ULTRADIAN OSCILLATION in SOMATOSTATIN BINDING SITES in the ARCUATE NUCLEUS of ADULT MALE RATS

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VI. REFERENCES
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In regard to the published manuscript [Tannenbaum GS, Farhadi-Jou F and Beaudet A (1993). Ultradian Oscillation in Somatostatin Binding in the Arcuate Nucleus of Adult Male Rats. Endocrinology V.133:PP.1029-1034] integrated into the present thesis:

The content of the sections entitled "Materials and Methods" and "Results", including the figures are, (under the supervision of Dr. G.S. Tannenbaum and Dr. A. Beaudet) totally and solely the contribution of F. Farhadi-Jou, resulting from data generated by experiments independently carried out and analysed by the candidate. (Please note, that the unbiased reporting of the results required that, during the analysis of the data, the candidate be blind with respect to the data regarding the plasma growth hormone profile presented in figure 3. Hence, this necessitated that the data for figure 3. be compiled with the generous assistance of Martine Lapointe). The remainder of the manuscript is the result of the collaborative contribution of all three authors, with Dr. G.S. Tannenbaum (who wrote the manuscript) and Dr. A. Beaudet, as primary and secondary supervisors respectively, providing the major input.

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I. ABSTRACT

In the adult male rat, growth hormone (GH) is secreted in an ultradian fashion at regular 3.3-h intervals from the anterior pituitary. Physiological and morphological evidence indicates that this pattern of secretion may be the result not only of an interaction of the stimulatory hormone, growth hormone releasing factor (GRF) and inhibitory hormone, somatostatin (SRIF), at the level of the pituitary, but also at the level of the hypothalamus. Consistent with this, SRIF binding sites have been demonstrated on a subpopulation of GRF-containing neurons in the arcuate nucleus. The aim of this study, in general, was to examine what role, if any, these bindings sites play in the temporal regulation of GH secretion at the level of the hypothalamus. More specifically, the possibility of the existence of a temporal relationship between the established ultradian oscillation of GH secretion from the anterior pituitary and postulated fluctuation in the number and/or density of SRIF binding sites in the arcuate nucleus was examined. In two separate experiments the ultradian rhythm of GH secretion was established in two different group of rats. In each experiment the rats were equally divided and decapitated at the times corresponding to peak (1100h) or trough (1300h) GH plasma levels. Next, 10um coronal brain sections beginning from the retrochiasmatic nucleus and ending at the mamillary recess of the third ventricle were processed for high resolution [\textsuperscript{125}I] SRIF radioautography. Both the binding density and the number of cells labelled were determined using computer assisted microdensitometry. The combined results of the two experiments revealed a 2 to 3 fold increase in the number of [\textsuperscript{125}I]SRIF cells labelled, concomitant with a 65% higher density throughout the rostrocaudal extent of the arcuate nucleus in rats decapitated at 1100h as compared to 1300h. Therefore, these results demonstrated an ultradian variation in SRIF binding to arcuate neurons in relation to the peaks and troughs of the GH rhythm. In conclusion, the results were interpreted as being due to one or more of the following: 1) Occupancy of SRIF receptors by endogenous ligand; 2) Changes in the affinity of SRIF receptors; 3) Changes in the number of SRIF receptors due to increases/decreases in their internalization/recycling or transcription/translation. In addition, the role of this rhythmicity in SRIF receptors in the generation of the ultradian rhythm of GH secretion was briefly examined.
II. RESUME

L'hormone de croissance (GH) du rat mâle adulte est libérée l'antéhypophyse, de façon ultradienne à interval les réguliers de 3.3h. Des preuves physiologiques et morphologiques indiquent que ce rythme de sécrétion peut être le résultat de l'interaction entre l'hormone stimulatrice, le facteur de libération de GH (GRF), et l'hormone inhibitrice, la somatostatine (SRIF), au niveau de l'hypophyse et de l'hypothalamus. En accord avec cette hypothèse, des sites de liaison SRIF ont été démontrés dans le noyau arqué sur des neurones GRF putatifs. L'objectif de cette étude est d'examiner si les sites de liaison démontrent aussi une variation ultradienne accompagnant celle de la concentration de GH dans le plasma. Dans deux expériences séparées, le rythme ultradien de la sécrétion de GH a été établi dans deux groupes de rats. Dans chaque expérience, les rats ont été divisés également et sacrifiés aux temps correspondant au maximum (11h) ou au minimum (13h) de la concentration de GH dans le plasma. Des sections coronales du cerveau de 10 μm en commençant par le noyau rétrochiasmatique jusqu'au récessus mammillaire du troisième ventricule ont été préparées pour la détection de [125I] SRIF. La densité de liaison ainsi que le nombre de cellules marquées ont été determinés à l'aide de la microdensitométrie informatisée. Le nombre des cellules marquées a augmenté du double et il y a eu également une augmentation de 65% de la densité chez les rats sacrifiés à 11h comparés à 13h. Les résultats ont été expliqués comme étant dus à: 1) la liaison du ligand endogène aux récepteurs SRIF; 2) des changements dans l'affinité des récepteurs SRIF; 3) des changements dans le nombre de récepteurs SRIF dus à une hausse/baisse au niveau de l'internalisation/recyclage ou de la transcription/translation.

En conclusion l'importance de ces résultats pour ce rythme pulsatile de GH a été élaboré.
III. INTRODUCTION

Interest in the processes underlying our own growth and development has probably occupied its predominant status in the sphere of human thought from the birth of the first homo sapiens. However, the first documented, at least quasi-scientific study on the topic can be traced back to only about two centuries ago, to Count Philibert Guereau de Montbeillard's 1777 longitudinal analysis of his children's linear growth patterns from birth to 18 years of age [see Tanner, 1978]. Since nearly as long ago, it has been understood that in the human species the process of growth has a multifactorial basis that depends on a variety of both biologic and sociologic variables, ranging from the genetic make up of the parents to the socioeconomic status of the child. Today, more than a century of clinical observation and scientific experimentation has determined that the final common pathway, or rate-determining step through which all of these factors are mediated is a complex neuroendocrine control system. In essence, growth is now understood to be the result of intricate relationships among the various constituents of system that includes: the central nervous system, specifically the hypothalamus; anterior pituitary gland; trophic factors; target glands; and peripheral tissues.

A. GROWTH HORMONE

1. Discovery and Isolation
The initial discovery of one of the major constituents of this system came from the clinical observations of Pierre Marie in 1886 [see Strobl and Thomas, 1994], who noted the enlargement of the pituitary gland in an acromegalic patient. However, the earliest scientific study on the elucidation of the function of the anterior pituitary is attributed to the noted neurosurgeon Harvey Cushing who, during the first decade of this century, provided the first experimental data linking it to growth in humans, canines and rodents [Cushing, 1909a & 1909b]. In the subsequent decade it was demonstrated that saline extracts of bovine anterior pituitary promoted and restored growth in both normal [Evans and Long, 1921] and hypophysectomized [Smith, 1927] rats, respectively. In 1944, Li and Evans isolated a peptide from bovine pituitaries, that unfortunately was unable to produce the same biologic response, i.e. the promotion of growth, in primates [Bennet et al, 1950]. Nevertheless, this finding led to the subsequent discovery and isolation of a very similar, but this time biologically potent factor from the pituitaries of patients treated for breast cancer by hypophysectomy, or from monkey and human cadavers [Li and Papkoff, 1956].

Similar substances have subsequently been isolated and characterized from the pituitary gland of several species ranging from fish, mice, rats, rabbits, cats, dogs, pigs, sheep, cows, rhesus monkeys, baboons, and of course humans [See Rudd, 1991]. These discoveries led to the conclusion that although the factors obtained from different species may exhibit species-specific biochemical differences, they are nevertheless part of the same family of factors named growth hormones (GHs)/ somatotropins that are responsible for the physiological process of growth in all vertebrates. This fact is well supported by the high positive correlation between the degree of cross-species growth-promoting efficacy and/or potency of these peptides and the contiguity of the species from which they were obtained on the phylogenetic ladder of evolution. This of course, in reality, is another representation of the degree of homology between the DNA of these species [Li, 1957].

2. Molecular Genetics: Growth Hormone Gene Expression and Synthesis
Consistent with this homology, it has now been shown that both the GH and Prolactin (PRL) genes are present in all vertebrates and hence are part of the same family [Barsh et al, 1983]. This has led to the hypothesis that this family originated from a common ancestor progenitor gene that underwent duplication approximately 350 million years ago to result in genes that are presently located on different chromosomes [Miller and Eberhardt, 1983]. Subsequent duplication of the GH gene in primates or the PRL gene in rodents gave rise to a cluster of five closely related placental lactogen (PL) genes that, in the human, span between 50 to 66.5 Kb of DNA along the proximal or long arm of chromosome 17 [Walker et al, 1991].

However, it has come as a surprise that these five genes are differentially expressed in a tissue specific manner despite the fact that their DNA, as well as their associated intervening intron and 5' flanking sequences, are highly homologous. In fact, they all share a 93.8% sequence identity in their first 464 base pairs at the 5' flanking terminal. In humans, the genes from this hGH/PL family are referred to as the GH normal gene (GH-N/GH-1), and GH variant or placental GH (GH-V/GH-2) gene, respectively. The remaining three are named the chorionic somatomammotropin or placental lactogen A, B, and Like genes (CS/PL-A),(CS/PL-B),(CS/PL-L), respectively [Selby et al, 1984].

Genetic deletion and mutation studies have shown that from these five genes the GH-N gene is the only one critical for normal postnatal growth [Cogan et al, 1993]. It is expressed exclusively in the anterior pituitary yielding both a major and minor form of GH. The major form which accounts for anywhere between 85 to 95 percent of the circulating immunoreactive GH in both children and adults is processed from a prohormone by cleaving a 26 amino acid signal sequence as it is being transferred across the rough endoplasmic reticulum into its storage granules. This yields a 22 KD, single chain 191 amino acid peptide which includes four cysteine residues at positions 53, 162, 182, and 189. The minor form which accounts for approximately the remaining 5 to 15 percent of the circulating GH results from the alternative splicing of the transcribed RNA, which results in a 20 KD variant that
lacks 15 of the internal amino acid residues from number 32 to 46 present in the major form [Baumann, 1991]

3. Pattern of Secretion

As alluded to above it has become apparent that although the biochemical structure of the GH's obtained from a variety of species may differ, there exists a striking similarity across species in the temporal pattern by which GH is secreted. The emerging consensus is that in nearly all mammalian species, GH, like all other pituitary hormones, is secreted in an episodic manner. This pattern of pulsatile secretion was recognized to occur in the human [Quabbe et al, 1966] well before the definite characterization of the two peptides that are now known to be responsible for its genesis.

The amplitude, frequency and regularity of these spontaneous, pulsatile GH secretory episodes varies over a wide scale. These variations are, to some degree, determined by the species, and the gender, age, and size of the individual [Hindmarsh et al, 1991; Ono et al, 1991; Painson and Tannenbaum, 1991]. In terms of gender, in the sexually mature male rat, GH secretion is characterized by a striking ultradian rhythm with high-amplitude GH pulses and an interpulse interval of 3.3 hours. This episodic secretion occurs several times over a 24 hour period and results in plasma GH levels which vary from trough levels that are below the detection level of almost all but the most sensitive radioimmunoassays, to crests as high as 200 ng/ml [Tannenbaum and Martin, 1976].

On the other hand, female rats exhibit more frequent, low amplitude GH pulses and elevated basal GH levels with no clear rhythm [Saunders et al, 1976]. In terms of species variation, GH peak amplitude levels vary from 400 - 800 ug/L in the guinea pig [Gabrielsson et al, 1990] to 4 - 6 ug/L in the rabbit [Minamitani et al, 1989]. In terms of age, this pulsatile release of GH manifests itself throughout the ontological development of nearly every mammalian species studied to date, including humans [Casanueva, 1992]. Characteristically
the mammalian foetus and newborn exhibit higher pulse frequencies, which decrease to their slowest just prior to sexual maturity. However, during sexual maturation it is the pulse amplitude which increases appreciably, and then once again slowly recedes with every decade during adulthood [Cara 1993; Corpas et al, 1993; Miller et al, 1993].

Interestingly, in rodents, this pulsatile mode of exposure to GH results in more pronounced physiological effects, than does continuous administration of the same total dosage [Isgaard et al, 1988]. Similar observations have been made in children in whom the longitudinal growth response to hGH replacement therapy was greater when the identical amount was administered in divided doses three to four times weekly rather than once per week [Frasier and Lippe, 1990]. In fact, this temporal pattern of release which occurs in human neonates, children, adolescents, and adults is believed to play a crucial, but as yet, imprecisely defined role in permitting the realization of all of GH's physiological actions [Goji, 1993]. Hence, even though not initially apparent, it was found that the pattern of spontaneous GH secretion in the male rat could serve as good model for the GH release pattern exhibited by man [Winer et al, 1990].

4. Physiological Actions

Presently, GH has become firmly established as the factor that plays the central and critical role in postnatal growth; both forms have been shown to be capable of stimulating growth in numerous species including, more recently, in transgenic mice [Szabo et al, 1995]. However, it has also been demonstrated that the two variants of GH differ slightly in their biological effects and, unlike other hormones, do not merely exert their effects on one specific target organ [Lewis et al, 1980]. Hence, in terms of present scientific nomenclature, the term "growth" is not solely a description of the linear extension of the long bones of the skeleton. In the context of the ontological maturation of nearly all mammalian species it also refers to an increase in bi-iliac distance, head circumference, and the weight of the reproductive organs. GH exerts these effects on its target tissues of bone, cartilage, and
skeletal muscle through both hypertrophy and hyperplasia by inducing differentiation and proliferation at the cellular level and protein synthesis at the molecular level.

In a series of critical experiments on hypophysectomized animals in 1957, Salmon and Daughaday demonstrated that GH stimulated the synthesis of another factor in the serum that in turn modulated cellular growth by acting in a more generalized fashion on both soft and skeletal tissue. Hence, two of these peptides were isolated from mammalian serum and named Somatomedin C (SM-C) and Multiple Stimulating Activator (MSA) [Daughaday et al, 1972]. In view of their marked homology with the @ & B regions of proinsulin, the preferred names for SM-C and MSA became insulin-like growth factor 1 and 2 (IGF-I, IGF-II) respectively. However, unlike GH, these were found not to be species specific, except in some non-mammalian species such as fish [Daughaday et al 1987].

The original somatomedin hypothesis proposed that GH stimulated hepatic production and secretion of IGF-I, which subsequently acted on target tissues. However, more recent evidence has suggested that the IGFs are also produced and act in an autocrine manner in many tissues [D'Ercole et al, 1984; Holly and Wass, 1989]. The present consensus on the temporal and spatial mechanism by which GH exerts its actions has become known as the synergistic dual effector theory. Evidence is accumulating to provide support for this theory, which proposes that GH is able to act on its targets both in a direct or paracrine fashion concomitant with an indirect or endocrine manner via the production of the IGFs from the liver [Cohick and Clemmons, 1993].

In addition to promoting somatic growth, GH plays significant roles in many other physiological systems. GH plays an important role in the regulation of metabolism and body composition. GH stimulates lipolysis, has a diabetogenic effect on carbohydrate metabolism and hence enhances gluconeogenesis in both the liver and muscle [Jorgensen et al, 1990]. GH has been shown to be play a major role in the homeostatic systems of the body by regulating renal plasma flow, glomerular filtration rate, proliferation of T-cells [Gala, 1991;
Gelato, 1993], and maintaining bone mineral density [Guler et al, 1989; Spencer et al, 1991]. Last, but not least, GH has profound effects on both male and female fertility, and reproduction not only through the development of the gonads, but also in inducing ovulation and sperm production [Spiteri-Grech and Nieschlag, 1992; Yoshimura et al, 1993].

Consequently, with the exception of the androgens and estrogens, GH is probably the most important hormone, not only because of its necessity for the ontological development of our own species, but also due to its significance to all species spanning the entire continuum of the phylogenetic ladder of evolution. However, the major focus of this review shall be on the molecular, biochemical and physiological regulation of GH synthesis and secretion, rather than on its myriad of functions.

B. NEUROENDOCRINE CONTROL of GH SECRETION by SOMATOSTATIN and GROWTH HORMONE-RELEASING FACTOR

The first report implicating the hypothalamus and indeed the central nervous system (CNS) as one of the major constituents of a neuroendocrine system responsible for the regulation of hormone secretion was published in 1947 by Green and Harris who described a portal pituitary blood supply that was densely innervated by cell bodies located within the hypothalamus. They proposed that this unique neuroanatomical arrangement was the basis for the control of pituitary hormone secretion by the hypothalamus.

However the first major evidence for the existence of a CNS control of GH secretion came from reports by Reichlin [1960, 1961] demonstrating that basal hypothalamic lesions impaired growth in young rats, depleted the content of GH in the pituitary, and lowered plasma GH levels. These finding were later corroborated by studies utilizing more
anatomically specific lesions limited to the ventromedial hypothalamic area that encroached on the arcuate nucleus, but spared the median eminence [Frohman and Bernardis, 1968]. Indirect evidence for the critical importance of the integrity of the CNS-pituitary axis in controlling GH secretion also came from clinical observations that patients with pituitary stalk transections had reduced plasma GH concentrations with partial or complete loss of the GH response to insulin-induced hypoglycaemia [Glick, 1970]. These observations were later confirmed by animal experiments, showing that lesions limited to only the median eminence of squirrel monkeys also resulted in failure of the GH response to insulin induced hypoglycaemia.

1. GH-Releasing Factor (GRF)
   a) Discovery and Isolation

   The original report providing evidence for an actual GH secretagogue of hypothalamic origin was published in 1964 by Deuben and Meites who demonstrated GH-releasing activity in median eminence extracts of the hypothalamus. Its effect on GH secretion was shown to be both potent and specific as it was able to cause a six-fold increase in the amount of GH released by cultured rat hemi-pituitaries, while the administration of extracts derived from the cerebral cortex failed to induce the release of any detectable amount of GH. These in vitro findings were soon followed by another laboratory demonstrating the presence of a GH-releasing factor in crude extracts of pituitary stalk-median eminence tissue by measuring the effect of systemic injection of these extracts on the GH content of the pituitary as estimated by the tibial epiphyseal cartilage bioassay in hypophysectomized rats [Krilich et al, 1965]. However, interestingly enough, the first report of a purified factor that would enhance growth of rats and cause the release of GH into the medium of pituitaries incubated in vitro had already been published in 1962 [Franz et al,]. The purification of a similar factor from extracts of sheep stalk median eminence by McCann's laboratory [Dhariwal et al, 1965 &1969], allowed others [Garcia and Geschwind, 1966] to demonstrate its potent GH-releasing ability in vivo, by administrating it to primates.
The isolation and complete characterization of what is now referred to as GH releasing factor/hormone (GRF/GHRH) or somatocrinin remained elusive until the description of the syndrome of ectopic GRF secretion [Zafar et al, 1979] and the preoperative identification of two acromegalic patients with pancreatic islet adenomas from which GRF was subsequently sequenced [Guillemin et al, 1982; Rivier et al, 1982]. Two of the three GRF forms, namely those containing 40 or 44 amino acids, that were found in the pancreatic tumours were subsequently identified in human hypothalamus [Bohlen et al, 1983]. The factor with equivalent function in the rat was found to be a 43 residue peptide with a free carboxy terminus which displayed up to 33% non-homology with that isolated from humans [Spiess et al, 1983]. Other studies [Badger et al, 1984; Barinaga et al, 1985] were able to demonstrated that this factor could independently stimulate GH secretion in vitro.

b) Molecular Genetics

In humans, the GRF gene has been localized to the region of the p12 band of chromosome 20 [Mayo et al., 1985; Rao et al, 1991] and exists as a single copy consisting of five exons spanning a 10 Kb domain. There is a single mRNA species of about 750 nucleotides that exhibits slight heterogeneity due to the presence of an alternate splice site one triplet downstream in the fifth exon. This mRNA codes for pre-pro GRF 107/108 amino acid precursors that undergo proteolytic processing and degradation of their 30 amino acid residue signal peptide to yield the pro-GRF peptide containing the 44 GRF residue peptide, an amidation signal, and a 30/31 carboxyl terminal peptide (GCTP).

Studies of GRF post-translational processing in pituitaries of mice expressing the hGRF transgene have demonstrated that subsequent to the removal of the signal peptide, the pro-GRF is cleaved by endopeptidases to a 45 amino acid peptide which contains the 44 amino acid GRF attached to a glycine and the GCTP [Brar et al, 1991]. Next, this 45 amino acid peptide is converted to the familiar 44 amino acid form containing an amidated NH₂ terminus by the enzyme that was first identified in relation to pro-opiomelanocortin processing,
peptidylglycine @- amidating monooxygenase. The 40 amino acid version of GRF is derived from this 44 amino acid form by cleavage of the four carboxyl terminal amino acid residues by endopeptidases that are present not only in the hypothalamus, but also in other neuronal tissue [Brar et al, 1991].

However, in other mammalian species studied thus far this 40 amino acid version has never been described and GRF always appears to exist in only one molecular form. This has been an unexpected finding since, as cited above, all the necessary converting enzymes are present in the mouse to express the hGRF transgene all the way to the 40 amino acid version. Consequently, although the 44 amino acid version seems to have become accepted as the more biologically important version in mammals biologically, both forms are still physiologically relevant in the human species, since studies have demonstrated an abundance of the 40 amino acid version in the human hypothalamus, and its comparable bioactivity in man [Ling et al, 1984]. Furthermore, the 40 amino acid version of GRF present in the human, like the GRF present in the species most often used to study its physiology i.e. the rodent, lacks the requirement of an amidated carboxyl terminus to maintain its potent secretagogue ability [Spiess et al, 1983]. In fact, the full biological activity of all GRFs remains intact in the species from which they were derived as long as the first 29 amino acids are present.

c) Ontogeny and Anatomic Localization

Immunohistochemical [Ishikawa et al, 1986; Rodier et al, 1990] and radioimmunoassay [Jansson et al, 1987] studies have shown that GRF is first detectable at embryonic day 18 in the rat. Consistent with this, in situ hybridization experiments [Chihara et al, 1994] have demonstrated the presence of GRF mRNA one day earlier. In terms of its localization within the CNS of both rodents and primates almost all of the GRF-containing neurons, with the exception a limited number in the cortex and amygdala, are located in the arcuate nucleus of the mediobasal hypothalamus [Ibata et al, 1986]. Indeed, the organization of hypothalamic GRF-containing neurons has been shown to be essentially similar in all adult
mammalian species studied thus far, including human [Bloch et al, 1983].

Nearly all of the axonal projections from these GRF perikarya project to the outer layer of the median eminence, suggesting that the primary, if not the only, function of GRF is to regulate GH secretion [Bohlen et al, 1983; Kita et al, 1985; Merchantaller et al, 1984]. The limited number of GRF-containing neurons that encapsulate the caudal part of the ventromedial nucleus in its dorsal extent, do not project to the median eminence but innervate other hypothalamic or perihypothalamic nuclei. Neuronal cell bodies extend to the periventricular zone and partially overlap both the tuberoinfundibular and ventromedial nuclei [Bloch et al, 1984].

d) Cellular Receptors

The focus of this thesis will be on the receptors for Somatostatin. However, for the sake of completion it should be noted that the receptor for GRF has been cloned in both rodents [Lin et al, 1992] pigs [Hsiung et al, 1993] and humans [Mayo, 1992]. Gaylinn et al [1993], reported the cloning of a cDNA encoding a human GRF receptor from an acromegalic pituitary cDNA library, which encodes a 423-amino acid protein that has seven putative transmembrane domains characteristic of G-protein-coupled receptors. In line with GRF's major site of action, Northern analysis indicated that this GRF receptor mRNA was most abundant in extracts of pituitary and was not detected in other tissues. Furthermore, it is intriguing that a recent report has demonstrated that the GRF receptor is regulated by GH since exogenous administration of GH suppresses the mRNA of GRF's receptor in the pituitary. However, GRF is necessary for the expression of its receptor, since suppression of GH secretion in rats administered GRF antibody was due to a decrease in the expression of the receptor for GRF [Horikawa et al, 1996]

2. Somatostatin (SRIF)
a) Discovery and Isolation

Although the concept of inhibitory factors or "chalones" controlling the release of substances from the anterior pituitary was suggested more than half a century ago, the discovery of the factor that inhibits the release of GH from the anterior pituitary came about more recently in a serendipitous manner. Ironically, it was identified by the group that was screening hypothalamic extracts for the factor possessing GH-releasing activity, who instead isolated a low molecular weight peptide concentrated in the median eminence with inhibitory activity [Kruilich et al, 1968]. Subsequent to further studies by this same group on the physiology and specificity of this factor for inhibiting GH secretion [Crighton et al, 1969; McCann et al 1968], its isolation and characterization was achieved by yet another laboratory that was also searching for the hypothalamic factor that stimulates GH release from pituitary cells. In 1973 Brazeau et al, isolated and characterized a 14 amino acid peptide from ovine hypothalamus that has a cyclic conformation due to an intramolecular disulfide bond linking two cysteine residues. It consistently and potently inhibited GH secretion from pituitary cells in culture, and hence was named Somatotropin-release inhibiting factor (SRIF) [Brazeau et al, 1973].

A second peptide consisting of 28 amino acids containing the complete sequence of the 14 amino acid peptide at its carboxyl terminus was subsequently discovered in the gut [Pradayrol et al, 1980] and the hypothalamus [Schally et al 1980]. Soon thereafter the potent inhibitory effect of this version of SRIF was also demonstrated [Brazeau et al, 1981]. In fact, Tannenbaum et al, [1982] were the first to show that SRIF-28 is a more selective and longer lasting inhibitor of both insulin and spontaneous GH secretion. These discoveries were shortly followed in the ensuing years by studies demonstrating the presence of both forms of SRIF in a number of neuronal populations throughout the entire neuroaxis, including the central and peripheral nervous system as well as in the endocrine glands of the stomach, intestine and pancreas [Johansson et al, 1984].
b) Molecular Genetics

In mammalian species, a single gene codes for both the 14 and 28 amino acid versions of SRIF, unlike in the fish where each peptide is encoded for by its own separate gene [Andrisani and Dixon, 1990]. The molecular cloning of the gene for human and rat SRIF [Shen and Rutter, 1984; Montminy et al, 1984] and the elucidation of the SRIF prohormone sequence revealed that SRIF mRNA is transcribed as part of a larger precursor molecule, named pre-prosomatostatin [Goodman et al, 1983]. In humans, the gene has been located on chromosome 3 [Naylor et al, 1983] where the mechanism for its expression appears to be very similar to that for other mammalian neuropeptides. The 565 bp pre-prosomatostatin cDNA contains an open-reading frame of 348 bp, coding for a 116 amino acid polypeptide. The cleavage of this prohormone into SRIF-14 and SRIF-28 requires the activity of endoproteolytic peptide-converting enzymes (PCs). In total, six of these putative processing enzymes, namely, Furin, PC1/PC3, PC2, PACE-4, PC4 and PC5/PC6 have been identified [Seidah et al, 1993]. The cleavage is believed to take place at mono- or dibasic residues, with other structural requirements still being necessary for the coordinated action of these endoproteases.

In general, it seems that prosomatostatin is processed variably according to the cell type expressing it and on whether it is being processed within a constitutive or regulated secretory pathway. In a constitutive pathway, e.g. in mucosal cells, prosomatostatin is processed by the action of PACE-4 to yield SRIF-28, the major form in this tissue type. On the other hand, in neuronal and pancreatic tissue, the main product is SRIF-14, which is generated by the endoproteolytic action of PC2 to a lesser extent PC1 [Brakch et al, 1995]. Apparently SRIF-28 arises by processing from a single precursor, however, it is not as clear whether most of SRIF-14 arises from the direct processing of prosomatostatin by dibasic cleavage or rather from further cleavage of SRIF 28 through a trypsin-like enzyme acting on the Arg-Lys site in position 13-14 of SRIF-28. On the other hand, it is quite clear that SRIF-14 and -28 are the only two biologically active factors that result from the processing of...
prosomatostatin. Studies utilizing molecular sieve filtration followed by radioimmunoassay have reported that, in brain tissue, SRIF-14 accounts for up to 70% of the total SRIF activity and SRIF-28 for the remaining 20 to 30% [Moyse et al, 1984]. However, some studies have shown that SRIF-28 has distinct physiological effects, being in some cases much more potent and longer acting than SRIF-14 [Mandarino et al, 1981; Tannenbaum and Patel, 1986]

c) Ontogeny and Anatomic Localization

As alluded to before, both forms of SRIF have been shown to be present in both neuronal and non neuronal tissues. In fact, Patel et al [1981] have reported that the non neuronal portion localized in the gut and pancreas amounts to more than 70% of the total body SRIF, which means that there is more SRIF in a single GI tract than in the half million hypothalami that were originally required for its isolation. In terms of the SRIF localized in neuronal tissue, there is an excellent match between its distribution, as visualized by immunocytochemistry or in situ hybridization, in lower mammals and primates, including humans [Bouras et al, 1987; Priestly et al, 1991].

Somatostatinergic neurons occur in high densities throughout the CNS. They are much more numerous and heavily stained in the telencephalon and diencephalon than in the mesencephalon and rhombencephalon. More specifically, SRIF-14 and/or -28 immunoreactive neurons have been shown to exist in both rat and primate, including human, olfactory bulb, all subdivisions of the cerebral cortex (layers II to VI), hippocampal formation and striatum. In the hippocampus they are predominantly found in the hilus of the dentate gyrus and the stratum oriens in the CA1, with only a few cells in the pyramidal layer [Bennet-Clark et al, 1980; Chan-Palay, 1987; Hendry et al, 1984].

A differential developmental pattern exists for SRIF expression between these CNS divisions. In the forebrain, the expression is continuous over ontological development with
only a slight decrease in adults after the peak level of immunoreactivity and mRNA levels are reached in the first or second postnatal week. On the other hand, in the lower brain stem, SRIF expression is transient subsequent to reaching a maximal level in the perinatal period [Maubert et al, 1994]. Consequently, in addition to its widely accepted function of as a neuromodulator/neurotransmitter in the CNS [Renaud et al, 1975], these studies suggest that SRIF may also play a further critical role as neuroregulator of neuronal differentiation, maturation and maintenance by acting as a neurotrophic and/or neurogenic agent in both the young [Gonzales et al, 1992], adult and aging brain [Epelbaum et al, 1986].

In the hypothalamus, in contrast to GRF's restricted localization, most areas possess an extensive network of SRIF containing fibers. Anatomically, distinct populations of immunoreactive perikarya are present in the suprachiasmatic, arcuate, ventromedial and both the anterior and parvocellular portion of the periventricular nuclei [Johansson et al, 1984]. Studies coupling retrograde tracing to immuno- cytochemistry have demonstrated that while nearly 80% of the SRIF neurons located in the periventricular nucleus send their efferents to the median eminence, the periventricular region receives no somatostatinergic afferents from any other hypothalamic nuclei [Kawano and Daikoku, 1988]. A study utilizing mechanical lesions and transections revealed the route through which SRIF is delivered to somatotrophs. Subsequent to projecting in a lateral direction to the lateral hypothalamus, SRIF-positive fibres travel for a short distance in the medial forebrain bundle and reenter the mediobasal hypothalamus at the level of the retrochiasmatic area to ultimately reach the external layer of the median eminence from which SRIF is liberated into the portal circulation [Epelbaum et al, 1981].

d) Cellular Receptors

The diverse actions of SRIF, outlined above, including its role in regulating GH secretion are mediated through membrane specific receptors. Various tyrosine-modified SRIF molecules have been used as iodinated ligands to study the binding sites recognizing
SRIF peptides. The first available radioligands were N-terminal tyrosine-modified ligands such as $^{125}$I[Tyr1] SRIF-14 that were extremely susceptible to degradation by aminopeptidases and endopeptidases present in brain membranes. Nevertheless, SRIF binding sites were originally characterized on a clonal pituitary cell line (GH4C1 cells) using radioligand binding studies with ($^{125}$I-Tyr1) SRIF [Schonbrunn and Tashjian, 1978]. This initial study was followed by the identification of putative receptors for SRIF in neural [Srikant and Patel, 1981b], endocrine, and exocrine tissues, as well as a large number other cultured cell lines such as AtT-20, and RINm5f [Heisler and Srikant, 1985; Sullivan and Schonbrunn, 1986].

Initially, monoiiodinated tyrosine-substituted SRIF agonists were shown to bind with nanomolar affinity to a single class of binding sites. Subsequently, pharmacological studies suggested the existence of multiple receptor subtypes with different affinities for various synthetic ligands such as SMS 201-995 (octreotide), MK 678 (seglitide), and CGP 23996. This was originally suggested by Srikant and Patel [1981a], who showed that the two different versions of SRIF, SRIF-14 and SRIF-28, had different affinities for rat brain versus pituitary receptors. Support for this view came from the utilization of the octapeptide SRIF agonist, octreotide, which in competition studies on rat brain membranes, demonstrated the heterogeneity of SRIF brain receptors. This agonist inhibited ($^{125}$I-Tyr1) SRIF binding to rat brain membranes in a biphasic manner; hence, it differentiated one binding site possessing a nanomolar affinity from another displaying a micromolar affinity for SRIF in the brain [Reubi, 1984; Tran et al, 1985]. Ultimately, two types of binding sites were characterized on the basis of their pharmacological properties, GTP dependence, pertussis toxin sensitivity, and desensitization [Raynor and Reisine, 1989; Raynor et al, 1991], the biological significance of which still remains to be determined. Therefore, two SRIF receptor types, A/I and B/II, were discriminated based on the former exhibiting a high affinity for the SRIF synthetic agonists octreotide and seglitide and being desensitizable, while the latter were not [Reubi, 1985].
In contrast to that observed in the brain, octreotide displaced SRIF binding to rat pituitary and pancreatic membranes in a monophasic manner. Subsequent studies confirmed that, although multiple SRIF receptor subtypes could be distinguished in the brain, the somatotrophs in the anterior pituitary exhibited only a single class of binding sites with a nanomolar affinity for octreotide [Epelbaum et al., 1987]. Unfortunately, multiple techniques used to isolate the SRIF receptors from various peripheral and neuronal tissues led to conflicting results concerning their sizes [Rens-Domiano and Reisine, 1992]. However, it became clear that the SRIF receptors were glycoproteins, sensitive to GTP and divalent cations, suggesting a coupling to GTP binding proteins [Epelbaum et al., 1994].

Subsequently, the development of in vitro radioautographic methods with an enhanced sensitivity, permitted localization of SRIF binding sites, in both human and rat brain with an enhanced sensitivity. These studies confirmed the regional distribution of SRIF binding sites anticipated from the binding studies performed earlier in brain membrane homogenates. They demonstrated that SRIF-1 sites labelled with \([^{125}\text{I}]-[\text{Tyr}3]\)octreotide and \([^{125}\text{I}]\)seglitide in the rat brain were almost identical, but were significantly different from SRIF-2 sites labelled by \([^{125}\text{I}]\)CGP 23996 [Epelbaum et al., 1985; Reubi and Maurer, 1985]. They also furnished substantial additional data concerning receptor distribution patterns. Film autoradiography, after incubation with tritiated or iodinated ligands, revealed a wide pattern of distribution of SRIF binding sites in the mammalian, including the human, brain [Reubi et al., 1986; Krantic et al., 1992]. Although some discrepancies were reported, they could be accounted for by the differences in radioligand or species of animal used. In terms of differences in hypothalamic binding sites, one group found moderate to dense binding in the peri- and para-ventricular zones [Uhl et al., 1985] in contrast to the initial radioautographic studies that failed to reveal such sites [Reubi and Maurer, 1985].

In order to better visualize hypothalamic binding sites, autoradiographic procedures were carried out either concomitant with treatments that were designed to deplete endogenous SRIF e.g. by cysteamine injections in vivo [Leroux et al., 1985], or subsequent
to *in vitro* preincubation of the brain sections in the presence of an excess GTP that served to dissociate the bound endogenous peptide from its binding site [Leroux et al, 1988]. These desaturation procedures resulted in an increase in the number of measurable binding sites. Therefore, GTP pretreatment of brain sections made it possible to detect new populations of binding sites at the hypothalamic level, resulting in SRIF-14 in observation of binding site populations in numerous hypothalamic areas including the preoptic area, the supraoptic nucleus, the fornix and optic tract. This regional distribution of SRIF binding sites in the human hypothalamus were later corroborated by a further study utilizing quantitative autoradiography [Najimi et al, 1991].

The final proof for the existence of SRIF receptor heterogeneity was provided recently. Five different SRIF receptor subtypes, designated as SSTR 1-5, have recently been described on the basis of consecutive molecular cloning studies. The first subtype, SSTR-1, was initially identified from human pancreatic islet cDNAs amplified using a reverse transcriptase polymerase chain reaction (PCR) procedure with degenerate oligonucleotides corresponding to the conserved regions in G protein coupled receptors (consisting of domains 3 and 4) followed by screening of human and mouse genomic libraries [Yamada et al, 1992a]. This was rapidly followed by cloning of SSTR-1 in the rat brain [Li et al, 1992]. The open reading frame corresponds to a 1173 bp long cDNA, encodes for the 3.8 to 4 kb SSTR-1 mRNA which results in a 391 amino acid protein.

The original SSTR-2 was identified in the human and mouse by screening corresponding genomic libraries with the human SSTR-1 [Yamada et al, 1992a]. It was subsequently sequenced in the rat brain [Kluxen et al, 1992] using an expression cloning strategy. It corresponds to a 1107 bp cDNA, that is transcribed into a 2.5 and 7.5 kb long messenger, and translated into a 369 amino acid protein. The diversity of the five SSTRs identified so far is further increased in that the SSTR-2 exists in two variant forms, generated by alternative splicing of the SSTR-2 mRNA [Vanetti et al, 1992]. The SSTR-2B form, which was cloned from NG 108-15 cells, encodes for a 23 amino acid-shorter protein, which
differs from the A form by 15 amino acids at its carboxyl terminus.

The third subtype, SSTR-3 was independently characterized from a mouse genomic library using both low stringency screening with the human SSTR-1 probe [Yasuda et al, 1992], and also by using the PCR method on a rat brain cDNA library [Meyerhof et al, 1992]. The 1284 bp open reading frame corresponds to a 4.4 kb messenger which encodes a protein of 428 amino acids. The human version of SSTR-3 was found to consist of 418 amino acids with a deletion in the third intracellular loop [Yamada et al, 1992b]. The fourth and fifth receptor subtypes, SSTR-4 & SSTR-5, were both also extracted from a rat genomic library by utilizing a similar PCR. The SSTR-4 cloning procedure employed oligonucleotide primers specific for mouse SSTR-1 and -2 which identified a 1152 bp cDNA that yields a 348 amino acid protein [Rohrer et al, 1993]. The fifth subtype, SSTR-5, was more recently cloned from a rat pituitary cDNA library using degenerate oligonucleotides corresponding to the second and sixth transmembrane domains. The 1230 long open reading frame leads to a 2.6 kb messenger which in turn encodes for a 383 amino acid protein [O'Caroll et al, 1992].

Although all five of these SSTRs exhibit a variance in their size, they share the similarity of an intronless organization with the exception of the 2B splice variant of the SSTR-2 subtype [Vanetti et al, 1992]. Nevertheless, they are indeed subtypes of the same family, since they all belong to the superfamily of G-protein coupled receptors with the highest homology found between their characteristic seven transmembrane domains [Probst et al, 1992]. The third intracellular loop that supposedly interacts with the G protein is relatively short in all of the SSTRs, with anywhere between 28 in SSTR-(1,2,4 & 5) and 31 to 39 residues in mouse and human SSTR-3 respectively. It is this portion of the protein which is strongly conserved between SSTR-1 & SSTR-4, SSTR-3 & SSTR-5 and SSTR-2 & SSTR-5, with amino acid identities of 68 and 65 percent respectively.

In fact it seems that SSTR-1 & SSTR-4 are the most closely related to each other within
the SSTR family, with a 61% amino acid sequence identity and a 78% similarity for that protein sequenced from the rat. The overall percentage of amino acid sequence identity among the cloned SSTRs family members is more than 90% between the same SSTR in different species, in contrast to only anywhere between 35% to 60% for different SSTRs in the same species. Therefore, as might be expected, each receptor subtype is encoded by a unique gene that, in humans, is localized on different chromosomes. All SSTRs posses multiple asparagine-linked glycosylation sites at the amino terminus, with the exception of SSTR-3 which displays a glutamic acid rich domain at the carboxyl terminal zone [Bell and Reisine, 1993].

Despite having only been cloned within the past few years, the pharmacological properties of the SSTRs have been studied extensively. Expression of these cloned receptors into cell lines that do not normally express any of them allowed functional radioligand binding studies using SRIF-14 & -28 and other structural analogues. The binding profile of short synthetic analogues, such as SMS 201-995, MK-678, RC-160, or BIM-23014 revealed a profile that seems to distinguish two related sub groups of receptors. Thus SSTR-2, SSTR-3 & SSTR-5 have been shown to share a high to intermediate affinity for MK-78 and SMS 201-95, and therefore probably represent the pharmacological equivalent of the previously defined SRIF-1 class of receptors. On the other hand, as may have been already suggested by structural similarities, SSTR-1 & SSTR-4 display very low to nonexistent affinity for these same analogues and likely correspond to the SRIF-2 class of receptors [Hoyer et al, 1995; Rens-Domiano et al, 1992; Schoeffter et al, 1995].

In contrast to their pharmacological profile, when expressed in CHO1, or COS (1 & 7) cell lines, all five SSTRs bind the endogenous ligands, SRIF-14 & -28, with similar high affinity in the nano to picomolar range [Yamada et al, 1992b]. SSTR-5 has turned out to be quite a unique subtype in that it is the only one of the five SSTRs to bind with a 10- to 50-
fold higher affinity to SRIF-28 than SRIF-14. In fact, the binding affinity of all five SSTRs sequenced from the rat, is measured at around 200 pM for SRIF-28, while only SSTR-5's affinity for SRIF-14 is lower at 5 nm [Raynor et al, 1993]. In addition, there exists a significant difference between rat and human SSTR-5, in terms of their binding affinity for octreotide [O'Carroll et al, 1992]. This functional disparity is accompanied by the largest structural diversity between any of the human and rat homologues tested to this date, registering at over 20% overall difference in amino acid sequence.

Determination of the expression patterns of these five receptors in both the peripheral and central nervous system tissues is pivotal for evaluating any distinctive physiological roles they might have. The cloning of these SSTRs has, unlike previous autoradiographic binding studies, made possible the future development of subtype specific agonist and antagonist probes so that their expression at the mRNA level can be investigated by in situ hybridization histochemistry, RNase protection assay and Northern and Southern blot analyses. Indeed the mRNAs for all five SSTRs subtypes have been shown to be expressed in the CNS, where SSTR 1-4 mRNA can be readily detected in adult rat brain by RNA blotting [Bruno et al, 1993]. Thoss et al, [1996] have performed an extensive study on the expression of all SSTRs' mRNA by in situ hybridization in the human brain and pituitary. They showed that the mRNAs for SSTR-1, SSTR-2 and SSTR-3 are mainly found in the cerebral cortex, whereas those for SSTR-4 and SSTR-5 appeared to be absent in these areas. SSTR-4 transcripts were present in the hippocampal formation and cerebellum while the one for SSTR-5 was only found in the cerebellum. In the pituitary, high levels of the mRNA for SSTR-2 and SSTR-5, but only low levels for SSTR-3, were detected.

It has become possible to elucidate the probable identity of the subtype of receptors in both the arcuate, and periventricular nuclei of the hypothalamus. The hypothalamus, like most areas in the CNS, expresses the mRNA for all of five SSTRs. A study by Beaudet et al, [1995] used in situ hybridization to visualize the distribution of the mRNA for SSTR-1 and SSTR-2 in the hypothalamus of both female and male rats. The film and light
microscopic autoradiograms of $^{35}$S-labelled cells, revealed a distribution that corresponded closely with that reported in the mouse hypothalamus by Breder et al. [1992]. However, they managed to reveal a more extensive pattern of distribution, especially for SSTR-2, than that reported by Perez et al. [1994]. The almost exclusive expression pattern for SSTR-1 in the paraventricular and periventricular nuclei suggested them to be autoreceptors, since they closely resembled that revealed by SRIF immunohistochemistry [Alpert et al, 1976; Johansson et al, 1984].

However, the hybridization pattern for both SSTR-1 and SSTR-2 was similar in the arcuate nucleus, in that although there was intense labelling throughout its entire rostrocaudal extent, the caudal pole exhibited a more intense signal than the rostral for both receptors. On the other hand, there was still a distinct difference in the pattern of nuclear labelling. The cells containing SSTR-1 mRNA tended to be concentrated more medially, next to the borders of the third ventricle, whereas those containing SSTR-2 mRNA were distributed more diffusely mediolaterally. Nevertheless, these observations are consistent with the finding by Patel et al, [1994] that the same cells can express multiple SRIF receptor genes, since there is probably an overlap between neuronal populations containing both receptors. In conclusion, it has become apparent that although SSTR mRNAs and binding sites in the human brain partly overlap, SRIF binding sites are generally more widely distributed. In a recent immunocytochemical study, Dournaud et al [1996], by utilizing a specific anti-peptide antibody to the SSTR-2A in the rat brain, were the first to show the widespread cellular distribution of SSTR-2A in the cerebral cortex and limbic structures.

Therefore, in terms of the regional pattern of distribution of the 5 subtypes in those brain regions pertinent to this review, the reported order of the density of labelling is as follows; Cortex: 1=2>>3=4=5, Hippocampus 4=1>2=3=4=5, Hypothalamus 1>2>3>5>4 and in the pituitary 2>1=3>5>4 [Bruno et al, 1993; Harrington et al, 1995; Kong et al, 1994]. Nevertheless, until the development of subtype-specific ligands or antibodies, the existence
of any degree of positive correlation between qualitative or quantitative mRNA expression and the actual presence and number of cellular receptors still remains an open question.

3. Physiological Significance of Somatostatin and Growth Hormone-Releasing Factor for GH Regulation

In the decades preceding the discovery and molecular characterization of GRF and SRIF, it was already evident that many stimuli, either endogenously originating in the CNS such as sleep, or exogenously impinging on it as in metabolic and physiological stressors such as hypo/hyperglycaemia and exercise, influence GH secretion from the anterior pituitary [Martin, 1976]. Nevertheless, in the ensuing decades following their discovery it has also become clear that the neurotransmitters that mediate the biological effects of these stimuli exert nearly all of their influence on GH synthesis and/or secretion through the contradictory actions of the two hypothalamic hypophysiotropic peptides. However, although it is now well established that both of these hypothalamic hormones are necessary for genesis of the pulsatile pattern of GH secretion, the specific role each plays and at how many locations each exerts its effects to generate this pattern is still the subject of some considerable study.

Several in vivo and in vitro experiments have been performed to investigate the specific role and contribution of endogenous SRIF and GRF in the generation and maintenance of the classical pulsatile pattern of GH secretion by artificially augmenting or diminishing their physiological action(s) via the infusion of the peptide(s) alone or in combination with anti-(SRIF or GRF) serum. The major thrust of nearly all of these studies is that most probably SRIF controls the timing of the GH pulse by acting as the physiological regulator of the GH trough periods, whereas GRF determines the magnitude of the GH peak [Sato et al, 1988].

25
a) Interaction of SRIF and GRF at the Pituitary Level

Data obtained from experiments performed *in vitro*, demonstrate that binding of GRF to its receptor on pituitary cells, results not only in the release of previously synthesized intracellular pools of GH, but also induces the synthesis of new GH at the transcriptional level. [Barinaga et al, 1983]. On the other hand, *in vitro* studies demonstrate that although SRIF does not inhibit the synthesis of GH [Barinaga et al, 1985], there is as much if not more evidence, to indicate that it is an essential component in maintaining the typical pulsatile pattern of GH secretion, by exerting an inhibitory effect on GH release. It has been shown that SRIF maintains GH pulsatility by operating during trough periods to optimize somatotroph response to successive pulses of GRF analogue [Sato et al, 1990].

The suggestion that periodic reductions of SRIF set the timing of GH secretory bursts, has received considerable support from a number of *in vitro* experiments utilizing dispersed rat pituitary cells demonstrating that GRF induced release is augmented by a simultaneous withdrawal of SRIF [Kraicer et al, 1986; Stachura et al, 1988; Weiss et al, 1987]. The role of SRIF in regulating GH secretion has been investigated by *in vitro* studies on cultured adenohypophyseal cells of spontaneously diabetic BB/W rats [Walsh and Szabo, 1988]. However, utilizing this model, it was unexpectedly found that GH responsiveness was enhanced to exogenous GRF administration. Therefore, it was concluded that under different temporal conditions SRIF may act in a paradoxically positive manner to sensitize somatotrophs to the actions of GRF [Tannenbaum et al, 1989].

Results obtained from *in vivo* studies utilizing passive immunization with anti-GRF serum, indicate that endogenous GRF plays a major and necessary role in the control of pulsatile secretion in rodents, ruminants and primates, including humans. Injection of GRF antiserum have been shown to virtually obliterate spontaneous GH pulses in male rats [Wehrenberg et al, 1982], and in cattle also result in decreased serum concentrations of both GH and IGF-I [Trout and Schanbacher, 1990]. A more recent study further reinforced the
crucial importance of GRF in generating GH pulses as administration of a GRF antagonist totally abolished them in humans [Jaffe et al, 1993].

In freely moving rats, passive immunization against SRIF results in an increase in GH trough levels without any change in either the amplitude of the GH secretory episodes or the overall mean plasma levels [Terry and Martin, 1981]. Furthermore, active immunoneutralization of SRIF in both sheep and growing pigs also led to a significant increase in basal and mean GH secretion, that were not accompanied by any change in the amplitude or frequency of GH pulses [Dubreuil et al, 1989; Varner et al, 1980]. In primates, such as in adolescent male baboons, active immunization against SRIF results in an increase in basal serum GH concentrations concomitant with no significant changes in the episodic bursts of GH [Steiner et al, 1978].

Furthermore, some of the evidence for the role SRIF in the generation of the ultradian rhythm of GH secretion have come indirectly from relying on nutritionally deprived rats as an in vivo model of increased SRIF tone [Tannenbaum et al, 1978]. Certain in vivo studies have reported that spontaneous episodes of GH release are markedly suppressed in 72-hr food-deprived rats [Tannenbaum et al, 1979]. Although the exact mechanisms mediating this response are still not clearly understood, several studies have demonstrated that passive immunization of these starved rats with SRIF antiserum restores GH secretory episodes [Hugues et al, 1986, Mounier et al, 1989].

To this date the only theory that has been successful at being able to integrate these results into a cohesive theory on SRIF/GRF interactions, at least with respect to the rat, was proposed by Tannenbaum and Ling [1984]. They proposed a model of asynchronous pulsatile secretion of GRF and SRIF, in which GH peaks are the consequence of pulses of GRF released during periods of SRIF quiescence, while conversely, trough GH values are postulated to occur at a time of elevated SRIF release concomitant to the period of minimal GRF activity. This hypothesis that SRIF and GRF, like GH, are released 180 degrees out of
phase in reciprocal 3-4 hour cycles received indirect support by the observations that the GH response to GRF is greater at times of spontaneous GH pulses than during GH troughs as a consequence of the changing SRIF tone [Clark and Robinson, 1985; Tannenbaum and Ling, 1984].

Consistent with this, pretreatment with SRIF augments the GH response to GRF analogues [Clayton and Bailey, 1987]. Furthermore, a critical study demonstrated that the actual measured levels of immunoreactive GRF and SRIF levels measured in the rat hypophyseal portal blood are consistent with this model [Plotsky and Vale, 1985]. In addition, a more recent study has provided support for the model at the molecular genetic level. In situ hybridization histochemistry revealed that the mRNA of SRIF and GRF in the periventricular and arcuate nucleus, oscillate in a reciprocal ultradian fashion, in phase with plasma GH [Zeitler et al, 1991].

Clinical studies have also suggested a similar episodic SRIF secretion in humans by observing that the GH response to GRF analogues is dependent on the GH secretory status at the time of the GRF analogue administration [Devesa et al, 1989]. Other studies in humans, have also provided support for the above theory that GH pulses are the result of an association between increased GRF secretion and the episodic diminution or withdrawal of endogenous SRIFs by demonstrating that GH pulses are maintained in the face of high and constant levels of GRF resulting either from secreting tumours or exogenous infusion [Vance et al 1985]. Consequently, the data from both animal and humans suggests that the endogenous secretion of SRIF is important in determining the ability of a somatotroph to respond adequately to repeated GRF analogue stimulation. Hence, optimal episodic GH secretion requires episodic GRF stimulation concomitant with a cyclic variation in SRIF tone to maximize a GH pulse and prevent GH release between pulses [Clark et al, 1988].

Reports of the existence of inter species discrepancies on the exact role of GRF and SRIF [Frohman et al, 1990] can be due to methodological differences, such as the inability
to achieve total immunoneutralization under *in vivo* conditions without the use of extremely high affinity antibodies. However, a more salient reason for these differences is due to the analysis of the data from the perspective that SRIF and GRF interact only at the level of the pituitary to generate the pulsatile pattern of GH secretion. As will be outlined below more recent studies have demonstrated that there is now enough evidence to consider that this temporal pattern of GH release may also be influenced by a delicate interplay of these two factors at the level of the CNS in the hypothalamus.

b) Interactions of SRIF and GRF at the Hypothalamic Level

Indeed, there is now considerable morphological and physiological evidence for an interaction between SRIF and GRF at the level of the CNS. In terms of morphological evidence, a subpopulation of SRIF-immunoreactive neurons has been visualized in the arcuate nucleus, that apparently do not significantly innervate the median eminence [Kawano et al, 1982]. Furthermore, immunohistochemical studies have localized a dense network of SRIF-positive axon terminals in the arcuate [Ohtsuka et al, 1983], which have been shown by physiological [Willoughby et al, 1989 b] and other ultrastructural studies [Daikoku et al, 1988; Horvath et al, 1989; Liposits et al, 1988; Willoughby et al, 1989 a] to directly contact the somato/dendritic arbors of GRF-containing neurons within the arcuate nucleus.

Further evidence comes from the report of a remarkable spatial similarity between the autoradiographic distribution of cells harbouring specifically labelled SRIF binding sites with the immunohistochemical distribution of GRF neurons within the arcuate nucleus of adult rats [Bertherat, et al 1991b]. In fact, cell counts confirmed that the rostrocaudal distribution of the two differentially labelled cell populations had coefficient of correlation of 0.8. These findings suggested that the SRIF binding sites may be directly associated with the perikarya of arcuate GRF neurons [Epelbaum et al, 1989]. Additional experiments provided evidence to support this conclusion. In two subsequent experiments, adjacent 5-6 um coronal sections were processed for SRIF radioautography and GRF immuno-histochemistry [McCarthy et al, 1992] or SRIF radioautography and GRF *in situ* hybridization [Bertherat et al, 1992].
Subsequent to quantification of the number of cells positive for each condition, and the superimposition of their spatial maps, it was discovered that in the ventrolateral portion of the arcuate nucleus over 30 percent of the cells were colabelled for SRIF binding sites and the presence of GRF or its mRNA.

Similarly, physiological evidence is also accumulating to suggest that SRIF can directly regulate GRF synthesis and/or release from arcuate neurons. Evidence for this comes from indirect studies where GH release was measured after passive immunization with anti-SRIF antibodies [Miki et al, 1988] and sequential intracerebroventricular (ICV) injections with highly specific antisera against SRIF [Thomas et al, 1985]. These studies demonstrated that an acute withdrawal of endogenous, or exogenously [Clark et al, 1988] administered SRIF triggers a discharge of GRF from the median eminence, indicating that intrahypothalamic SRIF can block GRF release.

A paradoxical elevation of GH secretion is elicited by the ICV administration of SRIF to either anesthetized [Abe et al, 1978] or freely behaving [Lumpkin et al, 1981; Tannenbaum and Patel, 1986] rats. This effect has been shown to be mediated through GRF release into the portal system, since it is abolished by neonatal treatment with monosodium glutamate, which selectively destroys arcuate neurons, including the tuberoinfundibular GRF system [Murakami et al, 1987]. Furthermore, although a selective pharmacological SRIF receptor antagonist is not yet available, it is possible to block some of the actions of SRIF by using cysteamine. This compound selectively interacts with the disulphide bond of the SRIF molecule, thereby reducing both its biological and immunological activities. Subsequent to depleting endogenous SRIF by cysteamine, Tannenbaum et al, [1990b] were able to demonstrate a striking increase in both the number and labelling density of GRF-immunoreactive cells in the arcuate nucleus concomitant with a decrease in GH release. Consistent with this, lesions of heavily stained SRIF immunoreactive neurons in the hypothalamic preoptic area, result in increased GH levels that are reduced by antibodies to GRF [Johanson et al, 1984; Katakami et al, 1988].
In another study, cysteamine treatment caused a two-fold increase in both the number of GRF mRNA labelled cells and specific SRIF binding [Bertherat et al, 1991a]. These observations were indeed further evidence for a direct inhibitory effect of SRIF on GRF, since the same effect was not observed along the base of the ventromedial nucleus where SRIF binding is not associated with GRF mRNA cells [Bertherat et al, 1991a]. In addition, peripherally administered SRIF strongly diminished GRF mRNA levels in the arcuate nucleus [Katakami et al, 1991]. In addition transections of the periventricular SRIF afferents to the arcuate nucleus have been shown to increase the number of GRF immunoreactive neurons therein [Daikoku and Tsuruo, 1990].

Similar transections also increased GRF release from a median eminence arcuate nucleus complex in vitro [Epelbaum, 1992]. Recent in vitro studies demonstrate that SRIF perifusion inhibits depolarization induced GRF release from the media eminence arcuate complex [Yamaushi et al, 1991]. Similar results have been reported from clinical studies on GRF secreting tumours. A high density of specific SRIF binding sites has been visualized by autoradiography in ectopic GRF secreting tumours from two patients with acromegaly. Interestingly, the effectiveness of therapy of these patients with octreotide was directly dependent on the density of SRIF binding sites. In vitro perifusion of a tumour fragment from these patients, indeed showed inhibition of GRF secretion by SRIF [Bertherat et al, 1994].

C. AIM of PRESENT INVESTIGATION

Growth Hormone (GH), with the exclusion of the sex hormones, is arguably the most important hormone throughout the species spanning the entire phylogenetic ladder of evolution. In most mammalian species it displays a pulsatile mode of secretion. Specifically in the male rat GH secretion is characterized by an endogenous ultradian rhythm with secretory bursts at regular 3.3 h intervals. Traditionally it has been accepted that this mode
of secretion is achieved by a complex interaction between the stimulatory growth hormone releasing factor (GRF) and inhibitory hormone somatostatin (SRIF), at the level of the anterior pituitary, from which GH is secreted. However, numerous physiological and morphological evidence indicate that SRIF and GRF may also interact at the level of the hypothalamus to regulate GH secretion. The emerging consensus on the nature of GH secretion, at least for the rat, is that not only GH itself, but also the SRIF and GRF oscillate temporally beginning from their transcription and translation at the genetic level to their ultimate secretion. The aim of this study was to further examine the significance of SRIF binding sites in the arcuate nucleus, for GH secretion. It was our intention to determine whether these binding sites also displayed a similar temporal variation concomitant to that displayed by GH, GRF, SRIF secretion in the rat. The rationale consisted of the qualitative and quantitative examination of [125I]-SRIF binding sites at times of plasma GH zenith and nadir via high resolution radioautography.
Ultradian Oscillation in Somatostatin Binding in the Arcuate Nucleus of Adult Male Rats*

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(A) ABSTRACT

The ultradian rhythm of GH secretion in the male rat is generated by the reciprocal cyclic release of somatostatin (SRIF) and GH-releasing factor (GRF) from the hypothalamus. We recently demonstrated the presence of high affinity \(^{125}\text{I} \text{SRIF} \) binding sites on a subpopulation of GRF-containing arcuate neurons in rat hypothalamus. In the present study, we tested the hypothesis that these binding sites undergo rhythmic fluctuations in parallel with those of GH. Adult male rats were killed at times associated with either a peak (1100 h) or trough (1300 h) period of the GH rhythm. The hypothalamus was serially sectioned from the retrochiasmatic nucleus, rostrally, to the mammillary recess of the third ventricle, caudally, and \(^{125}\text{I} \text{SRIF-binding sites were labelled in vitro using high resolution autoradiography.} \text{[}^{125}\text{I} \text{SRIF-labeled neuronal perikarya were counted at eight cross-sectional levels across the arcuate nucleus and binding densities were quantitated over each of these cross-sectional surfaces using computer-assisted microdensitometry. Both the number of labeled cells and the density of}^{125}\text{I} \text{SRIF binding varied as a function of the time of death. The average number of}^{125}\text{I} \text{SRIF-labeled cells per 10 \mu m-thick section was 2-to 3-fold higher in rats killed at 1100 h than in those killed at 1300 h. In addition, overall binding density levels were 65\% higher in animals killed at the onset of a GH peak than in those killed at the time of a GH trough. Both of these effects were apparent throughout the rostrocaudal extent of the arcuate nucleus. In contrast,}^{125}\text{I} \text{SRIF binding density in the cerebral cortex did not vary between 1100 h and 1300 h. These results demonstrate an ultradian variation in SRIF binding to arcuate neurons in relation to the peaks and troughs of the GH rhythm. Such rhythmicity in SRIF receptors might underlie a temporal responsiveness of arcuate GRF neurons to endogenous SRIF and may be an important mechanism by which SRIF modulates the rhythmic release of hypothalamic GRF in generation of the ultradian rhythm of GH secretion. \text{[}^{125}\text{I} \text{SRIF binding density in the cerebral cortex did not vary between 1100 h and 1300 h. These results demonstrate an ultradian variation in SRIF binding to arcuate neurons in relation to the peaks and troughs of the GH rhythm. Such rhythmicity in SRIF receptors might underlie a temporal responsiveness of arcuate GRF neurons to endogenous SRIF and may be an important mechanism by which SRIF modulates the rhythmic release of hypothalamic GRF in generation of the ultradian rhythm of GH secretion.\text{]}}\)
(B) Introduction

In the adult male rat, GH secretion is characterized by an endogenous ultradian rhythm, with high-amplitude GH secretory bursts occurring at regular 3.3 h intervals throughout a 24-h period; in the intervening trough periods, basal plasma GH levels are undetectable (1). Regulation of the episodic release of GH from the pituitary gland is achieved by the complex interaction of two hypothalamic hormones, a stimulatory GH-releasing factor (GRF) synthesized in the arcuate nucleus (2,3), and an inhibitory hormone, somatostatin (SRIF), originating from the periventricular nucleus (4). There is considerable evidence that, in the male rat, these two neurohormones are released in reciprocal 3-to 4-h cycles from the median eminence into the hypophyseal portal circulation to act upon the pituitary somatotropes (5,6). In addition, the cellular mRNA content for SRIF and GRF appears to vary as a function of the phase of GH secretion, suggesting that, like secretion, the synthesis of SRIF and GRF also varies rhythmically (7).

Physiological (8-11) and morphological (11-15) evidence is accumulating to indicate that SRIF and GRF may interact not only at the pituitary level, but also within the central nervous system to generate the ultradian rhythm of GH secretion. Some of these interactions could take place within the arcuate nucleus of the hypothalamus because it contains virtually all GRF-containing tuberoinfundibular neurons as well as a dense network of SRIF-positive axon terminals synapsing upon those cells (11-15). Moreover, we recently obtained direct
anatomical evidence for the presence of SRIF receptors on a subpopulation of these GRF-containing neurons (16) and Bertherat et al. (17) showed an association of SRIF binding sites with arcuate neurons expressing GRF mRNA. Both of these findings provide strong support for the concept of a central influence of SRIF on pathways associated with GRF.

In this context, it was tempting to speculate that the rhythmic oscillations in the secretion of hypothalamic SRIF, and hence that of GH, may be associated with parallel changes in SRIF binding to its receptors on arcuate neurons. To test this hypothesis, we measured cellular levels of [¹²⁵I] SRIF binding by high resolution autoradiography and compared values between groups of animals killed at times of peaks and troughs of the GH rhythm.

(C) Materials and Methods

(1) Animals and experimental design

Adult male Sprague-Dawley rats (200-250 g) were obtained from Charles River Canada (St. Constant, Quebec, Canada) and individually housed in an isolated room under a rigidly controlled 12-h light, 12-h dark cycle (lights on at 0600 h) in a temperature (22±C)- and humidity-controlled environment. They were given free access to rat chow (Ralston-
Purina, St-Louis, MO) and tap water, and were handled once daily. After at least 2 weeks acclimatization to the lighting cycle, the rats were killed by rapid decapitation.

In a first experiment, two groups of animals were killed at two different time points: 1100 h (n=7) and 1300 h (n=5). These times were chosen because they reflect typical peak and trough periods of GH secretion, respectively, in rats maintained under the above photoperiodic conditions, as previously established in this laboratory (1,5). The brains were immediately removed, frozen in isopentane at -40 C and stored at -80 C until use.

In the second experiment, a similar experimental design was used. However, in this experiment, the GH secretory profile of each animal was monitored before death. The rats were implanted with chronic intracardiac venous cannulae under sodium pentobarbital anesthesia (50 mg/kg, ip), 1 week before sampling, using a previously described technique (1). On the day of the experiment, blood samples were obtained every 15 min starting at 1000 h until the animals were killed by decapitation at either 1100 h (n=6) or 1300 h (n=6). The blood samples were centrifuged immediately after withdrawal and the plasma stored at -20 C until assayed for GH. At death, the brains were immediately removed, snap frozen and stored as above.

(2) Plasma GH assay

Plasma GH levels were determined in duplicate by double antibody RIA, using materials
supplied by the NIDDK (Bethesda, MD). The averaged plasma GH values are reported in terms of the rat GH reference preparation (rGH RP-2). The standard curve was linear between 0.62-320 ng/ml. The intra- and inter-assay coefficients of variation were 7.6% and 9.6%, respectively, for duplicate samples of pooled plasma containing a mean concentration of 7.4 ng/ml.

(3) \[^{125}I\] SRIF autoradiography

Brains were mounted on a cryostat chuck and serially sectioned in the coronal plane. Two series of sections, 10 µm-thick, were collected at 150 µm intervals from the retrochiasmatic area rostrally to the mammillary nucleus caudally. Sections were mounted on gelatin-subbed slides (one section/slide), with care being taken to place each section at an equal distance from the extremity of the slide (to ensure uniformity of subsequent emulsion coating). After overnight storage at -20 C, sections from the first series were: (1) preincubated for 15 min at room temperature in 0.17 M Tris-HCl buffer (pH 7.4) containing 0.2% BSA; (2) incubated for 40 min at room temperature in the same medium supplemented with 3 x 10^{-5} M MgCl_2 and 5 x 10^{-5} M Bacitracin (Sigma), and containing 0.25 nM \[^{125}I\] Tyr^a-D-Trp^8-SRIF-14 (\[^{125}I\]-SRIF; SA, 165 Ci/mmol; iodinated and purified as described in (16); (3) rinsed 3 x 5 min in ice-cold Tris buffer baths; and (4) fixed by immersion for 30 min in a 4% glutaraldehyde solution in 0.05 M PO_4 buffer (18). Sections from the second series were treated as above except for the second step which was carried out in the presence of 0.25 µM non-radioactive Tyr^a-D-Trp^8-SRIF-14 for determination of non-specific binding.
After fixation, sections were dehydrated in graded ethanols, defatted in xylene, rehydrated and coated by dipping in Ilford K-5 emulsion (Ilford, Moberley, Cheshire, UK) diluted 1:1 with distilled water. After 4 to 5 weeks of exposure, the autoradiograms were developed in Dektol (Eastman Kodak, Rochester, NY) fixed in Kodak Ektaflo (Eastman Kodak, Rochester, NY), stained with cresyl violet and coverslipped.

(4) Data analysis

Sections were analyzed at 25X under darkfield on a Leitz Dialux 20 microscope (Rockleigh, NJ) equipped with a CCD video camera coupled to a Bioquant computerized image analysis system (R & M Biometrics, Nashville, TN). All measurements were performed by an operator unaware of the animal’s group assignment. First, the number of $^{[125\text{I}]}$SRIF-labeled cells, identified as clusters of silver grains overlying cresyl violet-stained perikarya, were counted at each of eight cross-sectional planes of the ARC nucleus and were averaged over the eight levels for each rat within each experimental group. Second, $^{[125\text{I}]}$SRIF binding densities were measured by densitometry over the entire cross-sectional surface of the arcuate nucleus. Here again, the data were averaged over the eight levels for each rat within each experimental group after subtracting non-specific from total binding values and ensuring that all measurements fell within the linear part of a standard curve. This curve was established using brain paste standards as follows. Fresh brains were homogenized in Tris buffer and 30 µl aliquots containing predetermined amounts of tissue protein were mixed with graded concentrations of $^{[125\text{I}]}$SRIF, and then deposited inside a
grooved circle on gelatinized glass slides. The standards were then air dried, fixed in 4% glutaraldehyde, dehydrated and processed for autoradiography in parallel with tissue sections. Labelling densities were measured by densitometry for each standard sample and grey levels expressed as fmol per mg protein based on the concentration of \[^{125}\text{I}\]SRIF present in the sample. Tissue readings were subsequently matched against this curve for extrapolation of \[^{125}\text{I}\]SRIF binding values. Data (mean number of cells and specific binding densities per level) were compared between groups using Student's two-tailed t test. In addition, we compared the \[^{125}\text{I}\]SRIF binding density in the cerebral cortex of experimental groups.

(D) Results

(1) Exp 1

In keeping with earlier data (16-19), light microscopic autoradiographs of sections incubated with \[^{125}\text{I}\]SRIF alone (total binding) revealed the presence of selectively radiolabeled cells throughout the rostrocaudal extent of the arcuate nucleus (Fig. 1). As previously described, these labeled cells were mainly confined to the ventral aspect of the nucleus and stood out against a relatively heavy neuropil labelling in their surround. By contrast, sections incubated in the presence of non-radioactive SRIF-14 (non-specific binding) showed only weak and homogeneous background labelling (Fig. 2c).

Both the number and labelling density of \[^{125}\text{I}\]SRIF-labeled cells varied as a function
of the time of death. Thus, in animals killed at 1100 h, labeled cells were more numerous and stood out more clearly against the surround than in animals killed at 1300 h (Fig. 1). At high magnification, silver grain densities over individual labeled perikarya were also higher on average in sections from animals killed at 1100 h than from those killed at 1300 h (Fig. 2a,b). Quantitation of autoradiograms confirmed that the number of $^{125}$I SRIF-labeled cells per 10 μm-thick sections of the arcuate nucleus [18.9 ± 2.1 vs. 7.2 ± 1.2 (mean ± SEM); P<0.01] was significantly higher in rats killed at 1100 h than in those killed at 1300 h. Specific $^{125}$I SRIF binding density across the arcuate also was significantly higher (253.9 ± 17.9 vs. 174.7 ± 5.2 fmol/mg protein; P<0.01) at 1100 h than at 1300 h. In contrast, specific $^{125}$I SRIF binding density in the cerebral cortex did not vary between 1100 h and 1300 h (605.5 ± 12.6 vs. 593.8 ± 19.7 fmol/mg protein). Non-specific binding in the arcuate nucleus also remained virtually constant between the two time points in absolute terms and, therefore, ranged between 26% (1100 h) and 38% (1300 h) of the total in relative terms; in the cerebral cortex it was 21% of the total at both time points.

(2) Exp 2

Plasma GH measurements in this experiment confirmed that the brains of rats killed at 1100 h had been sampled at or immediately preceding a peak period of GH secretion, whereas the brains of animals killed at 1300 h had been sampled at the beginning of a GH trough (Fig. 3).

The pattern of autoradiographic labelling in the arcuate nucleus of these rats was the
same as that observed in experiment 1. Again, both the number of $[^{125}I]$SRIF-labeled cells (24.5 ± 2.1 vs. 9.7 ± 0.5; P<0.001) and specific labelling density of arcuate cross-sections (337.7 ± 20.2 vs. 195.0 ± 15.3; P<.001) were significantly greater in animals killed at the onset of a GH peak than in animals killed at the time of a GH trough. There was no evidence of temporal variation in $[^{125}I]$SRIF binding density in the cerebral cortex (738.5 ± 24.9 vs. 689.6 ± 30.2 fmol/mg protein). Non-specific binding was slightly higher than in the first experiment, ranging between 29% (peak) and 42% (trough) of total in the arcuate nucleus; in the cerebral cortex, it accounted for 26% of total at both time points.

Since experiments 1 and 2 were essentially the same, data from all animals in each experimental group (1100 h: n=13; 1300 h: n=11) were pooled for final analysis. As shown in Fig. 4, the number of $[^{125}I]$SRIF-labeled cells was 2- to 3- fold higher on average in rats killed at 1100 h than in those killed at 1300 h (21.5 ± 1.7 vs. 9.1 ± 0.5; P<0.001) (Fig. 4). This difference was apparent throughout the rostrocaudal extent of the nucleus and not confined to a specific sub-region (Fig. 4). Densitometric data further indicated the existence of a 1.6-fold disparity between the two groups of animals in overall $[^{125}I]$SRIF-binding densities across the entire arcuate surface (292.5 ± 17.6 vs. 185.5 ± 8.9 fmol/mg protein; P<0.001); here again, the effect was apparent throughout all levels of the nucleus (Fig. 4).

**(E) Discussion**

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The present study demonstrates the existence of an ultradian oscillation in the density of \([^{125}\text{I}]\text{SRIF}\) binding within the arcuate nucleus of the rat hypothalamus in synchrony with the pattern of GH secretion. While previous studies have demonstrated variations in neuropeptide/neurotransmitter binding in relation to other types of biological cycles, e.g., \(\mu\)-opioid binding in the medial preoptic area as a function of the estrous cycle (20), the present finding represents to our knowledge, the first example of receptor binding oscillations with such a short phase period (2 h) within the central nervous system.

The concomitant variations in the number of \([^{125}\text{I}]\text{SRIF}\)-labeled cells and density of \([^{125}\text{I}]\text{SRIF}\) binding over the entire arcuate cross-sectional surface, which were reproducible in two independent experiments, likely reflect a single underlying phenomenon, that is, a time-dependent variation in the binding of SRIF to arcuate neurons. Indeed, the changes in labeled cell numbers were clearly associated with parallel changes in the density of silver grains detected over their perikarya, and therefore, are more likely attributable to variations in the detection threshold of labeled cells than to the recruitment of different neuronal populations. In turn, oscillations in overall arcuate \([^{125}\text{I}]\text{SRIF}\) binding densities might reflect variations in \([^{125}\text{I}]\text{SRIF}\) binding onto both neuronal perikarya and locally arborizing processes of the same neurons.

As recalled in the introduction, combined autoradiographic and immunocytochemical (16) or in situ hybridization (17) experiments have respectively, demonstrated that an important fraction of arcuate neurons harboring high affinity \([^{125}\text{I}]\text{SRIF}\) binding sites actually
Correspond to neurons that contain and express GRF. It may, therefore be surmised that the \(^{125}\text{I} \) SRIF binding variations documented in the present study largely concern GRF neurons. This interpretation is in keeping with the fact that the \(^{125}\text{I} \) SRIF binding oscillations were in phase with those of GH and, by the same token, with the presumed release of GRF (5,6).

Because of technical limitations inherent to the size of the arcuate nucleus and to variations in \(^{125}\text{I} \) SRIF binding across its rostrocaudal extent, it was not possible to determine by Scatchard analysis whether the variations in \(^{125}\text{I} \) SRIF binding recorded in the present study were due to changes in the number or in the affinity of SRIF receptors. One possible interpretation of our findings is that these variations merely reflect variable occupancy of arcuate SRIF receptors by endogenous SRIF at the time of death. Previous autoradiographic studies have indeed suggested that a significant proportion of arcuate SRIF receptors might be normally occupied by the endogenous peptide (19). The temporal pattern of \(^{125}\text{I} \) SRIF binding observed here would conform to this interpretation, in that the lowest \(^{125}\text{I} \) SRIF binding densities were detected at the onset of a trough period in GH secretion, i.e., during expected peak periods of cyclic SRIF secretion in hypophysial portal blood (5,6). It is conceivable that at this time, there is also a simultaneous increase in SRIF release at synaptic junctions between SRIF axon terminals and GRF cells in the arcuate.

Alternatively, a change in either the number or the affinity of \(^{125}\text{I} \) SRIF binding could reflect alternating up- and down-regulation of arcuate SRIF receptors induced by a previous non-occupancy or occupancy of the receptors, respectively, by endogenous SRIF.
This could occur via either short-term (e.g., phosphorylation and/or internalization of the receptors; see 21 for a review) or long-term (e.g., change in receptor protein expression) regulatory mechanisms. Down-regulation and presumptive concomitant desensitization of SRIF receptors within the arcuate nucleus might, in turn, play a role in triggering the phasic release of GRF through disinhibition of GRF-containing cells.

A third possibility is that the oscillations reflect independent intrinsic fluctuations in expression of the SRIF receptor protein. The recent cloning of a family of SRIF receptors (22) that are highly expressed in the arcuate nucleus of both mouse (23) and rat (24) brain affords an opportunity to address this question, although it remains to be determined whether these receptor subtypes are the ones that are expressed in GRF cells. Finally, the observed oscillations may result from the feedback effect of circulating GH on SRIF receptor expression by hypothalamic GRF cells. Indeed, peripherally administered GH has been shown to affect GRF gene expression within these cells (25, 26). Whether SRIF receptor expression is regulated by circulating GH is a topic for further investigation.

Whichever mechanisms are involved, the present results strongly suggest the existence of an oscillating responsiveness of GRF arcuate neurons to endogenous SRIF. The synchrony between periods of heightened SRIF responsiveness and peaks of GH secretion further suggest that these oscillations are intimately involved in the regulation of GRF neuronal activity by endogenous SRIF and may, in particular, be an important mechanism by which SRIF modulates the rhythmic release of hypothalamic GRF in generation of the
ultradian rhythm of GH secretion.
(F) Acknowledgements

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(H) FIGURES
**Figure 4**

**Top Panel:**
- Y-axis: Number of $^{125}$I-SRP-Labeled Cells
- X-axis: Rostro-Caudal Level (μm)
- Two bars for each level:
  - Dark gray: 1100 hours (n=13)
  - Black: 1300 hours (n=11)
- P-value: P<0.001

**Bottom Panel:**
- Y-axis: $^{125}$I-SRP Binding Density (fmol/mg protein)
- X-axis: Rostro-Caudal Level (μm)
- Two bars for each level:
  - Dark gray: 1100 hours (n=13)
  - Black: 1300 hours (n=11)
- P-value: P<0.001
(I) FIGURE LEGENDS

Figure 1. Light microscopic autoradiograms of the arcuate nucleus of the hypothalamus labeled in vitro with $[^{125}I]SRIF$ in rats killed at 1100 h (a) and 1300 h (b); darkfield. In a, numerous intensely labeled $[^{125}I]SRIF$-labeled cells are apparent throughout the ventral aspect of the nucleus. In b, labeled cells are more sparse and less prominent against neuropil labeling. III, third ventricle; ME, median eminence. Scale bar, 150 μm.

Figure 2. High magnification of autoradiograms from the arcuate nucleus of rats killed at 1100 h (a) and 1300 h (b and c); brightfield. a and b, Total binding. $[^{125}I]SRIF$-labeled cells (arrows) are both more numerous and more reactive in sections from animals killed at 1100 h (a) than from those killed at 1300 h (b). c, Non-specific binding. Silver grain clusters are no longer evident, and neuronal labeling is markedly weaker than that in the total section for either time points. Scale bar, 15 μm.

Figure 3. Mean (±SEM) plasma GH profile of adult male rats killed at either the onset (1100 h; n=6) or the termination (1300 h; n=6) of a spontaneous GH secretory episode. Arrows indicate the times of death, and the number of animals sampled at each time point is shown in parentheses.

Figure 4. Distribution histogram (mean ± SEM) of the number of $[^{125}I]SRIF$-labeled cells (upper panel) and $[^{125}I]SRIF$ binding densities (lower panel) across the rostrocaudal extent of the arcuate nucleus of rats killed at 1100 h (hatched bars) and 1300 h (dark bars). Level values on the ordinate correspond to the distance in microns from the retrochiasmatic area [i.e., approximately from interaural 7.2 mm in Paxinos and Watson's stereotaxic atlas (27)]. Mean cross-sectional values are given at the right of the diagram (300-1350 μm).
V. GENERAL CONCLUSIONS

The purpose of this section is to present more evidence in support of the possible mechanisms, previously outlined in the Discussion, to explain the observed temporal fluctuations in $^{125}\text{I}}$-Tyr-D-Trp$^6$ SRIF (subsequently referred to as $^{125}\text{I}}$-SRIF) binding in the arcuate nucleus. In addition, the significance of the data for the physiological regulation of pulsatile GH secretion from the anterior pituitary, will be briefly touched upon.

A. MECHANISMS for the TEMPORAL FLUCTUATION of
$^{125}\text{I}}$-SOMATOSTATIN BINDING SITES

1. Occupancy of $^{125}\text{I}}$-Somatostatin Binding Sites by Endogenous Ligand

The first interpretation of the present findings is that the observed temporal fluctuations in $^{125}\text{I}}$-SRIF binding merely reflect temporal variations in the occupancy of arcuate SSTRs by endogenous SRIF at the different times of sacrifice. As others (Leroux and Pelletier, 1984) have also noted, this could indeed be the case, even though the brain sections were preincubated as per the radioautography protocol to clear the SSTRs of endogenous ligand. Theoretically, one should be able to determine the extent to which this explanation accounts for our observations by the in vivo administration of cysteamine to rats.

However, in practice this probably would not provide a definite answer since, although some studies (Leroux et al, 1985; Tran et al, 1984) have indeed been able to reveal more $^{125}\text{I}}$-SRIF binding sites in the rat hypothalamus; it is questionable as to what changes at the receptor level led to these results. The results could be due to decreasing SSTR occupancy because cysteamine altered the ring portion of SRIF and hence prevented ligand-receptor
interactions or, as Srikant and Patel [1984] have demonstrated in the cerebral cortex, be due to induced up regulation of SSTRs by cysteamine. For this reason, among many others which include the fact that cysteamine has been shown to not only be nonspecific in its action but also toxic [Millard et al, 1983], it was not utilized in this study.

On the other hand, one could take advantage of the fact that with the adenylate-cyclase mediated model by which SSTRs are thought to transduce their signals, binding of guanosine triphosphate (GTP) to the regulatory proteins would exert a negative heterotropic effect on the affinity of SSTR for SRIF. Taking advantage of this assumption Leroux et al [1988], demonstrated that pretreatment of brain sections with GTP caused the dissociation of endogenous SRIF from its receptors, allowing the detection of a new population of $^{125}\text{I}$-SRIF binding sites in the hypothalamus.

A preliminary study employing GTP in our radioautography protocol, as in the protocol used by Leroux et al [1988], was inconclusive, since the observed disparity in the $^{125}\text{I}$-SRIF binding density and the number of $^{125}\text{I}$-SRIF labelled cells between the two different time points were not significantly affected by this method. Nevertheless, this possibility cannot, and indeed need not, be totally ruled out to substantiate the importance of the present finding for the regulation of GH. In fact, this possibility can be construed as yet another confirmation for the theory that SRIF itself is secreted in an ultradian fashion not only in the portal blood [Plotsky and Vale, 1985], but also at the level of the arcuate nucleus.

2. Homologous and Heterologous Up- and Down-Regulation of Somatostatin Receptors

Biological systems have developed a number of adaptive mechanisms to facilitate the responsiveness of their cells to successive stimuli over time. One crucial method by which they achieve this, is by regulating the sensitivity of their receptors by changes in either the affinity of their receptors for their endogenous ligand and/or the number of receptors. In turn, the number of receptors on the plasma membrane available at any one time for binding might
be controlled by the internalization/recycling of already synthesized (and present) cellular receptors and/or by the regulation of their synthesis at the transcriptional and/or translational level [Collins et al, 1992].

Essentially, for G-protein coupled receptors this regulation occurs through three basic mechanisms. First, desensitization is mediated by receptor phosphorylation by G-protein receptor kinases and second-messenger kinases. This involves interaction of phosphorylated receptors with arrestins leading to the receptor being uncoupled from the heterotrimeric G protein that renders it ineffective at controlling effector molecules such as adenylate cyclase ion channels [Bohm et al, 1997]. Second, sustained but typically short term exposure (seconds to minutes) to the appropriate ligand, leads to the depletion from the cell surface of high-affinity receptors by their rapid removal through surface sequestration, and hence internalization into an intracellular compartment. Often, this process involves the recycling of the internalized receptors back to the plasma membrane, which is thought to contribute to the resensitization of cellular responses [Bohm et al, 1997]. The third mechanism of receptor downregulation is a form of desensitization that typically occurs during continuous long-term (hours to days) exposure of cells to the appropriate ligand. It is characterized by the depletion of the cellular receptor content, which is thought to be mediated by alterations in the rates of receptor degradation due to the decreased stability via reduction of the steady state mRNA and/or changes in their rate of synthesis (transcriptional and/or translational) [Bohm et al, 1997]. The exposure to the appropriate ligand that leads to the initiation of any or all of the above mechanisms can be agonist specific, i.e. the endogenous ligand, or agonist non-specific, referred to as homologous and heterologous downregulation, respectively [Bohm et al, 1997].

The observed temporal variations in SRIF binding sites can be explained by alternating up- and down-regulation of arcuate SRIF receptors. There is evidence that the functional effectiveness of SRIF receptors can be homologously and/or heterologously regulated by SRIF itself and other factors, respectively. In vivo and high affinity in vitro ligand binding studies have provided evidence for both the homologous [Presky and Schonbrunn, 1988;
Reisine and Axelrod, 1983; Smith et al, 1984) and heterologous regulation of SRIF receptors, by such factors as thyrotropin-releasing hormone, phorbol esters, glucocorticoids, thyroid hormone and serotonin [Hinkle et al, 1981; Munozacedo and Arilla, 1996; Osborne and Tashjian 1982; Schonbrunn, 1982; Schonbrunn and Tashjian, 1980]. Much of this work has been carried out on recombinant wild type or mutagenized receptors expressed in variety of cell lines. The data obtained from these studies should be judiciously applied to physiological systems, since it is debateable as to whether native receptor-bearing cells use the same or different mechanisms.

a) Changes in the Number of Somatostatin Receptors Via:

i) Internalization/recycling of existing plasma membrane receptors

The mechanisms involved in the regulation of the responsiveness of SRIF receptors need to be evaluated. The majority of the in vitro and in vivo studies have demonstrated that temporal fluctuations in SSTR binding over a period as short as hours to periods as long as weeks are more likely due solely to changes in the number of SRIF receptors rather than to concomitant or sole changes in their affinity [Reisine, 1985]. Therefore, the major focus in this dissertation shall be on mechanisms responsible for SRIF receptor up- and down-regulation via a change in receptor number.

It has been demonstrated that ligand mediated endocytosis/ internalization and surface re-expression of receptors via recycling to the plasma membrane, can modulate receptor function by reducing the number of cell surface receptors [Gruenberg and Howell, 1989]. Indeed, most peptide hormones and growth factors such as insulin and epidermal growth factor undergo rapid, receptor mediated internalization following binding to their cell surface receptor [Sorokin and Carpenter, 1993]. This has also been reported for many receptors, such as those for the members of the secretin receptor family, like VIP and CCK [Anteunis et al, 1989; Williams et al, 1982]. A number of in vitro and in vivo studies have also documented the internalization of various neurotransmitter and neuropeptide ligands with their receptors
into neural tissue. Some of the earlier studies reported internalization of a number of anterior pituitary neuropeptides such as gonadotropin and thyrotropin [Goldstein et al, 1985; Morel et al, 1985]. Recently, Stojilkovic and Catt, [1996] reported that in neuroendocrine cells, endothelin induces a rapid and marked desensitization of its signaling system, which is associated with extensive internalization of its receptors.

Numerous studies have also illustrated the receptor mediated internalization of neurotransmitters. The cholinergic denervation of the hippocampus by medial septal (MS) lesions [Ayyagari et al, 1995] and chronic treatment with phorbol dibutyrate leads to the internalization of muscarinic acetylcholine receptors in the cerebral cortex [Pediconi and Barrantes 1995]. Sorensen et al, [1997] showed that incubation of SH-SY5Y neuroblastoma cells with oxotremorine-M results in a time-dependent endocytosis of muscarinic acetylcholine receptors. It has been shown that the acute exposure of GABA(A)/benzodiazepine receptors to high concentrations of GABA in vivo, that occurs early after ischemia, results in receptor down-regulation [Alicke and Schwartzbloom, 1995]. In vitro studies have shown that this occurs by the rapid (30-45 min.) internalization of benzodiazepine receptors in cultured cerebral cortical neurons, with the degree of internalization being dependent on the concentration of agonist that the receptor is exposed to [Katsura et al, 1996].

The results from a number of recent studies suggest that many of the observations that have been made on the internalization and recycling of G protein-coupled receptors in in vitro transfected cell systems may be applicable to similar events that occur in the mammalian central nervous system in vivo. Mantyh et al, [1995] investigated this phenomenon in vivo, using substance P (SP)-induced internalization of the SP receptor (SPR). They showed that within one minute of a unilateral striatal injection of SP in the anesthetized rat, nearly 60% of the SPR-immunoreactive neurons within the injection zone display massive internalization of the SPR in cell bodies and dendrites. It has also been demonstrated that adenosine A(1) receptor desensitization in the rat brain by in vivo treatment with R-PIA corroborates the data from in vitro studies by suggesting a role for
coated vesicles in the internalization of G-protein coupled receptors [Ruiz et al, 1996]

A number of both in vitro and in vivo experiments from the candidate's laboratory have substantially added to the growing body of evidence suggesting that neuropeptide binding to G protein-linked receptors may result in the internalization of receptor-ligand complexes, followed by intracellular mobilization and degradation of the ligand into its target neural cells. Recently, by utilizing confocal microscopy Gaudriault et al [1997] were able to demonstrate that radiiodinated and fluorescent mu and delta opioid peptides are internalized in mammalian cells transfected with the corresponding opioid receptor according to a receptor-mediated time- and temperature-dependent mechanism.

Extensive studies in this laboratory have centred around the internalization of the tridecapeptide neurotensin (NT). It is useful to review the findings from these studies, since the utilization of similar techniques applied to the study of SRIF has yielded fruitful results. The synthesis of a fluorescent derivative of neurotensin provided a new tool that allowed markedly higher cell resolution than autoradiography using [I-125]-NT. By retaining the ability to internalize in vivo, it allowed both the regional and intracellular localization of neurotensin and its receptor by confocal microscopy [Faure et al, 1994]. Hence, by using confocal laser scanning microscopic Alonso et al [1994] were able to demonstrate the receptor-mediated internalization of NT into cholinergic neurons of the basal forebrain. Subsequently, Faure et al, [1995a] demonstrated a selective time- and temperature-dependent internalization of fluo-neurotensin in cells of the adult mammalian brain. They showed that internalization is receptor mediated, proceeds from the entire somatodendritic membrane of the cells, and utilizes endosome-like organelles which are mobilized from dendrites to perikarya. Confocal laser microscopic examination of superfused slices revealed that the fluoresceinylated neurotensin is internalized throughout the terminal and dendritic arborizations of mesostriatal dopamine cells [Faure et al, 1995b]. Recently it was discovered that the rapid internalization of this receptor-ligand complex in mesencephalic dopamine neurons is predominantly through only one of two (i.e the high-affinity) types of neurotensin receptors [Nouel et al, 1997a].

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Several studies have also demonstrated receptor-mediated endocytosis of SRIF in rat anterior pituitary cells [Drannin et al, 1985; Mentlein et al, 1989; Morel et al, 1983; Smith et al, 1984], AT20 cells [Morel et al, 1986; Mahy et al, 1988] as well as in pancreatic acinar cells [Viguerie et al, 1987]. With respect to the data presented here, one has to be clear whether this proposed internalization of SRIF receptors is possible within the time frame studied, and what the actual route and fate of the internalized radioligand-receptor complex is. In terms of the time frame, internalization of SRIF receptors fits in quite well with the results presented here. Quantitative electron microscopic radioautography studies have also reported the internalization of $[^{125}\text{I}]$ Tyr1-SRIF-14 in monolayer cultures of rat islet cells [Amherdt et al, 1989]. Consistent with our data, and some ways similar to neurotensin, they showed a time (within minutes)- and temperature- dependent internalization of the radioligand into endocytotic vesicles, then into multivesicular bodies, and finally into lysosomes.

Nevertheless, other investigators have showed that $[^{125}\text{I}-\text{Tyr}1]$SRIF-14 and $[^{125}\text{I}-\text{Tyr}11]$SRIF-14, unlike $[^{125}\text{I}]$ epidermal growth factor, are not rapidly internalized by GH4C1 rat pituitary cells and RINm5F insulinoma cells, respectively [Presky and Schonbrunn, 1986; Sullivan and Schonbrunn, 1986]. However, as even the authors themselves assert, internalization is probably dependent on the subtype of receptor involved and the cell line in which it is expressed. Furthermore, it has also been suggested that the radioligands used in these studies were prone to degradation by membrane proteases. Although, an in vivo study involving the administration of octreotide to both normal and mutant dwarf rats showed up-regulation of SRIF receptors, it failed to reach a conclusive answer on whether chronic exposure results in SRIF receptor desensitization, citing the need for a full dose-response study [Turner and Tannenbaum, 1995]. To clarify this issue one can take advantage of the stability of the cyclic SRIF agonist octreotide and in vivo imaging studies of SRIF receptors.

The recent development of techniques for the in vivo visualisation of SRIF receptors in human neoplastic tissues, has provided yet further evidence for the internalization of SRIF
receptors. The visualisation of the SRIF receptors is made possible by the intravenous injection of radioactive octreotide. Consequently, the persistence of the radioligands $^{[23]}$I-Tyr3 or $^{[11]}$In-DTPA-D-Phe1-octreotide beyond their biological half lives and hence the ability to visualize SRIF binding sites for more than 48 hours, has led to the conclusion that SRIF receptors are internalized with their ligand [Krenning et al, 1992].

It has been demonstrated that octreotide is highly resistant to degradation by pure enzymes and tissue homogenates [Bauer et al, 1982]. Consequently, by taking advantage of the stability of the parental compound the internalization of $^{[125]}$I-Tyr3-octreotide was investigated in AtT20/D16V mouse and cultured human GH-secreting pituitary tumour cells. This study was able to clearly demonstrate that a high amount of the radioligand was internalized by these two cell types. Furthermore, consistent with the results presented here, it was found that the radioactivity was still present in the cells after 4 hours of incubation and in addition it was still bound to the peptide [Hofland et al, 1995].

A study from this laboratory has managed to successfully adapt and apply, the techniques used to study receptor mediated internalization of neurotensin to that of SRIF, so as to try to bring some resolution to the discrepant results in the literature concerning the occurrence of such a mechanism for the tetradecapeptide somatostatin (SRIF). Nouel et al, [1997b] reinvestigated this question by comparing the binding and internalization of iodinated and fluorescent derivatives of the metabolically stable analog of SRIF, [D-Trp(8)]SRIF, in COS-7 cells transfected with complementary DNA encoding the SSTR-1 or SSTR-2A subtype. COS-7 cells transfected with complementary DNA encoding either SSTR-1 or SSSTR-2A revealed that the approximately 20% of specifically bound ligand of the former versus up to 75% of the latter were recovered inside the cells. The bound ligand was clustered into small endosome-like particles, which increased in size and moved toward the nucleus with time, which the authors thought suggestive of receptor-ligand complexes proceeding down the endocytic pathway.
The next question that needs to be addressed is the actual mechanism by which the process of internalization of ligand receptor complexes occurs. Some of the earliest data on mechanisms for receptor mediated ligand internalization was obtained from studies on single transmembrane segment receptors, such as insulin. The process begins with a local clustering/concentration of receptors in clathrin-coated pits which undergo endocytosis by being pinched off from the plasma membrane, to eventually form clathrin-coated vesicles. The ligands may then be targeted to late endosomes and lysosomes, where they are eventually degraded, while the receptors return to the plasma membrane, probably via carrier vesicles formed from the tubular part of the early endosomes [Pastan and Willingham, 1981; Keen, 1990]. Recent data indicate that this pathway may also be applicable to multiple transmembrane segment proteins such as the G-protein coupled receptors, such as the SSTRs [Garland et al, 1994; Roth et al, 1997]. Indeed, in a very recent study, Koenig et al [1997] reported that somatostatin receptors internalized in a temperature and time dependent manner in a neuroblastoma cell line via a mechanism involving clathrin coated pits.

On the other hand, G-protein-coupled receptors (GPCRs) represent a large family of proteins that have been shown to rapidly desensitize primarily as a consequence of receptor phosphorylation. Two families of serine/threonine kinases, second messenger dependent protein kinases and receptor-specific G-protein-coupled receptor kinases (GRKs), phosphorylate GPCRs and thereby contribute to receptor desensitization. Receptor-specific phosphorylation of GPCRs promotes the binding of cytosolic proteins referred to as arrestins, which function to further uncouple GPCRs from their heterotrimeric G-proteins. To date, the GRK protein family consists of six members, which can be further classified into subgroups according to sequence homology and functional similarities. The arrestin protein family also comprises six members, which are subgrouped on the basis of sequence homology and tissue distribution. One must appreciate that GRK-mediated phosphorylation and arrestin binding are not only involved in the functional uncoupling of GPCRs but are also intimately involved in promoting GPCR sequestration and, as such, likely play a role in receptor mediated ligand internalization [Ferguson et al, 1997].
A further question that deserves an answer would be the elucidation of structural signals in the receptor that regulate its internalization. Chabry et al [1995], have identified the amino acid sequences responsible for the internalization of the cloned rat brain neurotensin receptor, by carrying out site-directed mutagenesis of the cDNA encoding the neurotensin receptor. Mammalian COS 7 cells transfected with the wild type receptor showed a temperature-dependent intracellular accumulation of a fluorescent analog of neurotensin, whereas cells transfected with a receptor truncated at the carboxyl terminus showed a clustering of the fluorescent peptide at the cell surface. The endocytosis of the five rat somatostatin receptor subtypes SSTR1-5 was investigated in transfected HEK cells by biochemical ligand binding assays and confocal microscopic analysis [Roth et al, 1997]. In this study, delineation of sequence motifs responsible for internalization of SSTR3 revealed multiple serines and a threonine (Ser-341, Ser-346, Ser-351, and Thr-357) residue within the carboxy-terminal tail, of which Ser-351 and Thr-357 were the most effective ones. Step-wise truncation of the carboxyl terminus of wild-type SSTR4 revealed a motif of three amino acid residues Glu-Thr-Thr (SSTR4-330-332) that is responsible for preventing internalization and may be important in regulating the internalization of this receptor subtype.

Hence, in consideration of the above one can say with some certainty that the majority of the evidence is in favor of the ability of some SSTRs to be internalized in a variety of cell types. Therefore, it is not inconceivable that the process of receptor mediated internalization could indeed be one explanation of the mechanisms mediating the ultradian variation in SRIF binding sites reported here.

i) Increases/decreases in transcription and/or translation of new receptors

There is some evidence in the literature for the notion that changes in cellular responsiveness in a variety of systems can be modulated at the genetic level. Souaze et al, [1997] have reported that the incubation of cells for 6 h with the NT agonist, JMV 449, resulted in an increase of 270% in NTR mRNA levels. These changes were the direct result
of new NTR gene transcription, as indicated by run-on and half-life experiments. In addition, the transcriptional activation of the NTR gene was shown to be dependent on NT-receptor complex internalization and de novo protein synthesis. This was apparently followed by a second response, which was detected after prolonged exposure to JMV 449. In this case, a decrease of 70% was detected in NTR mRNA levels. Unlike the initial phase, this change was mediated by a post-transcriptional event, since the half-life of NTR mRNA from treated cells decreased by 50% as compared with control cells. Hence, NT agonists appear to regulate the synthesis of NTR mRNA. In HT-29 cells, this feedback is exerted by a biphasic response, with the phases being apparently independent and mediated by two separate mechanisms.

Similarly, Aleppo et al. [1997] tried to determine if homologous down-regulation of GHRH receptor number is due to a decrease in GHRH receptor synthesis. Their results indicate that GHRH inhibits the production of its own receptor by a receptor-mediated, cAMP-dependent reduction of GHRH-R mRNA accumulation. The possibility that SSTRs can be down regulated in vivo, at the transcriptional level has also been studied. Increased exposure to SRIF in vivo, leading to homologous down-regulation may be explained by a decrease in receptor concentration and expression of SSTR mRNAs [Haddock and Malbon, 1991]. Several pituitary tumour cell lines have been used to investigate the effect of chronic SRIF treatment on SRIF receptor binding and SSTR mRNA. Initial studies revealed that paradoxically, SRIF up regulates, rather than down regulates, its binding sites in GH4C1 cells [Presky and Schonbrunn, 1988]. This up-regulation was shown not to be the result of a change in affinity, but involve an increase in receptor biosynthesis controlled at the level of transcription, mRNA processing and/or translation.

However, in another study in which GH3 pituitary tumour cell lines were exposed to 1μM SRIF, the level of the mRNA for all SSTRs, with the exception of SSTR-2, was up-regulated. Although, like NT, SSTR-2 was reported to exhibit a biphasic response. This response involves a one and half times increase in mRNA levels after two hours, which then decreases to one half the original control levels by six hours, to only re-establish the original
level after 48 hrs of exposure to SRIF [Bruno et al, 1994a]. This observation is consistent with the previous study by Katakami et al [1985], reporting a decrease in pituitary SRIF receptor binding following the injection of a single dose of GH into rats. Furthermore, in vitro transfected SSTR-2 and -3, but not SSTR-4 African green monkey COS kidney cells or Chinese hamster ovary (CHO) cells could be homologously down regulated [Yasuda et al, 1992; Xu et al, 1993]. Further evidence for the in vivo homologous down regulation of pituitary SRIF receptors at the genetic level was provided by studies where a transient increase in endogenous SRIF (caused by GH injection) was followed by a transient decrease in pituitary SRIF receptor binding [Katakami et al, 1985].

The homologous regulation of transcription/translation of SRIF receptors has also been studied in prolonged food deprivation and diabetes mellitus in the rat which result in a loss of GH secretory episodes [Tannenbaum, 1981]. In both cases GH secretion can be restored, in part, by the in vivo administration of SRIF antiserum [Tannenbaum et al, 1978]. Therefore, in these models of SRIF hypersecretion it has been shown that decreased pituitary plasma membrane SRIF receptor binding is due to a decrease in receptor concentrations and not a decrease in affinity [Bruno et al, 1994b; Olchovsky et al, 1990]. In addition, pituitary cells from these animals incubated in vitro demonstrated resistance to the GH-suppressive effects of SRIF, which is consistent with the in vivo studies suggesting receptor down regulation [Walsh and Szabo, 1988]. Therefore, it is possible that the temporal variation in SRIF binding sites in the arcuate nucleus may be a result of homologous regulation of the rate of transcription/translation of the mRNA for some of the SSTRs identified to exist in the arcuate.

B. SIGNIFICANCE of the TEMPORAL FLUCTUATIONS in SOMATOSTATIN BINDING SITES for GH SECRETION from the ANTERIOR PITUITARY

It is important to discuss what the present findings mean for the secretion of GH from
the anterior pituitary. It is particularly important to discuss this point in light of the more significant results obtained in the second experiment. In experiment 2, in which the trough and peak levels of plasma GH were closely monitored, one can deduce that the greater the disparity between GH plasma levels, the more significant (i.e. larger) fluctuation in SRIF binding.

The data can be analyzed from basically two perspectives, i.e. it could be that the observed fluctuations in SRIF binding sites in the arcuate are either the cause or result of pulsatile GH secretion. In terms of the latter possibility it could be that these temporal fluctuations in SRIF binding are actually the result of a short or long loop feedback mechanisms whereby GH or the IGFs, respectively, regulate GH secretion from the pituitary. This alternative relies on the evidence that GH and/or IGF's would feedback on the arcuate to heterologously modulate SRIF receptor numbers (by inducing or changing the rate of their internalization/recycling or transcription/translation) or feedback to the periventricular nucleus to regulate the secretion and/or synthesis of SRIF which would then homologously modulate the internalization or synthesis of its receptor(s) in the arcuate [Richardson and Twente, 1986].

However, before one can propose that such mechanisms occur under normal physiological conditions to regulate SRIF binding sites in the arcuate, at least two criteria must be fulfilled. First, is GH/IGFs in the plasma able to cross the blood brain barrier and eventually get access to the hypothalamus. Indeed there is evidence that GH can travel to the hypothalamus from the plasma across the blood brain barrier as was recently demonstrated in a study by Johansson et al [1995]. The second prerequisite for this theory is the existence of receptors for GH and/or IGFs at the sites necessary for GH and/or the IGFs to exert their effects. Receptors for both IGF-I and IGF-II have been identified in both the hypothalamus and pituitary of rats [Lesniak et al, 1988]. However, both in vitro [Goodyear et al, 1984; Yamashita and Melmed, 1986] and in vivo [Harel and Tannenbaum 1992 a,b] studies have not clearly established the role of the IGFs in regulating GH release at the level of the hypothalamus.
On the other hand, binding studies have demonstrated hGH binding to rat brain homogenates and its binding sites in the brain [Waters et al, 1990]. Other studies have reported that GH receptors are expressed in several areas of the human CNS, with strikingly high levels reported in the choroid plexus and hypothalamus [Minami et al, 1993]. GH receptor mRNAs have been shown to exist, by in situ hybridization, in both the periventricular and arcuate nuclei [Burton et al, 1992]. Furthermore, the site of GH feedback inhibition is most probably not the pituitary gland, as both in vitro [Kraicer et al, 1988] and in vivo [Abe et al, 1983; Tannenbaum, 1980] studies have suggested a hypothalamic site of action by showing that the central administration of rat GH results in a marked suppression of spontaneous GH pulses.

Several lines of evidence suggest a more prominent role for hypothalamic SRIF. Hypophysectomy has been shown to decrease the expression of the SRIF gene in cells of the periventricular nucleus [Rogers et al, 1988] and ICV injections of GH leads to a marked increase in the release of SRIF into hypophyseal portal blood [Chihara et al, 1981]. Hence, it is quite possible that GH can feedback to the periventricular nucleus to modulate the transcription of SRIF, which then exerts its effect on the arcuate nucleus [Yamaushi et al, 1991] by homologously modulating its own receptors either by internalization or at the transcriptional level, to result in the ultradian oscillation in their binding sites presented here.

Of course, the second possibility is that GH actually has its effect directly on the neurons in the arcuate nucleus that bear the binding sites that were observed to fluctuate temporally in this study. Support for this view comes from a report that systemic administration of a large dose of GH induces c-fos expression in the arcuate and periventricular neurons of hypophysectomized rats [Minami et al, 1992]. Furthermore, more recent studies from our laboratory have also revealed that hypophysectomy can specifically modulate the mRNA levels of those two SSTRs i.e. 1 and 2 that are expressed in the arcuate nucleus [Guo et al, 1996]. Two weeks after hypophysectomy (HPX) there was an over 50% reduction in both the number and labeling density of SSTR-1 and SSTR-2. Administration of recombinant hGH for seven days post HPX augmented both the cell number and labelling.
density of only SSTR-1mRNA [Guo et al, 1996].

On the other hand the most intriguing explanation for the observed variations in SRIF binding would be that these fluctuations are not the result of the pulsatile secretion of GH, but actually its cause. In other words intrinsic oscillations in those neurons in the arcuate bearing these SRIF binding sites serve as the pacemakers of the GH-IGF neuroendocrine axis by being responsible for the genesis or setting of the periodicity of GH secretion from the anterior pituitary. This could be accomplished by the temporal modulation of the transcription/translation of the genome in those neurons responsible for those SSTRs present on their membrane. Hence if these binding sites represent SSTRs on GRF neurons, they would then be able to modulate the secretion of GRF in a similar ultradian pattern.
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IMAGE EVALUATION
TEST TARGET (QA-3)

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