P = 0.64$; Figure 2, bottom panel). Perifusion with medium containing 60 m*M* KCl increased secretion of growth hormone from all chambers, indicating that somatotropes were viable at the end of the experiment.

DISCUSSION

In the current experiment, activation of serotonergic receptors did not alter secretion of growth hormone from either anterior pituitary cells alone or in co-culture with posterior pituitary cells. Our laboratory showed previously that the serotonin receptor agonist quipazine had no effect on secretion of growth hormone from dispersed bovine anterior pituitary gland cells (Gaynor et al., 1996). However, Balsa and coworkers (1998) hypothesized that posterior pituitary cells (melanotropes) mediated the effects of serotonin on secretion of growth hormone in the rat, and reported that serotonin increased secretion of growth hormone from rat anterior pituitary gland cells co-cultured with posterior pituitary gland cells in medium containing corticosterone.

Secretion of growth hormone is calcium-dependent. Glucocorticoids modulate calcium channel subunit mRNA expression as well as calcium currents in clonal GH_3 cells (Fomina et al., 1993; Fomina et al., 1996). However, Balsa

and coworkers reported the addition of corticosterone to media had no effect on serotonin-induced secretion of growth hormone from rat anterior pituitary cells alone. Similar results were obtained in the current experiment with bovine anterior pituitary cells.

Balsa and coworkers (1998) reported that for serotonin to induce secretion of growth hormone, rat anterior pituitary cells had to be co-cultured with posterior pituitary cells in medium supplemented with corticosterone, Therefore, suggesting that something from the posterior pituitary cells in combination with corticosterone was mediating the effects of serotonin on secretion of growth hormone. Balsa and co-workers initially speculated that αMSH may be a mediator. However, they did not observe an increase in αMSH secretion from posterior pituitary cells cultured in medium containing corticosterone and treated with serotonin. This led Balsa and coworkers to speculate that something that has yet to be identified was secreted from the posterior pituitary cells in response to serotonin, in combination with corticosterone, ultimately inducing the secretion of growth hormone from anterior pituitary cells.

I tested the effects of a nonspecific serotonin receptor agonist, quipazine, on secretion of growth hormone from bovine anterior pituitary cells alone and anterior pituitary cells co-cultured with posterior pituitary cells with added glucocorticoids. My results contrast those of Balsa and coworkers (1998) in that the serotonin receptor agonist did not induce secretion of growth hormone from anterior pituitary cells co-cultured with posterior pituitary cells in medium containing hydrocortisone. One possibility for the differing results is that the

mechanism of serotonin-induced secretion of growth hormone is different in cattle than in rats. Another possibility is that Balsa and coworkers conducted their experiment in a static culture system with cells that had been in culture for 72 h. I used a perifusion system to reduce the possibility of negative feedback on the secretory cells and more closely mimic an in vivo environment for the cells. I also used cells that had been in culture for only 24 h. It is also possible that by the time I conducted the experiment the posterior pituitary cells could have died. I tested the viability of the somatotropes with KCL at the end of the experiment, but I did not assay the KCL samples for αMSH, to confirm that the posterior pituitary cells were still viable.

The data from this experiment support the conclusion of Gaynor and coworkers (1996) in that the effects of serotonin receptor agonist on secretion of growth hormone in cattle are not directly on the anterior pituitary gland. In addition, these data demonstrate that in cattle the effects of quipazine on secretion of growth hormone are not mediated by posterior pituitary cells. Therefore, I rejected the model proposed in Figure 1. These data combined with those of Gaynor and co-workers led me to hypothesize that the effects may be mediated via hypothalamic hormones.

CHAPTER 2

Serotonin Induced Secretion of GHRH and SS

INTRODUCTION

In Chapter 1, I concluded that serotonin does not induce secretion of growth hormone directly from the pituitary gland in cattle, and speculated that hypothalamic hormones could be mediating serotonin's effects. The central serotonergic system is the largest brain system known with projections throughout the neural axis (Azmitia and Whitaker-Azmitia, 1991). The hypothalamus, which controls most anterior pituitary gland functions, contains large numbers of serotonergic terminals and fibers (Conrad et al., 1974; Bobillier et al., 1976; Bobillier et al., 1979; Azmitia and Segal, 1978). Several serotonin receptor subtypes have been identified in the hypothalamus (5-HT_{1E}, 5-HT_{2B}, 5-HT_{2C} and 5-HT_7) and have been reviewed by Barnes and Sharp (1999). Therefore, it is possible that hypothalamic hormones mediate serotonin's effects on secretion of growth hormone. However, exactly which hypothalamic hormone(s) mediate serotonin-induced secretion of growth hormone is not known.

Secretion of growth hormone is controlled mainly by GHRH which induces and SS which inhibits secretion (Thorner et al., 1998). Therefore, it would seem reasonable to speculate that the effects of serotonin on secretion of growth hormone could be mediated by GHRH and(or) SS. In fact, in the rat, GHRH has been implicated as a mediator of serotonin-induced secretion of growth hormone (Murakami et al., 1986). However, other hypothalamic peptides (TRH and PACAP) also have been implicated in serotonin-induced secretion of growth

hormone in the rat (Chen and Ramirez, 1981; Yamauchi et al., 1996). I hypothesized that GHRH and SS mediate serotonin-induced secretion of growth hormone in cattle. A proposed model of this hypothesis is depicted in Figure 3. My objective was to determine if activation of serotonin receptors would affect release of GHRH or SS from cultured bovine hypothalamic slices.

MATERIALS AND METHODS

Perifusion of Bovine Hypothalamic Slices

Brain tissue was obtained from cattle killed at a local abattoir within 10 min of death. Tissue was processed and cultured as described in a previous report (West et al., 1997a) with slight modification. Briefly, brains with attached, intact pituitary stalks were removed from the cranial cavity. A block of tissue containing the thalamus, hypothalamus and pituitary stalk was dissected from each brain with a scalpel. Coagulated blood and blood vessels were carefully removed with scissors and forceps from the pituitary stalk and dorsal surface of the block of tissue. The block was then bisected along the sagittal midline through the thalamus, third ventricle and pituitary stalk. Using a custom made jig, one 1-mm thick parasagittal slice was cut from the medial surface of each hemihypothalamus. All extrahypothalamic tissue was removed, and each hypothalamic slice was rinsed in ice-cold oxygenated CMF-HBSS. Each hypothalamic slice was then placed in a 5 ml perifusion chamber and filled with oxygenated, ice-cold minimal essential medium-α (pH 7.4, MEM-α; Life Technologies Inc.) supplemented with 25 m*M* HEPES, 0.1% bovine serum

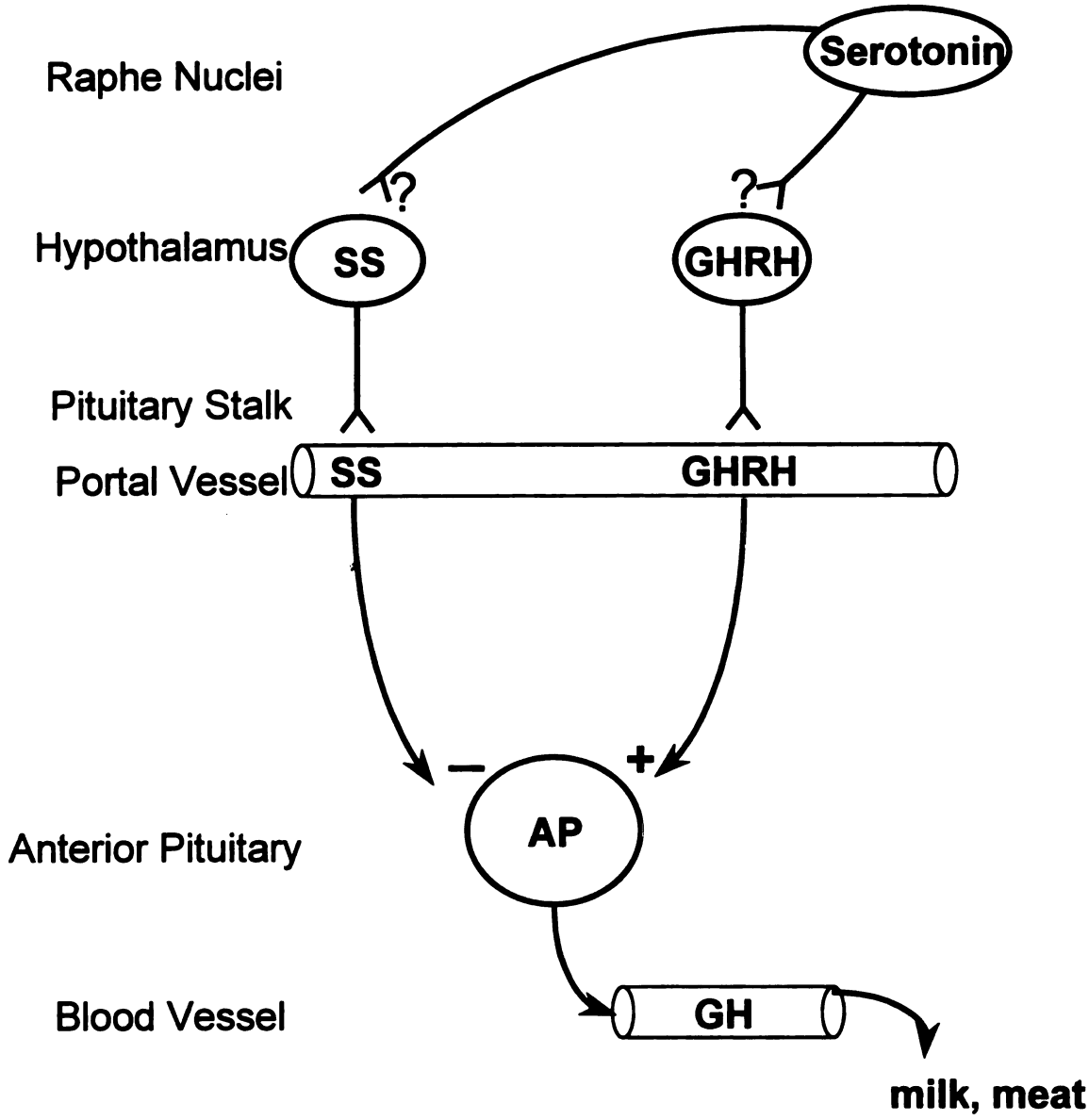


Figure 3 Proposed model for GHRH and SS mediating serotonin-induced secretion of growth hormone in cattle. Serotonergic neurons (oval) project axons to hypothalamus that terminate on GHRH and SS neurons (ovals). Terminals of GHRH and SS neurons are juxtaposed to portal vessels which transport them to the anterior pituitary gland (AP) where they stimulate and inhibit secretion of growth hormone, respectively.

albumin (A3859; Sigma Chemical Co.), penicillin (10 U/ml), streptomycin (10 μg/ml), ascorbic acid (1 g/L; Merck and Co. Inc., Rathway, NJ), bacitracin (25 mg/L; Sigma Chemical Co.), diprotin A (10 mg/L; Peninsula Laboratories, Belmont, CA) and soybean trypsin inhibitor (150 mg/L; Sigma Chemical Co.) that had been sterilized by filtration. Chambers were then placed in a Kool Mate™ Series Thermoelectric Cooler and Warmer (Igloo Products Corp., Houston, TX) that was maintained at approximately 4° C and transported back to the laboratory.

Chambers containing hypothalamic slices were randomly assigned to treatments and connected to peristaltic pumps in an incubator at 37°C. Slices were perifused with oxygenated MEM-α at a rate of 0.15 ml/min for 120 min to establish stable basal secretion of GHRH and SS. After this initial period, slices were used for experimental protocols. Treatments were dissolved in medium and filtered into separate vials as described in **Chapter 1: Perifusion of Bovine Anterior and Posterior Pituitary Cells.** During experiments, medium effluent was collected in 3-ml fractions inside the incubator at 37° C. Fractions were promptly removed from the incubator and stored at –20°C until assayed for GHRH and SS as described by West and coworkers (1997a).

Experimental Protocols

This experiment was conducted across two consecutive weeks and two trips to the local abattoir. Each week, sagittal hypothalamic slices (1 mm thick) were perifused with MEMα at a flow rate of 0.15 ml per min. Medium effluent

was collected at 20-min intervals for 180 min. Pre-treatment samples were collected at 20, 40 and 60 min. Treatments included control medium, or quipazine at either 10^{-6} M, 10^{-8} M, or 10^{-10} *M* dissolved in medium. Treatments were given from 61 to 80 min. Slices were then perifused with medium from 81 to 180 min. The treatments were then re-randomized and slices perifused with medium for 120 min to allow the tissue to recover. Beginning at 300 min the experiment was then repeated. At the end of the second replicate each week, slices were perifused with medium containing 60 m*M* KCl from 181 to 200 min, to depolarize the secretory cells and determine tissue viability, then medium for an additional 40 min. Growth hormone-releasing hormone and SS were measured in all samples. Data from the first and second replicates each week were pooled and aligned by time of treatment. Data from both weeks were also pooled (n = 3 and 5 per treatment for weeks one and two respectively).

Statistical Analysis

Areas under the response curve for GHRH and SS were calculated for the interval between 61 and 120 min using the trapezoidal rule and subjected to analysis of variance using Mixed Models procedures of SAS (SAS, 1999). To account for differences in pre-treatment concentrations, concentrations of GHRH and SS at 60 min were used as covariates in their respective statistical analysis. Week of experiment, replicate, treatment and covariate were included in the model. Potassium chloride-stimulated release was calculated as the average concentration of GHRH or SS in the three samples following KCl treatment.

Comparisons between average baseline concentrations (average concentration from 20 to 60 min), and KCl-stimulated concentrations were conducted using Student's paired t-test (Gill, 1978).

RESULTS

Effect of Quipazine on Secretion of GHRH and SS from bovine Hypothalamic Slices

Profiles of concentrations of GHRH and SS in medium effluent are presented in Figures 4 (top panel) and 5 (top panel) respectively. Compared with hypothalamic slices perifused with medium alone, perifusion with medium containing 10^{-10} *M* of quipazine, increased AUC for GHRH ($P = 0.02$; Figure 4 bottom panel). However, higher doses of the quipazine (10^{-6} and 10^{-8} M) did not increase AUC for GHRH.

Perifusion of hypothalamic slices with medium containing quipazine did not affect AUC for SS compared with hypothalamic slices perifused with medium alone (Figure 5; bottom panel). All slices responded to perifusion with medium containing 60 m*M* KCl by releasing both GHRH and SS, indicating that the tissue was viable at the end of the experiment.

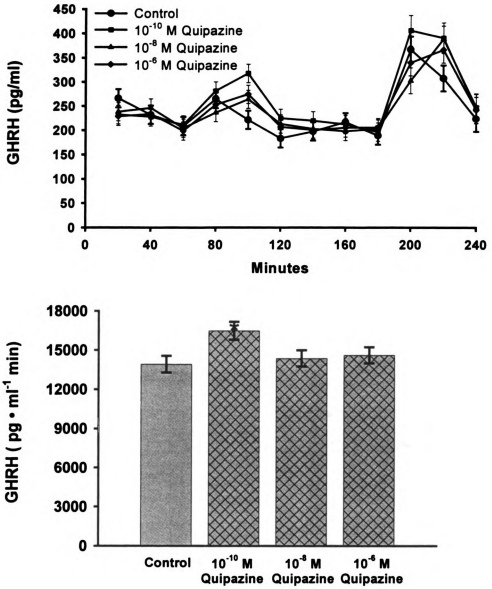


Figure 4. Effect of quipazine on secretion of GHRH from bovine hypothalamic slices. TOP PANEL; Data presented are least squares mean concentrations (± pooled standard error of mean; SEM) of GHRH in medium effluent from bovine hypothalamic slices perifused with control medium (control; n=8), 10^{-6} *M* quipazine (n=8), 10^{-8} *M* quipazine (n=8), or 10^{-10} *M* quipazine (n=8). Treatments were imposed from 61 to 80 min (indicated by the box on the X axis) and slices were exposed to 60 m*M* KCl from 181 to 200 min (indicated by the box on the X axis) to test tissue viability. **BOTTOM PANEL;** Statistical analysis was performed on least squares mean AUC (± pooled SEM) for GHRH calculated from 61 to 120 min and adjusted for pre-treatment concentrations using the concentration of GHRH at 60 min as a covariate. Quipazine at 10^{-10} *M* induced a greater AUC than the AUC of controls ($P = 0.02$).

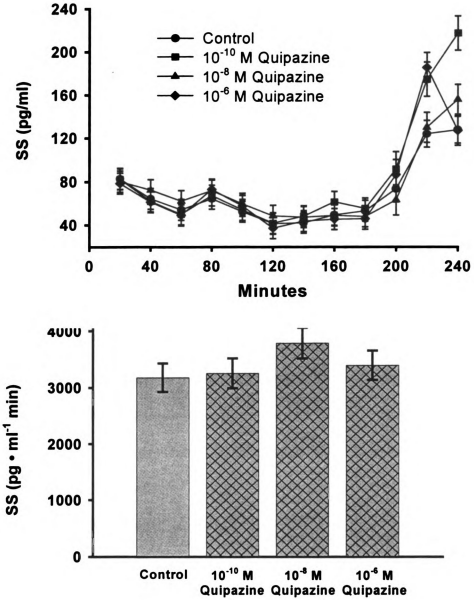


Figure 5. Effect of quipazine on secretion of SS from bovine hypothalamic slices. TOP PANEL; Data presented are least squares mean concentrations (± pooled standard error of mean; SEM) of SS in medium effluent from bovine hypothalamic slices perifused with vehicle medium (control; n=8), 10^{-6} *M* quipazine (n=8), 10^{-8} *M* quipazine (n=8), or 10^{-10} *M* quipazine (n=8). Treatments were imposed from 61 to 80 min (indicated by the box on the X axis) and slices were exposed to 60 m*M* KCl from 181 to 200 min (indicated by the box on the X axis) to test tissue viability. **BOTTOM PANEL;** Statistical analysis was performed on least squares mean AUC (± pooled SEM) for SS calculated from 61 to 120 min and adjusted for pre-treatment concentrations using the concentration of SS at 60 min as a covariate. Least squares means were not different from controls.

DISCUSSION

Hypothalamic control of growth hormone secretion is mediated mainly by GHRH and SS (Thorner et al., 1998). In this experiment, I tested the hypothesis that GHRH and (or) SS mediate the effects of serotonin on growth hormone secretion in cattle. The serotonin receptor agonist, quipazine, at the lowest dose tested (10^{-10} M), significantly increased AUC for GHRH compared with controls, indicating an increase in GHRH release. However, higher doses of quipazine had no effect on AUC for GHRH compared with controls. Thus, the response in secretion of GHRH was not a typical dose response. In addition, all slices, including controls, demonstrated a slight increase in GHRH and SS secretion during the time that treatments were imposed (Figures 4 and 5). In the current experiment, activation of serotonin receptors with 10^{-10} *M* quipazine increased AUC 18% over that of controls. This increase is very small compared to other receptor agonists that induce secretion of GHRH.

In past experiments using a similar culture system, the α_2-adrenergic receptor agonists clonidine and guanabenz each increased secretion of GHRH in a dose responsive manner. The minimal dose of each agonist required to increase AUC of GHRH was 10^{-6} M. Additionally, clonidine and guanabenz at the highest dose tested (10^{-4} M) each increased AUC of GH 2-fold compared with controls (West et al., 1997a). Therefore, it is possible that the small increase in GHRH secretion induced by 10^{-10} *M* quipazine in the current experiment was merely an artifact of the culture system.

The increase in GHRH secretion in vitro contrast with the effects of quipazine and GHRH on secretion of growth hormone in vivo. For example, a previous report using a meal-fed steer model (feed available for 2 h each day) shows that injection of quipazine either 1 h before feeding or 1 h after feeding induced large increases in concentrations of growth hormone in serum (Gaynor et al., 1995). Furthermore, the increases were of similar magnitude before versus after feeding. In contrast, injection of GHRH induces a much greater increase in concentrations of growth hormone in serum before compared with after meal-feeding of steers (Moseley et al., 1988; McMahon et al., 2000). If GHRH is the mediator of serotonin-induced secretion of growth hormone, then GHRH-induced secretion of growth hormone would be expected to be of a similar magnitude before and after meal-feeding. The serotonin receptor agonist that I used in the current experiments was biologically active because the same lot of material induced secretion of growth hormone in vivo (**Chapter 3**).

In conclusion, serotonin receptor agonist-induced secretion of growth hormone in cattle is not mediated via GHRH or SS from the hypothalamus. Therefore, I rejected the model proposed in Figure 3.

CHAPTER 3

Role of TRH and PACAP in Serotonergic Regulation of Growth Hormone Secretion

INTRODUCTION

In Chapters 1 and 2, I concluded that serotonin receptor agonist-induced secretion of growth hormone in vivo was neither a direct effect of the agonist on the pituitary gland nor mediated via change in secretion of GHRH or SS from the hypothalamus. However, two other hypothalamic neuropeptides have been implicated in serotonin-induced secretion of growth hormone. Chen and Ramirez (1981) reported that serotonin induced secretion of TRH from superfused rat hemi-hypothalami in a dose responsive manner. However, in cattle, hemi-hypothalami are too large to culture as one piece . To overcome this problem, slices of bovine hypothalamic tissue (600 to 1000 μm thick) have been cultured and medium effluent assayed for GHRH and SS. However, TRH neuronal cell bodies are located in the paraventricular nucleus with axons projecting laterally around the fornix then dorsally through the retrochiasmatic area to the median eminence. Due to the course of TRH axons from the paraventricular nucleus to the median eminence, it is impossible to cut a slice thin enough to maintain tissue viability while maintaining axonal integrity. Therefore, experiments similar to Chen and Ramirez (1981) could not be conducted with bovine hypothalami. However, in this chapter, I describe in vivo experiments designed to test the possibility that TRH mediates serotonin-induced secretion of growth hormone.

A second hypothalamic neuropeptide that may mediate serotonin-induced secretion of growth hormone is PACAP. Pituitary adenylate cyclase-activating polypeptide increases secretion of growth hormone from cultured bovine anterior

pituitary cells (Hashizume et al., 1994). Evidence for PACAP's role in serotonergic regulation of growth hormone comes from a report by Yamauchi and coworkers (1996). In rats, intravenous injection of PACAP induced a dose responsive increase in concentrations of growth hormone in plasma. The PACAP-induced increase in growth hormone secretion was blocked with a truncated form of PACAP called (PACAP (6-38)), which is a PACAP receptor antagonist (Robberecht et al., 1992). Prior injection with PACAP (6-38) also blocked 5-hydroxytryptophan- (serotonin precursor) induced growth hormone secretion in these rats. Thus, blockade of PACAP receptors inhibited serotonin-induced secretion of growth hormone indicating that PACAP could be mediating serotonin-induced secretion of growth hormone.

To determine if either TRH or PACAP were possible mediators of serotonin-induced secretion of growth hormone in cattle (Figure 6), I used the meal-fed steer model. In this model, growth hormone secretion is synchronized among a group of steers by allowing them ad libitum access to feed for only 2 h each day. Growth hormone-releasing hormone-induced secretion of growth hormone is suppressed 2 h after feeding compared with 2 h before feeding (Moseley et al., 1988; McMahon et al., 2000). In contrast, Gaynor and coworkers (1996) reported that injection of a serotonin receptor agonist either 1 h before or 1 h after feeding induced quantitatively similar increases (AUC and peak responses) in concentrations of growth hormone in serum. Therefore, if either TRH or PACAP is the mediator of serotonergic regulation of growth hormone, then their administration shortly before feeding would be expected to increase

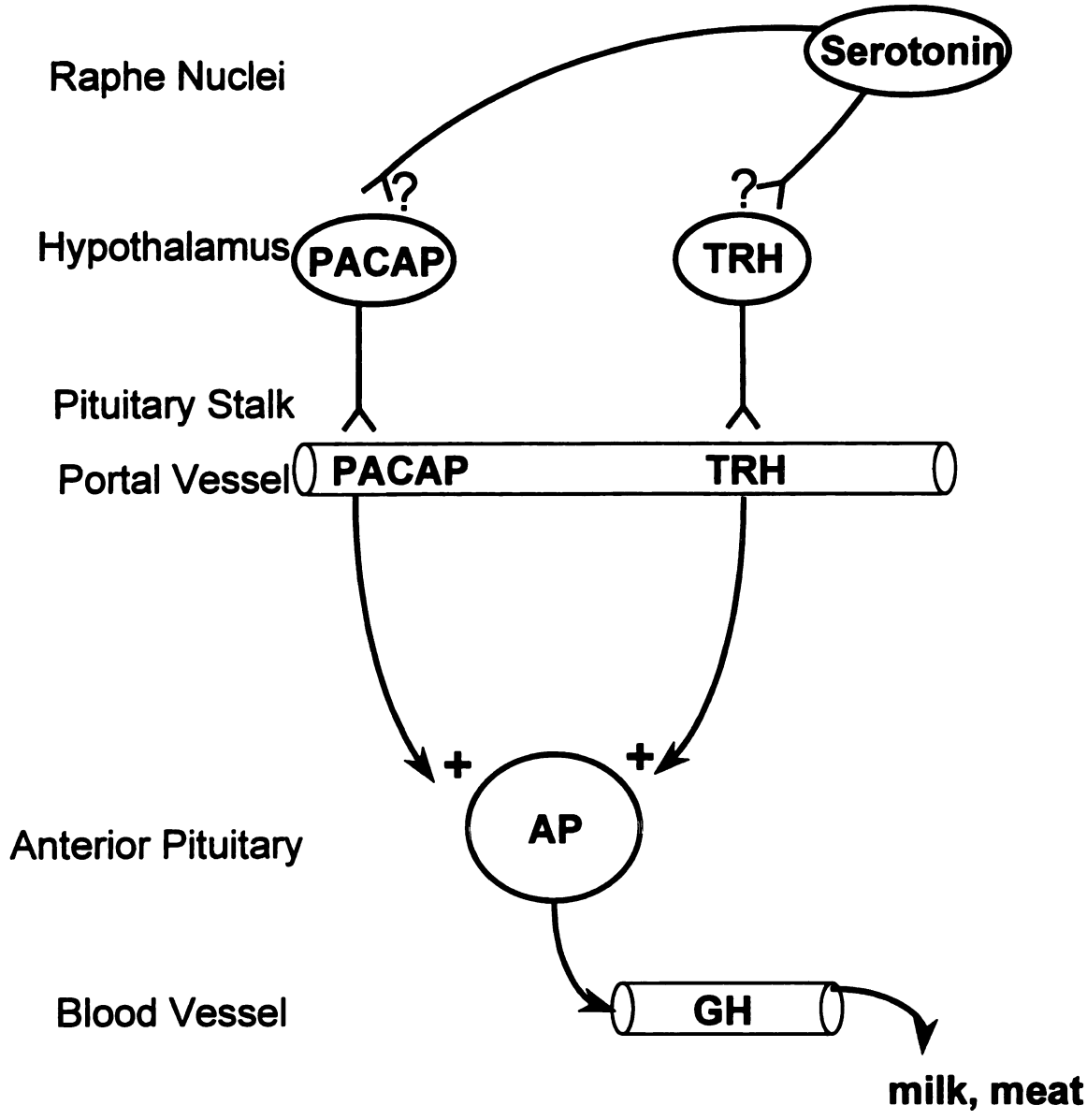


Figure 6. Proposed model for TRH and PACAP mediating serotonin-induced secretion of growth hormone in cattle. Serotonergic neurons (oval) project axons to hypothalamus that terminate on TRH and PACAP neurons (ovals). Terminals of TRH and PACAP neurons are juxtaposed to portal vessels which transport them to the anterior pituitary gland (AP) where they stimulate secretion of growth hormone.

concentrations of growth hormone in serum to a magnitude similar to their administration after feeding. I hypothesized that injection of TRH or PACAP before and after feeding would induce secretion of growth hormone to similar magnitudes. Thus, my objectives were to determine: 1) if activation of serotonin receptors with a nonspecific agonist would induce similar increases in growth hormone secretion before and after feeding to confirm the work of Gaynor and coworkers (1996), 2) a dose of TRH that would maximally stimulate secretion of growth hormone before feeding, 3) if the dose of TRH determined in objective 2 would induce secretion of growth hormone to similar magnitudes before and after feeding, 4) a dose of PACAP that would maximally induce secretion of growth hormone before feeding and 5) if the dose of PACAP from objective 4 would stimulate growth hormone secretion to similar magnitudes before and after feeding.

MATERIALS AND METHODS

Management of Animals

Holstein steer calves were housed in rooms maintained at 20 ± 1° C with 18 h of light and 6 h of dark each day. Steers were fed a pelleted diet balanced to meet nutrient requirements (NRC, 1989) that contained 18% crude protein and 19.6% acid detergent fiber (Land O'Lakes, Indianapolis, IN). Beginning at 8 to 10 weeks of age, steers were adapted to meal-feeding over a 14-d period by gradually decreasing the amount of time feed was offered from 24 h to 2 h each

day. Thereafter, steers were allowed free access to feed from 1000 to 1200 h each day. Water was available at all times. Steers were maintained on the meal-feeding regimen for at least 14 d before an experiment was conducted. Steers were weighed once a week for calculating drug doses.

Blood Sample Collection and Processing

Steers were fitted with indwelling jugular catheters (18 Ga Micro-renethane®; Braintree Scientific Inc., Braintree, MA) at least 18 h before the experiment began. A sterile solution of 3.5% sodium citrate was used to maintain catheter patency. Blood samples (8 ml) were collected with 10 ml syringes (Becton Dickenson & Co., Rutherford, NJ) and catheters immediately flushed with 3.5% sodium citrate. Blood was transferred to 16 by 100 mm glass tubes and stored at room temperature for 6 h and then at 4° C for 18 h. Blood samples were then centrifuged at 1500 X g for 25 min and serum was harvested and stored at –20° C until assayed. Concentrations of growth hormone in serum were determined by radioimmunoassay as described previously (Gaynor et al., 1995).

Experimental Protocols

Experiment 1 was designed to test the effect of quipazine before and after feeding on concentrations of growth hormone in serum. Meal-fed Holstein steers (21 ± 0.6 weeks old, 131 ± 8 kg BW) were randomly assigned to receive 0.2 mg quipazine/kg BW (Gaynor et al., 1995) intravenously 1 h before feeding (0900 h; n=4) or 1 h after feeding (1300 h; n=4) in a crossover design. Blood samples were collected at 0800, 0820, 0840, 0900, 0905, 0910, 0915, 0920, 0930, 0940

and 1000 h from steers injected with quipazine at 0900 h. Blood samples were collected at 1200, 1220, 1240, 1300, 1305, 1310, 1315, 1320, 1330, 1340 and 1400 h from steers injected with quipazine at 1300 h. The following day treatments were reversed between the two groups of steers. Each steer served as its own control by comparing pre-treatment with post-treatment concentrations of growth hormone in serum.

Experiment 2 was a TRH dose-response experiment to determine the minimal dose of TRH that would induce maximal secretion of growth hormone. Six meal-fed Holstein steers (13.2 ± 0.5 weeks old, 100 ± 5 kg BW) were used in a 6 X 6 Latin Square design, balanced for residuals, to test the effects of six doses of TRH (0, 0.1, 0.3, 1, 3 and 10 μg TRH/kg BW) on secretion of growth hormone on six different days. Each steer received each treatment one time and treatment days were 48 h apart. On each treatment day, TRH (Sigma Chemical CO., St. Louis, MO) was dissolved in 0.9% saline solution, and each steer received it's assigned dose of TRH intravenously at 0900 h (before feeding). Blood samples were collected at 0800, 0820, 0840, 0900, 0905, 0910, 0915, 0920, 0930, 0940 and 1000 h.

Experiment 3 was conducted to test the effect of TRH injection before and after feeding on concentrations of growth hormone on serum. Dose of TRH used was 0.3 μg/kg BW as determined in the second experiment. This third experiment was conducted in two replicates with eight Holstein steers per replicate. In replicate one, steers averaged 20 ± 0.5 weeks of age and 156 ± 8 kg BW. In replicate two, steers averaged 15 ± 0.6 weeks of age and 100 ± 6 kg

BW. Each replicate was a crossover design with steers randomly assigned to receive an intravenous injection of TRH either 1 h before feeding (0900 h) or 1 h after feeding (1300 h) on the first treatment day. Times of TRH administration were reversed the following day. Blood samples were collected at 0800, 0820, 0840, 0900, 0905, 0910, 0915, 0920, 0930, 0940 and 1000 h from steers injected with TRH at 0900 h, and 1200, 1220, 1240, 1300, 1305, 1310, 1315, 1320, 1330, 1340 and 1400 h from steers injected with TRH at 1300 h.

Experiment 4 was a PACAP-38 dose-response experiment conducted in two replicates to determine the minimal dose of PACAP-38 that would maximally stimulate secretion of growth hormone. Each replicate used five Holstein steers in a 5 X 5 Latin Square design to test the effects of five doses of PACAP-38 (Synpep Corp., Dublin, CA) on secretion of growth hormone. The first replicate tested doses of 0, 0.1, 0.3, 1, and 3 μg PACAP-38/kg BW. Steers in replicate one averaged 14 ± 0.6 weeks of age and 96 ± 3 kg BW. Because the highest dose of PACAP-38 (3 μg/kg BW) was the only dose that increased concentrations of growth hormone in serum, I repeated the experiment with doses of 0, 1, 3, 10 and 10 μg PACAP-38/kg BW. The 10 μg PACAP-38/kg BW dose was used twice to complete the 5 X 5 Latin Square which provided an n of 10. Steers in replicate two averaged 17 ± 0.5 weeks of age and 111 ± 4 kg BW. In both replicates, treatment days were 48 h apart and on each treatment day PACAP-38 was dissolved in 0.9% saline solution and respective treatments were injected intravenously at 0900 h. Blood samples were collected at 0800, 0820,

0840, 0900, 0905, 0910, 0915, 0920, 0930, 0940 and 1000 h. Data from replicates 1 and 2 were pooled for final statistical analysis.

Experiment 5 was conducted to determine the effect of PACAP-38 administered 1 h before and 1 h after feeding on secretion of growth hormone. Eight Holstein steers (20.5 ± 1 week of age and 145 ± 9 kg BW) were used in a crossover design where they were randomly assigned to receive an injection of PACAP-38 (3 μg/kg BW, determined in Experiment 4) either before or after feeding on the first day of sampling. Treatments were reversed on the second day of treatment. Blood samples were collected at 0800, 0820, 0840, 0900, 0905, 0910, 0915, 0920, 0930, 0940 and 1000 h from steers injected with PACAP at 0900 h, and 1200, 1220, 1240, 1300, 1305, 1310, 1315, 1320, 1330, 1340 and 1400 h from steers injected with PACAP-38 at 1300 h.

Statistical Analysis

In Experiments 1, 2 and 3, AUCs of growth hormone were calculated for 60 min following treatment using the trapezoidal rule. In Experiment 4 and 5, AUCs were calculated for 40 min following treatment because growth hormone secretion had returned to basal levels. In all five experiments, the highest concentration of growth hormone in serum during the 60 min period following treatment was determined as the peak concentration of growth hormone. Both AUC and peak concentrations were subjected to analysis of variance using Mixed Models procedures of SAS (SAS, 1999). In Experiments 3 and 4, data for AUCs and peak concentrations of growth hormone were transformed by natural logarithm (LN) to achieve homogeneous variance and normality. To account for

differences in pre-treatment concentrations of growth hormone in serum in all experiments, concentrations of growth hormone in serum immediately before treatment were used as covariates in the statistical analysis. The statistical model for Experiments 1 and 2 included the covariate, animal, treatment and sampling day. In Experiment 3, the model included the covariate, animal, replicate, treatment, sampling day, and interactions of experiment by sampling day and experiment by treatment. The statistical model for Experiment 4 included the covariate, replicate, animal within replicate, day within replicate and treatment. The statistical model for Experiment 5 included the covariate, animal and treatment. Sampling day was not significant ($P > 0.25$); therefore, it was removed from the statistical analysis to add another degree of freedom to the error term.

RESULTS

Effect of Quipazine Before and After Feeding on Secretion of Growth Hormone (Experiment 1)

All serum samples from this experiment were assayed for growth hormone in a single assay with an intra-assay coefficient of variation of 11.6%. Basal secretion of growth hormone was greater before feeding than after. Injection of quipazine increased concentrations of growth hormone in serum both before and after feeding ($P < 0.05$; Figure 7). Furthermore, injection of quipazine 1 h after feeding

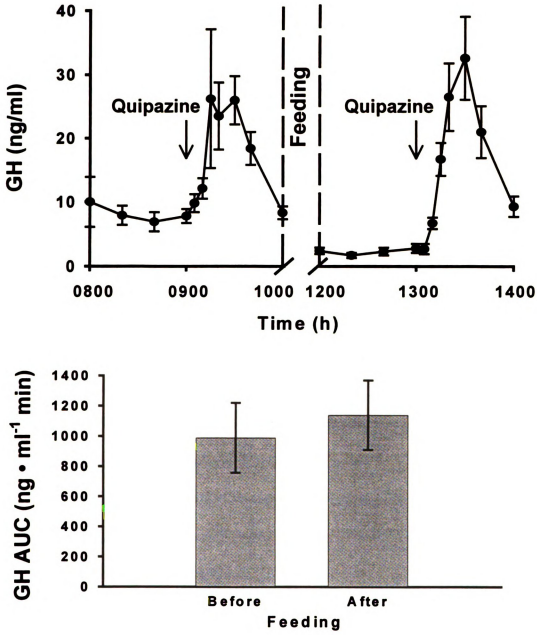


Figure 7. Effect of quipazine on secretion of GH in meal-fed steers. TOP PANEL; Data presented are mean concentrations (± standard error of mean; SEM) of growth hormone in serum of steers injected with 0.2 mg serotonin receptor agonist (quipazine)/kg BW either 1 h before (n=8) or 1 h after (n=8) feeding. Arrows indicate times of injection. **BOTTOM PANEL;** Statistical analysis was performed on least squares mean AUC (± pooled SEM) calculated over 60 min from time of injection and adjusted by covariance using concentrations of growth hormone in serum immediately before injection of quipazine. Least squares mean AUC before versus after feeding was not different ($P = 0.7$).

induced a similar growth hormone AUC increment as injection of quipazine 1 h before feeding ($P = 0.71$; Figure 7). Injection of quipazine before and after feeding induced similar increments in peak concentrations of growth hormone (Figure 8).

Effect of Various Doses of TRH on Secretion of Growth Hormone (Experiment 2)

In this experiment, all serum samples were assayed for growth hormone in a single assay with an intra-assay coefficient of variation of 15.8%. Compared with controls, injection of TRH at 0.1 and 10 μg/kg BW did not significantly increase AUC of growth hormone, while TRH at 0.3, 1 and 3 μg/kg BW significantly increased AUC of growth hormone (Figure 9). Compared with controls, 0.3 μg TRH/kg BW was the only dose that significantly increased least squares mean peak concentrations of growth hormone in serum (Figure 10).

My objective was to determine a minimal dose of TRH that would maximally stimulate secretion of growth hormone. Therefore, 0.3 μg TRH/kg BW was chosen to be used in Experiment 3.

Effect of TRH Before or After Feeding on Secretion of Growth Hormone (Experiment 3)

In this experiment, serum samples were assayed for growth hormone in a two separate assays with intra-assay coefficients of variation of 7.1% and 11.2% and an inter-assay coefficient of variation of 7.1%. As in Experiment 1, basal secretion of growth hormone was greater before feeding than after. Injection of TRH increased concentrations of growth hormone in serum both before and after

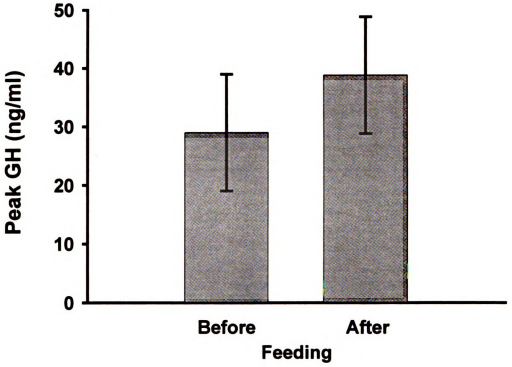


Figure 8. Effect of quipazine on peak concentrations of GH in meal-fed steers. Data presented are least squares mean peak concentration (± pooled standard error of mean) of growth hormone in serum of steers injected with 0.2 mg serotonin receptor agonist (quipazine)/kg BW either 1 h before (n=8) or 1 h after (n=8) feeding. Peak concentration of growth hormone following injection of quipazine was not different before versus after feeding (P = 0.55).

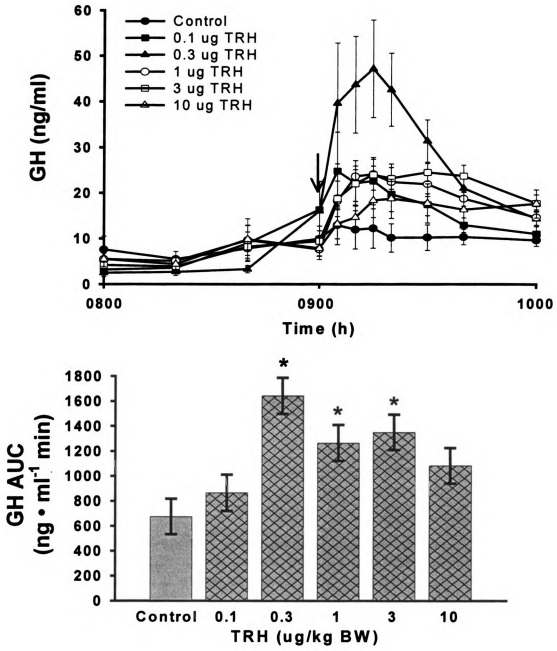


Figure 9. Effect of various doses of TRH on secretion of GH in meal-fed steers. TOP PANEL; Data presented are mean concentrations (± standard error of mean, SEM) of growth hormone in serum of steers injected with saline (control; n=6) or TRH (0.1, 0.3, 1, 3 or 10 μg/kg BW; n=6 per dose). Arrow indicates time of injection. **BOTTOM PANEL;** Statistical analysis was performed on least squares mean AUC (± pooled SEM;) calculated over 60 min from time of injection and adjusted by covariance using concentrations of growth hormone in serum immediately before injection. Least squares means with * differ from control ($P < 0.02$).

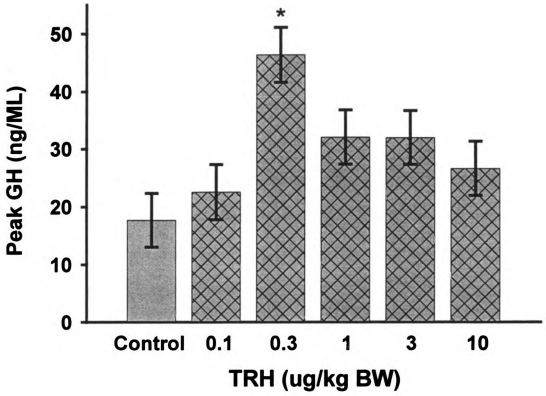


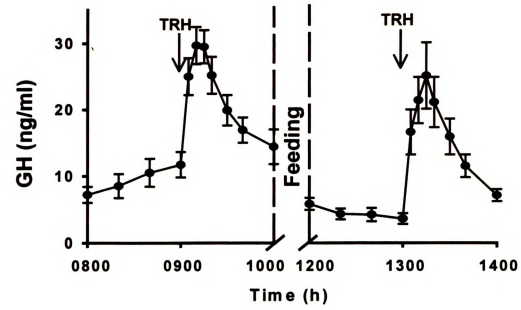
Figure 10. Effect of various doses of TRH on peak concentrations of GH in meal-fed steers. Data presented are least squares mean peak concentrations (± pooled standard error of mean) of growth hormone in serum of steers after injection of saline (control; n=6) or TRH (0.1, 0.3, 1, 3 or 10 μg/kg BW; n=6 per dose). Least squares mean with * differs from control ($P < 0.01$).

feeding ($P < 0.05$; Figure 11), and the magnitude and pattern of the increase was similar at both times. Injection of TRH after feeding induced a similar increase in growth hormone AUC as injection of TRH before feeding ($P = 0.22$; Figure 11).

Injection of TRH increased least squares mean peak concentrations of growth hormone to similar magnitudes before versus after feeding (Figure 12).

Effect of Various Doses of PACAP on Secretion of Growth Hormone (Experiment 4)

In this experiment, serum samples were assayed in two separate assays with intra-assay coefficients of variation of 11.9% and 9.0% and an inter-assay coefficient of variation of 13.7%. When adjusted for pre-treatment differences in concentrations of growth hormone in serum, injection of PACAP-38 at 0.1, 0.3, 1 and 10 μg/kg BW did not significantly increase growth hormone AUC compared with controls. However, injection of PACAP-38 at 3 μg/kg BW tended to increase growth hormone AUC ($P = 0.09$; Figure 13). When adjusted for differences in pre-treatment concentrations of growth hormone in serum, peak concentrations of growth hormone after injection of 0.1, 0.3 and 1 μg PACAP-38/kg BW were not different from control (Figure 13). However, compared with controls, injection of 3 or 10 μg PACAP-38/kg BW increased mean peak concentrations of growth hormone in serum. Since my objective was to determine a minimal dose of PACAP-38 to maximally stimulate secretion of growth hormone, 3 μg PACAP-38/kg BW was used in the subsequent experiment.



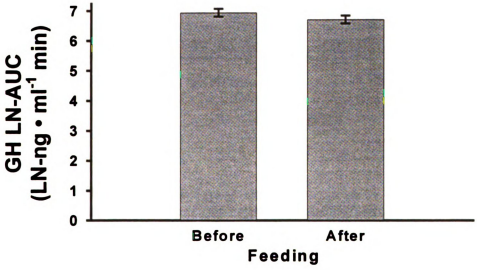


Figure 11. Effect of TRH on secretion of GH before and after feeding in meal-fed steers. TOP PANEL; Data presented are mean concentrations (± standard error of mean; SEM) of growth hormone in serum of steers injected with 0.3 μg TRH/kg BW either 1 h before (n=16) or 1 h after (n=16) feeding. Arrows depict times of injection. **BOTTOM PANEL;** Statistical analysis was performed on least squares means AUC transformed by natural logarithm (± pooled SEM;GH LN-AUC;) calculated over 60 min from time of injections and adjusted by covariance using concentrations of growth hormone in serum immediately before injection of TRH. Least squares mean LN-AUC before versus after feeding was not different (P = 0.22).

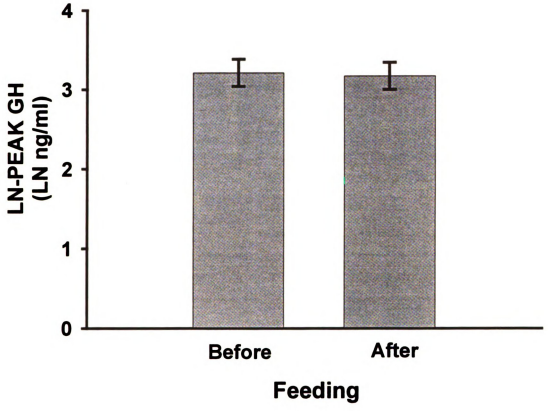


Figure 12. Effect of TRH on peak concentrations of GH before and after feeding in meal-fed steers. Data presented are least squares mean peak concentrations (± pooled standard error of mean) of growth hormone in serum (transformed by natural logarithm) of steers injected with TRH (0.3 μg/kg BW) either 1 h before (n=16) or 1 h after (n=16) feeding. LN-Peak concentration of growth hormone following injection of TRH before versus after feeding was not different (P = 0.85).

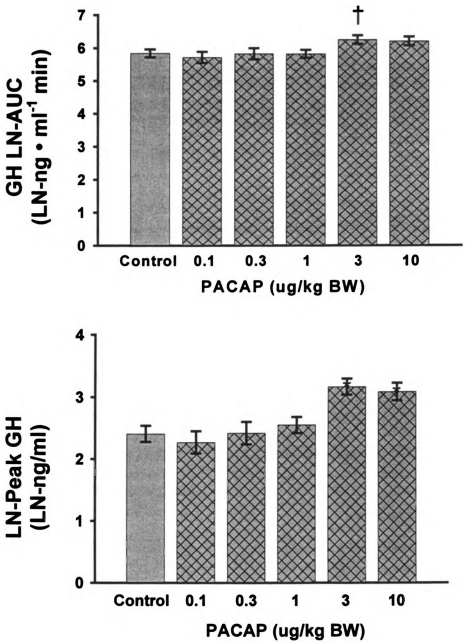


Figure 13. Effect of various doses of PACAP on secretion of GH in meal-fed steers. TOP PANEL; Data presented are least squares mean AUC (± pooled standard error of mean; SEM) transformed by natural logarithm (LN-GH AUC). **BOTTOM PANEL;** least squares mean peak concentrations (± pooled SEM) of growth hormone in serum (transformed by natural logarithm; LN-Peak) of steers injected with saline (control; n=10) or PACAP (0.1 (n=5), 0.3 (n=5), 1 (n=10), 3 (n=10) or 10 (n=10) μg/kg BW) calculated over 40 min from time of injection and adjusted by covariance using concentrations of growth hormone in serum immediately before injection. Least squares means with * differ from control ($P < 0.01$), † differs from control ($P < 0.1$).

Effect of PACAP Before and After Feeding on Secretion of Growth Hormone (Experiment 5)

All serum samples from this experiment were assayed for growth hormone in a single assay with an intra-assay coefficient of variation of 11.6%.Two steers that were supposed to receive an injection of PACAP-38 1 h after feeding were removed from the data set because a vial of PACAP-38 was accidentally broken. Therefore, the number of steers that received PACAP-38 after feeding was 6. As reported in Experiments 1 and 3, basal secretion of growth hormone was greater before feeding than after. Injection of PACAP-38 increased concentrations of growth hormone in serum both before and after feeding ($P < 0.05$). However, injection of PACAP-38 1 h before feeding induced greater AUC of growth hormone than injection of PACAP-38 1 h after feeding ($P = 0.05$; Figure 14). Injection of PACAP 1 h before feeding induced a greater least squares mean peak concentration of growth hormone than injection 1 h after feeding (Figure 15).

DISCUSSION

Basal secretion of growth hormone was greater before feeding than after in Experiments 1, 3 and 5 which confirms the work of Moseley and coworkers (1988) and Gaynor and coworkers (1996) showing that feeding suppresses secretion of growth hormone. In Experiment 1 of this chapter I repeated the work of Gaynor and coworkers (1996). My results confirm their findings, that

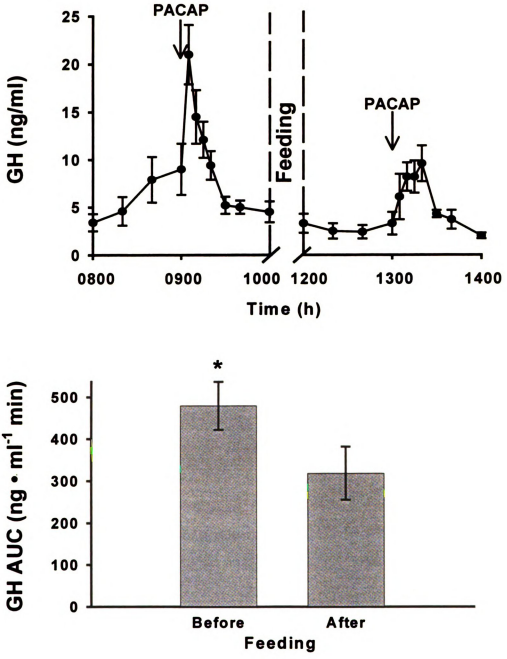


Figure 14. Effect of PACAP on secretion of GH before and after feeding in meal-fed steers. TOP PANEL; Data presented are mean concentrations (± standard error of mean; SEM) of growth hormone in serum of steers injected with 3 μg PACAP/kg BW either 1 h before (n=8) or 1 h after (n=8) feeding. Arrows indicate times of injection. **BOTTOM PANEL;** Statistical analysis was performed on least squares mean AUC (± pooled SEM) calculated over 60 min from time of injection and adjusted by covariance using concentrations of growth hormone in serum immediately before injection of PACAP. Least squares mean AUC before feeding differs from AUC after feeding ($P = 0.05$).

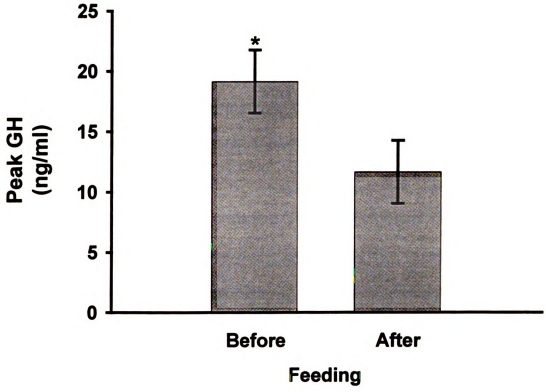


Figure 15 Effect of PACAP on peak concentrations of GH before and after feeding in meal fed steers. Data presented are least squares mean peak concentration (± pooled standard error of mean) of growth hormone in serum of steers injected with PACAP (3 μg/kg BW) either 1 h before (n=8) or 1 h after (n=8) feeding. Peak concentration of growth hormone following injection of PACAP was greater before versus after feeding ($P = 0.002$)

administration of a nonselective serotonin receptor agonist stimulates secretion of growth hormone to similar magnitudes before and after feeding in meal-fed steers. These data suggest that the pituitary gland does not become refractory after feeding to serotonin-induced secretion of growth hormone. Since quipazine does not directly affect secretion of growth hormone from cultured anterior pituitary cells, it was prudent to investigate the roles of other known hypothalamic neuropeptides as possible mediators of serotonin-induced growth hormone secretion.

Previous experiments showed that GHRH-induced secretion of growth hormone is much greater before versus after feeding (Moseley et al., 1988; McMahon et al., 2000). In contrast, quipazine induces similar increases in secretion of growth hormone before and after feeding. Furthermore, I showed that quipazine does not affect secretion of GHRH from cultured hypothalamic slices (Chapter 2). Collectively, these two experiments suggested it is unlikely that GHRH is the mediator of serotonin-induced secretion of growth hormone. Therefore, some other hypothalamic neuropeptide must mediate serotonin's effects on secretion of growth hormone. In this chapter, I tested the likelihood of two other hypothalamic peptides as mediators of serotonin-induced secretion of growth hormone, namely TRH and PACAP.

Earlier work in our laboratory determined that the minimal dose of TRH required to maximally stimulate secretion of prolactin from the anterior pituitary gland of cattle was between 50 and 100 μg per heifer (Vines, 1976). However, Vines (1976) also reported that maximal secretion of growth hormone was not

achieved even with the highest dose of TRH tested. Therefore, it was necessary for me to conduct a dose-response experiment to determine a minimal dose of TRH that would maximally stimulate secretion of growth hormone. In Experiment 2, I determined that 0.3 μg TRH/kg BW was the minimal dose of TRH to maximally increase both peak concentrations of growth hormone and AUC calculated over a 60-min period after injection of TRH. This dose of TRH is similar to the dose of TRH required to maximally stimulate secretion of prolactin determined by Vines (1976). In fact, when Vines' doses are converted to a per kg BW basis using the median weight of the heifers in his experiment, the 50 μg dose used converts to approximately 0.36 μg TRH/kg BW. The highest dose of TRH that Vines administered was approximately 0.73 μg/kg BW. In the current experiment there were three doses (1, 3 and 10 μg/kg BW) larger than the 0.3 μg/kg BW. Compared with controls, 0.3 μg TRH/kg BW was the only dose that increased peak concentrations of growth hormone, although 0.3, 1 and 3 μg TRH/kg BW increased AUC of growth hormone.

An interesting observation from this experiment is that the doses of TRH greater than 0.3 μg/kg BW increased release of growth hormone for a longer time period. In fact concentrations of growth hormone in serum after administration of TRH at doses of 1, 3 and 10 μg/kg BW were still greater than controls 60 min after injection. One explanation for this is that TRH receptors are internalized in response to agonist binding (Drmota et al., 1998b). In another report, TRH at 5 X 10^{-10} *M* did not induce receptor internalization while TRH at 5 X 10^{-9} induced massive receptor internalization. Internalization occurred within 3 to 5 min and

was maintained for 30 min (Drmota et al., 1998a). In the current experiment it appears that the higher doses induced a prolonged release of growth hormone. Perhaps the higher doses induced receptor internalization and desensitization. If circulating concentrations of TRH were still high when the receptors were recycled, they could be reactivated and prolong secretion of GH. I speculate that if I had collected blood samples for an additional hour, AUC of growth hormone would have been similar in steers receiving TRH at doses from 0.3 to 10 μg/kg BW. Therefore, I concluded that 0.3 μg/kg BW was an optimal dose in that it was the minimal dose that would maximally stimulate secretion of growth hormone. Thus, I decided to use the 0.3 μg/kg BW dose in subsequent experiments.

Based on the following information I hypothesized that some hypothalamic peptide other than GHRH or SS mediates quipazine's actions on secretion of growth hormone. Activation of serotonin receptors induces similar increases in growth hormone secretion before versus after feeding, and this effect is not exerted directly on the anterior pituitary gland, Clonidine, an α2-adrenergic receptor agonist induces secretion of GHRH (West et al., 1997a). Both GHRH and clonidine induce greater secretion of growth hormone before versus after feeding (Moseley et al., 1988; Gaynor et al., 1993; McMahon et al., 2000). If TRH is the mediator of serotonin receptor agonist-induced secretion of growth hormone, then TRH-induced secretion of growth hormone would be expected to be of similar magnitude before and after feeding. In Experiment 3 of this chapter, TRH was administered to meal-fed steers before and after feeding. Indeed, injection of TRH before and after feeding induced similar peak concentrations of

growth hormone and AUC. These data suggested that TRH could be the mediator of serotonin-induced growth hormone secretion in cattle.

Another neuropeptide that could mediate serotonin-induced secretion of growth hormone is PACAP. Treating bovine anterior pituitary cells with PACAP increased secretion of growth hormone (Hashizume et al., 1994). However, when sheep were treated with PACAP, there was no effect on concentration of growth hormone in serum (Sawangjaroen and Curlewis, 1994). The effects of PACAP on secretion of growth hormone in cattle have not been investigated. In Experiment 4 of this chapter, PACAP was administered at six different doses. When the data were adjusted for differences in pre-treatment concentrations of growth hormone, the 3 μg/kg BW dose was the only dose that tended to increase growth hormone AUC ($P < 0.1$). However, both the 3 and 10 μg/kg BW dose increased peak concentrations of growth hormone ($P < 0.01$). Thus, I concluded that PACAP does increase concentrations of growth hormone in serum of cattle. The discrepancy between my data in cattle and the results of Sawangjaroen and coworkers (1994) in sheep may be explained by dose and (or) injection protocol. Sawangjaroen infused a total of 10 n*M* PACAP over a 10-min period into the carotid artery. I administered doses that ranged from 2.2 n*M* to 220 n*M* for a 100 kg BW steer as a bolus injection into the jugular vein. The two highest doses (66 and 220 n*M* for a 100 kg steer) were the only doses that increased growth hormone secretion.

Injection of PACAP induced a greater AUC of GH before feeding than after. Because quipazine induces similar increments in secretion of growth

hormone before and after feeding, and PACAP does not, I suggest that PACAP is not the mediator of serotonin-induced secretion of growth hormone. These results are explainable. Feeding also suppresses GHRH-induced secretion of growth hormone. Both GHRH and PACAP activate adenylate cyclase and increase intracellular cAMP (Mayo et al., 1995; Wong et al., 1995; Miyata et al., 1989). If the post-feeding suppression of growth hormone secretion is due to inhibition of adenylate cyclase then it is reasonable that PACAP-induced secretion of growth hormone would also be suppressed after feeding. In contrast, TRH uses a phospholipase C second messenger system (Aragay et al., 1992; Hsieh and Martin, 1992; Svoboda et al., 1996), and it induces release of similar amounts of growth hormone before and after feeding. In addition, serotonin induces secretion of TRH from rat hypothalamic tissue in culture (Chen and Ramirez, 1981).

In conclusion, these results support the hypothesis that TRH mediates serotonin-induced secretion of growth hormone and further suggest that PACAP is not a mediator of serotonin-induced secretion of growth hormone. Therefore, I rejected the portion of the model proposed in Figure 6 depicting serotonergic neurons stimulating the secretion of PACAP which in turn stimulates secretion of growth hormone from the anterior pituitary gland.

CHAPTER 4

Serotonin-Induced Secretion of Growth Hormone in Hyperthyroid Meal-fed Steers

INTRODUCTION

Thus far, evidence described in this dissertation suggests that TRH may mediate serotonin-induced secretion of growth hormone in cattle. However, there has been no direct test of this hypothesis. One way to test this hypothesis would be to use a TRH receptor antagonist to block TRH receptors on the anterior pituitary gland, then challenge with a serotonin receptor agonist and measure subsequent increments in secretion of growth hormone. Benzodiazepines have been shown to inhibit TRH-induced secretion of thyroid stimulating hormone (TSH) and growth hormone from perifused rat pituitary glands (Roussel et al., 1986). In prior experiments not described in this thesis, I attempted to block TRH receptors in meal-fed steers with a benzodiazepine called chlordiazepoxide. In vitro, chlordiazepoxide is a competitive TRH receptor antagonist (Simasko and Horita, 1984; Drummond, 1985; Joels and Drummond, 1989). In vivo, chlordiazepoxide has anxiolytic and sedative effects (Baldessarini, 1993). During my attempts to block TRH receptors in meal-fed steers, chlordiazepoxide was more potent as a sedative than as a TRH receptor antagonist. Therefore, chlordiazepoxide was not a suitable TRH receptor antagonists to test my hypothesis.

Another way to block TRH effects on the anterior pituitary gland would be to use negative feedback of 3, 3', 5-triiodo-L-thyronine (T_3) to down regulate TRH receptors on the anterior pituitary gland. In addition, T_3 feeds back negatively on the hypothalamus.

In the anterior pituitary gland and on GH_3 cells, T_3 inhibits expression of TRH receptors (Lean et al., 1977; Perrone and Hinkle, 1978). In pituitary tumor cell lines, T_3 increases the rate of growth hormone mRNA accumulation (Samuels and Shapiro, 1976; Seo et al., 1977; Martial et al., 1977). However, in GH_3 cells, the concentration of T_3 required to induce a half maximal increase in growth hormone mRNA synthesis is 45-fold greater than the concentration required to induce a half maximal loss of TRH receptors (Hinkle et al., 1979). Therefore, it should be possible to down regulate TRH receptors without affecting synthesis of growth hormone.

Within the hypothalamus, T_3 inhibits synthesis of TRH mRNA in neurons in the paraventricular nucleus. These neurons control the secretion of TSH from the anterior pituitary gland. This effect is selective, because T_3 has no effect on TRH mRNA synthesis in TRH neurons in other regions of the brain (Segerson et al., 1987; Koller et al., 1987; Dyess et al., 1988).

Hormonal hyperthyroidism has been induced in cattle with daily injections of T_3 dissolved in corn oil (Thrift et al., 1999). Daily injections of T_3 increased concentrations of T_3 and decreased concentrations of 3, 3', 5, 5'-tetraiodo-L-thyronine (thyroxin; T_4) in serum to below the detection limit of the assay. Since T_4 is the predominant form released from the thyroid gland, these data indicate that T_3 injections effectively shut down the thyroid axis.

If daily injections of T_3 can effectively shut down the thyroid axis, negative feedback must be exerted at the level of the hypothalamus and (or) the pituitary gland, or even at the level of the thyroid gland (Figure 16). Negative feedback at

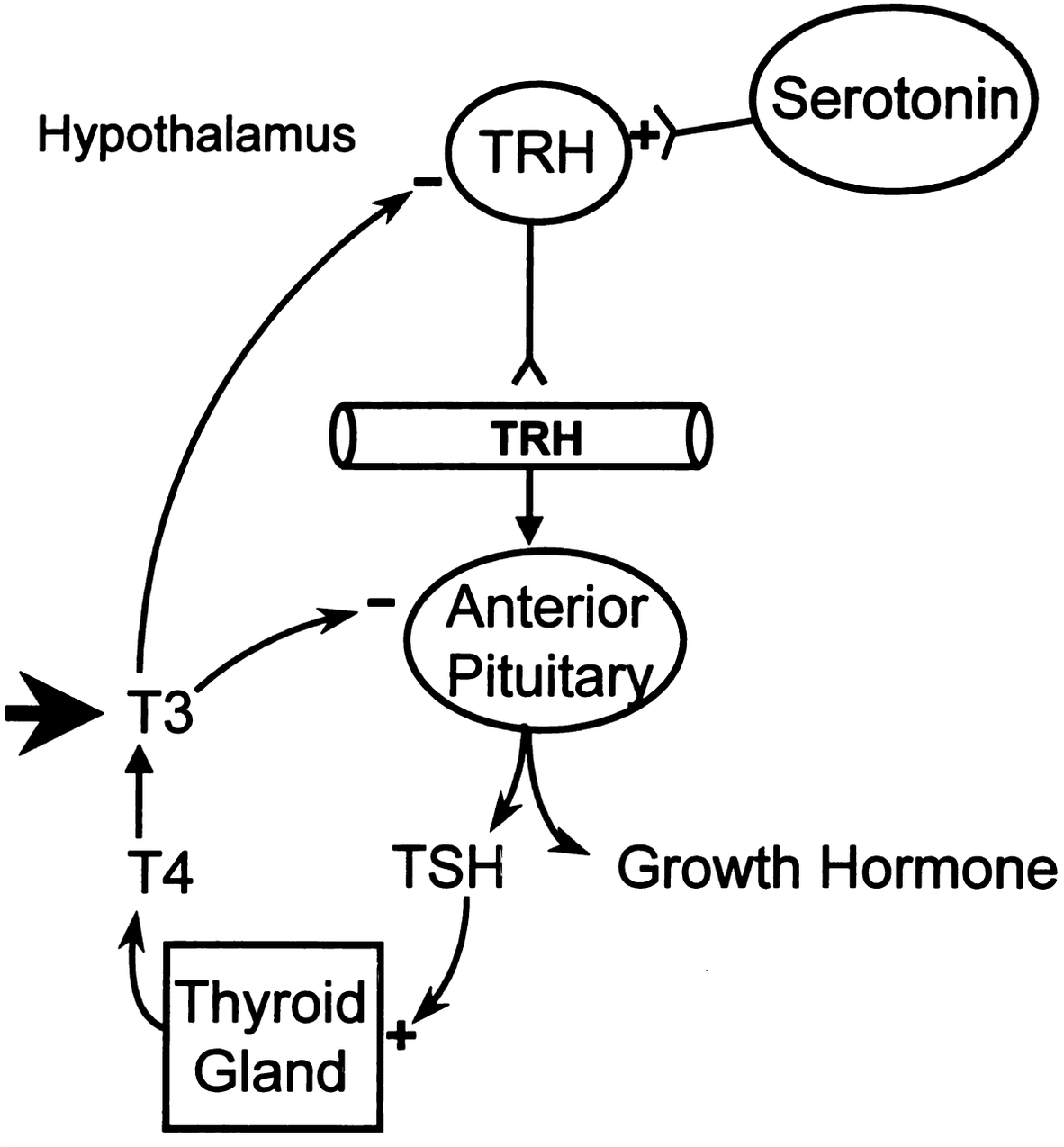


Figure 16. Model of negative feedback of 3,3',5-triiodo-L-thyronine (T_3) on thyrotropin releasing-hormone (TRH) neurons in the hypothalamus and the anterior pituitary gland. Injecting exogenous T_3 (bold arrow) will increase negative feedback on TRH neurons and (or) the anterior pituitary gland thereby reducing thyroid stimulating hormone (TSH) secretion from the anterior pituitary gland and 3, 3',5, 5'-tetraiodo-L-thyronine (T_4) secretion from the thyroid gland as well as the ability of serotonin to induce secretion of growth hormone from the anterior pituitary gland.

the level of the hypothalamus would decrease TRH synthesis and release, which may be induced by afferent neurons such as serotonin. Negative feedback at the pituitary gland could cause a decrease in TSH synthesis and (or) TRH receptor expression. If daily injections of T_3 decrease either TRH synthesis or TRH receptor expression and TRH is the mediator of serotonin-induced secretion of growth hormone, then the increase in growth hormone secretion induced by a serotonin receptor agonist should be suppressed in steers chronically treated with T_3. I hypothesized that daily injections of T_3 would inhibit TRH- and quipazine-induced secretion of growth hormone. Therefore, my objectives were to determine if daily injections of T_3 affected: 1) basal secretion of growth hormone in meal–fed steers, 2) releasable amounts of growth hormone from the anterior pituitary gland by measuring GHRH-induced secretion of growth hormone, 3) TRH-induced secretion of growth hormone and 4) quipazine-induced secretion of growth hormone.

MATERIALS AND METHODS

Management of Animals

Holstein steer calves were adapted to meal-feeding and housed as described in Chapter 3.

Blood Sample Collection and Processing

Blood samples were collected, processed and assayed as described in Chapter 3. In addition, selected blood samples were also assayed for total T_4

using a commercially available kit (Diagnostic Products Corp., Los Angeles, CA) validated for bovine serum (Williams et al., 1987).

Experimental Protocols

Experiments in this chapter were designed to test the effects of daily T_3 (Sigma Chemical Co., St Louis, MO) injections on basal, GHRH-, TRH- and quipazine-induced secretion of growth hormone. In the first experiment Holstein steers (138.5 ± 6 kg BW: 20 ± 0.5 weeks of age) were injected subcutaneously each day, 2 h before meal-feeding (1000 to 1200 h) with T_3 (3 μg/kg BW; n=4) dissolved in corn oil to induce negative feedback on the hypothalamic-pituitary-thyroid axis thereby decreasing TRH receptor expression and(or) TRH synthesis in the hypothalamus. Controls (n=4) received corn oil. Fresh T_3 was initially dissolved in 100% ethanol to a concentration of 5 mg/ml then diluted with corn oil to a final concentration of 500 μg/ml every third day. Ethanol was also added to corn oil, at the same ratio as that used to dissolve T_3. Injection volumes were adjusted for changes in BW once per week to maintain a constant dose of T_3. Two days before commencement of T_3 treatment (day 0), all steers were fitted with indwelling jugular catheters. On days –1, +5 and +10 relative to initiation of T_3 treatment, blood samples were collected at 20-min intervals from 0800 h to 1400 h to be assayed for growth hormone to determine if T_3 treatment affected basal secretion of growth hormone in meal-fed steers. Samples collected at 0900 h were also assayed for total T_4 concentrations to determine secretory activity of the thyroid gland as an index of the effectiveness of T_3 treatment.

After 20 d of T_3 treatment, meal-fed steers were again fitted with indwelling jugular catheters. On day +21, steers (within thyroid status; i.e. within corn oil and T_3–treated groups) were randomly assigned to receive an intravenous injection of either GHRH (0.2 μg/kg BW) dissolved in sterile water or sterile water at 0900 h in a crossover design. The following day treatments were reversed. Blood samples were collected at 0800, 0820, 0840, 0900, 0905, 0910, 0915, 0920, 0930, 0940, 1000 and 1300 h on each treatment day.

On day +24 of T_3 treatment, meal-fed steers (within thyroid status) were randomly assigned to receive an intravenous injection of either TRH (0.3 μg/kg BW) dissolved in sterile 0.9% saline solution or sterile 0.9% saline solution at 0900 h in a crossover design. The following day treatments were reversed. Blood samples were collected at 0800, 0820, 0840, 0900, 0905, 0910, 0915, 0920, 0930, 0940, 1000 and 1300 h on each treatment day.

On day +27 of T_3 treatment, meal-fed steers (within thyroid status) were randomly assigned to receive an intravenous injection of either quipazine (0.2 mg/kg BW) dissolved in sterile 0.9% saline solution or sterile 0.9% saline solution 0900 h in a crossover design. The following day treatments were reversed. Blood samples were collected at 0800, 0820, 0840, 0900, 0905, 0910, 0915, 0920, 0930, 0940, 1000 and 1300 h on each treatment day.

Sera harvested from blood samples from 0800 to 1000 h on days +21, +22, +24, +25, +27 and +28 were assayed for growth hormone. Sera from blood samples taken at 0900 and 1300 h on days +21, +22, +24, +25, +27 and +28 were assayed for total T_4.

The previous experiment was repeated with another group of meal-fed steers. However, in this experiment the dose of T_3 was doubled, and the order in which the growth hormone secretagogues were administered was rearranged to control for any carry over effects. Holstein steers (108.8 ± 4 kg BW: 16.5 ± 0.4 weeks of age) were injected subcutaneously each day with either corn oil (n=4) or T_3 (6 μg/kg BW; n=4) dissolved in corn oil. Fresh T_3 and vehicle were prepared every third day as in the first experiment.

After 22 d of T_3 treatment, meal-fed steers were fitted with indwelling jugular catheters. On day 23, steers (within thyroid status) were randomly assigned to receive an intravenous injection of either quipazine (0.2 mg/kg BW) dissolved in sterile 0.9% saline solution or sterile 0.9% saline solution at 0900 h in a crossover design. The following day treatments were reversed. Blood samples were collected at 0800, 0820, 0840, 0900, 0905, 0910, 0915, 0920, 0930, 0940, 1000, 1100, 1200, 1300, 1400 and 1500 h on each treatment day.

On day 26 of T_3 treatment, meal-fed steers (within thyroid status) were randomly assigned to receive an intravenous injection of either GHRH (0.2 μg/kg BW) dissolved in sterile water or sterile water at 0900 h in a crossover design. The following day treatments were reversed. Blood samples were collected at 0800, 0820, 0840, 0900, 0905, 0910, 0915, 0920, 0930, 0940, 1000, 1100, 1200, 1300, 1400 and 1500 h on each treatment day.

On day 29 of T_3 treatment, meal-fed steers (within thyroid status) were randomly assigned to receive an intravenous injection of either TRH (0.3 μg/kg BW) dissolved in sterile 0.9% saline solution or sterile 0.9% saline solution at

0900 h in a crossover design. The following day treatments were reversed. Blood samples were collected at 0800, 0820, 0840, 0900, 0905, 0910, 0915, 0920, 0930, 0940, 1000, 1100, 1200, 1300, 1400 and 1500 h on each treatment day.

Sera harvested from all blood samples collected from 0800 to 1000 h were assayed for growth hormone. Sera harvested from blood samples collected at 0800, 0900, 1000, 1100, 1200, 1300, 1400 and 1500 h were assayed for total T_4.

Statistical Analysis

To determine the effects of T_3 treatment on basal secretion of growth hormone, total AUC of growth hormone secretion was calculated from 0800 to 1400 h for corn oil- and T_3-treated steers on days –1, 5 and 10 relative to initiation of T_3 treatment in the first experiment (day 0). Total AUC and concentrations of total T_4 were subjected to analysis of variance using Mixed Models procedures of SAS (SAS, 1999). The statistical model included steer nested within T_3 treatment, T_3 treatment, day and T_3 treatment by day interaction. Total AUC of growth hormone and total T_4 concentrations of control and T_3-treated steers were compared on each day.

Secretion of growth hormone in response to growth hormone secretagogues (GHRH, TRH and quipazine) was estimated by calculating total AUC of growth hormone from 0900 h (time of injection) to 1000 h using the trapezoidal rule. Secretion of growth hormone (AUC) in response to an injection of GHRH, TRH or a quipazine in the steers injected with 3 μg T_3/kg BW (first experiment) was compared to the responses in growth hormone secretion to an

injection of GHRH, TRH or a quipazine in the steers injected with 6 μg T_3/kg BW (second experiment) using a students t-test (Gill, 1978). The dose of T_3 (3 vs. 6 μg/kg BW) did not affect secretion of growth hormone in response to GHRH (P = 0.83), TRH (P = 0.61) or quipazine (P = 0.86). Therefore, data were pooled and subsequent analyses were conducted to compare T_3-treated steers (regardless of dose) with steers injected with corn oil.

Total AUC of growth hormone was calculated from 0900 to 1000 h for GHRH, TRH and quipazine challenges and subjected to analysis of variance using Mixed Models procedures of SAS (SAS, 1999). The statistical model included concentration of growth hormone at 0900 as a covariate when significant (P < 0.25), steer nested within T_3 treatment, experiment, T_3 treatment, challenge, T_3 treatment by challenge interaction and day when significant (P < 0.25). Data for basal secretion of growth hormone and peak concentrations of growth hormone from the TRH challenge and quipazine were transformed by natural logarithm to achieve homogeneous variance before statistical analysis.

RESULTS

Effects of T_3 on basal, GHRH-, TRH- and Quipazine-Induced Secretion of Growth Hormone and Total T_4

In the first experiment in which half of the steers were injected with 3 μg T_3/kg BW, serum samples from days –1, +5 and +10 were assayed for growth hormone in a single assay with an intra-assay coefficient of variation of 14.6%.

Total AUC of growth hormone from 0800 to 1400 h for T_3-treated steers was not different from corn oil-injected steers on days –1, +5 or +10 of T_3 treatment (Figure 17).

In contrast, by day +5, T_3-treated steers had significantly lower concentrations of total T_4 in serum compared with corn oil-injected steers (Figure 18). On day +10, concentrations of total T_4 in serum of T_3-treated steers were below the detection limit of the assay (1 μg/dl).

Serum samples from the GHRH challenges (from first and second experiments) were assayed for growth hormone in two separate assays with intra-assay coefficients of variation of 11.9% and 10.0% and an inter-assay coefficient of variation of 11.7%. Serum samples from the TRH challenges (from first and second experiments) were assayed for growth hormone in two separate assays with intra-assay coefficients of variation of 6.1% and 8.1% and an inter-assay coefficient of variation of 12.1%. Serum samples from quipazine challenges (from first and second experiments) were assayed for growth hormone in two separate assays with intra-assay coefficients of variation of 9.5% and 8.6% and an intra-assay coefficient of variation of 8.8%. All serum samples assayed for total T_4 (first and second experiments) were assayed in two assays with intra-assay coefficients of variation of 0.4% and 4.5% and an inter-assay coefficient of variation of 6.5%.

The profile of concentrations of growth hormone in serum (pooled data from first and second experiments) of corn oil-treated steers injected with sterile

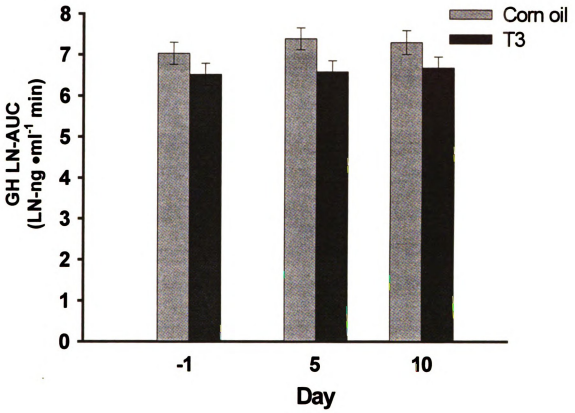


Figure 17. Effect of daily injections of T_3 on basal secretion of GH in meal-fed steers. Data presented are least squares mean AUC (± pooled standard error of mean) of growth hormone of corn oil-injected (control; n=4) and T_3-treated (3 μg/kg BW; n=4) meal-fed steers. The AUCs were calculated from 0800 to 1400 h on days –1, 5 and 10 relative to initiation of injections, and transformed by natural logarithm (GH LN-AUC). Injection of T_3 did not affect least square mean GH LN-AUC on days –1, 5 or 10 ($P > 0.16$).

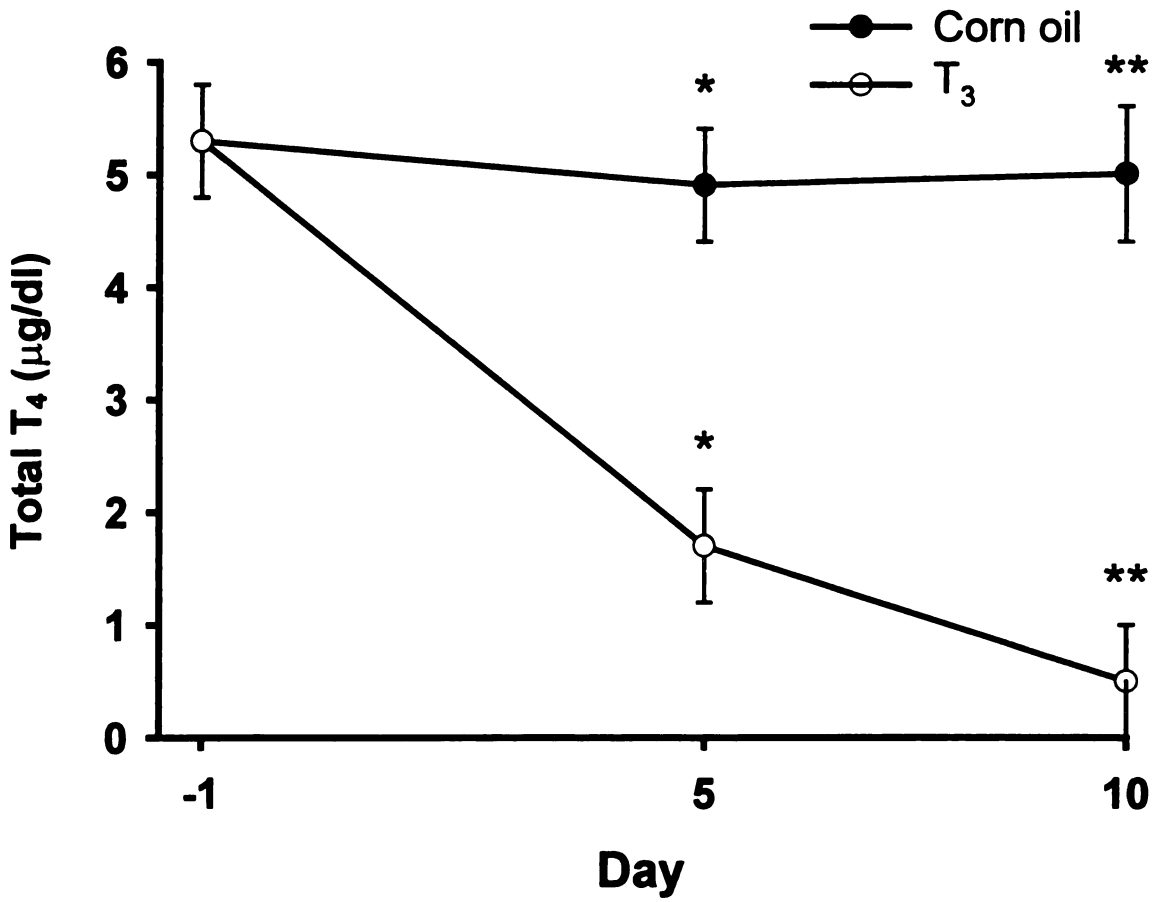


Figure 18. Effect of daily injections of T_3 on concentrations of total T_4 in meal-fed steers. Data presented are least squares mean concentrations (± pooled standard error of mean) of total T_4 in serum of corn oil-injected and T_3-treated (3 µg/kg BW) meal-fed steers on days –1, 5 and 10 relative to initiation of injections. Compared with corn oil-injected steers, daily injection of T_3 reduced concentration of total T_4 in serum on d 5 and 10. Points with * differ $P = 0.001$. Points with ** differ $P = 0.0001$.

water was similar to that of T_3-treated steers injected with sterile water (Figure 19; top panel). In addition, the profile of concentrations of growth hormone in serum of corn oil-treated steers injected with GHRH was similar to that of T_3-treated steers injected with GHRH. The treatment (GHRH or sterile water) by thyroid status (T_3- or corn oil-treated) interaction was not significant ($P = 0.55$). In corn oil-treated steers, compared with injection of sterile water, injection of GHRH markedly increased AUC of growth hormone (Figure 19; bottom panel). Likewise, in T_3-treated steers, compared with injection of sterile water, injection of GHRH markedly increased AUC of growth hormone. Compared with corn oil-treated steers injected with GHRH, injection of T_3-treated steers with GHRH induced secretion of growth hormone to a similar magnitude as indicated by both AUC (Figure 19, bottom panel) and mean peak concentrations of growth hormone (Figure 20).

Similar to results shown in Figure 15, compared with injection of corn oil, chronic T_3 markedly reduced concentrations of total T_4 in serum (Figure 21). In corn oil-treated steers, injection of sterile water did not affect concentrations of total T_4 in serum at 1300 h compared with 0900 h. Similarly, in T_3-treated steers, injection of sterile water did not affect concentrations of total T_4 in serum at 1300 h compared with 0900 h. In corn oil-treated steers, injection of GHRH did not affect concentrations of total T_4 in serum at 1300 h compared with 0900 h. Additionally, in T_3-treated steers, injection of GHRH did not affect concentrations of total T_4 in serum at 1300 h compared with 0900 h.

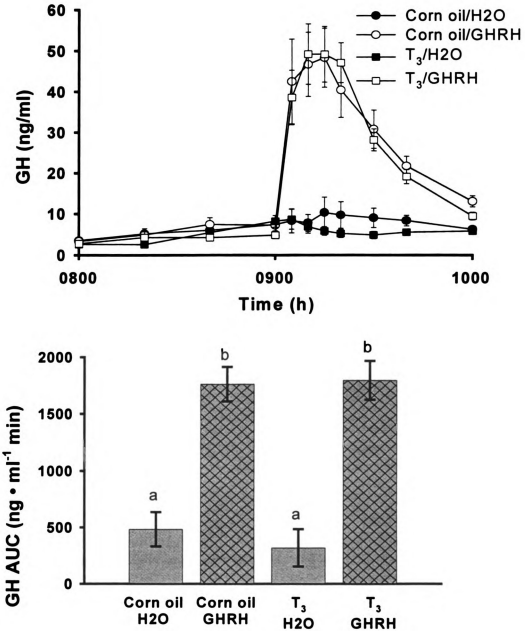


Figure 19. Effect of daily injections of T_3 on GHRH-induced secretion of GH in meal-fed steers. TOP PANEL; Data presented are mean concentrations (± standard error of mean; SEM) of growth hormone in serum of corn oil-injected and T_3-treated meal-fed steers after intravenous injection of sterile water (H2O) or GHRH dissolved in H2O (0.2 μg/kg BW) at 0900 h. Arrow indicates time of injection. **BOTTOM PANEL;** Statistical analysis was performed on least squares mean AUCs (± pooled SEM) calculated from 0900 to 1000 h. Bars with different letters differ $P < 0.001$.

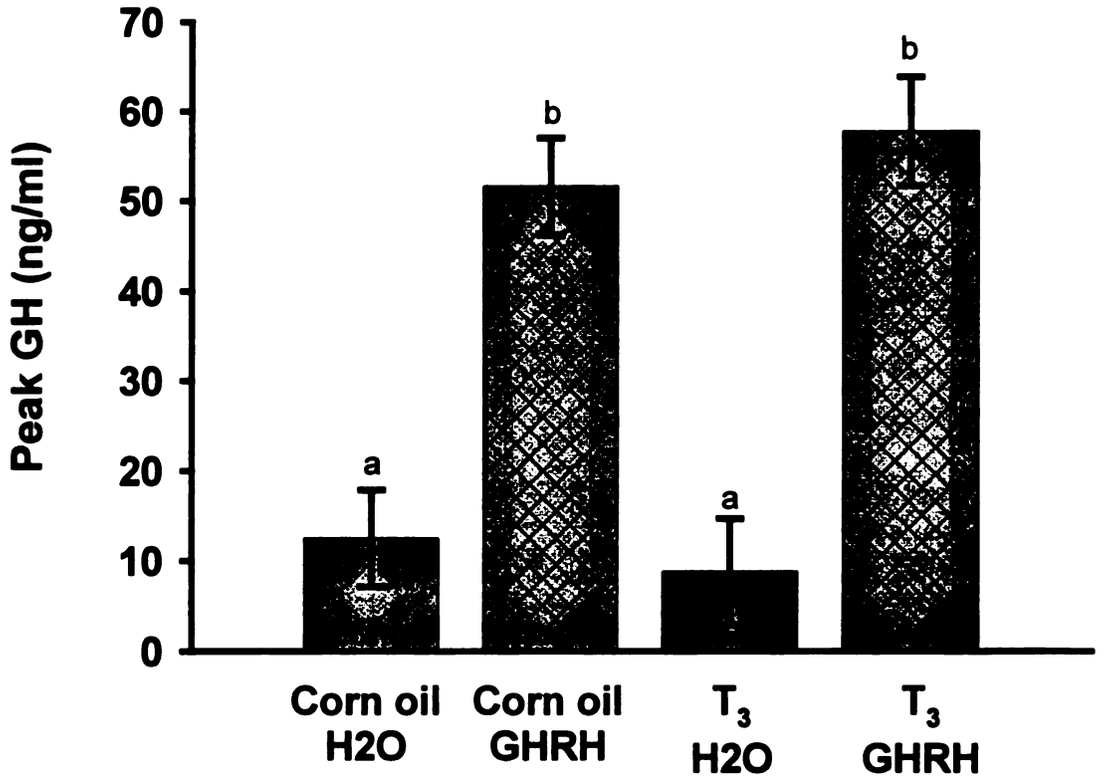


Figure 20 Effect of daily injections of T_3 on GHRH-induced peak concentrations of GH in meal-fed steers. Data presented are least squares mean peak concentrations (± pooled standard error of mean) of growth hormone in serum of corn oil-injected and T_3-treated meal-fed steers after intravenous injection of sterile water (H2O) or GHRH dissolved in H2O (0.2 μg/kg BW) at 0900 h. Bars with different letters differ $P < 0.001$.

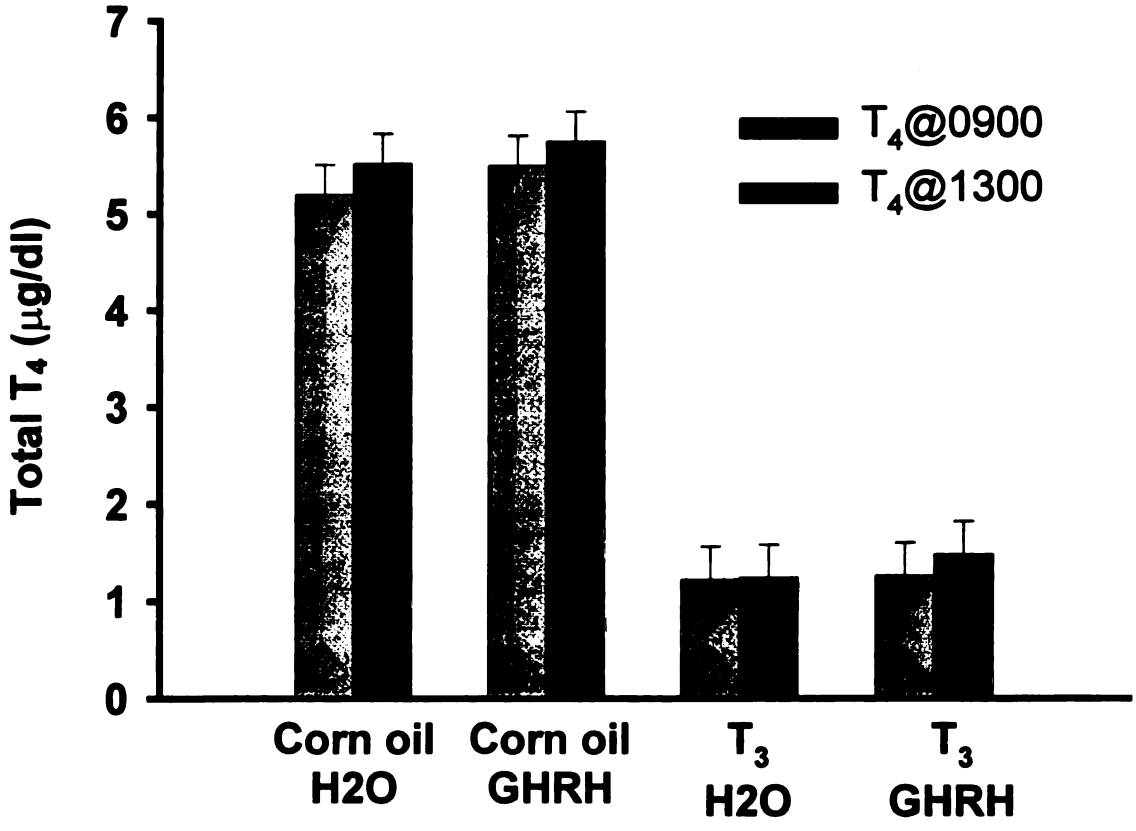


Figure 21. Effect of daily injections of T_3 on GHRH-induced secretion of T_4 in meal-fed steers. Data presented are least squares mean concentration (± pooled standard error of mean) of total T_4 in serum of corn oil-injected and T_3-treated meal-fed steers immediately before (0900 h) and 4 h after (1300 h) intravenous injection of sterile water (H2O) or GHRH dissolved in H2O. Injection of H2O did not affect concentrations of total T_4 in serum of corn oil-injected or T_3-treated steers. Injection of GHRH did not affect concentrations of total T_4 in serum of corn oil-injected or T_3-treated steers.

The profile of concentrations of growth hormone in serum (pooled data) of corn oil-treated steers injected with saline was similar to that of T_3-treated steers injected with saline (Figure 22; top panel). Likewise, the profile of concentrations of growth hormone in serum of corn oil-treated steers injected with TRH was similar to that of T_3-treated steers injected with TRH. The treatment (TRH or saline) by thyroid status (T_3- or corn oil-treated) interaction was not significant (P = 0.34). However, the main effect of thyroid status tended to affect secretion of growth hormone in response to injection of TRH (P = 0.1). In corn oil-injected steers, compared with injection of saline, injection of TRH increased AUC (Figure 22; bottom panel) as well as mean peak concentrations of growth hormone (Figure 23). In contrast, in T_3-treated steers, compared with injection of saline, injection of TRH did not increase AUC of growth hormone (Figure 22, bottom panel). However, with respect to mean peak concentrations of growth hormone in T_3-treated steers, injection of TRH increased mean peak concentrations to 2.9 ± 0.2 LN-ng/ml compared with 2.0 ± 0.2 LN-ng/ml in saline injected steers (Figure 23). Compared with corn oil-treated steers injected with TRH, injection of T_3-treated steers with TRH increased secretion of growth hormone to similar magnitudes as indicated by both AUC (Figure 22;bottom panel) and mean peak concentrations of growth hormone (Figure 23).

Concentrations of total T_4 in serum were greater in corn oil-treated steers than in serum of T_3-treated steers. In corn oil-treated steers, injection of saline did not affect concentrations of total T_4 in serum at 1300 h compared with 0900 h (Figure 24). Similarly, in T_3-treated steers, injection of saline did not affect

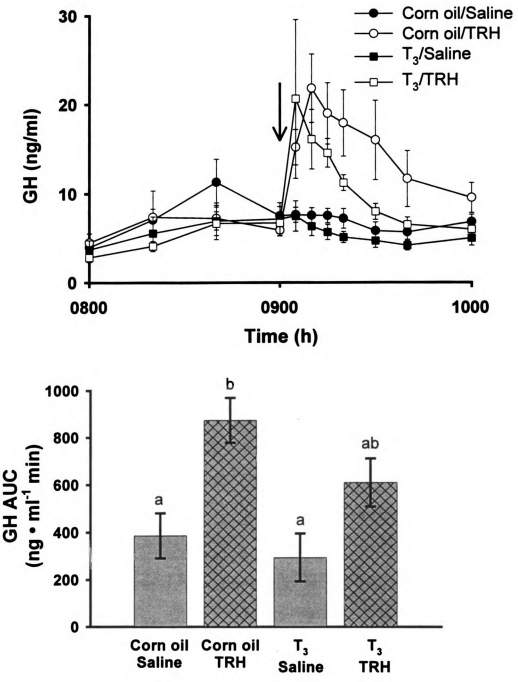


Figure 22. Effect of daily injections of T_3 on TRH-induced secretion of GH in meal-fed steers. TOP PANEL; Data presented are mean concentrations (± standard error of mean; SEM) of growth hormone in serum of corn oil-injected and T_3-treated meal-fed steers after intravenous injection of 0.9% saline or TRH dissolved in 0.9% saline (0.3 μg/kg BW) at 0900 h. Arrow indicates time of injection. **BOTTOM PANEL;** Statistical analysis was performed on least squares mean AUCs (± pooled SEM) calculated from 0900 to 1000 h. Bars with different letters differ $P < 0.01$.

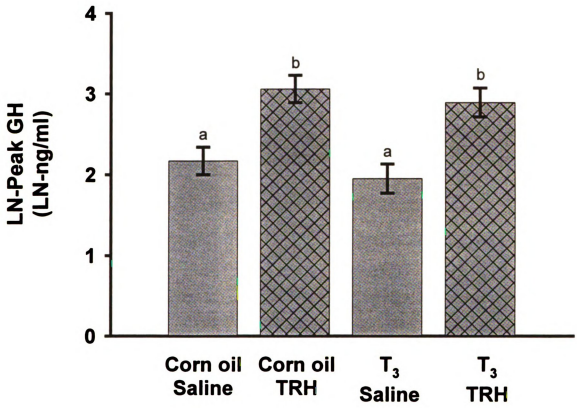


Figure 23. Effect of daily injections of T_3 on TRH-induced peak concentrations of GH in meal-fed steers. Data presented are least squares mean peak concentrations (± pooled standard error of mean) of growth hormone transformed by natural logarithm (LN-Peak GH) in serum of corn oil-injected and T_3-treated meal-fed steers after intravenous injection of sterile saline or TRH dissolved in saline (0.3 μg/kg BW) at 0900 h. Bars with different letters differ $P <$ 0.01.

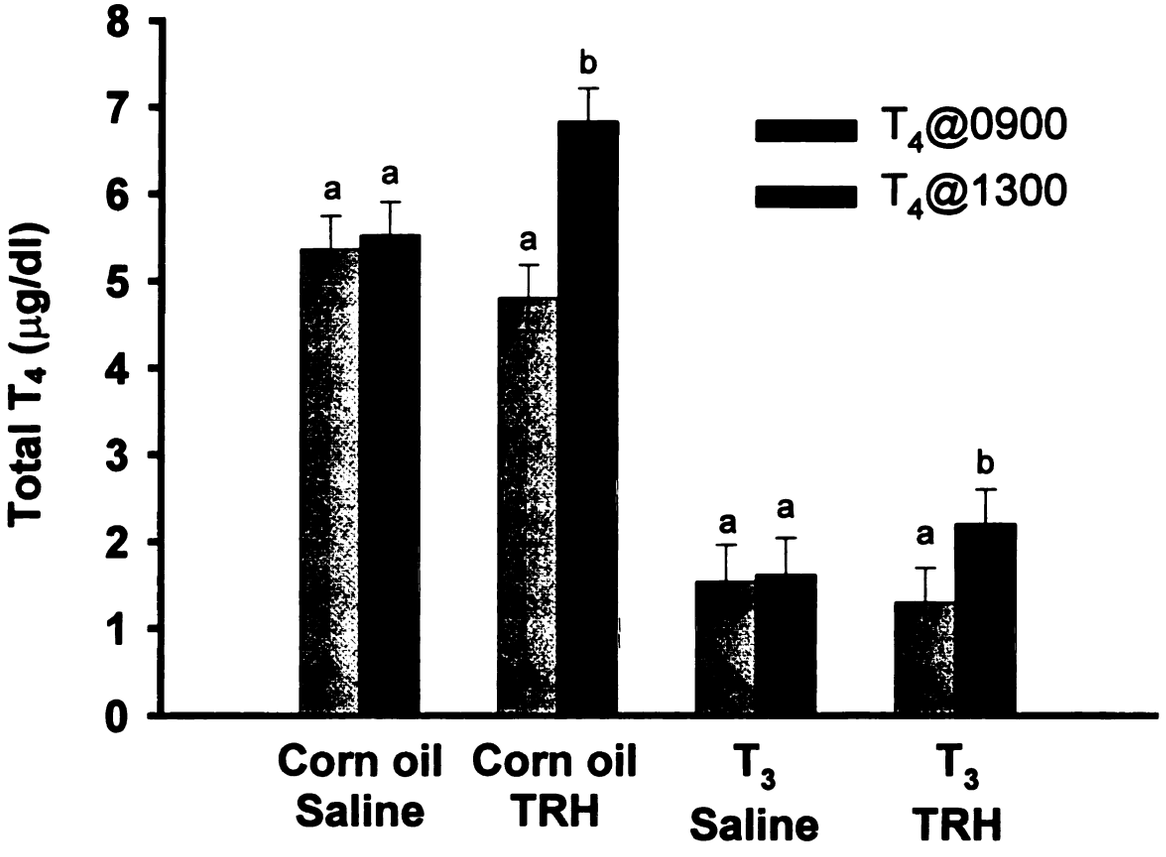


Figure 24. Effect of daily injections of T_3 on TRH-induced secretion of T_4 in meal-fed steers. Data presented are least squares mean concentrations (± pooled standard error of mean) of total T_4 in serum of corn oil-injected and T_3-treated meal-fed steers immediately before (0900 h) and 4 h after (1300 h) intravenous injection of sterile 0.9% saline or TRH dissolved in 0.9% saline. Injection of saline did not affect concentrations of total T_4 in serum of corn oil-injected or T_3-treated steers. Injection of TRH increased concentrations of total T_4 in serum of both corn oil-injected and T_3-treated steers at 1300 h. Within treatment, bars with different letters differ $P < 0.01$.

concentrations of total T_4 in serum at 1300 h compared with 0900 h. As expected, in corn oil-treated steers, injection of TRH increased concentrations of total T_4 in serum at 1300 h compared with 0900 h. In addition, in T_3-treated steers, injection of TRH also increased concentrations of total T_4 in serum at 1300 h compared with 0900 h.

The profile of concentrations of growth hormone in serum (pooled data) of corn oil-treated steers injected with saline was similar to that of T_3-treated steers injected with saline (Figure 25; top panel). The profile of concentrations of growth hormone in serum of corn oil-treated steers injected with quipazine had a pattern similar to that of T_3-treated steers injected with quipazine. The treatment (quipazine or saline) by thyroid status (T_3- or corn oil-treated) interaction was significant ($P = 0.07$). In corn oil-treated steers, compared with injection of saline, injection of quipazine increased secretion of growth hormone as indicated by both AUC (Figure 25; bottom panel) and mean peak concentrations of growth hormone (Figure 26). In contrast, in T_3-treated steers, compared with injection of saline, injection of the quipazine did not increase AUC of growth hormone (Figure 25; bottom panel). However, with respect to mean peak concentrations of growth hormone in T_3-treated steers, injection of quipazine increased mean peak concentrations to 2.9 ± 0.2 LN-ng/ml compared with 2.0 ± 0.2 LN-ng/ml in saline injected steers (Figure 26). Compared with corn oil-treated steers injected with quipazine, injection of T_3-treated steers with quipazine increased both AUC (Figure 25; bottom panel) and mean peak concentrations of growth hormone to similar magnitudes (Figure 26).

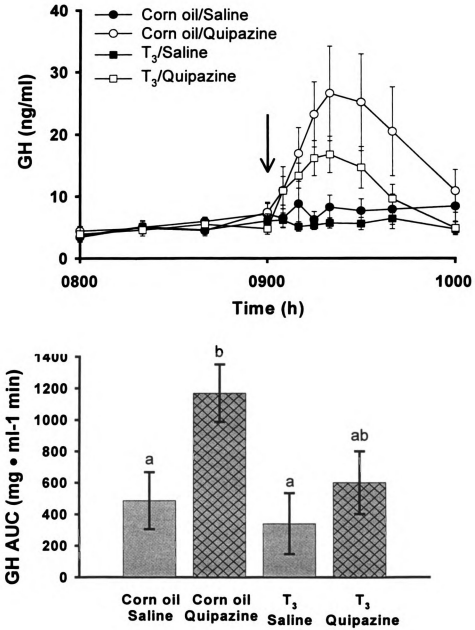


Figure 25. Effect of daily injections of T_3 on quipazine-induced secretion of GH in meal-fed steers. TOP PANEL; Data presented are mean concentrations (± standard error of mean; SEM) of growth hormone in serum of corn oil-injected and T_3-treated meal-fed steers after intravenous injection of 0.9% saline or a serotonin receptor agonist (quipazine) dissolved in 0.9% saline (0.2 mg/kg BW) at 0900 h. Arrow indicates time of injection. **BOTTOM PANEL;** Statistical analysis was performed on least squares mean AUCs (± pooled SEM) calculated from 0900 to 1000 h. Compared with injection of saline, injection of quipazine increased GH AUC in corn oil-injected but not T_3-treated steers. Bars with different letters differ $P < 0.05$.

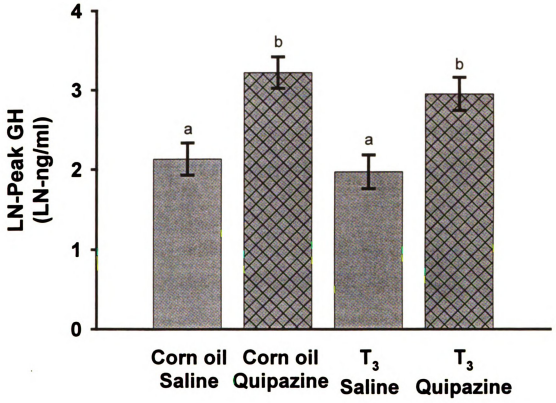


Figure 26. Effect of daily injections of T_3 on quipazine-induced peak concentrations of GH in meal-fed steers. Data presented are least squares mean peak concentrations (± pooled standard error of mean) of growth hormone transformed by natural logarithm (LN-Peak GH) in serum of corn oil-injected and T_3-treated meal-fed steers after intravenous injection of sterile saline or serotonin receptor agonist (quipazine) dissolved in saline (0.3 μg/kg BW) at 0900 h. Bars with different letters differ $P < 0.01$.

Concentrations of total T_4 in serum of corn oil-treated steers were greater than in serum of T_3-treated steers. In corn oil-treated steers, injection of saline did not affect concentrations of total T_4 in serum at 1300 h compared with 0900 h (Figure 27). Likewise, in T_3-treated steers, injection of saline did not affect concentrations of total T_4 in serum at 1300 h compared with 0900 h. In corn oil-treated steers, injection of quipazine did not affect concentrations of total T_4 in serum at 1300 h compared with 0900 h. Similarly, in T_3-treated steers, injection of quipazine did not affect concentrations of total T_4 in serum at 1300 h compared with 0900 h.

DISCUSSION

Injection of the serotonin receptor agonist, quipazine, induces secretion of growth hormone in cattle (Gaynor et al., 1996). However, the mechanism by which the serotonin receptor agonist induces secretion of growth hormone is not known. An earlier experiment by Gaynor and coworkers (1996) and in vitro experiments described in Chapter 1 of this dissertation suggests that the effects of the serotonin receptor agonist are not directly on the pituitary gland. In other experiments utilizing in vitro and in vivo approaches, I have collected data that suggest that the effects of serotonin on secretion of growth hormone are not mediated by SS, GHRH or PACAP. However, the experiments that I conducted in Chapter 3 of this thesis suggest that TRH could be mediating serotonin-induced secretion of growth hormone.

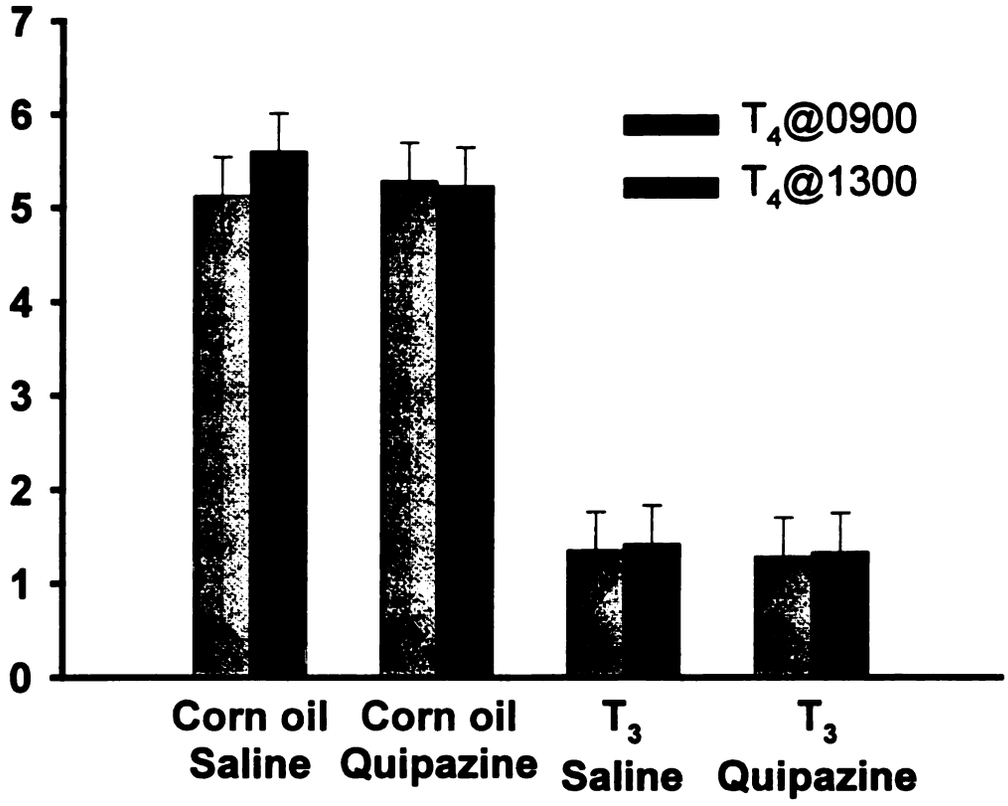


Figure 27. Effect of daily injections of T_3 on quipazine-induced secretion of T_4 in meal-fed steers. Data presented are least squares mean concentrations (± pooled standard error of mean) of total T_4 in serum of corn oil-injected and T_3-treated meal-fed steers immediately before (0900 h) and 4 h after (1300 h) intravenous injection of sterile 0.9% saline or a serotonin receptor agonist (quipazine) dissolved in 0.9% saline. Injection of saline did not affect concentrations of total T_4 in serum of corn oil-injected or T_3-treated steers. Injection of quipazine did not affect concentrations of total T_4 in serum of corn oil-injected or T_3-treated steers.

Therefore, my hypothesis in Chapter 4 was that TRH mediates serotonin receptor agonist-induced secretion of growth hormone. To test this hypothesis, I had to block the effects of TRH on secretion of growth hormone. To do this, I used negative feedback of T3 on the hypothalamus and pituitary to decrease TRH synthesis and secretion and(or) TRH receptor expression in the anterior pituitary gland. Previous experiments demonstrated that T3 feeds back at the level of the hypothalamus to down regulate TRH synthesis (Segerson et al., 1987; Koller et al., 1987; Dyess et al., 1988) as well as at the level of the anterior pituitary gland to down regulate TRH receptors (Lean et al., 1977; Perrone and Hinkle, 1978). A recent report (Thrift et al., 1999) showed that injecting heifers with 2 mg T_3/d induced negative feedback on the thyroid axis and decreased serum concentrations of T_4 to below detection levels of their assay. In this chapter, I also decreased concentrations of T_4 in serum of cattle to below detectable levels by injecting 3 μg T_3/kg BW daily.

The hormone T_3 also increases synthesis of growth hormone mRNA (Samuels and Shapiro, 1976; Seo et al., 1977; Martial et al., 1977). However, at least in GH_3 cells, the concentration of T_3 required to increase growth hormone mRNA is 45-fold greater than the concentration required to down regulate TRH receptors (Hinkle et al., 1979). Another report demonstrated that hyperthyroidism in cattle induced by injecting T_4 increased concentrations of T_3 in plasma by 65% but did not affect basal secretion of growth hormone (Hannon and Trenkle, 1991). In my experiment I chose to inject T_3 so that I could measure total T_4 in serum as an indicator of thyroid gland secretory activity. In my

experiment, injecting T_3 down regulated the thyroid axis in meal-fed steers without affecting basal secretion of growth hormone throughout the 28 and 30 days of injecting T_3, in the first and second experiments respectively.

Even though daily injections of T_3 did not affect basal secretion of growth hormone, it remained to be determined if daily injections of T_3 affected release of growth hormone in the bovine anterior pituitary gland. To my knowledge no one has tested the effects of hyperthyroidism on GHRH-induced secretion of growth hormone in cattle. However, in the rat, hyperthyroidism does not affect GHRH-induced secretion of growth hormone (Dieguez et al., 1986; Root et al., 1986). Data from this chapter indicate that hyperthyroidism does not affect GHRH-induced secretion of growth hormone in cattle.

Similarly, T_3-induced hyperthyroidism did not affect TRH-induced secretion of growth hormone in meal-fed steers. However, the AUC of growth hormone following injection of TRH was numerically less in T_3-treated steers. These data contrast results reported in rats (Chihara et al., 1976). Because basal, GHRH-, and TRH-induced secretion of growth hormone in meal-fed steers were not affected by injections of T_3, it appears that the anterior pituitary gland's ability to synthesize and secrete growth hormone is not affected. The failure of T_3-treatment to decrease TRH-induced secretion of growth hormone suggests that the TRH receptors on the anterior pituitary were not down regulated by T_3-treatment. It is possible that injections of T_3 at doses greater than 6 μg/kg BW would in fact down regulate TRH receptor expression and inhibit TRH-induced secretion of growth hormone.

There are several reports suggesting that serotonin plays a role in regulating secretion of growth hormone. In the rat, concentrations of growth hormone in serum are highly correlated with serotonin turnover (ratio of metabolite, 5-hydroxyindoleacetic acid to serotonin) in the brain (Smythe et al., 1982). Similar results have been observed in cattle (Gaynor and coworkers, unpublished). Previous experiments in this dissertation and those of Gaynor et al. (1996) demonstrated that the effects of a serotonin receptor agonist on secretion of growth hormone are mediated via hypothalamic hormones. Chen and Ramirez (1981) reported that serotonin induced secretion of TRH from perifused rat hemihypothalami. In this chapter, I demonstrated that inhibition of the hypothalamic-pituitary-thyroid axis with negative feedback inhibits but does not completely block serotonin receptor agonist-induced secretion of growth hormone in cattle. However, inhibition of the hypothalamic-pituitary-thyroid axis with negative feedback did not inhibit TRH-induced secretion of GH. One interpretation of these data is that injections of T_3 inhibited TRH mRNA transcription and translation within the hypothalamus but not expression of TRH receptors on the anterior pituitary gland. These data suggested that TRH may be one of several mediators of serotonin-induced secretion of growth hormone in cattle.

It is possible that some other hypothalamic peptide that was not examined in this dissertation may play a role, in concert with TRH, in mediating serotonin-induced secretion of growth hormone. Support for this hypothesis comes from a report by Gaynor and coworkers (1996) in which meal-fed steers were pretreated

with either yohimbine or idazoxan (α_2-adrenergic receptor antagonists) and then injected with quipazine (serotonin receptor agonist). Pretreatment with these α_2-adrenergic receptor antagonists partially blocked serotonin receptor agonist-induced secretion of growth hormone. These data indicate that serotonergic and α_2-adrenergic neuronal systems work in concert to regulate secretion of growth hormone.

In this chapter, the T_4 data obtained after injection of the serotonin receptor agonist do not support the hypothesis that TRH mediates serotonin receptor agonist-induced secretion of growth hormone. However they do not negate it either. If TRH mediates serotonin receptor agonist-induced secretion of growth hormone, then the serotonin receptor agonist should also increase secretion of TSH and ultimately increase serum concentrations of total T_4. In this chapter, both vehicle-injected controls and T_3-treated steers responded to TRH with an increase in serum concentrations of total T_4. However, injection of quipazine did not increase serum concentrations of total T_4 in either corn oil-injected controls or T_3-treated steers. One explanation for these results may be that when we determined the dose of serotonin receptor agonist, we determined a dose optimal for inducing secretion of growth hormone (Gaynor et al., 1996) not TSH or T_4 secretion. In the rat approximately 61% of the cells that contain TRH receptor mRNA are somatotropes (Konaka et al., 1997). If serotonin receptor agonist-induced secretion of growth hormone is mediated by TRH, perhaps the dose required to induce TSH secretion and ultimately T_4 secretion is different from the dose required to induce secretion of growth hormone.

In conclusion, these results demonstrate that daily injections of T_3 negatively feed back on the thyroid axis and reduces serum concentrations of total T_4. I also demonstrated that daily injections of T_3 reduces, but doesn't completely abolish quipazine-induced secretion of growth hormone. These data support the hypothesis that TRH mediates, in part, serotonin receptor agonist-induced secretion of growth hormone in cattle.

SUMMARY AND CONCLUSIONS

My overall goal in this dissertation was to understand the mechanisms by which serotonin induces secretion of growth hormone in cattle. Specifically, my objectives were to: 1) determine if a serotonin receptor agonist (quipazine) acts directly on the anterior pituitary gland to induce secretion of growth hormone in cattle and; 2) determine the role of SS, GHRH, TRH and PACAP in mediating the effects of serotonin receptor agonist-induced secretion of growth hormone in cattle. To address my objectives I used both in vitro and in vivo approaches.

In a previous experiment, Gaynor and coworkers (1996) reported that quipazine did not induce secretion of growth hormone from dispersed bovine anterior pituitary cells in a perifusion culture system. In contrast, a more recent report by Balsa and coworkers (1998) demonstrated that serotonin induced secretion of growth hormone from rat anterior pituitary cells co-cultured with posterior pituitary cells in a static system with medium supplemented with corticosterone. The requirement for corticosterone can be explained because glucocorticoids are required for expression of voltage gated calcium channels (Fomina et al., 1993; Fomina et al., 1996), However, Balsa and coworkers were unable to explain the requirement for posterior pituitary cells for serotonin induced-secretion of growth hormone, and speculated that there was an unknown factor from posterior pituitary cells that mediated the effects of serotonin on secretion of growth hormone.

In chapter 1 I reported that quipazine does not induce secretion of growth hormone from bovine anterior pituitary cells co-cultured with posterior pituitary cells in medium supplemented with cortisol. Results presented in Chapter 1 also showed that the serotonin receptor agonist did not directly induce secretion of growth hormone from perifused dispersed anterior pituitary cells. Therefore, my data support the conclusion of Gaynor et al (1996) that serotonin does not directly induce secretion of growth hormone from the anterior pituitary gland in cattle. In addition I demonstrated that the posterior pituitary gland does not mediate the effects of serotonin on the secretion of growth hormone. Collectively, the data supported the idea that the effects of serotonin on secretion of growth hormone are mediated by hypothalamic hormones.

Murakami and coworkers (1986) reported that serotonin induced-secretion of growth hormone in rats was inhibited by passive immunization against rat GHRH, indicating that GHRH mediates serotonin-induced secretion of growth hormone in rats. In Chapter 1, I used a perifusion culture system to test the effects of quipazine on secretion of GHRH and SS from bovine hypothalamic tissue. Activation of serotonin receptors did not increase secretion of GHRH or decrease secretion of SS from perifused bovine hypothalamic slices. Therefore, I concluded that the effects of serotonin on growth hormone in cattle were not mediated by either GHRH or SS in cattle. This conclusion is supported by in vivo work from Moseley and coworkers (1988) and Gaynor and coworkers (1996). Both of these experiments utilized meal-fed steers. Moseley and coworkers reported that GHRH-induced secretion of growth hormone is greater before than

after feeding, while Gaynor and coworkers reported that serotonin receptor agonist-induced secretion of growth hormone is similar before versus after feeding. If GHRH was mediating the effects of serotonin receptor agonists, then GHRH-induced secretion of growth hormone would be expected to be similar before and after feeding.

Previous reports have implicated both PACAP (Yamauchi et al., 1996) and TRH (Chen and Ramirez, 1981) as possible mediators of serotonin-induced secretion of growth hormone in rats. Yamauchi et al (1996) reported that prior treatment of rats with a PACAP receptor antagonist suppressed serotonin-induced secretion of growth hormone. Chen and coworkers (1981) perifused rat hemihypothalami, treated them with medium containing serotonin and measured TRH in the medium effluent. Chen and coworkers reported that serotonin increased secretion of TRH from perifused hemihypothalami. In Chapter 2, I determined the minimal doses of TRH and PACAP required to maximally stimulate secretion of growth hormone in meal-fed steers. I demonstrated that activation of serotonin receptors induced secretion of growth hormone before feeding when concentrations of growth hormone in serum are normally high, as well as after feeding when concentrations of growth hormone in serum are low, and that the magnitude of response in growth hormone secretion was similar before and after feeding. If either PACAP or TRH is mediating the effects of serotonin then they too would be expected to induce similar amounts of growth hormone secretion before and after feeding. Using the doses of TRH and PACAP determined in Chapter 2, I tested the effects of TRH and PACAP before

and after feeding. Injection of PACAP before feeding induced greater secretion of growth hormone than injection of PACAP after feeding. Therefore, I concluded that PACAP probably does not mediate serotonin-induced secretion of growth hormone in cattle. In contrast, injection of TRH before feeding induced secretion of similar amounts of growth hormone as injection of TRH after feeding. Thus, TRH could be mediating serotonin receptor agonist-induced secretion of growth hormone.

In Chapter 4, I developed and utilized a T_3 negative feedback paradigm to down regulate TRH synthesis and(or) TRH receptor expression. Previous reports indicate that T_3 inhibits expression of TRH receptors in the pituitary gland (Lean et al., 1977; Perrone and Hinkle, 1978) as well as TRH synthesis in the paraventricular nucleus of the hypothalamus (Segerson et al., 1987; Koller et al., 1987; Dyess et al., 1988). When meal-fed steers were injected daily with T_3, endogenous secretion of T_4 was inhibited indicating that this paradigm was effective in down regulating the thyrotropic axis. However, daily injections of T_3 did not affect TRH-induced secretion of growth hormone, which, indicates that TRH-receptors on the anterior pituitary gland were not down-regulated. In contrast, when steers were injected with quipazine, growth hormone secretion was inhibited in T_3-treated steers compared with control steers that received corn oil. These data indicate that down regulation of TRH synthesis inhibits serotonin-induced secretion of growth hormone. However, T_3-treatment did not completely abolish serotonin receptor agonist-induced secretion of growth hormone,

indicating that TRH may not be the sole mediator of serotonin-induced secretion of growth hormone.

In conclusion, the serotonin receptor agonist does not act directly on the pituitary gland to induce secretion of growth hormone. Additionally, the data reported in this dissertation suggests that serotonin-induced secretion of growth hormone in cattle is mediated by TRH, while at the same time supporting the conclusion that the effects of the serotonin receptor agonist on secretion of growth hormone are not mediated by SS, GHRH or PACAP. I propose the model in Figure 28 as the mechanism of serotonin induced secretion of growth hormone in cattle. Specifically, serotonin neurons activate TRH neurons and induce the secretion of TRH into portal vessels. Portal vessels then transport TRH to the anterior pituitary where it induces the secretion of growth hormone.

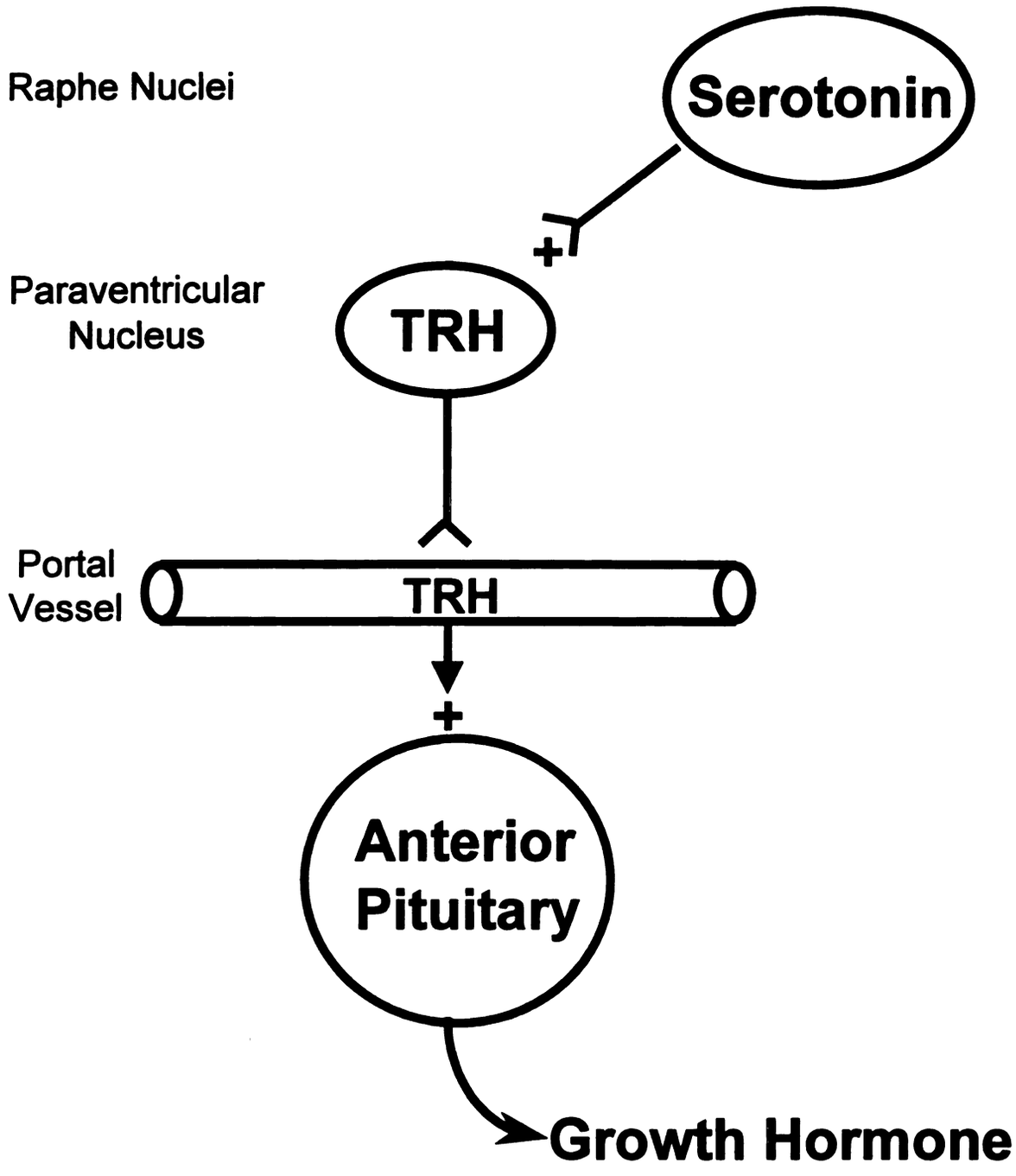


Figure 28. Proposed model for serotonergic regulation of growth hormone secretion in cattle. Ovals with connected lines represent serotonin and thyrotropin releasing hormone (TRH) neurons. The cylinder represents portal vessels between the hypothalamus and the anterior pituitary gland, which is represented by the large circle. The model depicts the neuronal pathway in which serotonin may induce secretion of growth hormone. Serotonergic neurons stimulate TRH neurons. In turn, TRH is secreted into portal vessels which transport it to the anterior pituitary gland where it stimulates secretion of growth hormone.

IMPLICATIONS

The data presented in this dissertation lends support to the hypothesis that TRH is a mediator of serotonin-induced secretion of growth hormone in cattle. However, further investigation is required to determine if other hypothalamic peptides are involved in the serotonergic regulation of growth hormone secretion, and whether or not this would be an appropriate neuroendocrine system to target in order to increase endogenous secretion of growth hormone.

Future research to determine if serotonin receptors are present on TRH neurons and exactly which serotonin receptor is involved would aid in identifying compounds to specifically increase secretion of growth hormone. Ultimately, we may be able to modulate serotonin receptors and increase growth and lactation with orally active compounds similar to serotonin reuptake inhibitors that are currently used in humans.

Since hyperthyroidism did not completely inhibit serotonin-induced secretion of growth hormone, it is possible that other hypothalamic peptides are involved in serotonergic regulation of growth hormone secretion in cattle. Two possible peptides are galanin and ghrelin (endogenous growth hormone releasing peptide-6 receptor agonist). Using specific receptor antagonists to block the action of these hormones at the anterior pituitary, then challenging the steers with a serotonin receptor agonist and measuring growth hormone would determine if either of these hormones were involved in serotonergic regulation of

growth hormone secretion. Alternatively, development of a portal vessel cannulation technique in cattle would allow scientists to measure the hypothalamic hormones in portal blood and determine if injection of a serotonin receptor agonist increases secretion of any of these hypothalamic hormones. Granted, this technique has already been developed in sheep, but prior research shows that activation of serotonin receptors does not increase secretion of growth hormone in sheep. Therefore, sheep would not be an acceptable model.

The fore-mentioned research would further our goal of understanding neuroendocrine regulation of growth hormone secretion. However, investigating the mechanism of feeding-induced suppression of growth hormone secretion in cattle would also improve our understanding of regulation of growth hormone secretion in cattle. Specific questions to answer might be what is the mechanism by which somatotropes become refractory to GHRH after feeding and does feeding reduce the neuroendocrine signals that stimulate secretion of growth hormone. Answering these questions may ultimately lead to methods to improve growth and lactation by increasing endogenous secretion of growth hormone.

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