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Growth Hormone Secretagogues and Hypothalamic Networks

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Growth hormone secretagogues (GHSs) act at distinct levels to control growth hormone (GH) secretion. At the pituitary level they reinforce or extend a tonic GH–releasing-hormone (GHRH)-induced activated state by mobilizing intracellular Ca^{2+} store. At the hypothalamic level GHS actions are more complex than originally anticipated. Chronic treatments with GHS result in loss of responsiveness to the secretagogues, an effect probably accounted for by indirect negative feedback of GHS stimulated plasma GH levels over GHRH release. Moreover, intracerebroventricular treatments with GHS can have paradoxical, inhibitory effects on GH secretion. Several mechanisms can account for such dual effects. GHS receptors were found to extend far beyond the arcuate nucleus and are mainly coexpressed by GHRH, somatostatin, and neuropeptide Y (NPY) neurons. Activation of GHRH neurons by GHS can be direct or indirect. Indeed using antisense strategy we found that sst1 are physiological activators of arcuate GHRH neurons and we propose that activation of SRIH arcuate interneurons by GHS can increase GHRH neuron activity. Moreover, GHS can stimulate distinct populations of NPY neurons having opposite effects on GH secretion: arcuate NPY interneurons, act as indirect facilitators of GHRH release, whereas, on the contrary, a different subset of NPY neurons projecting to the periventricular hypothalamus (those also involved in mediating leptin effects on GH) seems able to activate SRIH release.

Key Words: Growth hormone secretagogues; somatostatin; neuropeptide Y; leptin, β-endorphin; arcuate nucleus.

Introduction

The physiology of growth hormone secretagogues (GHSs) is more complex than initially anticipated, when growth hormone (GH)–releasing peptide or nonpeptide analogs were considered as simple activators of growth hormone–releasing hormone (GHRH) neurons *(1,2)* and, in synergy with GHRH, of pituitary somatotropes *(3–5)*. Paradoxical effects of the secretagogues have now been observed, which seem to depend on the species, the duration, and the mode of administration (e.g., GHSs can inhibit GH after intracerebroventricular administration) *(6)*. In addition, GHS targets are more diversified than formerly believed; recent cloning of the receptor *(7)* and subsequent *in situ* hybridization studies have shown that the receptor is expressed in several hypothalamic nuclei, as well as in other brain structures. Cloning of ghrelin *(8)*, an endogenous ligand of GHS receptors (GHS-Rs) produced in the digestive tract and exhibiting neurohormone-like actions, has revealed that the GHS system is likely to have global peripheral and central effects.

Before analyzing major properties of hypothalamic neuronal networks involved in processing GHS information, we consider the pituitary mechanisms underlying GHS stimulation, because the pattern of somatotrope responses to hypothalamic inputs can contribute to the understanding of their regulation.

GHS Actions on the Pituitary

In addition to GHRH and somatostatin, the major regulators of pituitary growth hormone release, GHSs have been shown to stimulate GH release and to markedly potentiate the action of GHRH *(3,9)*. Furthermore, GHRH is not able to release GH during troughs of episodic GH secretion, suggesting that its action can be completely antagonized by somatostatin, whereas GHSs can still elicit limited peaks of GH secretion under these conditions (Tannenbaum, personal communication). In parallel, reversal by somatostatin of GHRH-induced cyclic adenosine monophosphate (cAMP) accumulation in primary culture of pituitary cells is blunted by simultaneous addition of GHS.

The presence of GHS-R mRNA has been described in the pituitary of many species on the basis of reverse transcriptase polymerase chain reaction (RT-PCR) or *in situ* hybridization studies *(10,11)*. Their concentration seems to be species dependent, and to increase progressively during

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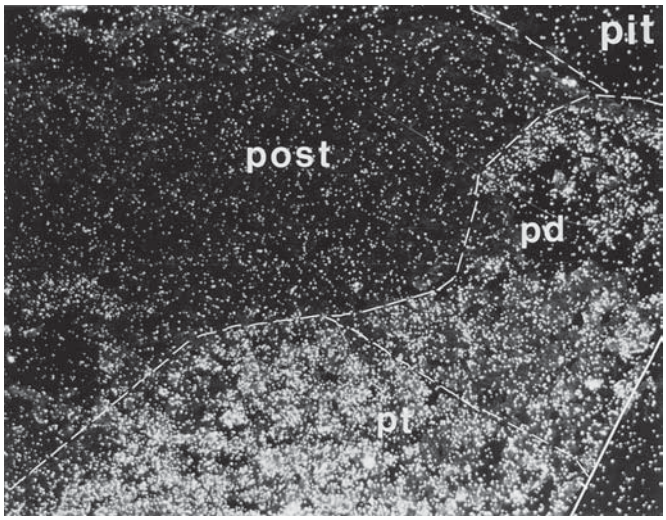

Fig. 1. Distribution of GHS-R mRNA in the Lemurian pituitary. Note the abundant expression and the important population of GHS-R-expressing cells. pd, pars distalis; pit, pituitary; post, posterior pituitary; pt, pars tuberalis.

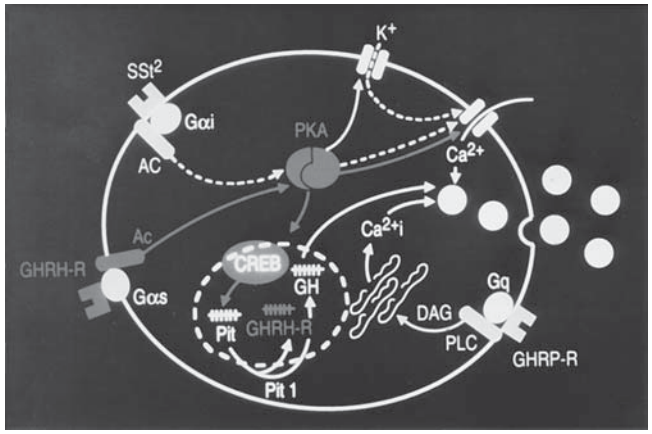

Fig. 2. Schematic representation of a pituitary somatotrope showing the mode of action and the partial antagonism of GHRH, GHSs, and somatostatin receptors. CREB, cAMP responsive transcription factor; DAG, diacylglycerol; Gai, ao, aq..., subsets of heterotrimeric G proteins; PLC, phospholipase C. Solid and broken lines refer to stimulatory and inhibitory intracellular pathways, respectively.

development until puberty *(12)*. The Lemurian pituitary, e.g., shows intense labeling extending to the anterior and intermediate lobes (Fig. 1). In the rat, all cells responding to GHSs have been identified as somatotropes *(13)*. In humans, however, GHS-R mRNA has also been described in lactotropes and corticotropes, but only under pathologic conditions *(14,15)*.

GHRH and somatostatin act by stimulating and inhibiting cAMP accumulation, respectively. Increased cAMP results in activation of voltage-dependent Ca^{2+} channels, a process that both triggers exocytosis of prestored GH containing granules and activates CREB, a cAMP-responsive transcription factor that induces expression of the GH gene (Fig. 2). The effects of GHRH stimulation and somatostatin inhibition are not entirely symmetrical. Somatostatin blocks GHRH-induced cAMP accumulation and GH exocytosis but is unable to reverse GHRH-induced CREB activation and GH gene expression. (If somatostatin did antagonize GHRH-induced GH gene expression, intermittent inhibition of GH biosynthesis by recurrent somatostatin release would probably not allow the pituitary to accumulate enough GH to release the large amounts of hormone—at least 20% of the gland's content—released within each secretory episode.)

Mechanisms Underlying GHS Potentiation of Pituitary GH Responses to GHRH

The addition of GHRH to somatotropes in culture results in long-lasting activation of the cells, as shown by the persistence of trains of spikes and Ca^{2+} oscillations for relatively long periods after the stimulus has been removed. Those persisting oscillations are interrupted by the addition of somatostatin but resume spontaneously as soon as somatostatin is washed out from the medium *(16)*.

By contrast, GHSs do not affect directly trigger cAMP but activate a phospholipase C coupled to the GHS-R by an αq G-protein isotype. Their ionic mode of action on somatotropes exhibits a few differences with respect to that of GHRH: GHSs depolarize the membrane by a dose-dependent effect on Na^+ influx *(17)* and evoke Ca^{2+} oscillations and internal $[Ca^{2+}]_i$ mobilization (Fig. 3A). The latter effect is obtained only at higher doses of GHS and is sensitive to depletion of intracelluar Ca^{2+} stores by thapsigargin, whereas the former is mainly observed at low doses of GHSs and is sensitive to protein kinase C (PKC) inhibitors and PKC depletion *(18)*. Therefore, GHRH and GHSs can both activate voltage-dependent L-type Ca^{2+} channels and evoke rhythmic Ca^{2+} entry, but only GHSs are able to induce internal $[Ca^{2+}]_i$ mobilization.

The addition of GHSs to somatotropes in culture further potentiates action potentials previously stimulated by GHRH and increase both their frequency and amplitude (Fig. 3B).

The following conclusions can be inferred from these data. First, somatostatin is the major pacemaker of GH episodic release, because it is required to silence somatotropes activated by intermittent, recurrent GHRH supply. Regular supply of GHRH can thus be considered a permissive condition, mostly concerned with maintaining somatotropes in an activated state in the absence of inhibitory somatostatin signals. The level of this activated state depends on the intensity and, possibly, the frequency of GHRH fluxes to the pituitary. Second, potentiation of GHRH by GHSs results from the additivity of their effects on Ca^{2+} activation, and from additional mobilization of $[Ca^{2+}]_i$ by GHSs. Furthermore, the Na^+-dependent depolarizing effect of GHSs further sensitizes the cell toward additional depolarizing stimuli. By this mechanism, GHSs

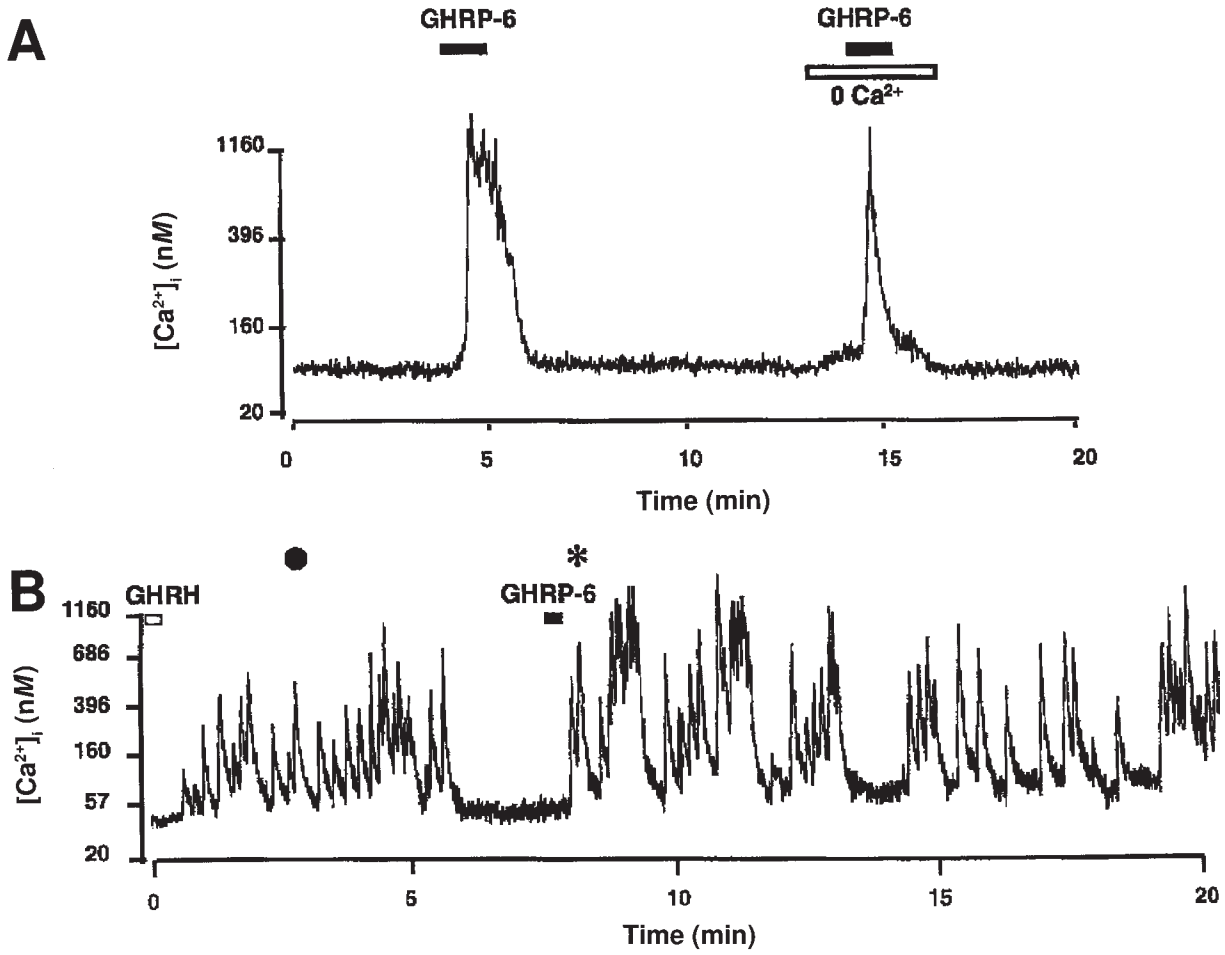

Fig. 3. (A) Application of GHRP-6 (100 n*M*, 1 min) evokes a biphasic response in somatotropes, with an initial peak of $[Ca^{2+}]_i$ followed by a plateau of oscillations. When applied in a calcium-free medium, only the peak response is preserved, suggesting that it results from release of internal Ca^{2+} stores. **(B)** Application of GHRH (30 n*M*, 40 s) triggers transient $[Ca^{2+}]_i$ oscillations (●). Subsequent application of GHRP-6 on the same cell evokes persistent bursts of $[Ca^{2+}]_i$ oscillations (*).

can contribute to the enhancement of the GHRH-activated state of somatotropes. Third, incomplete reversal by somatostatin of GHS potentialization of GHRH-induced GH secretion probably results because only Ca^{2+} channel activation, but not $[Ca^{2+}]_i$ mobilization, can be reversed by somatostatin. In addition, potentiation of cAMP accumulation after combined GHRH/GHS treatments is likely to result, as in other cell models *(19)*, from indirect adenylate cyclase stimulation by PKC or Ca^{2+}/calmodulin kinase, a process not mediated (as for somatostatin) by Gαi proteins.

GHS Actions on Hypothalamic Networks

As already reviewed, the synergy between GHSs and GHRH—still controversial on GH release by somatotropes but well documented on activation of $[Ca^{2+}]_i$ oscillations in somatotropes—can be partly explained by GHS potentiation at the pituitary level. Stimulation of GHRH neurons by GHSs, a mechanism that was already postulated on the basis of early studies on GHS actions, accounts for the remaining part of that synergy.

The situation, however, is not as simple as suggested by the initial model of GHS action, which postulated that only GHRH neurons were targets of GHSs. After the GHS-R was cloned *(7)* and probes for *in situ* hybridization or PCR amplification synthesized, the distribution of GHS-R was found to extend far beyond the arcuate nucleus. In that structure, GHS-Rs are mainly coexpressed by neuropeptide Y (NPY) neurons *(20)*, but also by GHRH and somatostatin neurons *(21)*, as well as by a few β-endorphin neurons *(22)*. In addition, GHS-Rs are abundant in the ventromedial nucleus, a structure involved in the regulation of food intake behavior, and in several other brain structures *(23)*.

Analysis of the distribution of GHS-Rs, however, also reveals important species differences. For instance, GHS-Rs in the ventromedial nucleus are less abundant in primates than in rodents, and GHS-Rs are expressed in hypothalamic structures as supraoptic nuclei in mice but not in rats or other studied species.

The intricate diversity of arcuate neurons, however, seems to correspond to very strict articulation rules among them, which provide a limited leeway only for neuronal

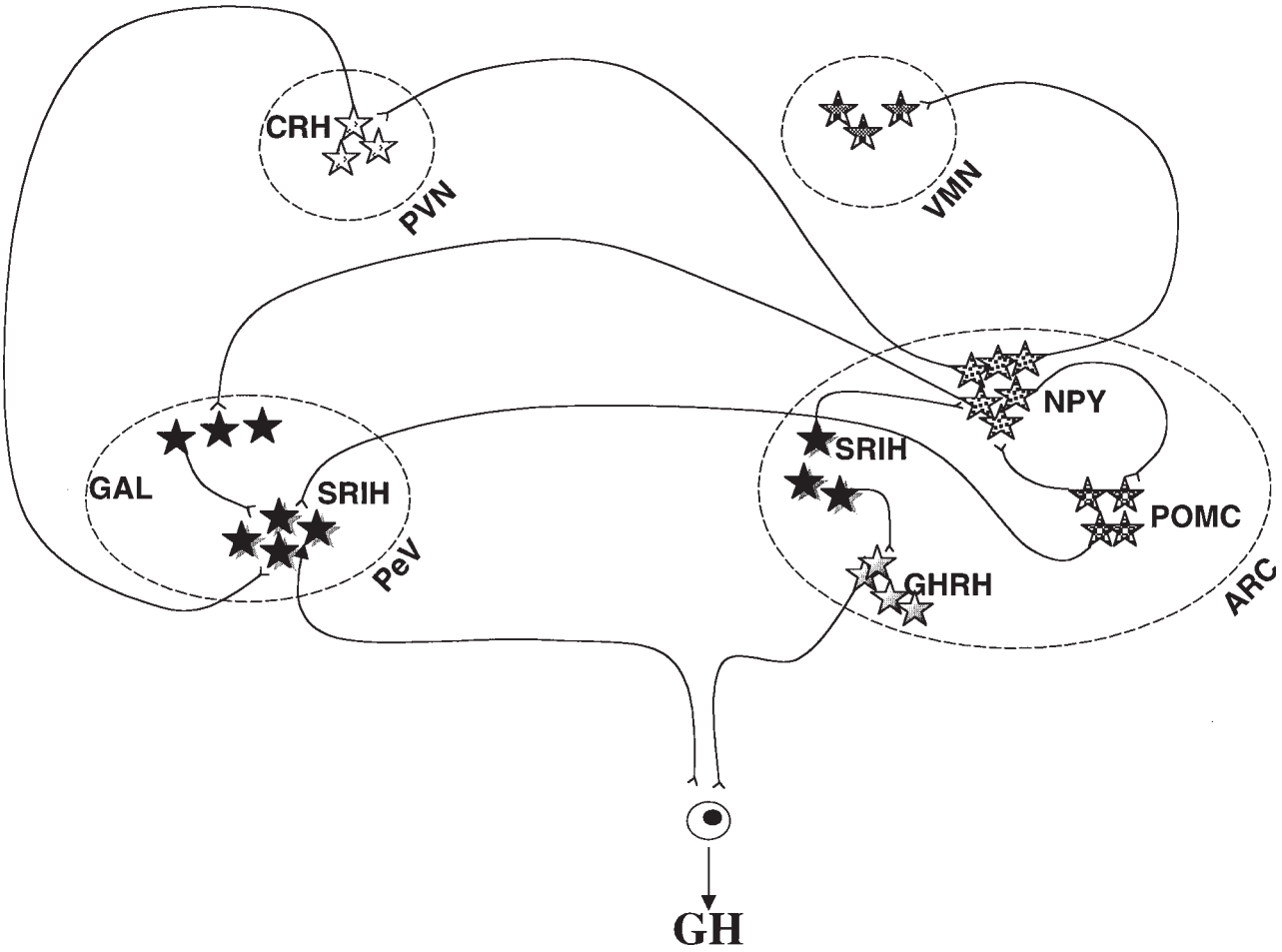

Fig. 4. Schematic representation of neuronal networks involved in GH regulation. ARC, arcuate nucleus; PeV, periventricular area; PVN, paraventricular nucleus; VMN, ventromedial nucleus; CRH, corticotropin-releasing hormone; GAL, galanin; NPY, neuropeptide Y; POMC, proopiomelanocortin; SRIH, somatostatin.

interactions. Most GHRH neurons project to the median eminence but do not seem to provide a significant innervation to other arcuate neurons *(24)*. In addition to GHS-Rs *(21)*, they express sst1 and sst2 somatostatin receptors (each subtype being present in about 15% of GHRH cells) *(25)* but are not directly innervated by either NPY neurons or from the major population of somatostatin neurons present in the periventricular hypothalamus (their major somatostatin input is owing to local, intraarcuate somatostatin interneurons).

Neuroanatomy of Arcuate Connections

Within the arcuate nucleus, NPY neurons themselves seem to project only to other NPY neurons (homologous NPY/NPY interactions) and to proopiomelanocortin neurons; these two targets have been shown to express distinct NPY receptor subtypes (Y1 for the homologuous connections and Y2 for the other) *(26)*. In addition, they project to the ventromedial nucleus and to the periventricular area, where they seem able to affect hypophysiotropic somatostatin neurons (either directly or via galanin neurons). Two distinct populations of NPY neurons are involved, the first in relaying leptin inputs to the hypothalamus (leptin strongly inhibits NPY synthesis in these neurons, a prerequisite for both anorexigenic and GH stimulatory effects of leptin, *[27]*), and the second in relaying GHS inputs *(20)*. Expression of leptin and GHS-Rs on arcuate NPY neurons shows very little overlap *(28)*. In parallel, NPY neurons expressing GHS and leptin receptors exhibit distinct projection territories; many arcuate leptin-containing neurons project to the paraventricular nucleus, whereas GHS-containing neurons do not *(29)*. Figure 4 presents a schematic representation of the area covered by GHS-Rs and of projections of corresponding neurons.

Physiologic responses to GHSs do not fit a simple model of univocal GHRH neuron stimulation. At the level of the central nervous system, GHSs are also able to trigger feeding behavior and to affect slow-wave sleep *(30,31)*. For instance, GHSs are no longer able to stimulate GH release after sustained intracerebroventricular infusion *(32)*. Under conditions of infusion of low GHRP-6 concentrations (10 n*M*), however, food intake behavior and body growth

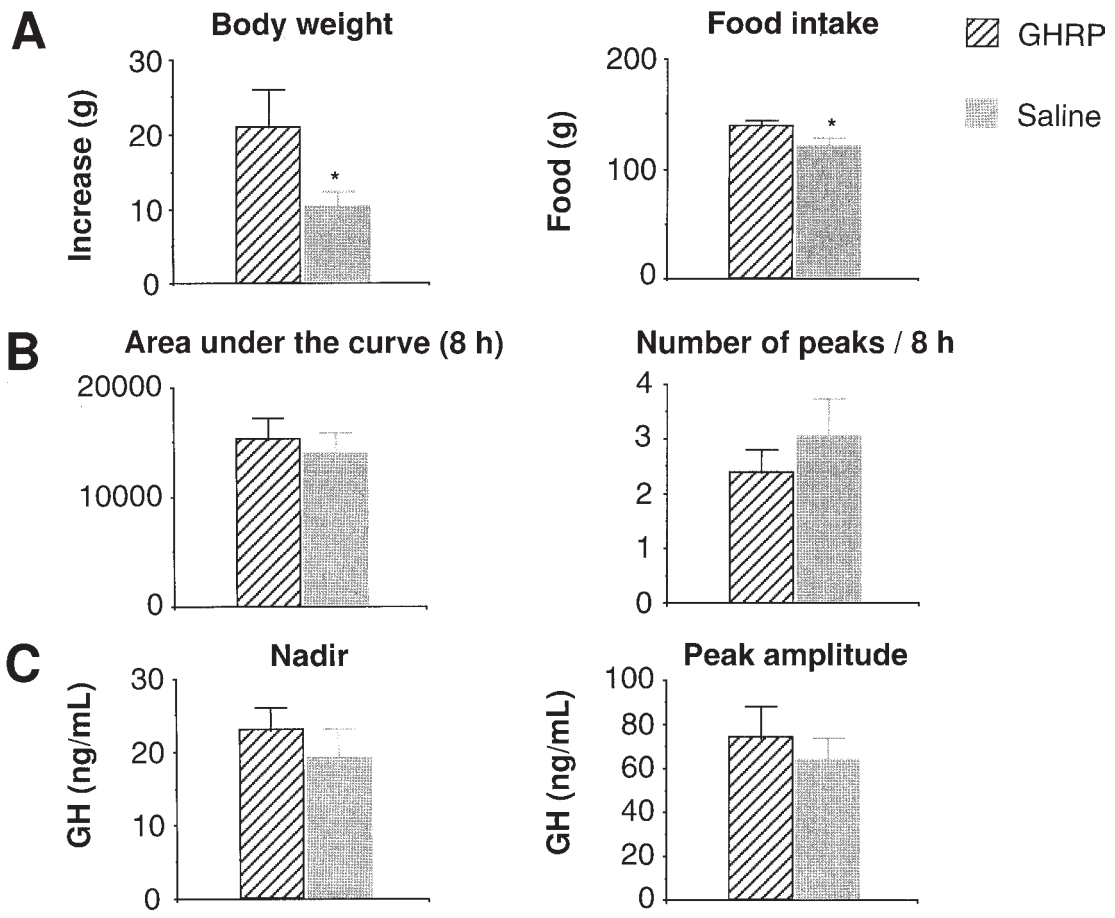

Fig. 5. Effect of a 6-d infusion of low concentrations of GHRP-6 (10 n*M*) on body growth, food intake **(A)** and GH secretion, **(B,C)** at the end of the treatment.

are still stimulated by GHS after 6 d, a time when GH is no longer stimulated by the secretagogue (Fig. 5). Persistence of growth stimulation thus seems independent of GH stimulation, an observation suggesting that progressive loss of GH stimulation under sustained administration of GHS does not only result from receptor desensitization. Other observations showing that intracerebroventricular administration of GHSs can result in a paradoxical inhibition of GH secretion *(6)* are not compatible with the simple model of GHRH stimulation by GHSs, and suggest that alternate mechanisms are involved, or at least that hypothalamic GH regulatory mechanisms can adapt to sustained GHS infusion and compensate the resulting GH overstimulation.

Animal Models Exhibiting Decreased GHS-R Activity

A comparable conclusion can be drawn from transgenic mice with an invalidated sst2 receptor, a model that exhibits reduced GH feedback on hypothalamic neurons *(33)* as well as decreased hypothalamic GHS-R mRNA concentrations and blunted GH responses to GHSs (Fig. 6A,B). Nevertheless, the overall secretion of GH is not significantly impaired in these animals, in spite of attenuated GHS inputs to the hypothalamus.

Taken together, these experiments confirm that normal GH pulsatility can be achieved in the absence of significant GHS inputs. Although compensating mechanisms that allow the GH oscillator to operate under these conditions are not yet fully understood, they are likely to involve complex feedback effects of GHSs on GHRH and somatostatin neurons.

Hypotheses to Account for the Paradoxical, Negative GH Responses to GHS

As expected from early experiments on GHS actions, the acute effects of GHSs first result in stimulation of GHRH neurons. By contrast, the negative actions of GHSs on the hypothalamic networks controlling GH release, such as those involved in GH inhibition after chronic treatment with GHS *(34)* or after their intracerebroventricular administration *(6)*, are likely to involve another subset of NPY neurons, more directly concerned with the pituitary actions of leptin. These interactions are represented in Fig. 7; they involve, in particular, NPY neurons known to stimulate somatostatin release from periventricular somatostatin neu-

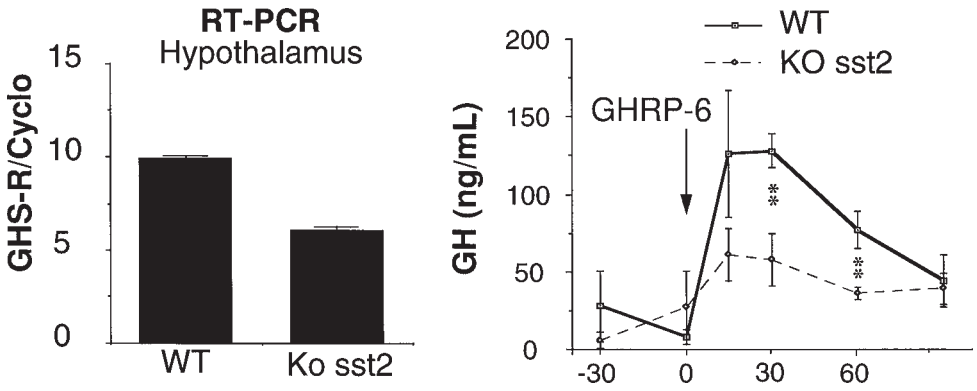

Fig. 6. GHS-R expression and responsiveness to GHSs in mice with an invalidated sst2 receptor gene. **(A)** Semiquantitative assessment with respect to cycloheximide (cyclo) of GHS-R mRNA by RT-PCR in +/+ and –/– mice; **(B)** concomitant responses to a standard dose of GHRP-6 (40 μg).

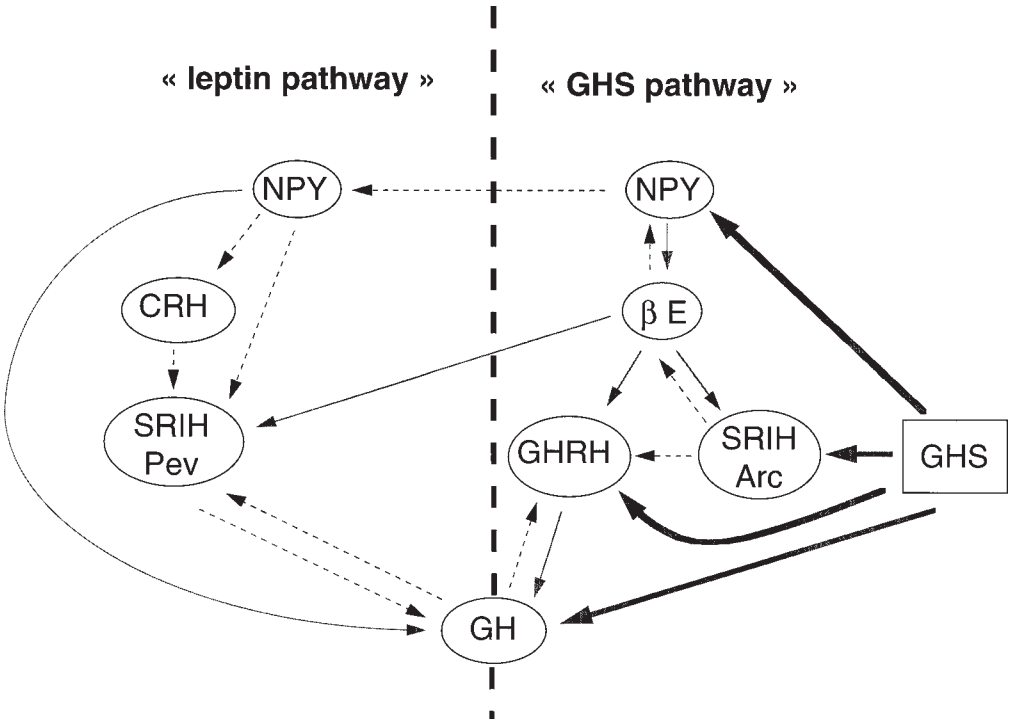

Fig. 7. Schematic representation of the dimorphic arcuate NPY neuronal population that may account for "paradoxical" GH responses to GHSs. Stimulatory effects of GHSs are mediated by both direct (through GHS-R expressed in somatotropes and in a subpopulation of the GHRH neurons themselves) and indirect (through NPY neurons influencing indirectly GHRH neurons via β-endorphin neurons) actions. This pathway, shown on the right, is labeled GHS pathway. A few arcuate somatostatin interneurons may also be involved. By contrast, negative effects of GHSs on GH are represented on the left, labeled leptin pathway. They involve NPY neurons more directly concerned with leptin regulatory pathways, which project to periventricular (PeV) somatostatin neurons and are able to trigger somatostatin release into the hypothalamo-hypophyseal portal system. Solid and broken lines represent putative stimulatory and inhibitory pathways, respectively.

rons, an interaction also observed under in vitro conditions *(35)*. Activation by GHSs of this subset of NPY neurons is probably more indirect than that of NPY neurons of the leptin pathway; they may result from homologous NPY/NPY connections within the arcuate nucleus demonstrated by neuroanatomic studies *(26)*.

One should also consider that both GHSs and leptin have been shown to exhibit dual effects on the electrical arcuate neurons *(36)*, an observation that may also account for some of their paradoxical effects on GH secretion. In addition, two other mechanisms can account for secondary, negative paradoxical effects of GHSs on GH secretion: GH feedback on hypothalamic neurons themselves and intra-arcuate somatostatin-GHRH connections.

GH itself has been shown to feed back on key regulatory neurons of GH-controlling hypothalamic networks *(37)*.

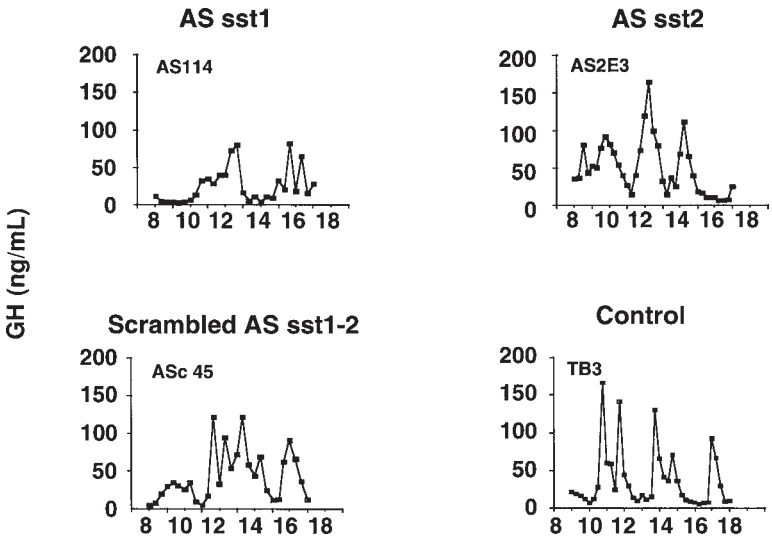

Fig. 8. Effect of intracerebroventricular infusion of oligonucleotides on GH pulsatility. Note that GH pulses are only impaired after sst1 antisense infusion. AS sst1/2, antisense directed against the receptor's messenger; scrambled AS, mixed scrambled oligonucleotides against both receptors.

GH levels are able to both turn off GHRH neurons and turn on periventricular and arcuate somatostatin neurons *(33)*, the latter process itself able to inhibit GHS activation of its target in the arcuate nucleus *(29,36,38)*. GH hyperstimulation resulting from chronic administration of GHSs can also be expected to shift the balance between GH stimulatory and inhibitory inputs in favor of the latter.

Additional Neuronal Involvement Downstream of GHS-Rs: Role of Somatostatin-GHRH Interactions

Arcuate somatostatin interneurons, also known to provide important inputs to GHRH neurons (which coexpress sst1 and sst2 somatostatin receptors) *(25)*, could also be involved in paradoxical responses to GHS. Experiments based on intracerebroventricular infusion of selective receptor antisense oligonucleotides suggest that sst1 inputs to GHRH neurons are necessary for normal activation of GHRH *(39)*. Infusion of an antisense sequence directed against sst1 mRNA strongly decreases GH pulse amplitude (Fig. 8), whereas sense oligonucleotides, or anti-sst2 antisense oligonucleotides are ineffective (Fig. 8). A likely consequence of this is that sst1-mediated, intraarcuate somatostatin inputs are necessary for appropriate operation of GHRH neurons. Under these conditions, disruption of sst1-mediated inputs is likely to impair recurrent firing of GHRH neurons and, subsequently, result in lowered maintenance of the somatotropic tone.

Although indirectly documented by several experimental data, the aforementioned explanations are still hypothetical. Their validation should now be made easier by the identification of endogenous ligands to the GHS-R, which will address more directly the problem of the physiologic relevancy of endogenous stores of the secretagogue.

Conclusion

GHSs seem to act as modulators of GH control mechanisms rather than as straightforward secretagogues. Neuronal networks on which their modulatory action is exerted include in-built, self-control systems, compensating potential overstimulation (resulting, e.g., from chronic GHS supply) by attenuating other positive inputs to GH release, or increasing the relative importance of negative inputs.

GHS modulations can be observed at various levels: on somatotrope cells, on which they reinforce or extend a tonic GHRH-induced activated state; and at the level of GH feedback, during which GHS receptors desensitize, at least in the pituitary, in order to limit the risk of overstimulation. Together with other mechanisms, in particular changes in the basal somatostatin activity over time, they play a role in age-dependent decreased GH activity.

Interestingly, some effects of GHSs overlap with those of leptin; variations in GH secretion levels are able to interfere with serum leptin levels *(40)* and leptin receptor gene expression *(41)*. The role of GHSs, as that of leptin, is probably more global than the one that can be inferred from GH and food intake behavior modulation. Both hormones are involved in overall coordination of complex functions, of which GH regulation represents only one aspect. Leptin primarily specializes in adapting growth, as well as growth and reproductive hormones, to the availability of food. This role even predominates in nonmammalian vertebrates, in which growth can be suspended or markedly delayed during food shortage periods, an observation suggesting that leptin is more likely to have been selected as an adaptive mechanism against food shortages rather than as a hormone of satiety (an evolutionary link resulting in a leptin-dependent thrifty behavior *(42)*. Leptin is also important for

adapting metabolic functions to food availability, through metabolic rather than growth promoting actions of GH, and has been assumed to play a specific role during pregnancy, for maternal nutrient partitioning and optimization of fetal growth *(43)*.

By contrast, temporal variations in the intensity of the GH response to GHSs suggest that activation of endogenous GHSs may have particular importance for a fine modulation of GH requirements at the time of puberty *(44)*. In addition, the secretagogues are probably of physiologic relevance throughout the growth period of young animals, a role that should soon be confirmed when tools become available to measure variations in endogenous GHS ligands.

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