Specific receptors for synthetic GH secretagogues in the human brain and pituitary gland

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Abstract

In vitro studies have been performed to demonstrate and characterize specific binding sites for synthetic GH secretagogues (sGHS) on membranes from pituitary gland and different human brain regions. A binding assay for sGHS was established using a peptidyl sGHS (Tyr-Ala-hexarelin) which had been radioiodinated to high specific activity at the Tyr residue. Specific binding sites for ¹²⁵I-labelled Tyr-Ala-hexarelin were detected mainly in membranes isolated from pituitary gland and hypothalamus, but they were also present in other brain areas such as choroid plexus, cerebral cortex, hippocampus and medulla oblongata with no sex-related differences. In contrast, negligible binding was found in the thalamus, striatum, substantia nigra, cerebellum and corpus callosum. The binding of ¹²⁵I-labelled Tyr-Ala-hexarelin to membrane-binding sites is a saturable and reversible process, depending on incubation time and pH of the buffer. Scatchard analysis of the binding revealed a finite number of binding sites in the hypothalamus and pituitary gland with a dissociation constant (K_d) of $(1.5 \pm 0.3) \times 10^{-9}$ and $(2.1 \pm 0.4) \times 10^{-9}$ mol/l respectively. Receptor activity is sensitive to trypsin and phospholipase C digestion, suggesting that

protein and phospholipids are essential for the binding of ¹²⁵I-labelled Tyr-Ala-hexarelin. The binding of ¹²⁵I-labelled Tyr-Ala-hexarelin to pituitary and hypothalamic membranes was displaced in a dose-dependent manner by different unlabelled synthetic peptidyl (Tyr-Ala-hexarelin, GHRP2, hexarelin, GHRP6) and non-peptidyl (MK 0677) sGHS. An inhibition of the specific binding was also observed when binding was performed in the presence of [D-Arg¹-D-Phe⁵-D-Trp^{7,9}-Leu¹¹]-substance P, a substance P antagonist that has been found to inhibit GH release in response to sGHS. In contrast, no competition was observed in the presence of other neuropeptides (GHRH, somatostatin, galanin or Met-enkephalin) which have a known influence on GH release.

In conclusion, the present data demonstrate that sGHS have specific receptors in human brain and pituitary gland and reinforce the hypothesis that these compounds could be the synthetic counterpart of an endogenous GH secretagogue involved in the neuroendocrine control of GH secretion and possibly in other central activities.

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Introduction

Growth hormone (GH) secretion is regulated by two neuropeptides: GH-releasing hormone (GHRH), which exerts stimulatory effects on GH secretion, and somatostatin, which exhibits an inhibitory influence (see Müller & Nisticò 1989 for review). GH release can also be stimulated by a group of synthetic oligopeptides termed growth hormone-releasing peptides (e.g. GHRP1, GHRP2, GHRP6 and its 2-methyl-D-Trp derivative hexarelin) which act through a mechanism different from that of GHRH (Bowers *et al.* 1991, Akman *et al.* 1993, see also Korbonits & Grossman 1995, Ghigo *et al.* 1997 for reviews). These synthetic GH secretagogues (sGHS),

although modelled from Met-enkephalins, have lost the opioid activity and stimulate GH secretion of pituitary somatotroph cells both *in vitro* and *in vivo* in a variety of species, including rat, pig and human (Momany *et al.* 1981, Bowers *et al.* 1984, Bowers 1993, Deghenghi *et al.* 1994, Ghigo *et al.* 1994, Arvat *et al.* 1995). Since all these peptides have an oral bioavailability lower than 1%, a number of non-peptidyl sGHS (L-692,429, L-692,585 L-700,653 and MK 0677), with structures more amenable to chemical modification and optimization of oral bioavailability, have been developed and studied in both animals and man (Cheng *et al.* 1993, Gertz *et al.* 1993, Smith *et al.* 1993, Jacks *et al.* 1994, Chang *et al.* 1995). Among them, the spiroindoline derivative, MK 0677, proved to be the

strongest stimulator of GH secretion (Patchett *et al.* 1995). It allowed the identification and cloning of a specific G-protein-coupled receptor that mediates the activity of peptidyl and non-peptidyl sGHS (Howard *et al.* 1996). Such a receptor does not show significant homology with any other receptors known so far and it has been found expressed in the pituitary and hypothalamus of different mammalian species (Howard *et al.* 1996, Pomès *et al.* 1996, Pong *et al.* 1996). These data supported the hypothesis that an endogenous sGHS-like ligand exists and is involved in the control of GH secretion (Bowers *et al.* 1991). In fact, the presence of GHS receptors (sGHS-R) in the pituitary gland and hypothalamus agrees with data for animals and humans showing that the GH-releasing activity of sGHS is the result of direct stimulation of somatotroph cells (Smith *et al.* 1993, Akman *et al.* 1993, Wu *et al.* 1994) and of a more important action at the hypothalamic level (Bowers *et al.* 1991, Fletcher *et al.* 1994, Fairhall *et al.* 1995, Hickey *et al.* 1996). The mechanisms of action underlying the GH-releasing activity of peptidyl and non-peptidyl sGHS seem to involve the antagonism of somatostatinergic pathways at both the pituitary and hypothalamic level as well as the stimulation of GHRH-secreting neurones (Conley *et al.* 1995, Hickey *et al.* 1996). Interestingly, the activity of sGHS is not fully specific since a slight stimulatory effect on prolactin, adrenocorticotrophin and cortisol levels as well as influences on the control of sleep and food intake have also been demonstrated (Jacks *et al.* 1994, Locke *et al.* 1995, Frieboes *et al.* 1995, Arvat *et al.* 1997). These actions could take place via activation of specific receptors at levels other than the hypothalamo-pituitary system. In agreement with this hypothesis, peptidyl sGHS-binding sites have already been detected in the forebrain of rat and pig (Codd *et al.* 1988, Veeraragavan *et al.* 1992).

Based on the foregoing, the aim of the present research was to study the distribution and biochemical characterization of sGHS-R in the human pituitary gland and brain regions.

Materials and Methods

Chemicals

Hexarelin (His-D-2Me-Trp-Ala-Trp-D-Phe-Lys-NH₂) was kindly supplied by Pharmacia & Upjohn (Stockholm, Sweden). GHRP6 (His-D-Trp-Ala-Trp-D-Phe-Lys-NH₂), Tyr-Ala-hexarelin, GHRP2 (D-Ala-D-βNal-Ala-Trp-D-Phe-Lys-NH₂) and MK 0677 (N-[1(R){[1,2-dihydro-1-methanesulphonylspro-3H-indole-3,4'-piperidin]-1'-yl]-2-(phenylmethoxy)-ethyl]-2-amino-2-methylpropanamide methanesulphonate) were supplied by Europeptides (Argenteuil, France). Met-enkephalin (endorphin 1-5), human GHRH (GHRH 1-44), human galanin, somatostatin (somatostatin 1-14) and [D-Arg¹-D-Phe⁵-D-Trp^{7,9}-Leu¹¹]-substance P, were purchased from Bachem, Feinchemikalien AG Bubendorf, Switzerland. Tyr-Ala-

hexarelin was radiolabelled with ¹²⁵I-labelled sodium using an Enzymobead iodination kit (Bio-Rad, Mississauga, Ontario, Canada). ¹²⁵I-Labelled Tyr-Ala-hexarelin was purified by reversed-phase HPLC on a Vydac C₁₈ column utilizing a gradient elution with 0.1% trifluoroacetic acid and acetonitrile-containing eluents. The addition of Tyr-Ala to the hexarelin molecule made it possible to obtain a highly specific radioligand with the same biological activity as hexarelin. This has been recently demonstrated by our group in neonatal rats, where Tyr-Ala-hexarelin stimulates GH secretion 6.8-fold over the control, reaching a level comparable with that produced by hexarelin (Ong *et al.* 1998). The average specific activity of ¹²⁵I-labelled Tyr-Ala-hexarelin was 2200 Ci/mmol (*n*=3). Phospholipase C, trypsin and other chemicals of analytical grade were purchased from Sigma Chemical Company, St Louis, MO, USA.

Human tissues

Pituitary gland and brain tissues were obtained at autopsy from 18 subjects (nine males and nine females, ranging in age from 42 to 79 years, median age 61 years) who had died from various causes (eight from cardiovascular diseases, four from trauma and six from neoplasms) and were submitted to autopsy for diagnostic purposes between January and November 1996. Tissue removal had been given ethical approval by our hospital committee. Cases were selected from a larger series based on the availability of fresh brain tissue and absence of extensive cerebral lesions. All subjects had no history of neurological or endocrinological disease nor was any primary nervous system or endocrine disease found at autopsy. Post-mortem examination was performed 23–48 h after death (mean 28 h) but a 1 day interval was most usual. Small samples of hypothalamus, thalamus, hippocampus, striatum, substantia nigra, frontal cerebral cortex, medulla oblongata, cerebellum and corpus callosum were dissected out on to ice according to the atlas of Nieuwenhuys *et al.* (1981). Choroid plexuses were removed from lateral and third ventricles after sectioning the corpus callosum. A small fragment of each tissue was excised for histopathological analysis, fixed in formalin and processed routinely. The pituitary gland and the different brain regions were immediately frozen at –35 °C and kept frozen for 2 weeks to 2 months until processed for membrane preparation and binding studies.

Membrane preparation

Frozen pituitary gland and different brain regions were thawed and homogenized with an UltraTurrax T-25 (Janke and Kunkel, Staufen, Germany) for 1 min at 4 °C in 0.32 mol/l sucrose solution. After centrifugation at 500 g for 10 min the pellet was discarded and the supernatant centrifuged at 30 000 g for 30 min. The resulting pellet was resuspended in ice-cold buffer (50 mmol/l Tris-HCl,

Table 1 Specific binding of ^{125}I -labelled Tyr-Ala-hexarelin to membranes from pituitary gland and different human brain regions. Values are means $\pm$ S.E.M. of nine subjects

Tissue	Specific binding of ^{125}I -labelled Tyr-Ala-hexarelin (% of radioactivity added/0.1 mg protein)	
	Male subjects	Female subjects
Pituitary gland	12.5 $\pm$ 0.4 ^{a,b}	12.8 $\pm$ 0.4 ^{a,b}
Hypothalamus	13.4 $\pm$ 0.5 ^{a,b}	13.1 $\pm$ 0.6 ^{a,b}
Choroid plexuses	7.4 $\pm$ 0.2 ^a	7.2 $\pm$ 0.2 ^a
Cerebral cortex	5.6 $\pm$ 0.2 ^a	6.2 $\pm$ 0.3 ^a
Hippocampus	5.5 $\pm$ 0.4 ^a	5.8 $\pm$ 0.3 ^a
Medulla oblongata	4.7 $\pm$ 0.4 ^a	4.4 $\pm$ 0.6 ^a
Thalamus	0.9 $\pm$ 0.4	1.1 $\pm$ 0.3
Striatum	1.0 $\pm$ 0.2	0.9 $\pm$ 0.3
Substantia nigra	0.8 $\pm$ 0.3	0.8 $\pm$ 0.2
Cerebellum	1.0 $\pm$ 0.4	0.7 $\pm$ 0.3
Corpus callosum	0.7 $\pm$ 0.4	1.0 $\pm$ 0.3

^a $P < 0.001$ vs all other brain regions; ^b $P < 0.01$ vs choroid plexuses, cerebral cortex, hippocampus and medulla oblongata.

2 mmol/l EGTA, pH 7.3) and immediately used to determine protein content (Lowry *et al.* 1951) and for binding studies.

Binding studies

^{125}I -Labelled Tyr-Ala-hexarelin-binding studies were performed as previously described by Codd *et al.* (1989). For single point binding assay, tissue membranes (corresponding to 100 μg protein) were incubated in triplicate at 0 °C for 60 min with approximately 0.5×10^{-9} mol/l ^{125}I -labelled Tyr-Ala-hexarelin in a final volume of 0.5 ml assay buffer (50 mmol/l Tris, 2 mmol/l EGTA, 0.1% BSA, 0.03% bacitracin, titrated with HCl to pH 7.3). Parallel incubations, where 2.5×10^{-6} mol/l unlabelled Tyr-Ala-hexarelin was also present, were used to determine non-specific binding which was subtracted from total binding to yield specific binding values.

To establish binding site specificity, different sGHS (GHRP2, GHRP6, hexarelin, MK 0677) and various neuropeptides with a known effect on GH release such as GHRH, somatostatin, galanin, Met-enkephalin and [D-Arg¹-D-Phe⁵-D-Trp^{7,9}-Leu¹¹]-substance P were also used in displacement assays with ^{125}I -labelled Tyr-Ala-hexarelin. The binding reaction was terminated by adding 4 ml ice-cold assay buffer followed by rapid filtration over Whatman GF/B filters presoaked with 0.5% polyethyleneimine to prevent excessive binding of radioligand to the filters. Filters were rinsed 3 times with assay buffer and the radioactivity bound to membranes was measured by a Packard auto-gamma counter. A filter blank (binding of radioligand to glass fibre filters during the filtration procedure in the absence of membranes) was calculated for all incubations (between 3.5 and 4.5% of radioactivity added) and subtracted from the binding value. Only 1% of

radioligand bound to blank filters was displaced by unlabelled Tyr-Ala-hexarelin. Thus tissues were considered to be sGHS-R-positive when a specific binding of 1% or more was observed. Specific binding was calculated as the difference between binding in the absence and presence of excess unlabelled Tyr-Ala-hexarelin and was expressed as a percentage of the total radioactivity added.

In some assays, receptor-binding saturation studies were conducted by incubating tissue membranes with increasing concentrations ($(0.15\text{--}10) \times 10^{-9}$ mol/l) of radioligand in the absence and presence of a fixed amount (2.5×10^{-6} mol/l) of unlabelled Tyr-Ala-hexarelin. Saturation isotherms were transformed using the method of Scatchard (1949) and the dissociation constant (K_d) and number of binding sites (B_{max}) were calculated with the GraphPAD Inplot program (GraphPAD Software, San Diego, CA, USA).

Statistical analysis

Data are expressed as means $\pm$ S.E.M. unless otherwise noted. Significant differences between groups were assessed by one-way ANOVA followed, when appropriate, by Duncan's multiple range test (Steel & Torrie 1960).

Results

Binding of ^{125}I -labelled Tyr-Ala-hexarelin to membranes from human brain and pituitary gland

The results of a survey of specific binding of ^{125}I -labelled Tyr-Ala-hexarelin to membranes from pituitary gland and different brain regions of adult male and female subjects

are summarized in Table 1. Binding varied greatly with the tissue examined. The highest ^{125}I -labelled Tyr-Ala-hexarelin binding activity was observed in the hypothalamus and pituitary gland of both sexes. Tyr-Ala-hexarelin binding was recorded in all specimens of these tissues, with binding values ranging from 11.2 to 17.4% for hypothalamus (11.2–15.0% for males and 11.2–17.4% for females) and from 11.0 to 14.9% for pituitary gland (11.0–14.9% for males and 11.3–14.0% for females). The specific binding in the hypothalamus or pituitary gland represented 52–67% of total radioactivity bound. In addition, specific binding was also clearly present in the choroid plexuses, cerebral cortex, hippocampus and medulla oblongata, with values that were about one half of those found in the hypothalamus and pituitary gland. Other brain regions tested, such as thalamus, striatum, substantia nigra, cerebellum and corpus callosum, did not exhibit significant binding of ^{125}I -labelled Tyr-Ala-hexarelin. Differences in the specific binding values were statistically significant for the hypothalamus, pituitary gland, choroid plexuses, cerebral cortex, hippocampus and medulla oblongata vs the other brain regions ($P < 0.001$ in both sexes) and for hypothalamus and pituitary gland vs choroid plexuses, cerebral cortex, hippocampus and medulla oblongata ($P < 0.01$ in both sexes). Specific binding of ^{125}I -labelled Tyr-Ala-hexarelin to membranes from pituitary gland or different brain regions was not correlated with the cause of death and the time elapsed from death to autopsy (23–48 h) in both sexes. Moreover, at histopathological examination, tissues were all well preserved both from an architectural and cytological point of view.

Biochemical characteristics of binding

To determine whether the binding of ^{125}I -labelled Tyr-Ala-hexarelin to tissue membranes shows the properties typical of ligand–receptor interaction, the binding of radiotracer was investigated in more detail in tissues (hypothalamus or pituitary gland) that displayed the highest levels of specific binding. As shown in Fig. 1a, the specific binding of ^{125}I -labelled Tyr-Ala-hexarelin to membranes of these tissues increased with the time of incubation, reaching equilibrium after 60 min at 0°C and declining slowly after 90 min of incubation. The specific binding of radiolabelled Tyr-Ala-hexarelin to pituitary and hypothalamic membranes was reversible; once formed the complex tracer–membranes could be quickly dissociated by adding a large excess (2.5×10^{-5} mol/l) of unlabelled Tyr-Ala-hexarelin. About 50% of radiotracer specifically bound to the pituitary and hypothalamic membranes was dissociated within 60 min at 0°C (Fig. 1b).

A study of specific binding as a function of membrane protein concentration indicated that the binding was proportional to the protein up to at least $100\text{ }\mu\text{g}/\text{tube}$. Therefore all our incubations were performed with about $100\text{ }\mu\text{g}$ protein/tube (Fig. 2). Specific binding of ^{125}I -

Figure 1 Effect of incubation period on the association and dissociation of ^{125}I -labelled Tyr-Ala-hexarelin binding. Association studies (a) were performed by incubating pituitary (○) and hypothalamic (●) membranes of female subjects with ^{125}I -labelled Tyr-Ala-hexarelin in the absence or presence of 2.5×10^{-6} mol/l unlabelled Tyr-Ala-hexarelin at 0°C and calculating the specific binding at the indicated times. Dissociation studies (b) were carried out by incubating membranes at 0°C with 0.5×10^{-9} mol/l radioligand in the absence or presence of 2.5×10^{-6} mol/l unlabelled Tyr-Ala-hexarelin. After 60 min (indicated as zero time) a large excess (2.5×10^{-5} mol/l) of unlabelled Tyr-Ala-hexarelin was added to the tubes, and samples were assayed for radioactivity specifically bound to the membranes at the indicated time intervals. Values are means $\pm$ S.E.M. ($n=3$).

labelled Tyr-Ala-hexarelin to pituitary gland and hypothalamic membranes occurred over a relatively wide range of pH. The maximal binding was observed at pH 5.6 and declined to half-maximal values at pH 4.5 and 8.6. However, varying the pH of the incubation from pH 5.6

Figure 2 Specific binding of ^{125}I -labelled Tyr-Ala-hexarelin to pituitary (○) and hypothalamic (●) membranes of male subjects as a function of membrane protein. Each point represents the mean of two separate experiments.

Figure 3 Effect of pH on specific binding of ^{125}I -labelled Tyr-Ala-hexarelin to pituitary (○) and hypothalamic (●) membranes from male subjects. Binding conditions were as described in Materials and Methods except that various buffers were used: 50 mmol/l sodium acetate (pH 4.5–5.6), 50 mmol/l Tris-HCl (pH 6.4–7.3), 50 mmol/l sodium bicarbonate (pH 8.0–8.6). All pH values are the final pH in the incubation medium. Values are means $\pm$ S.E.M. ($n=3$).

to 7.3 did not alter the binding significantly. Therefore the experiments were carried out at pH 7.3 (Fig. 3).

Brief exposure (5 min) of pituitary membranes to enzymes that disrupt protein structure such as trypsin

Figure 4 Saturation of ^{125}I -labelled Tyr-Ala-hexarelin binding to hypothalamic membranes of male subjects. Saturation experiments were performed by incubating a fixed amount of membrane protein (100 µg/tube) with increasing concentrations of radiolabelled Tyr-Ala-hexarelin alone (■, total binding) or plus 2.5×10^{-6} mol/l unlabelled Tyr-Ala-hexarelin to define non-specific binding (▲). Specific binding values (○) were obtained by subtracting non-specific binding from total binding. Upper panel: Scatchard analysis of saturation experiments. Values are means $\pm$ S.E.M. ($n=3$).

(50 µg/ml) resulted in a marked decrease (-68%) in the specific binding of radiolabelled Tyr-Ala-hexarelin, suggesting that protein is a functionally important part of the binding site. Phospholipase C (50 µg/ml) also caused a significant decrease (-28%) in binding, suggesting that phospholipids may also play an important role in the binding of Tyr-Ala-hexarelin.

Experiments using increasing concentrations of radio-iodinated Tyr-Ala-hexarelin ranging from 0.15×10^{-9} to 10×10^{-9} mol/l revealed evidence of saturable specific binding in the hypothalamus of three male subjects which yielded sufficient amounts of tissue membranes (Fig. 4).

Figure 5 Displacement of ^{125}I -labelled Tyr-Ala-hexarelin from hypothalamic membranes of female subjects by Tyr-Ala-hexarelin ($\circ$), GHRP2 ($\bullet$), hexarelin ($\square$), GHRP6 ($\blacksquare$), MK 0677 ($\diamond$), [D-Arg¹-D-Phe⁵-D-Trp^{7,9}-Leu¹¹]-substance P ($\blacklozenge$), GHRH (*), galanin (∇), somatostatin ($\blacktriangledown$) and Met-enkephalin (+). Binding assays were conducted as described in Materials and Methods. The ordinate represents binding as a percentage of control (specific binding in the absence of unlabelled competitor). Values are means $\pm$ S.E.M. of three separate experiments.

Scatchard analysis (upper panel Fig. 4) indicated the presence of a single class of high-affinity sites with a K_d of $(1.5 \pm 0.3) \times 10^{-9}$ mol/l and a $B_{\max}$ of 1110 ± 123 fmol/mg protein. Similar specific high-affinity binding was also detected in pituitary membranes of the same subjects, indicating a K_d of $(2.1 \pm 0.4) \times 10^{-9}$ mol/l and a $B_{\max}$ of 963 ± 172 fmol/mg protein.

Specificity of binding

The specificity of ^{125}I -labelled Tyr-Ala-hexarelin binding was established by determining the ability of different compounds to compete with the radioligand for the hypothalamic binding sites (Fig. 5). Both unlabelled peptidyl (Tyr-Ala-hexarelin, hexarelin, GHRP2, GHRP6) and non-peptidyl (MK 0677) sGHS completely displaced radiolabelled Tyr-Ala-hexarelin from binding sites, but MK 0677 was significantly less potent than peptidyl sGHS. The IC_{50} values (mean $\pm$ S.E.M. of three separate experiments) were $(1.2 \pm 0.1) \times 10^{-8}$ mol/l for Tyr-Ala-hexarelin, $(1.6 \pm 0.2) \times 10^{-8}$ mol/l for GHRP2, $(1.8 \pm 0.2) \times 10^{-8}$ mol/l for hexarelin, $(2.4 \pm 0.3) \times 10^{-8}$ mol/l for GHRP6 and only $(1.1 \pm 0.3) \times 10^{-7}$ mol/l for MK 0677. [D-Arg¹-D-Phe⁵-D-Trp^{7,9}-Leu¹¹]-substance P, a substance P antagonist that has been found to inhibit the GH release in response to sGHS both *in vivo* and *in vitro* (Bitar *et al.* 1991, Cheng *et al.* 1994), also displaced this binding, but its IC_{50} value was about 3 times higher than that of MK 0677. In

contrast, no competition was observed in the presence of other neuropeptides (GHRH, somatostatin, galanin or Met-enkephalin) that have a known effect on GH release. The pattern of displacement specificities in the pituitary gland and other sGHS-R-positive brain areas resembled that of the hypothalamus (data not shown).

Discussion

In the present study, we have demonstrated the properties of specific receptors for sGHS in the human brain and pituitary gland. sGHS-Rs have been identified by radio-receptor analysis using a peptidyl sGHS (Tyr-Ala-hexarelin) which had been radioiodinated to high specific activity (>2000 Ci/mmol) at the Tyr residue. The highest specific binding of ^{125}I -labelled Tyr-Ala-hexarelin was found in the hypothalamus and pituitary gland and a lower but significant binding was also detected in the choroid plexuses, cerebral cortex, hippocampus and medulla oblongata, whereas negligible binding was observed in the striatum, substantia nigra, thalamus, cerebellum and corpus callosum. No sex differences in radiolabelled Tyr-Ala-hexarelin binding to either pituitary gland or brain regions were observed, suggesting that sGHS-Rs were not influenced by sex hormones. This finding is in agreement with previous evidence showing that the GH-releasing activity of sGHS is not sex dependent (Bowers 1993, Ghigo *et al.* 1994).

^{125}I -Labelled Tyr-Ala-hexarelin binding shows many of the properties typical of ligand-receptor interaction (Reichert 1988). These include high affinity, saturability and reversibility. As described for other peptides (Furchgott 1978, Reichert 1988), the rate and extent of binding increased if the concentration of either radioligand or receptors was increased. ^{125}I -Labelled Tyr-Ala-hexarelin binding was also dependent on time of incubation, and exhibited an optimum pH. Moreover, loss of receptor activity caused by trypsin and phospholipase C treatments indicates that protein and phospholipid constituents are structural components that are essential for receptor activity. The binding specificity of ^{125}I -labelled Tyr-Ala-hexarelin to pituitary or hypothalamic membranes is very similar to that observed in the rat and pig tissues (Sethumadavan *et al.* 1991, Veeraragavan *et al.* 1992, Pomès *et al.* 1996, Pong *et al.* 1996); radioligand binding is displaced by unlabelled peptidyl sGHS (Tyr-Ala-hexarelin, hexarelin, GHRP2, GHRP6), non-peptidyl sGHS (MK 0677) and by a substance P antagonist such as [D-Arg¹-D-Phe⁵-D-Trp^{7,9}-Leu¹¹]-substance P, but not by other neuropeptides (GHRH, galanin, somatostatin or Met-enkephalin) that have a known effect on GH release. The displacement of ^{125}I -labelled Tyr-Ala-hexarelin by [D-Arg¹-D-Phe⁵-D-Trp^{7,9}-Leu¹¹]-substance P parallels its antagonist actions on GH secretion, since this peptide has been found to selectively inhibit GH release in

response to non-peptidyl and peptidyl sGHS both *in vivo* and *in vitro* (Bitar *et al.* 1991, Cheng *et al.* 1994). Our competition experiments also demonstrate that the different peptidyl sGHS studied here showed a similar efficacy in displacing ^{125}I -labelled Tyr-Ala-hexarelin, whereas MK 0677 was about 5–9 times less effective than these molecules. This agrees with previous clinical studies showing that hexarelin, GHRP2 and GHRP6 stimulate GH secretion *in vivo* with equal potency and that they are clearly more active than MK 0677 (Korbonits & Grossman 1995, Arvat *et al.* 1997, Ghigo *et al.* 1997).

The presence of specific sGHS-R in the human pituitary strengthens the hypothesis that this tissue may be an important target of sGHS. This is in agreement with other data in humans showing that these synthetic compounds release GH from the pituitary gland (Renner *et al.* 1994, Lei *et al.* 1995). Our observation that there are considerable amounts of sGHS-R in the hypothalamus indicate that this region may be a further target of sGHS. This agrees with other data showing that sGHS activate in GH-deficient rats and mice distinct subpopulations of hypothalamic arcuate neurones, as reflected by increased electrical activity (Dickson *et al.* 1995) and by the detection of Fos protein in cell nuclei (Dickson *et al.* 1995, Sirinathsinghji *et al.* 1995). More recently, evidence has been presented that the arcuate neurones activated by systemic or intracerebroventricular injections of GHRP6 are mainly of the neurosecretory type and contain GHRH or neuropeptide Y (Dickson *et al.* 1996, Dickson & Luckman 1997).

The present data also show, for the first time, the presence of sGHS-R in the choroid plexuses. As reported for other peptides (Pardridge 1986), these binding sites may act as transport proteins, translocating peptidyl sGHS from the blood to the cerebrospinal fluid. This mechanism could serve to transport sGHS molecules through the blood–brain barrier and should allow their interaction with other brain areas such as cerebral cortex, hippocampus and medulla oblongata, where we found considerable amounts of sGHS-R.

In conclusion, the present data demonstrate that sGHS have specific receptors in human pituitary and brain and reinforce the hypothesis that these compounds could be the synthetic counterpart of an endogenous GH secretagogue involved in the neuroendocrine control of GH secretion and possibly in other central activities.

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