Chapter 2

General introduction

Part of this review has been published:

Ghrelin, an endogenous growth hormone secretagogue with diverse endocrine and nonendocrine effects

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General introduction - Part I

Pituitary growth hormone secretion and its regulation, and the diverse endocrine and nonendocrine effects of synthetic growth hormone secretagogues and ghrelin

1. Nomenclature of the canine pituitary gland

According to the Nomina Anatomica Veterinaria, the canine pituitary gland is composed of two main parts 1) the adenohypophysis and 2) the neurohypophysis (Hullinger, 1993). The pituitary gland is suspended from the midline of the hypothalamus by a cylindrical stalk. This stalk is an extension of the median eminence of the hypothalamus, and is called the pars proximalis neurohypophysis (also called the infundibulum). The third ventricle continues as an invagination into the infundibulum. The pars proximalis neurohypophysis is continuous with the distal enlargement, the pars distalis neuohypophysis, which is the major portion of the neurohypophysis (Figure 1).

The adenohypophysis can be divided into two functional units 1) the anterior lobe (pars infundibularis adenohypophysis and pars distalis adenohypophysis) and 2) the pars intermedia (pars intermedia adenohypophysis). In the dog, the largest portion of the anterior lobe (AL) lays ventrorostral to the pars distalis neurohypophysis, which is almost entirely surrounded by the AL. The canine AL also extends as a cuff or collar around the pars proximalis neurohypophysis and even envelops part of the median eminence (Figure 1).

The pars intermedia (PI) is in direct contact with the pars distalis neurohypophysis and is separated from the AL by the hypophyseal cleft or cavity, which is a remnant of the embryonic Rathke's pouch.

The AL is populated by at least five highly differentiated types of endocrine cells, which are classified according to the trophic hormones they produce: somatotrophic cells secreting growth hormone (GH), lactotrophic cells secreting prolactin (PRL), thyrotrophic cells secreting thyroid-stimulating hormone (TSH), gonadotrophic cells secreting luteinizing hormone (LH) and follicle-stimulating hormone (FSH), and corticotrophic cells synthesizing the precursor molecule pro-opiomelanocortin (POMC), which gives rise to adrenocorticotrophic hormone (ACTH) and related peptides. Somatotrophic cells account for

50 % or more of the endocrine AL cells, with the other cell types each representing about 5-15 % of the AL cell population (Rijnberk, 1996; Meij, 1997).

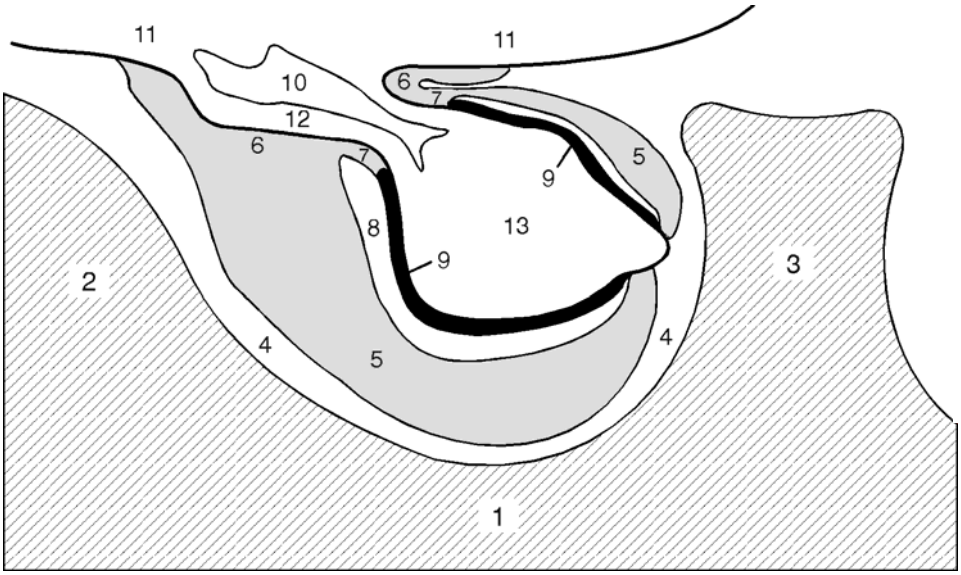

Figure 1. Schematic illustration of the median sagittal section through the canine pituitary gland (adapted from Meij, 1997). Left is rostral, right is caudal. 1 = sphenoid bone, 2 = tuberculum sellae, 3 = dorsum sellae, 4 = pituitary fossa, 5 = pars distalis adenohypophysis, 6 = pars infundibularis adenohypophysis, 7 = transitional zone, 8 = hypophyseal cleft or cavity, 9 = pars intermedia adenohypophysis, 10 = third ventricle, 11 = hypothalamus (median eminence), 12 = pars proximalis neurohypophysis, 13 = pars distalis neurohypophysis.

2. The hypothalamic-pituitary axis

The hypothalamic-pituitary axis constitutes the main axis of the neuroendocrine system of the body. In this axis, the pituitary is an essential regulatory interface integrating signals from the periphery and brain to control vital functions such as growth, reproduction, lactation, basal metabolism and the stress response (Treier and Rosenfeld, 1996). Hormonal control of pituitary gene expression and cellular proliferation is initiated during embryogenesis and continues through adulthood. At all stages, the cells of the pituitary have a remarkable ability to proliferate in response to demand for a specific hormone.

The hypothalamic-pituitary axis consists of three major systems: 1) a neuroendocrine system connected to an endocrine system by a portal circulation, 2) a neurosecretory pathway, and 3) a direct neural pathway that regulates endocrine secretion. The neuroendocrine system connects clusters of peptide- and monoamine-secreting cells in the anterior and middle

portion of the ventral hypothalamus to the AL (Swanson, 1987). Releasing and inhibiting factors such as GH-releasing hormone (GHRH), somatostatin (SS), thyrotrophin-releasing hormone (TRH), corticotrophin-releasing hormone (CRH), and gonadotrophin-releasing hormone (GnRH), are transported along nerve fibres from the hypothalamus to the median eminence. From the median eminence these factors are released into the capillary vessels of the hypothalamic-pituitary portal system and are transported to the pituitary to regulate the secretion of hormones from the AL (Figure 2). Specificity is achieved by the presence of specific receptors on individual types of AL cells. In addition to the hypothalamic hypophysiotrophic hormones, the secretion of AL hormones is regulated by feedback from target organs such as the thyroid, adrenals, and gonads (Figure 2).

The neurosecretory pathway is involved in osmoregulation through the production of vasopressin, and in parturition and nursing through the secretion of oxytocin. The two neurohypophyseal hormones are synthesised by populations of magnocellular neurons grouped in the paraventricular and supraoptic nuclei of the hypothalamus (Swanson, 1987), from which axons extend through the pituitary stalk and terminate in the neurohypophysis on fenestrated blood vessels. Vasopressin and oxytocin are stored in secretory granules within these nerve terminals and are released by exocytosis into the bloodstream in response to appropriate stimuli.

The pituitary PI is poorly vascularized and is directly innervated by predominantly dopaminergic nerve fibres from the hypothalamus. This direct neural control is mainly inhibitory in nature. Despite high levels of bioactive ACTH in the canine PI (Halmi et al., 1981), the main hormone secreted by the PI is α-melanocyte-stimulating hormone (α- MSH) (Figure 2).

3. Regulation of growth hormone secretion

3.1 Secretion and effects of growth hormone

In mammals, secretion of GH from the adenohypophysis is regulated by two hypothalamic hormones with antagonistic actions: a stimulatory GHRH that is produced in the arcuate nucleus, and an inhibitory hormone, SS, synthesized in the paraventricular nucleus (Figure 3) (Plotsky and Vale, 1985).

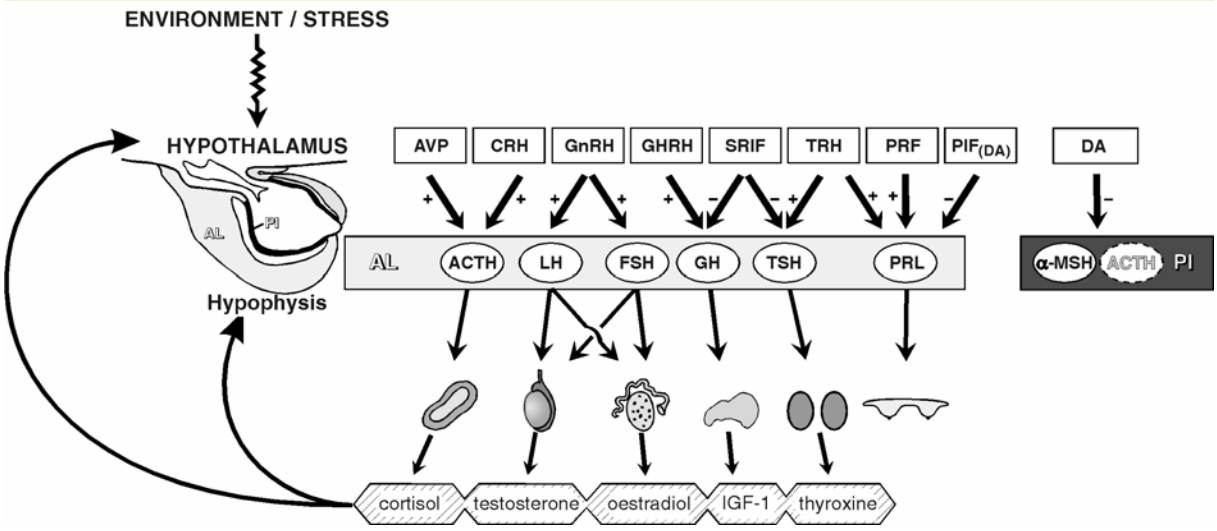

Figure 2. Simplified diagram of the hypophysiotrophic regulation of the secretion of hormones in the adenohypophysis, and with some target glands. Modified from Meij (1997).

Both hormones are transported from the hypothalamus to target cells in the pituitary gland via the hypothalamo-hypophyseal portal system in the median eminence. Alternation in secretion of GHRH and SS is responsible for the pulsatile pattern of GH release (Tannenbaum and Ling, 1984; Kooistra et al., 2000a). Measurement of GHRH and SS in hypophyseal-portal blood in humans and animals reveals that the episodic pattern of secretion of GHRH and SS does not fully account for all pulses of GH secretion (Frohman et al., 1992). The amplitude and frequency of GH secretory pulses are regulated by a complex array of external and internal stimuli, including body compostion, age, sleep, gender, disease status, menstrual cycle phase, genetic background, and nutritional status (Vigneri et al., 1976; Ho et al., 1988; Van Cauter et al., 1998). Furthermore, the secretion of GH is influenced by several hormones, such as progesterone, thyroid hormones, and glucocorticoids.

Growth hormone, a 191 amino acid single-chain polypeptide, mediates growth and metabolic functions through binding with the GH receptor. Growth hormone forms complexes with two peripheral GH-receptor components, leading to dimerization of the receptor, an event that is necessary for subsequent GH signalling (Hoech and Mukku, 1994). Growth hormone receptor dimerization elicits an intracellular phosphorylation cascade involving the JAK (Janus kinase)/STAT (signal transducers and activators of transcription) pathway (Xu et al., 1996).

The liver contains abundant GH receptors. Several other peripheral tissues, including muscle and fat, also express modest amounts of GH receptors (Barnard and Waters, 1997). In

contrast to most other pituitary hormones, the action of GH is not confined to a single target tissue and the hormone has both slow anabolic and rapid catabolic activities (Eigenmann et al., 1984). The catabolic effects are exerted via direct interaction with target cells, resulting in enhanced lipolysis in fat cells, and restriction of glucose transport across the cell membrane, caused by anti-insulin activity (Eigenmann et al., 1984; Casanueva, 1992; Carrel and Allen, 2002). The anabolic effects (i.e., growth and cell proliferation) of GH are exerted indirectly, mainly mediated by growth factors known as insulin-like growth factors (IGFs) or somatomedins (Figure 3) (Daughaday et al., 1972). The liver is the primary source of circulating IGFs. Growth hormone also promotes the production of IGFs in peripheral tissues (e.g., muscle, bone, cartilage, kidney, and skin), where they appear to have autocrine and paracrine effects (Daughaday et al., 1972).

Insulin-like growth factors have approximately 50 % sequence similarity with insulin (Tamura et al., 1989). In contrast to free circulating insulin, IGFs are bound to plasma proteins, which prolongs their half-life and contributes to their long-term growth-promoting effects. Circulating IGFs are important determinants of body size, because they stimulate protein synthesis, chondrogenesis, and body growth. Insulin-like growth factor-I has an inhibitory effect on GH secretion, most likely by stimulating the release of SS and by a directly inhibitory influence at the level of the pituitary gland (Figure 3) (Ceda et al., 1987). Additionally, GH has a negative feedback effect on its own production at the level of the hypothalamus (Figure 3) (Pelligrini et al., 1996).

3.2 Effects of gender and age on growth hormone secretion

Gender- and age-related differences in GH secretion have been established in various mammalian species. It appears that the sexually dimorphic pattern of GH secretion differs considerably between species. Before puberty, there is no apparent difference in GH secretion patterns between males and females in both humans (Zadik et al., 1985) and rats (Eden, 1979; Gabriel et al., 1992). In sheep, however, the plasma profiles of GH are already sexually dimorphic before puberty (Gatford et al., 1996). In humans (Zadik et al., 1985) and rats (Eden, 1979; Gabriel et al., 1992), GH secretion increases gradually till puberty. After puberty, GH release differs between males and females in most mammalian species. Mean concentrations of GH and IGF-I in blood are higher in adult males than in adult females in rats (Eden, 1979; Gabriel et al., 1992), mice (MacLeod et al., 1991), cattle (Plouzek and Trenkle, 1994), and horses (Thompson et al., 1994). In contrast, in primates mean circulating concentrations of IGF-I are higher in females than in males (Zadik et al., 1985; Ho et al.,

1987). Also the characteristics of GH pulses differ after puberty between males and females, albeit with some variation between species (Plouzek and Trenkle, 1994; Thompson et al., 1994).

In adulthood, the plasma GH concentration declines with increasing age in both primates (Finkelstein et al., 1972; Zadik et al., 1985; Ho et al., 1987; Corpas et al., 1993; Arvat et al., 1997a) and rodents (Sonntag et al., 1980; Crew et al., 1987). In humans it has been estimated that plasma GH decreases by 14 % with each decade (Iranmanesh et al., 1991). In both humans (Ho et al., 1987; Veldhuis et al., 1995) and rats (Sonntag et al., 1980) the amplitude of GH pulses is significantly lower in old individuals than in young individuals, whereas the frequency of GH pulses does not change with age. Several factors affect GH secretion in the elderly, such as age-related changes in body composition, reduced physical fitness, and medication use. Obesity is probably the strongest inhibitor of GH secretion (Veldhuis et al., 1995). Information on the effects of gender and age on the plasma profile in dogs is scarce. A recent study demonstrates that, in agreement with many other mammalian species, ageing in dogs is associated with a reduction in GH secretion. However, in contrast with findings in other mammalian species, no sex-related differences were detected in the pulsatile plasma profile in dogs (Lee, 2004).

3.3 Growth hormone secretion in endocrine disease

The pattern of GH secretion may also change in hyper- or hyposecretion syndromes, such as pituitary-dependent hyperadrenocorticism and pituitary dwarfism.

3.3.1 Pituitary-dependent hyperadrenocorticism

Cushing's disease or pituitary-dependent hyperadrenocorticism (PDH) is the most common endocrine disorder in the dog. Pituitary-dependent hyperadrenocorticism is most often caused by a corticotroph adenoma that may originate in the AL or the PI (Peterson et al., 1986). These corticotroph tumours produce an excessive amount of ACTH, resulting primarily in hypersecretion of glucocorticoids and in hyperplasia of the two inner zones of the adrenal cortices.

Glucocorticoids are important physiological regulators of GH synthesis and secretion. In humans and rats, glucocorticoids enhance GH gene transcription (Evans et al., 1982; Karin et al., 1990) and increase the number of pituitary GHRH receptors (Seifert et al., 1985; Ohyama et al., 1997). Consequently, both spontaneous and GHRH-induced GH secretion are stimulated by acute administration of dexamethasone (Casanueva et al., 1990). A minimum

level of cortisol is essential for normal GH production. Individuals with hypoadrenocorticism (Addison's disease) may become GH-deficient because of diminished GH synthesis (Allen, 1996). In humans with persistently elevated secretion of glucocorticoids, as in Cushing's disease, both the spontaneous and the stimulated GH secretion are blunted (Takahashi et al., 1992). Pituitary-dependent hyperadrenocorticism in dogs has many similarities to Cushing's disease in humans (Kemppainen and Peterson, 1993). Consequently, changes in spontaneous and stimulated pituitary GH secretion may also be expected in dogs with PDH.

3.3.2 Pituitary dwarfism

In dogs, congenital GH deficiency or pituitary dwarfism is the most striking example of adenohypophyseal hormone deficiency. Congenital GH deficiency has been mentioned to occur in different dog breeds, including Saarloos Wolfshounds and Carelian bear dogs. However, the condition is encountered most often as a simple, autosomal, recessive inherited abnormality in the German shepherd dog (Andresen and Willeberg, 1976).

Functionally, German shepherd dwarf dogs have a combined pituitary hormone deficiency. An absolute deficiency of GH, PRL, and TSH is associated with an impaired release of gonadotrophins, whereas ACTH secretion is preserved (Kooistra et al., 1998; Kooistra et al., 2000b). The abnormality in these dwarfs is most likely caused by a mutation in a developmental transcription factor that precludes effective expansion of a pituitary stem cell after the differentiation of the corticotroph cells (Kooistra et al., 2000b). To date, sequence analysis of genomic DNA from German shepherd dwarfs has not revealed causative mutations in candidate genes (Lantinga-van Leeuwen et al., 2000a; Lantinga-van Leeuwen et al., 2000b; Van Oost et al., 2002).

4. Ultradian pulsatile hormone secretion

4.1 Biological relevance of ultradian pulsatile hormone secretion

It is now generally accepted that all adenohypophyseal hormones are secreted in an ultradian pulsatile fashion and that this pattern of secretion represents an important component of neuroendocrine signalling (Negro-Vilar et al., 1987; Brabant et al., 1992). The ultradian patterns of adenohypophyseal hormone release are primarily driven by hypothalamic signals acting on responsive pituitary cells, which are modulated in turn by intrapituitary paracrine and autocrine factors, and also by systemic feedback of hormones derived of target organs.

Pulsatile hormone secretion allows for encoding information not only in the absolute concentration of the ligand but also in a time-dependent fashion. The pulsatile secretion of adenohypophyseal hormones is essential for target-cell regulation and is reproducible under comparable environmental conditions (Brabant et al., 1990). Physiological and pathophysiological situations may modulate pulsatile secretion patterns by altering the pulse amplitude, pulse frequency, or both (Brabant et al., 1992).

4.2 Analysis of pulsatile growth hormone secretion

The pulsatile pattern of GH secretion can be determined by collecting blood samples serially over a period of hours or days. The samples are assayed and the results are plotted graphically to enable recognition of the pulsatile secretion pattern. Different methods can be used to analyse the data. Traditionally, the graphs have been given to a blinded scorer who identifies the peaks by visual inspection. With this technique it is difficult to state precisely the criteria used for pulse identification, which complicates communicating among different centres studying similar problems and points to the need for more objective methods (Merriam and Wachter, 1982). Nowadays, a number of computer programmes are available for computer-assisted analysis of pulsatile secretion patterns. Commonly used detection methods such as the Pulsar programme (Merriam and Wachter, 1982) employ mathematical assumptions to identify pulses in time series of hormone concentrations. The statistical parameters used have to be adjusted for each hormone and different sampling frequencies.

Several methodological aspects should be borne in mind when interpreting patterns of pulsatile hormone secretion. Analysis of the profile of pulsatile hormone secretion requires that detection methods are precise and sensitive. A high intra-assay coefficient of variation will make it difficult to distinguish significant hormone pulses from assay noise. Secondly, hormone-specific responses are determined by the bioactivity of the hormone. However, plasma GH concentrations are measured by an immunoassay rather than by a bioassay. Thirdly, pulsatile plasma profiles are measured as a relatively small number of points, because “on-line” measurement of GH is not yet available. The shorter the sampling interval the higher the chance that significant GH pulses are detected. Therefore, the Pulsar programme is not able to extract the “true” underlying pattern of information. Nevertheless, the method is an objective way to compare different data sets (Brabant et al., 1992).

5. Synthetic growth hormone secretagogues

In 1975, before the discovery of GHRH, the GH-releasing properties of enkephalins were reported (Bowers et al., 1977). Chemical modification of the structure of met-enkephalin led to development of a highly potent GH-releasing hexapeptide, GHRP-6 [(His-D-Trp-Ala-Trp-D-Phe-Lys-NH_2)^3] in 1980 (Bowers et al., 1984). One of the most remarkable properties of GHRP-6 was the strong GH-releasing activity induced following oral administration (Bowers et al., 1984). The hexapeptide was the basic structure from which synthetic GH secretagogues (GHSs), of either peptidergic structure such as hexarelin or non-peptidergic structure such as MK-0677 (Ghigo et al., 1994; Chapman et al., 1996), were subsequently produced. Current synthetic GHSs are highly bioavailable and may be administered via intravenous, intramuscular, intranasal, subcutaneous, oral, and transdermal routes (Casanueva and Dieguez, 1999). Because GH is a large protein that must be administered via injection or inhalation, administration of synthetic GHSs is often preferred over administration of GH. In addition, GHSs induce a more physiologic pulsatile profile of GH release (Laron, 1995; Casanueva and Dieguez, 1999). For example, a single orally administered dose of MK-0677 increases mean 24-h plasma GH concentrations (Chapman et al., 1996; Jacks et al., 1996; Smith et al., 1997).

The synthetic GHSs have potent GH-releasing activity in several species, including humans, mice, rats, swine, goats, cows, and dogs (Hayashida et al., 2001; Bhatti et al., 2002; Bhatti et al., 2006 in press). In humans, nearly all synthetic GHSs induce the release of more GH than GHRH (Casanueva and Dieguez, 1999). However, the hormone-releasing action of synthetic GHSs is not specific in all instances (Casanueva and Dieguez, 1999). In humans, synthetic GHSs such as GHRP-6 also have a stimulatory effect on the secretion of PRL, ACTH, and cortisol (Massoud et al., 1996; Casanueva and Dieguez, 1999; Arvat et al., 2001). Newer selective GHSs, such as ipamorelin, do not have ACTH- or PRL-releasing actions (Raun et al., 1998; Broglio et al., 2002).

Interest in GHSs faded after the isolation and characterization of GHRH in 1982 (Guillemin et al., 1982; Rivier et al., 1982), but was later revived when it was discovered that GHSs operated through receptors that are different from those for GHRH (Howard et al., 1996; Guan et al., 1997; Casanueva and Dieguez, 1999). Growth hormone secretagogues and GHRH have strongly synergistic actions, which indicates that synthetic GHSs are not physiologic surrogates of GHRH (Bowers et al., 1990). In 1996, the GHS-receptor (GHS-R), a G-protein-coupled seven-transmembrane receptor, was identified (Pong et al., 1996). This receptor has been cloned from cells of the pituitary gland in humans (Howard et al., 1996; McKee et al., 1997a) and rats (McKee et al., 1997b).

Two types of GHS-Rs, which are presumably the result of alternate processing of pre-mRNA, have been identified and designated as receptors 1a and 1b (Howard et al., 1996; Smith et al., 1997). The human GHS-R 1a shares 96 and 93 % sequence identity with rat and pig receptors, respectively. The existence of this receptor can be traced to animals in the pre-Cambrian era because amino acid sequences highly similar to those in the human GHS-R 1a have been detected in teleost fish (Palyha et al., 2000). These observations indicate that the GHS-R 1a is highly conserved across species and likely has an essential biological function. This receptor is largely confined to somatotroph cells in the pituitary gland and to several hypothalamic nuclei (e.g., the supraoptic, arcuate, and paraventricular nuclei) in humans and rats (Howard et al., 1996; Guan et al., 1997; Smith et al., 1997; Shuto et al 2001). The presence in the pituitary gland of mRNA coding for GHS-R 1a indicates that GHSs can act directly on somatotrophs to stimulate GH release. This is in accordance with an earlier observation (Cheng et al., 1993) that GHSs are able to directly stimulate GH release from rat pituitary cells *in vitro*. The hypothalamic localization of the GHS-R 1a, especially in the supraoptic and paraventricular nuclei, supports the notion that GHSs may also indirectly regulate GH release by interacting with GHRH-producing neurons, SS-producing neurons, or both, in the hypothalamus (Dickson et al., 1995). The GHS-R 1a is also expressed in other areas of the brain and certain peripheral tissues (Papotti et al., 2000), indicating that GHSs may also be involved in other physiologic functions (Guan et al., 1997; Kojima et al., 2001). The importance of the widespread expression of GHS-R 1b in endocrine and non-endocrine tissues has not been determined (Howard et al., 1996; Gnanapavan et al., 2002).

The GHS-Rs are distinct from the GHRH receptor (Howard et al., 1996; Guan et al., 1997; McKee et al., 1997a). Although binding of GHRH to the GHRH receptor increases cAMP in somatotroph cells and stimulates GH release via activation of the kinase A pathway, the binding of ghrelin and synthetic GHSs to the GHS-R 1a activates the phospholipase C signalling pathway, leading to an increase in inositol triphosphate and protein kinase C activation, followed in turn by release of calcium from intracellular stores (Pong et al., 1996). Unlike GHS-R 1a, GHS-R 1b does not bind ghrelin or synthetic GHSs, and its function awaits clarification (Howard et al., 1996; McKee et al., 1997a; Gnanapavan et al., 2002).

6. Ghrelin

The 1999 discovery of the endogenous or natural ligand of the GHS-R, termed ghrelin (*ghre* is the proto-Indo-European root of the word grow, and *relin* indicates release), provided

a new dimension to GH research (Figure 3) (Kojima et al., 1999). The usual sequence of discovery in endocrinology is isolation of a hormone, cloning of its receptor, and development of analogues of the hormone for clinical use. With ghrelin, this sequence was reversed: first, analogues were synthesized, then the receptor was cloned, and lastly, the natural ligand of the orphan receptor was isolated.

Ghrelin releases GH *in vitro* and *in vivo*. The 28-amino acid peptide was isolated from the stomach, where its expression is higher than in any other tissue (Kojima et al., 1999). Although this source may initially seem strange, it should be remembered that most circulating SS is synthesized primarily in the gut and pancreas and that GHRH was first isolated not from the hypothalamus but from a pancreatic tumour (Casanueva and Dieguez, 2002). Thus, the 3 neurohormones (i.e., SS, GHRH, and ghrelin) responsible for regulation of GH secretion are highly expressed in gastrointestinal tissues.

In humans, rats, and domestic animals, expression of ghrelin mRNA and the ghrelin peptide have been primarily detected in the entero-endocrine or *X/A-like* cells of the fundic gland in the stomach (Hayashida et al., 2001; Tomasetto et al., 2001) and have now been renamed Ghr-cells. The cells containing ghrelin do not communicate with the lumen of the fundic gland. Like all entero-endocrine cells, they are positioned adjacent to capillaries, indicating that their primary action is secretion of hormone into plasma and not into the intestinal lumen (Date et al., 2000).

The degree of structural heterogeneity of ghrelin among species appears to be minor, suggesting that there is little functional heterogeneity. Such preservation of structure throughout evolution reflects the physiologic relevance of the peptide (Tomasetto et al., 2001; Casanueva and Dieguez, 2002). For example, human and rat ghrelin differ in only two amino acids (Table 1) (van der Lely et al., 2004). Alternative splicing of mRNA segments encoding ghrelin yields two different peptides, ghrelin and des-Gln14-ghrelin (Hosoda et al., 2000). The latter is homologous with ghrelin except for the absence of a single glutamine residue. Des-Gln14-ghrelin is expressed in the stomach in low quantities (Kojima et al., 2001), but, like ghrelin, it increases the intracellular concentration of calcium in cells that express the GHS-R 1a and increases plasma GH concentrations (Kojima et al., 1999; Hosoda et al., 2000).

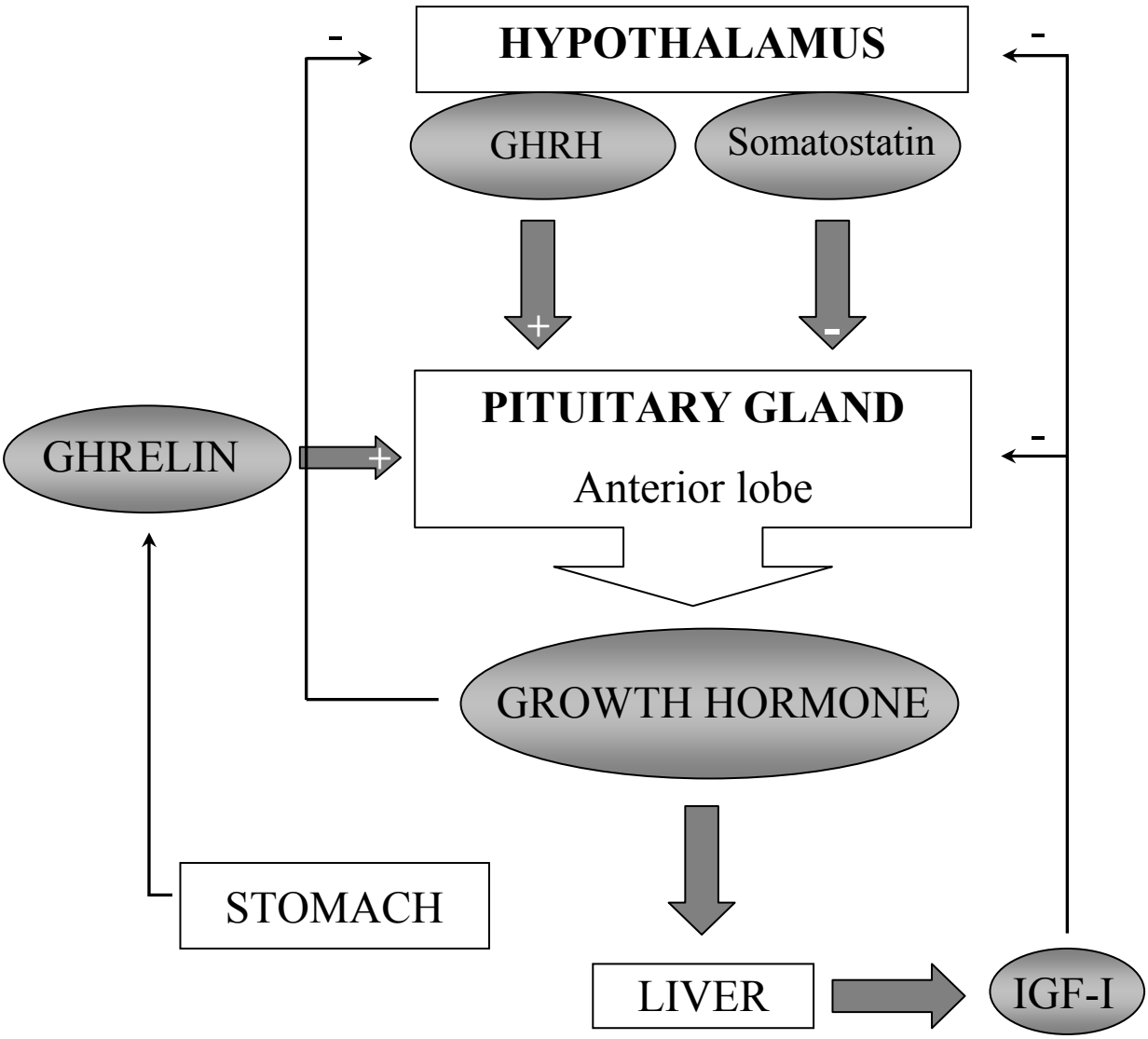

Figure 3. Regulation of the hypothalamic-pituitary-growth hormone (GH) axis. Growth hormone secretion by the pituitary gland is stimulated by growth hormone releasing hormone (GHRH) and is inhibited by somatostatin (SS). Negative feedback control of GH secretion is exerted at the pituitary and hypothalamic level by insulin-like growth factor-I (IGF-I). Growth hormone itself exerts a short-loop negative feedback by activation of SS neurons. The gastric peptide ghrelin is the natural ligand for the GH secretagogue receptor that stimulates GH secretion at the pituitary level.

Table 1. Primary structure of ghrelin from domestic mammalian species. Adapted from van der Lely et al. (2004) with permission *(Copyright 2004, The Endocrine Society).* Bold indicates sites at which a residue is different from that in the human peptide.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	Species
G	S	S	F	L	S	P	E	H	Q	R	V	Q	Q	R	K	E	S	K	K	P	P	A	K	L	Q	P	R	Human
G	S	S	F	L	S	P	E	H	Q	**K**	**T**	Q	Q	R	K	E	S	K	K	P	P	A	K	L	Q	P	R	Gerbil
G	S	S	F	L	S	P	E	H	Q	**K**	**A**	Q	Q	R	K	E	S	K	K	P	P	A	K	L	Q	P	R	Mouse
G	S	S	F	L	S	P	E	H	Q	**K**	**A**	Q	Q	R	K	E	S	K	K	P	P	A	K	L	Q	P	R	Rat
G	S	S	F	L	S	P	E	H	Q	**K**	**L**	Q	Q	R	K	E	S	K	K	P	P	A	K	L	Q	P	R	Dog
G	S	S	F	L	S	P	E	H	Q	**K**	V	Q	Q	R	K	E	S	K	K	P	A	A	K	L	**K**	P	R	Pig
G	S	S	F	L	S	P	E	H	Q	**K**	**L**	Q	Q	R	K	E	**A**	K	K	P	**S**	**G**	**R**	L	**K**	P	R	Cattle
G	S	S	F	L	S	P	E	H	Q	**K**	**L**	Q	Q	R	K	E	**P**	K	K	P	S	**G**	**R**	L	**K**	P	R	Sheep
G	S	S	F	L	S	P	**T**	**Y**	**K**	**N**	**I**	Q	Q	**Q**	K	**D**	**T**	**R**	K	P	**T**	A	**R**	L	**H**	**R**	R	Chicken

Before being secreted, *n*-octanoic acid is added to the third serine residue of ghrelin and des-Gln14 ghrelin (Figure 4) (van der Lely et al., 2004). This acylation step, unique in mammalian species, is essential for binding to and activating the GHS-R 1a (Bednarek et al., 2000) and hence for the peptide's GH-releasing action. The acylation is most likely also necessary for the other endocrine actions of the ghrelin molecule (Kojima et al., 2001; Broglio et al., 2003). Addition of the *n*-octanoyl group confers a hydrophobic property to the N terminus of the peptide. It has been suggested that the octanoylation of ghrelin is critical to the peptide's ability to cross the blood-brain barrier. It may also facilitate distribution of the peptide in the brain although there are presently no data to support this speculation (Horvath et al., 2001). Non-acylated ghrelin is found in far greater quantities in human serum than acylated ghrelin, but it seems to be devoid of any endocrine activity. However, this peptide does have certain nonendocrine actions, such as cardiovascular and anti-proliferative effects and these are probably mediated through binding to a novel, as yet unidentified, GHS-R subtype (Cassoni et al., 2004). Non-acylated ghrelin is able to inhibit proliferation of human prostate cancer cell lines and neoplastic cell growth in thyroid, breast and lung tumours. Also, cardioprotective and negative inotropic effects have been described (Date et al., 2000; Cassoni et al., 2004).

Lower amounts of ghrelin have been detected in various other tissues, including the intestines (Date et al., 2000), the pituitary gland (Korbonits et al., 2001), the hypothalamus (Kojima et al., 1999; Date et al., 2000), the kidney (Mori et al., 2000), the placenta (Gualillo et al., 2001), the heart (Casanueva and Dieguez, 2002), the testes (Barreiro et al., 2002), the thyroid gland (Kanamoto et al., 2001), the pancreas (Volante et al., 2002a), the lung (Volante, 2002b), the ovary (Gyatan et al., 2003), the immune system (Hattori et al., 2001), and

neoplastic tissue (Papotti et al., 2000). The physiologic importance of ghrelin as a paracrine factor in these tissues is the subject of current research. An endocrine role for non-stomach-derived ghrelin is thought to be unlikely. Removal of the stomach in humans and rats decreases the plasma concentration of ghrelin by approximately 65 % and 80 %, respectively (Date et al., 2000; Ariyasu et al., 2001). However, plasma ghrelin concentrations gradually increase after gastrectomy (Hosoda et al., 2003). Taken together, these findings indicate that the stomach is the major source of circulating ghrelin but other tissues may contribute to the secretion of ghrelin in a compensatory manner (Moller et al., 2003).

6.1 Endocrine effects of ghrelin

Ghrelin has pronounced, dose-related GH-releasing actions that are more marked in humans than in animals (Smith et al., 1997; Kojima et al., 1999; Arvat et al., 2000; Date et al., 2000; Seoane et al., 2000; Takaya et al., 2000; Arvat et al., 2001; Ghigo et al., 2001; Hayashida et al., 2001; Bhatti et al., 2002; Bhatti et al., 2006 in press). The GH-releasing activity of ghrelin is greater *in vivo* than *in vitro*, because ghrelin and GHRH act synergistically, consistent with the fact that their actions are at least partially mediated via different mechanisms (Smith et al., 1997; Tannenbaum and Bowers, 2001). Nevertheless, GHRH activity is required for full expression of ghrelin's GH-releasing activity (Smith et al., 1997; Tannenbaum and Bowers, 2001). The GH response to ghrelin is inhibited, although not completely, by GHRH receptor antagonists and by hypothalamo-pituitary disconnection (Hickey et al., 1996; Popovic et al., 2003). This supports the assumption that the effect of ghrelin on GH secretion is primarily mediated by GHRH-secreting neurons at the level of the hypothalamus (Bowers et al., 1991; Smith et al., 1997; Tannenbaum and Bowers, 2001; Popovic et al., 2003).

In anaesthetized rats, intravenously administered ghrelin stimulates GH release without affecting the secretion of other adenohypophyseal hormones (Kojima et al., 1999). Also, in cultured rat pituitary cells, ghrelin stimulates GH release in a dose-dependent manner without affecting the release of other pituitary hormones, even at high concentrations (Kojima et al., 1999). However, in healthy humans, ghrelin is not fully specific for GH release, because it also has stimulatory effects on lactotroph and corticotroph cells (Arvat et al., 1997b; Peino et al., 2000; Takaya et al., 2000; Arvat et al., 2001). The effect of ghrelin on PRL secretion is independent of gender and age, and likely results from direct stimulation of somatomammotrophs (Renner et al., 1994; Arvat et al., 1997b; Takaya et al., 2000; Muccioli et al., 2002).

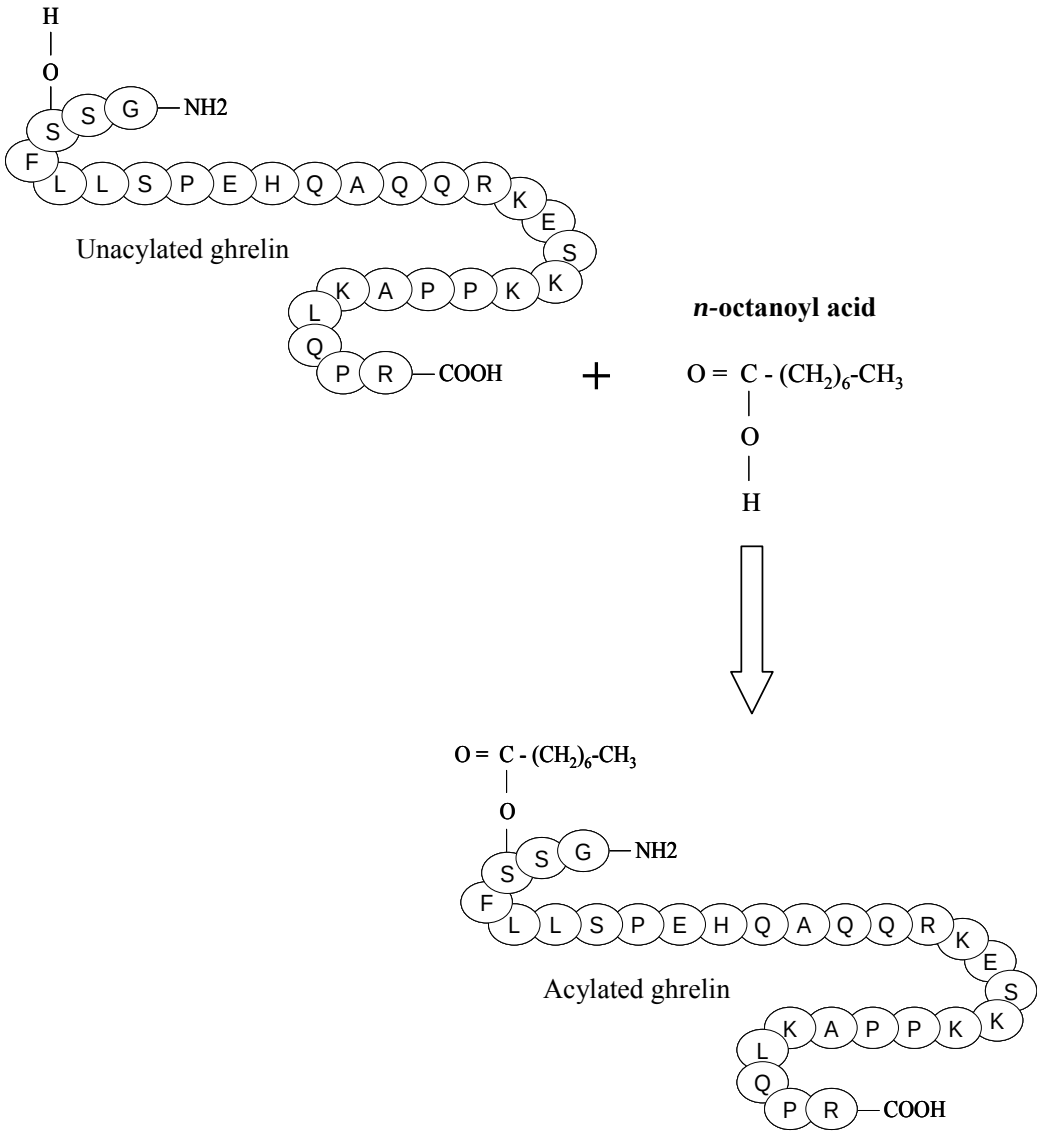

Figure 4. Acylation of the ghrelin molecule. A hydroxyl group on the serine residue at position 3 of the ghrelin molecule is octanoylated. This esterification is unique to mammals and is essential for ghrelin binding to and activating the growth hormone secretagogue receptor type 1a and, consequently, for the growth hormone-releasing action of ghrelin. The other endocrine actions of ghrelin are also likely dependent on the acylation of the peptide. Figure adapted from van der Lely et al. (2004) with permission *(Copyright 2004, The Endocrine Society).*

In rats (Kojima et al., 1999), synthetic GHSs do not stimulate PRL release. This species-related difference may be explained by differences in the number of somatomammotrophs in various species, with humans having a high proportion of those cells (Frawley and Boockfor, 1991; Raun et al., 1998). The mechanism by which ghrelin stimulates the pituitary-adrenocortical axis is still unknown, but may be mediated via the hypothalamus, because the stimulatory effect is lost after sectioning of the pituitary stalk (Loche et al., 1995). Ghrelin may also interact with hypothalamic peptides that control ACTH release, probably via arginine vasopressin (Thomas et al., 1997; Korbonits et al., 1999; Broglio et al., 2003).

6.2 Orexigenic actions and role in energy homeostasis

Evidence for the involvement of ghrelin in the regulation of appetite was first described in humans. Healthy human volunteers reported hunger after administration of ghrelin in a clinical study in which GH release was analyzed (Arvat et al., 2000). In rodents, ghrelin stimulates food intake and increases body weight while reducing mobilization of adipose stores (Tschop et al., 2000; Wren et al., 2000; Shintani et al., 2001). The effects of ghrelin on food intake are likely mediated through mechanisms other than those implicated in GH regulation, compatible with the concept of distinct GHS-R subtypes (Tschop et al., 2000; Toogood and Thorner, 2001).

Traditionally, adipocytes have been viewed as energy depots that store triglycerides during feeding and release fatty acids during fasting to provide fuel for other tissues. However, it has become clear that adipose tissue has major integrative physiologic functions, including the secretion of numerous proteins (Friedman an Halaas, 1998; Miner, 2004). The realization that adipose tissue functions as an endocrine organ has important implications for our understanding of the associations between excess body fat and pathologic states like insulin resistance and type-2 diabetes mellitus (Miner, 2004).

An important finding that linked central regulation of metabolism to peripheral energy stores was the discovery of the adipose hormone, leptin (from the Greek root *leptos*, meaning thin). Leptin, a peptide hormone discovered in 1994, is produced principally by white adipose tissue (Zhang et al., 1994). Leptin crosses the blood-brain barrier to act via receptors in the arcuate nucleus of the hypothalamus to inhibit the release of orexigenic neuropeptides and stimulate the release of anorexigenic neuropeptides (Friedman and Halaas, 1998; Neary et al., 2003). There is a direct relationship between plasma leptin concentrations and percentage body fat. Plasma leptin levels in humans are generally proportional to adipose mass. A reduction in leptin levels occurs because of loss of adipose mass such as anorexia nervosa,

diet- or exercise-induced weight loss, or starvation. Concentrations of circulating leptin decrease rapidly within 12h after initiation of starvation, whereas concentrations increase in response to overfeeding. Thus, plasma leptin concentrations reflect adipose tissue mass and provide a signal that informs the central nervous system about the body's energy reserves (Friedman and Halaas, 1998).

The similarities and complementary interplay of actions between leptin and ghrelin are intriguing. The effects of ghrelin on metabolism appear to be the opposite to those of leptin (Tschop et al., 2000; Bowers, 2001; Kojima et al., 2001; Spiegelman and Flier; 2001). Leptin reduces food intake and selectively reduces fat mass without altering lean body mass (Farooqi et al., 1999). Ghrelin, in contrast, increases food intake and selectively enhances fat mass (Tschop et al., 2000).

Ghrelin stimulates food intake in rodents when administered via central (intracerebroventricular) or peripheral (subcutaneous) routes, although the effect is more powerful after central administration (Tschop et al., 2000). There is evidence that the appetite-stimulating effects of ghrelin are mediated by secretion of 2 potent orexigenic hypothalamic hormones (neuropeptide Y and agouti-related peptide) and by inhibition of pro-opiomelanocortin and α-melanocyte-stimulating hormone (α-MSH) (Dickson et al., 1997; Hewson and Dickson, 2000; Kamegai et al., 2001; Cowley et al., 2003). Furthermore, the orexigenic action of ghrelin is eliminated when neuropeptide Y and agouti-related peptide are antagonized (Kamegai et al., 2001). By stimulating the release of orexigenic peptides and neurotransmittors, ghrelin mediates a novel regulatory circuit regulating energy homeostasis (Cowley et al., 2003; Kohno et al., 2003; Olszewski et al., 2003; Riediger et al., 2003; Seoane et al., 2003).

In humans and rats, concentrations of circulating ghrelin decrease in chronic (obesity) (Tschop et al., 2001) and acute (caloric intake) (Cummings et al., 2001) states of positive energy balance, whereas ghrelin levels increase in states of negative energy balance (e.g., fasting) (Tschop et al., 2000). In cattle, plasma ghrelin concentrations are low 1h after feeding and then return to the pre-feeding concentration (Hayashida et al., 2001). In sheep, the preprandial ghrelin surge is higher in animals fed twice daily than in animals fed four times daily, highlighting the influence of different feeding regimens on ghrelin concentrations (Sugino et al., 2002). The preprandial rise and postprandial fall in plasma ghrelin concentrations suggest a possible role for ghrelin as a hunger signal, triggering meal initiation (Cummings et al., 2001). Because ghrelin is a potent stimulator of GH release, these observations are in accordance with the low plasma GH concentrations associated with

obesity (Bowers, 1993) and the high concentrations observed in the malnutrition and fasting states (Dieguez and Casanueva, 1995).

It may be concluded that nutritional state is an important determinant of plasma ghrelin concentration (Tolle et al., 2002). Ghrelin peptide reaches ghrelin receptors in the hypothalamo-pituitary region via the general circulation, where it stimulates GH release and regulates energy homeostasis. It is unclear whether ghrelin must cross the blood-brain barrier to influence the activity of these central structures (van der Lely et al., 2004). In the general circulation, ghrelin is bound to high-density lipoproteins in the serum and presumably to other proteins, such as albumin, as well. Ghrelin may also signal the brain directly, by activating the afferent portion of the vagal nervous system as either an endocrine or a paracrine signal, at the level of the stomach. Ghrelin-responsive GHS-Rs are expressed on gastric vagal nerves, and vagotomy prevents some of the effects of ghrelin on energy balance. On the other hand, the extent and direction of ghrelin transport across the blood-brain barrier may be determined by its unique primary structure (Banks et al., 2002). There is still debate on the routes by which ghrelin in the peripheral circulation activates receptors in the central nervous system of different species.

6.3 Gastric prokinetic action

Ghrelin induces strong prokinetic activity in the stomach (Masuda et al., 2000; Asakawa et al., 2001). The peptide dramatically accelerates gastric and intestinal emptying in rats, and circulating ghrelin concentrations are correlated with gastric emptying time in humans (Masuda et al., 2000). In addition, ghrelin stimulates gastric acid secretion (Asakawa et al., 2001).

In this context, it is interesting to consider the structural and functional similarities between ghrelin and motilin (Folwaczny et al., 2001). In addition to their prokinetic effect on the gastrointestinal tract, both peptides have orexigenic properties (Garthwaite, 1985) and stimulatory effects on pituitary GH release (Samson et al., 1984). Also, the G-protein-coupled receptors of ghrelin and motilin have a high degree of structural homology (Feighner et al., 1999). In contrast to ghrelin, motilin is primarily expressed in the small intestine (Brown et al., 1971). Motilin stimulates motor activity in the gastric antrum and proximal portion of the duodenum, and plays a key role in the regulation of interdigestive motility (Itoh, 1997).

The gastrokinetic effects of ghrelin and motilin may prove beneficial in the treatment of postoperative gastric ileus. In humans and other mammalian species, abdominal surgery and attendant manipulation of the viscera inhibit gastric emptying and digestive motor

activity, which may result in postoperative ileus. Attempts to stimulate smooth muscle activity with various prokinetics (eg, cisapride and acetylcholine) are often unsuccessful (Asakawa et al., 2001). In rats, ghrelin reverses postoperative gastric ileus (Masuda et al., 2000). Further studies may elucidate the pharmacologic potential of ghrelin and motilin in gastroenterologic applications.

6.4 Effects on the endocrine pancreas

Ghrelin and GHS-R 1a mRNA are expressed in endocrine cells of the pancreas (Guan et al., 1997; Date et al., 2002; Gnanapavan et al., 2002; Rindi et al., 2002). Expression of ghrelin has been reported in the pancreatic α-cells (Date et al., 2002), although other investigators have reported that ghrelin is expressed in the pancreatic β-cells (Volante et al., 2002a). Ghrelin is not co-expressed with any known islet-derived hormone; thus, ghrelin-producing cells may be a newly recognized type of islet cell (Wierup et al., 2002).

Published information regarding the effect of ghrelin on insulin secretion in humans and rats is conflicting (Caixas et al., 2002; Date et al., 2002; Lee et al., 2002). However, most findings suggest a negative association between ghrelin concentrations and insulin secretion (Broglio et al., 2001; Cummings et al., 2001; Tschop et al., 2001; Adeghate and Ponery, 2002; Date et al., 2002). In humans, ghrelin induces a significant increase in plasma glucose concentrations and a decrease in insulin secretion (Broglio et al., 2001; Broglio et al., 2003). Coupled with the observation that treatment with GHSs, particularly the non-peptidyl derivatives, induces hyperglycaemia and insulin resistance in the elderly and in obese human patients, those findings suggest that ghrelin has an important role in the regulation of insulin secretion and glucose metabolism (Svensson et al., 1998; Muller et al., 2001).

In healthy humans, hyperglycaemia suppresses both baseline plasma concentrations of GH and GH release induced by GHRH (Masuda et al., 1985). The mechanism of the hyperglycaemia-induced decrease in circulating GH is unclear. Acute hyperglycaemia substantially decreases plasma ghrelin concentrations in healthy humans (Nakagawa et al., 2002). Because ghrelin markedly stimulates GH secretion, the hyperglycaemia-induced suppression of GH release may be caused, at least partly, by the decrease in plasma ghrelin concentrations (Casanueva, 1992).

6.5 Cardiovascular effects

Ghrelin receptors are widely distributed in cardiovascular tissues. In humans and rats, GHS-R 1a mRNA has been detected primarily in the heart, coronary arteries, and aorta

(Nagaya et al., 2001, Gnanapavan et al., 2002). Ghrelin is synthesized and secreted by isolated human cardiomyocytes, in which it likely has paracrine or autocrine effects and may protect the cells from apoptosis (Iglesias et al., 2004).

Growth hormone improves cardiac performance in experimentally induced heart failure (Yang et al., 1995; Fazio et al., 1996). In one study, prolonged treatment with GHSs protected aged rats against cardiovascular damage and improved cardiac performance after myocardial infarction, and enhanced left ventricular contractility in pigs with dilated cardiomyopathy (Muccioli et al., 2002). Long-term ghrelin administration improves cardiac contractility and cardiac output and reduces systemic vascular resistance in humans with chronic heart failure (Nagaya et al., 2001). Furthermore, it induces myocardial growth, improving the structure and function of the left ventricle (Nagaya et al., 2003; Nagaya et al., 2004). Interestingly, hexarelin, acylated ghrelin, and even unacylated ghrelin all prevent doxorubicin-induced death in cultured cardiomyocytes (Filigheddu et al., 2001). Because unacylated ghrelin does not activate the GHS-R 1a (Bednarek et al., 2000), these data indicate that another subtype of GHS-R exists in cardiac tissue and that unacylated ghrelin has some biological activity (Muccioli et al., 2002). Thus, long-term administration of ghrelin may become a treatment strategy for patients with heart failure (Nagaya et al., 2003).

6.6 Anti-proliferative effects

GHS-Rs are also found in human neoplastic tissues, such as mammary gland tumours and thyroid carcinoma cells (Cassoni et al., 2001; Kanamoto et al., 2001). Ghrelin and GHSs inhibit cell proliferation in thyroid tumour cells (Kanamoto et al., 2001; Cassoni et al., 2002) and breast cancer cells (Cassoni et al., 2001). Nonacylated ghrelin also exerts anti-proliferative actions (Cassoni et al., 2001). Because unacylated ghrelin is unable to bind to the GHS-R 1a, these data suggest that the anti-proliferative effects of acylated and unacylated ghrelin on cancer cells are mediated via a GHS-R subtype that is different from GHS-R 1a (Muccioli et al., 2001).

6.7 Conclusion

The isolation and characterization of ghrelin are landmarks in GH research and represent a major advancement in our understanding of GH regulation. Ghrelin is a gastric peptide that is active in the central nervous system, where it is involved in regulation of GH secretion and control of food intake. The widespread expression of GHS-Rs in central and

peripheral tissues suggests that ghrelin may have many endocrine, paracrine, and possibly autocrine effects.

Future challenges lie in improving our ability to diagnose and treat the different diseases associated with altered GH secretion. For example, the potential use of ghrelin in GH deficiency requires investigation. In addition, ghrelin or ghrelin analogues may be useful in pathologic catabolic states such as wound and fracture healing, osteoporosis, severe burns, sepsis, excessive inflammation, multiple organ failure, and weakness in critically ill patients, all conditions in which the administration of moderate doses of GH has been effective (Van den Berghe 2000; Petersenn 2002). The orexigenic actions of ghrelin and its analogues may be harnessed to treat the pathologic forms of anorexia that accompany cancer and ageing (Torsello et al., 1998). Whether ghrelin antagonists can be used to reduce food intake and be developed as a treatment for obesity remains to be investigated.

General introduction - Part II

Progesterone and synthetic progestins used for oestrus prevention in the bitch and their systemic effects

1. Progesterone and synthetic progestins

Natural progesterone is biosynthesized and secreted by partial luteinized granulosa cells during the preovulatory LH peak and consequently the corpus luteum during the luteal phase of the oestrous cycle and serves to prepare the genital tract for the reception and development of the fertilized ovum. The name progesterone comes from *pro*, *ges*tation, *ster*ol, and *one*, indicating this first recognized function of the steroid hormone. However, apart from the genital tract and ovaries, also brain, bone, and especially the mammary gland (Lantinga-van Leeuwen et al., 2000c) are important target organs for progesterone action. The terms progestin, progestagen, and progestogen are used interchangeably to refer to any of the manufactured steroids with progestational activity and derived from progesterone or related steroids. Progestins are widely used in companion animal medicine and the main use involves the control of the reproductive cycle (Briggs, 1983; Romagnoli and Concannon, 2003).

A progestational agent, capable of maintaining pregnancy in ovariectomized animals and of causing endometrial gland secretion, was identified, isolated, and eventually synthesized in the first decades of the 20th century. In the 1930s, it was discovered that progesterone administered by intramuscular injection was capable of blocking ovulation in rabbits. A quest for progesterone-like substances that could control reproduction ensued, leading to the synthesis and characterization of a number of progestins with potential contraceptive application. These included medroxyprogesterone acetate (MPA), melengestrol acetate, megestrol acetate, and others. In the 1960s, some progestins being evaluated for use in humans were found to induce mammary tumours in Beagle bitches during toxicity studies (Frank et al., 1979; Gräf and El Etreby, 1979; Edgren, 1994). Thereafter, long-term animal studies became a requirement by the Food and Drug Administration and the World Health Organization for any progestin to be marketed for human use (Johnson, 1989; Jordan, 1994). In veterinary medicine, depot-injectable MPA rapidly became, and remained for a few

decades, the most widely used progestin in Europe. In the United States, however, it was quickly withdrawn from the veterinary market because of a high incidence of uterine disease reported in dogs administered MPA. Other progestins that were developed as potential human contraceptives have also been marketed for contraceptive use in dogs and/or cats e.g. oral megestrol acetate, oral MPA, oral delmadinone acetate, oral clormadinone acetate, and depot-injectable proligestone (Romagnoli and Concannon, 2003). In a variety of *in vivo* and *in vitro* assays in various species, such compounds have been examined for relative biopotency, bioavailability, oestrogenic activity, and androgenic activity. Often, however, different compounds have not been evaluated in the same way, and therefore relative differences are not clear-cut. Species differences in potency and efficacy are also known to exist.

2. Mechanism of action of progestins

In most if not in all target tissues, progesterone and synthetic progestins diffuse through the cell membrane, and bind to intracellular receptors. In most mammalian species two progesterone receptor (PR) variants are known, the 51-94 kDa PR-A and the larger 116-120 kDa PR-B form. PR-A and PR-B share the same hormone- and DNA-binding domains and differ only in the length of the amino terminus (Graham and Clarke, 1997). After binding of progesterone, the progesterone-PR complex binds to a progesterone-response-element in the nuclear genome, resulting in suppression or activation of transcription and eventual translation of specific gene sequences regulated by progesterone. The translation products include structural and secretory proteins, enzymes, and other regulatory proteins. It is also likely that in some tissues, progesterone, similar to oestradiol, can bind to membrane receptors and has cellular effects in response to binding to membrane receptors (Graham and Clarke, 1997; Romagnoli and Concannon, 2003).

3. Reproductive effects of progestins

In addition to its progestational activity in maintaining pregnancy, progesterone was initially characterized and assayed based on its ability to increase uterine weight (acting synergistically with oestrogen) and its ability to increase endometrial glandularity and endometrial secretory activity (Nelson et al., 1982). Numerous studies on progesterone and progestins have demonstrated that their administration can have actions that can be classified

as progestational, anti-oestrogenic, anti-androgenic, anti-gonadotrophic, and/or contraceptive (Romagnoli and Concannon, 2003).

The pregnancy supporting or progestational actions of progesterone and synthetic progestins include the following: stimulation of endometrial gland development and secretion, promotion of cervical closure, suppression of uterine motility by depressing myometrial sensitivity and contractility directly and by decreasing the availability of myometrial oxytocin receptors, and stimulating proliferation of mammary tissue, especially lobulo-alveolar tissue (Nelson et al., 1982; Mol et al., 1995a,b). Prior progestin administration reduces or prevents oestrogen-induced phenomena including vaginal bleeding, oestrus behaviour, and ciliation of the oviduct by suppressing the synthesis of oestrogen receptors normally stimulated by exposure to oestrogens. Progestin administration reduces, inhibits, or reverses some effects of androgens, including libido, possibly by interfering with androgen or other steroid receptors' reponses to their own hormone ligands. Progestin administration can also suppress follicle development and prevent ovulation. The exact mechanism of the contraceptive activity of progestins is still unclear. In many species there is evidence that contraceptive progestins reduce serum concentrations of gonadotrophins. However, there is little information about the effects of progestins on gonadotrophin secretion in dogs. In one study high doses of MPA administered to Beagle bitches for several months did not reduce the increased concentrations of LH in ovariectomized bitches nor did it lower LH concentrations in intact bitches (McCann et al., 1987). In another study high doses of megestrol acetate did not suppress basal gonadotrophin secretion during anoestrus, nor was the pituitary hypersecretion of LH and FSH in ovariectomized bitches suppressed (Colon et al., 1993). The contraceptive activity of progestins may involve the prevention of increases in gonadotrophin secretion above basal values. In addition, there may be a direct negative effect on follicle development in the ovary (Colon et al., 1993).

The progestins most frequently used for oestrus prevention in the dog are proligestone and MPA. Because the drug cannot be rapidly withdrawn after injection of the depot progestin, care must be taken to use the lowest possible effective dosing regimen. The single injection dosage recommended by the manufacturer for proligestone ranges from 10 mg/kg for a dog of about 60 kg, to 30 mg/kg for one of 3 kg, s.c., and for MPA the single injection dose is 2 mg/kg (maximum 60 mg), s.c. (Schaefers-Okkens, 1996). They should be administered during anoestrus about one month before the expected follicular phase. The first oestrus after the use of proligestone in the majority of bitches can be expected within 9-12 months; after MPA administration it may be up to 2-3 years. Medroxyprogesterone acetate

can also be administered orally, 5 mg once daily (10 mg for large dogs during the first 5 days) for as long as oestrus prevention is wanted or for a maximum of 21 days. The recurrence of oestrus may vary from 2-9 months (Schaefers-Okkens, 1996). In the United States the advise dosage for megestrol acetate, a progestin which probably has a stronger progestagenic effect than MPA, is 0.5 mg/kg orally once daily for 32 days starting during anoestrus, or 2 mg/kg for 8 days starting at the onset of pro-oestrus (Schaefers-Okkens, 1996).

4. Additional effects of progestins

Side effects have been observed after prolonged (6-12 months or longer), chronic use of progestins such as during the course of chronic toxicity studies. They are also reported with varying severity and varying or unknown frequency during the course of treatment with recommended contraceptive doses.

4.1 Induction of mammary growth hormone secretion

In the 1970s acromegalic features were reported to occur in some dogs used in toxicological studies on long-term treatment with progestins (Tucker, 1971; Sloan et al., 1975). Initially it was denied that GH might be involved in the development of the physical features reminiscent of acromegaly (Hansel et al., 1977). In 1980 confirmation that progestin administration can lead to increased circulating GH concentrations was obtained (Concannon et al., 1980). The elevated plasma GH levels declined after cessation of progestin administration (Rijnberk et al., 1980). This phenomenon appeared not to be confined to exogenous progestins, as an excess of GH was also found in bitches during the luteal phase of the oestrous cycle (Concannon et al., 1980; Eigenmann et al., 1983; Rutteman et al., 1987). The absence of a pulsatile pattern in plasma GH concentrations after progestin treatment pointed to autonomous GH production, which only could be inhibited by treatment with the antiprogestin RU 38486 (Watson et al., 1987). This autonomous release was further substantiated by the fact that GHRH did not stimulate and SS did not inhibit GH expression and release (Selman et al., 1991). The progestin-induced increase of plasma GH concentration was associated with elevated plasma concentrations of IGF-I (Selman et al., 1994a).

Because the GH overproduction could not be attributed to a pituitary tumour or an ectopic neoplastic production of GHRH, as in other species, and because cessation of progestin administration resulted in gradual normalization of the circulating GH levels, an extra-pituitary site of GH production was looked for. Measurement of GH concentrations in

tissue homogenates revealed that the progestin-induced GH excess in the dog originated from foci of hyperplastic ductular epithelium of the mammary gland (Selman et al., 1994a; van Garderen et al., 1997). Further evidence came from the arterio-venous gradient over the mammary gland and the fast decrease of plasma GH concentrations after complete mastectomy (Selman et al., 1994a). RT-PCR analysis revealed the expression of the gene encoding GH in normal mammary tissue and in benign and malignant mammary tumours (Mol et al., 1995b). The expression of the GH gene was also documented for feline and human mammary tissue, indicating that the phenomenon of mammary GH expression is not unique for the dog (Mol et al., 1995a; Mol et al., 1996). Immunohistochemical analysis and in situ hybridization revealed that both immunoreactive GH and GH mRNA were present in normal and tumourous mammary epithelial cells. Growth hormone-containing secretory granules could also be demonstrated in epithelial cells by immunoelectron microscopy (van Garderen et al., 1997). Sequence analysis has revealed that the gene encoding GH in the mammary gland is identical to the pituitary GH gene (Mol et al., 1995a; Mol et al., 1995b). The regulation of extra-pituitary GH gene expression is largely unknown. There are at least two differences between the regulation of GH expression in the mammary gland and that in the pituitary gland. First of all, the synthesis and release of mammary GH are highly dependent upon progesterone (Selman et al., 1994a; Lantinga-Van Leeuwen et al., 1999). Secondly, expression of mammary GH is independent of the transcription factor Pit-1, as is GH expression in bone marrow (Lantinga-Van Leeuwen et al., 1999).

The co-localization of GH and the progesterone receptor in the canine mammary gland supports the concept that ligand-activated progesterone receptors may play a direct role in GH gene promotor activation (Lantinga-van Leeuwen, 2000c). The presence of progesterone receptors in mammary gland tissue of dogs opens possibilities for a targeted endocrine therapy with progesterone receptor blockers in dogs with progestin-induced mammary-derived GH overproduction.

4.2 Increased incidence of mammary tumours

The role of progestins in the pathogenesis of human breast cancer has been highly debated (van Leeuwen 1991; Pike et al., 1993). Several epidemiologic studies have linked the use of contraceptive agents during adolescence or before a full-term pregnancy to a higher risk of developing breast cancer at a young age (Pike et al., 1981; van Leeuwen et al., 1991). The question of whether this increased risk is attributable to the oestrogen or progestin content of the contraceptives has not been answered satisfactorily, but there is good reason to

assume that this increased risk is due to the progestin component (Lee et al., 1987; Kay and Hannaford, 1988). This assumption is supported by observations that the highest proliferation rates of mammary epithelium are found in the progesterone-dominated luteal phase of the menstrual cycle and in women receiving progestin-only formulations of contraceptives, indicating a strong mitogenic action of progestins upon mammary epithelium (Horwitz, 1991; Clarke and Sutherland, 1990).

In the dog, prolonged administration of oestrogens does not increase the incidence of mammary tumours (Rutteman, 1992), but treatment of female dogs with progestins at high dosages induces a dose-dependent mammary tumour development (Casey et al., 1979; Misdorp 1991; Rutteman, 1992). Also, endogenous ovarian steroids appear to promote mammary tumourigenesis in dogs, as ovariectomy, even performed at an advanced age, protects against mammary tumour formation (Misdorp 1988). As mentioned above, GH gene expression has been demonstrated in neoplastic mammary tissue of the dog (Mol et al., 1995b). The expression in normal tissue is stimulated by progestins and might mediate the progestin-stimulated development of canine mammary tumours. Progestin-induced GH probably participates in the cyclic development of the mammary gland but may promote mammary tumourigenesis by stimulating proliferation of susceptible, and sometimes transformed, mammary epithelial cells. Progestin-induced mammary GH might function as an autocrine or paracrine growth factor, in view of the presence of the GH receptor that has been demonstrated in the normal canine mammary gland and in mammary tumours of dogs (van Garderen and Schalken, 2002).

In young queens exogenous progestins, but also endogenous progesterone, may cause extensive proliferation of mammary duct epithelium and stroma, leading to the so-called fibroadenomatous hyperplasia (Hayden and Johnson, 1986). Growth hormone mRNA has been demonstrated in mammary tissues of cats with progestin-induced fibroadenomatous changes (Mol et al., 1995b).

4.3 Increased incidence of uterine pathology

Teunissen (1952) reported that progestins may induce the development of cystic endometrial hyperplasia (CEH) in bitches. In line with this observation CEH is frequently seen in bitches treated repeatedly with progestins for prevention of oestrus (Sokolowski and Zimbelman 1974; Goyings et al., 1977). Cystic endometrial hyperplasia may also develop spontaneously in the luteal phase of the oestrous cycle of middle-aged or elderly bitches, i.e., bitches that have gone through several luteal phases (Dow, 1958). The luteal phase of the

oestrous cycle of the bitch differs from that of most other mammals because it is characterized by a prolonged increase of plasma concentrations of progesterone, irrespective of pregnancy (Concannon et al., 1975).

Uterine pathology involves proliferation of the glandular endometrium and cystic dilatation of the endometrial glands with endometrial fluid accumulated in their lumen. Cystic endometrial hyperplasia may be an incidental finding and the natural incidence of CEH is not known. Cystic endometrial hyperplasia predisposes the uterus to infection and can result in pyometra.

From studies in which CEH was induced experimentally in the dog (Dow, 1959), it has become clear that metoestrus or progesterone influence in general seems to be required or at least predisposes to development of CEH. This is remarkable because in women, cows, ewes, mares, and sows endometrial hyperplasia is associated only with excess stimulation by oestrogen from cystic follicles, oestrogenic implants or granulosa cell tumours (Potter et al., 1991). In women it is well known that progesterone induces endometrial atrophy (Seidman et al., 1997), while in dogs progestin treatment has been associated with endometrial hyperplasia (Anderson et al., 1965; Fidler, 1966). The exposure of captive wild felids to progestins is a strong risk factor for development of uterine carcinoma (Harrenstien et al., 1996). In contrast, endometrial carcinomas in dogs, even under progestin treatment, are rare (Kennedy, 1993). This suggests that the canine endometrium responds in a different way to progesterone compared to other species.

As mentioned before, locally produced mammary GH most likely plays a autocrine and/or paracrine role in the progestin-induced proliferation of mammary epithelium (Mol et al., 1995b). Because of the similarity of the progestin-induced epithelial changes in both the mammary gland and the uterus, it is reasonable to assume that GH is also involved in the development of progestin-induced CEH. Although immunoreactive GH was found in the uterine epithelial cells of progestin treated dogs, the absence of mRNA encoding GH in uterine tissue as shown by RT-PCR suggests that this immunoreactive GH does not originate in the uterus (Kooistra et al., 1997). This finding refutes the hypothesis that local production of GH is involved in the pathogenesis of progestin-induced CEH. However these findings do not exclude the possibility that GH plays a role in the pathogenesis of progestin-induced CEH, whereby progestins promote the expression of membrane-bound GH receptors of uterine epithelial cells. In combination with the increase in progestin-induced circulating mammary-derived GH this would explain the presence of GH in uterine epithelial cells. The presence of mRNA encoding the GH receptor has already been demonstrated in the human

uterus (Sharara and Nieman, 1995). The possible role of progestin-induced mammary-derived GH in the pathogenesis of CEH warrants further investigation.

4.4 Prolonged pregnancy

This occurs if progestins are administered subcutaneously at the onset of the follicular phase and the bitch or queen is mated. The gestation will be prolonged and a caesarean section may be needed, unless a progesterone receptor antagonist, such as mifepristone or aglépristone, is given (Schaefers-Okkens, 1996).

4.5 Insulin resistance and diabetes mellitus

The progestin-induced GH excess may give rise to glucose intolerance, which may lead to "exhaustion" of the pancreatic β-cells and subsequently diabetes mellitus (Eigenmann et al., 1983). Diabetes mellitus is a common finding in acromegaly in humans, cats and dogs and can be explained by the diabetogenic properties of GH, leading to insulin resistance (Eigenmann and Rijnberk, 1981).

The occurrence of the afore mentioned side-effects is, with the exception of 'prolonged pregnancy', largely dependent upon total progestin exposure. With the advised dosage regimens the exposure may be higher with MPA and megestrol acetate than with proligestone, the latter being a rather weak progestagen (Schaefers-Okkens, 1996).

4.6 Suppression of the hypothalamic-pituitary-adrenal axis

Besides an effect on the progesterone receptor, MPA also has intrinsic glucocorticoid properties due to the relatively high affinity of MPA for the glucocorticoid receptor (Selman et al., 1996). Suppression of the hypothalamic-pituitary-adrenocortical axis by MPA has been reported in both humans (Willemse et al., 1990) and dogs (Selman et al., 1994b; Selman et al., 1996). In man the use of high doses of MPA may even lead to Cushing's syndrome (Simononski et al., 1989).

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