Fasting-induced changes in the hypothalamic-pituitary-GH axis in the absence of GH expression: lessons from the spontaneous dwarf rat

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Abstract

Fasting results in a reciprocal shift in hypothalamic neuropeptide Y (NPY) and GH-releasing hormone (GHRH) expression in the adult male rat. It is hypothesized that the fasting-induced rise in NPY is responsible for the GHRH decline and subsequent attenuation of pulsatile GH release. Fasting also leads to a decrease in circulating IGF-I, attributed to both reduced GH release and peripheral GH resistance. Although pituitary GH output is suppressed in the fasted rat, we report herein that pituitary GHRH receptor (GHRH-R) and GH secretagogue receptor (GHS-R) mRNA levels are increased, while pituitary expression of the somatostatin receptor subtype 2 (sst2) and 5 (sst5) is decreased, as determined by real-time reverse transcription (RT)-PCR. A shift in the expression of pituitary receptor subtypes to favor GH synthesis and release may be due, at least in part, to a decline in GH/IGF-I negative feedback. In order to test this hypothesis, we compared hypothalamic and pituitary response to fasting (72 h) in normal male rats and rats with isolated GH deficiency (spontaneous dwarf

rats (SDR)). Circulating GH levels were undetectable in SDR, and IGF-I levels were less than 10% of normal controls. Fasting stimulated NPY mRNA levels in SDR; however, the rise in NPY mRNA levels was not accompanied by a fall in GHRH mRNA, as observed in fasted normal rats. In fact, GHRH mRNA levels paradoxically rose in the fasted SDR to 135% of fed controls. At the pituitary level, fasting did not alter sst2 and sst5 mRNA levels in SDR but did stimulate the expression of GHRH-R and GHS-R to 165% and 149% of fed controls, respectively. These results demonstrate that the fasting-induced changes in pituitary expression of sst2 and sst5, but not GHRH-R and GHS-R, are GH/IGF-I dependent. In addition, these results argue against the theory that the negative association of NPY and GHRH expression observed following fasting represents a simple cause-and-effect relationship and suggest that GH, either directly or indirectly, mediates the effects of fasting on hypothalamic GHRH expression.

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Introduction

Growth hormone (GH) secretion is affected by fasting in all mammalian species studied to date (Tannenbaum *et al.* 1979, Ho *et al.* 1988, Fairhall *et al.* 1990, Thomas *et al.* 1990, Henricks *et al.* 1994, Stoving *et al.* 1999). However, the GH-secretory response to reduced food intake is species dependent. In humans, nutrient deprivation results in a rise in circulating GH (Ho *et al.* 1988, Riedel *et al.* 1995, Stoving *et al.* 1999) accompanied by a paradoxical decrease in insulin-like growth factor (IGF)-I due to the GH resistance of peripheral target tissues (Ho *et al.* 1992, Gianotti *et al.* 2000). The increase in GH release is associated with an increase in pituitary sensitivity to

growth hormone-releasing hormone (GHRH) and a decrease in pituitary sensitivity to somatostatin (SRIF) (Gianotti *et al.* 1999). In contrast to the fasted human, the fasted male rat shows dramatically reduced pulsatile GH release (Tannenbaum *et al.* 1979, Bruno *et al.* 1990, Janowski *et al.* 1993). The fasting-induced fall in GH is thought to be due to a decrease in hypothalamic GHRH expression (Bruno *et al.* 1990, Ghigo *et al.* 1997, Vuagnat *et al.* 1998, Carro *et al.* 1999) brought about by the inhibitory actions of neuropeptide Y (NPY) (Minami *et al.* 1998, Korbonits *et al.* 1999). However, consistent with the fasted human, food deprivation in the rat leads to peripheral GH resistance and reduced IGF-I (Bornfeldt *et al.* 1989), and increased pituitary sensitivity to GHRH

Table 1 Primers used in the generation of riboprobes for RPA

Name	GenBank acc. no.	Primer sequence	Signal size	
			Full-length riboprobes	Protected fragments
GHRH	M73486	Sense: 5'- AGA TGC CCA GCC TGA GGT CA -3' Antisense: 5'- <u>GGG AGA</u> ATA TAC TAA TGT GCT CCT CCG CTG AAA GCT TCA TC-3'	431 bp	410 bp
SRIF	M25890	Sense: 5'- GAA GGA GAC GCT ACT GGA GT -3' Antisense: 5'- <u>GGG AGA</u> GTG CTC CAC GGT AAC CTG GGT TCG AGT TGG CAG AC-3'	395 bp	374 bp
NPY	M15880	Sense: 5'- TCG CTC TAT CCC TGC TCG TGTG -3' Antisense: 5'- <u>GGG AGA</u> GGA TCA TCA TCG TAC GTC TGC CTG TCC CAC CCA ATG-3'	362 bp	341 bp
β-Actin	NM_031144	Sense: 5'- ATG GGT CAG AAG GAC TCC TAC G -3' Antisense: 5'- <u>GGG AGA</u> ACT AGC TGA CCG ATT AGT GGT ACG ACC AGA GGC ATA C-3'	330 bp	309 bp

Gene-specific primers are shown in bold. Underlined sequences represent the transcription initiation start site, whereas those in italic are nonspecific and therefore nonhybridizable.

(Sugihara *et al.* 1996). The fasting-induced increase in GHRH sensitivity is associated with an increase in pituitary GHRH receptor (GHRH-R) mRNA and an increase in pituitary GHRH binding (Sugihara *et al.* 1996), with a concomitant decline in pituitary SRIF receptor expression and ligand binding (Bruno *et al.* 1994). The rise in GHRH-R mRNA may be mediated by the reduction in GH/IGF-I negative feedback since IGF-I has been shown to suppress directly GHRH-R mRNA levels in primary pituitary cell cultures (Sugihara *et al.* 1999), and we have observed an increase in GHRH-R mRNA in rats with isolated GH deficiency (spontaneous dwarf rats (SDR)) (Kamegai *et al.* 1998a) and in mice with reduced circulating IGF-I (liver-specific IGF-I knockout mice) (Wallenius *et al.* 2002). In order to test whether fasting-induced fall in GH/IGF-I is responsible for fasting-induced changes in pituitary receptor expression, we compared the hypothalamic/pituitary response to fasting in normal rats and GH-deficient SDR.

Materials and Methods

Animals

Male SDR (3–5 months; 87–106 g) and age-matched normal Sprague-Dawley rats (307–408 g; Harlan, Indianapolis, IN, USA) were used in the present study. SDR were first described by Okuma (1984) and found to lack GH. This defect was later attributed to a point mutation in the GH gene that creates a premature, in-phase stop codon (Takeuchi *et al.* 1990). Both strains of rats were housed under controlled environmental conditions (12-h light and 12-h dark). Animals were fasted or

supplied food *ad libitum*. Both groups were allowed free access to water. Seventy-two hours after food withdrawal, animals were killed by decapitation, and anterior pituitaries, hypothalami and sera were collected. Experiments were conducted according to the principles and procedures outlined in the NIH Guide for the Care and Use of Laboratory Animals and approved by the University of Illinois at Chicago Animal Care Committee.

Measurement of GH, IGF-I and corticosterone concentrations

Serum GH concentrations were measured by homologous RIA using an established assay system, as previously described (Frohman & Bernardis 1968). Total serum IGF-I levels were assayed using a human IGF-I RIA kit (Nichols Institute Diagnostics, San Juan Capistrano, CA, USA) after acid/ethanol extraction according to the manufacturer's instructions. Serum corticosterone concentrations were assessed using the OCTEIA corticosterone electroimmunoassay (EIA) (Alpco Diagnostics, Windham, NH, USA).

RNA isolation and RNase protection assay (RPA) of hypothalamic GHRH, SRIF and NPY mRNA

Total hypothalamic and pituitary RNA was recovered using standard procedure previously reported (Kamegai *et al.* 1998a,b). One microgram of rat hypothalamic RNA was reverse transcribed using Superscript II reverse transcriptase (Life Technologies, Inc., St Louis, MO, USA) with an oligo d(T) primer. To generate riboprobes, complementary DNA (cDNA) was amplified by PCR using primers for rat GHRH, SRIF, NPY or β-actin. The

Table 2 Sequences of primers and hybridization probes used in real-time RT-PCR

Name	GenBank acc. no.	Primer sequence	Size (bp)
GAPDH	X02231	Sense: 5'-CATGCCATCACTGCCACTCAG-3' Antisense: 5'-AGCCCAGGATGCCCTTTAGTG-3'	292
β-actin	NM_031144	Sense: 5'-GCCCTAGACTTCGAGC-3' Antisense: 5'-CGGATGTCAACGTCAC-3' Probe 1: 5'-TGGAGAAGAGCTATGAGCTGCC(Fluo)-3' Probe 2: 5'-(Red 705)ACGGTCAGGTCATCACTATCG(Phosphate)-3'	212
sst1	X62314	Sense: 5'-AGAGTCGTGCCAACAG-3' Antisense: 5'-GGAGTTGGCGACGTTA-3' Probe 1: 5'-AGTAGTGCGTGACAGCGA(Fluo)-3' Probe 2: 5'-(Red 640)CAGCGCCCCCTGTTTAGCA(Phosphate)-3'	378
sst2	M93273	Sense: 5'-CCCCTATGTAATCTCGT-3' Antisense: 5'-TGCAAGAGGGATGCTG-3' Probe 1: 5'-TCCTGTGTTTGTAAACCAAGTAGC(Fluo)-3' Probe 2: 5'-(Red 640)GTTTCATGTGCGTCTAGTATAGGTGG(Phosphate)-3'	225
sst3	X63574	Sense: 5'-TAAGGCACGAGTACCAGACAC-3' Antisense: 5'-ACTTGGGCTCTGATTAGGAG-3'	300
sst4	M96544	Sense: 5'-TGTCCCTCATCTCAGCTATG-3' Antisense: 5'-CTCAGCACCTCCAGTTGTTTC-3'	137
sst5	L04535	Sense: 5'-CGCCAAGATGAAGACAGTTAC-3' Antisense: 5'-TGACCATCAGGCAGAAGATAC-3'	197
GHRH-R	L01407	Sense: 5'-AGGGGCTGTGAAAAGGGACTG-3' Antisense: 5'-CAGTGTCTCTAAAAGCCAGTT-3'	545
GHS-R	U94321	Sense: 5'-GTCTACGTGATAGCCAATG-3' Antisense: 5'-CCGTTGGGTCATACACT-3' Probe 1: 5'-AGCATGGAGGGTGAGAAGT(Fluo)-3' Probe 2: 5'-(Red 640)TCCTGGGAAGTCTGATCCACT(Phosphate)-3'	357

antisense primers were modified to contain a 17-base T7 RNA polymerase recognition sequence (5'-TAATACG ACTCACTATA-3'), a 6-base transcription initiation sequence and 15 bases of nonspecific (not hybridizable) sequence appended at the 5' end. The nonspecific sequence was added to the primer to allow for the differentiation of protected and unprotected probes after RNase digestion. The amplified PCR products were used as templates for *in vitro* transcription performed using the MAXIscript kit (Ambion, Inc., Austin, TX, USA) in the presence of [α - ^{32} P]CTP. Radiolabeled riboprobes were gel-purified before use. The primer sequences and the expected sizes of full-length riboprobes and protected fragments are summarized in Table 1. RPA was performed using Ambion, Inc.'s HybSpeed RPA kit, following the manufacturer's instructions, with minor modifications. In a single reaction, probes for GHRH (2×10^4 c.p.m.; specific activity, 1×10^9 c.p.m./ μ g), SRIF (1×10^4 c.p.m.; specific activity, 3×10^8 c.p.m./ μ g), NPY (2×10^4 c.p.m.; specific activity, 9×10^8 c.p.m./ μ g) and β -actin (4×10^3 c.p.m.; specific activity, 8×10^7 c.p.m./ μ g) were incubated for 20 min at 68 °C in

10 μ l of HybSpeed Hybridization Buffer containing 50% of the total RNA isolated from a single hypothalamus or 50 μ g of yeast RNA. Unhybridized probes were removed by treating the reactions with RNase A/T1 (1 μ g/20 units) mix for 1 h at 37 °C. Protected fragments were separated on a 5% polyacrylamide/8 M urea gel. Gel was dried on chromatography paper and exposed to a phosphorimage screen. Band intensity was evaluated by image analysis software (Molecular Dynamics, Sunnyvale, CA, USA).

Northern blot analysis of GH mRNA

Pituitary GH mRNA levels were measured by Northern blot analysis in pituitary extracts from normal rats. Pituitary total RNA (2 μ g) was electrophoresed on a 1% agarose-1.2% formaldehyde gel. After overnight transfer of the RNA to a Nytran membrane (Schleicher and Schuell, Keene, NH, USA), hybridization with ^{32} P-labeled GH or β -actin riboprobes was performed in 50% formamide at 65 °C overnight. Generation of riboprobes is described above. The sequences of gene-specific primers for PCR

Figure 1 Real-time RT-PCR analysis using LightCycler. Representative standard curve from 10¹ to 10⁷ copies of β -actin (A) and GHS-R (C) template. A plot of fluorescence against cycle number demonstrates discrimination down to 10¹ copies of β -actin (B) and GHS-R (D) template.

amplification of GH (GenBank acc. no. V01237) were as follows: sense; 5'-CTGGCTGCTGACACCTACAAA-3', antisense; 5'-CAGGAGAGCAGCCCATAGTTT-3'. After exposure to a phosphorimage screen, hybridization signals were quantitated by image analysis software. SDR pituitary samples were excluded from this analysis because GH mRNA is not detectable in this animal model due to a point mutation in the GH gene which introduces a premature stop codon resulting in truncated transcripts that are unstable (Ookuma 1984, Takeuchi *et al.* 1990).

Real-time reverse transcription (RT)-PCR of pituitary SRIF receptor subtypes, GHRH-R, and GHS-R mRNA

SRIF receptors (sst1-sst5), GHRH-R and GHS-R mRNA levels were quantified by real-time PCR using β -actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels as internal standards. Specifically, total pituitary RNA (1 μ g) was reverse transcribed using the Superscript II reverse transcriptase (Life Technologies, Inc., St Louis, MO, USA) with random hexamer priming. The resultant complementary DNA (cDNA) was amplified using the LightCycler (Roche Diagnostics Ltd, Lewes, UK). Real-time PCR analysis was carried out with SYBR Green I and primers (for GAPDH, sst3, sst4, sst5 and GHRH-R) or hybridization

probes and primers (for β -actin, sst1, sst2 and GHS-R). The PCR primer and hybridization probe sequences used in this study are summarized in Table 2.

PCR reactions with SYBR Green I and primers were performed in a 20- μ l volume capillary with 2- μ l RT product, 0.5 μ M primers and 4 mM MgCl₂. *Taq* polymerase, PCR buffer, dNTPs and SYBR Green I dye were included in the LightCycler-FastStart DNA Master SYBR Green I mix (Roche Diagnostics Ltd, Lewes, UK). Thermal cycling profile consisted of preincubation step at 95 °C for 10 min followed by 40 or 50 cycles of 95 °C denaturation step for 10 s, 56 °C (for sst3, sst4 and sst5) or 60 °C (for GAPDH and GHRH-R) annealing step for 5 s, and 72 °C extension step for 20 s. After each extension step, the temperature was raised to 84 °C (for sst4), 85 °C (for sst3 and sst5) or 87 °C (for GHRH-R and GAPDH) to measure SYBR Green I fluorescence at a temperature 2 °C below the product *T_m* and above the *T_m* of the primer-dimers to prevent the interference of nonspecific primers-dimers. At the end of the PCR, a melting curve analysis was performed by gradually increasing temperature from 65 °C to 95 °C (0.1 °C/s) to confirm the amplification specificity of the PCR products.

PCR amplification using hybridization probes and primers was carried out in 20 μ l reaction mixture containing a master mixture (LightCycler-FastStart DNA Master hybridization probes; Roche Diagnostics Ltd), 4 mM

Figure 2 Comparison of the absolute levels of GH-stimulatory (GHRH-R and GHS-R; upper panel) and GH inhibitory (sst1–5; lower panel) receptor mRNA, from pituitaries of fed normal rats and SDR. Pituitary receptor expression was measured by real-time RT-PCR assay. Data are mean $\pm$ S.E.M., n =five animals/group for normal rats and four animals/group for SDR. ** P <0.01.

MgCl₂, 0.3 μ M primers, 0.1 μ M each probe and 2 μ l cDNA in a capillary. The reaction mix was preincubated at 95 °C for 10 min followed by 40 to 50 cycles of; 95 °C (10 s) for denaturation, 52 °C (10 s) for annealing and 72 °C (20 s) for extension.

The level of expression of each mRNA and their estimated crossing points (C_p) in each sample were determined relative to the standard preparation using the LightCycler computer software (v. 3.5). The PCR standards for each sst1, sst2, sst3, sst4, sst5, GHRH-R, GHS-R, β -actin and GAPDH consisted of a known number of molecules of PCR product, purified using a gel extraction kit (Qiagen, Inc., Valencia, CA, USA) and quantified spectrophotometrically. Standards were made to a concentration of 10^8 copies/ μ l. PCR amplification was performed with a series of standards prepared by successive dilutions, and a linear standard curve was automatically generated. A standard curve was constructed for each PCR run. All samples to be compared were run in the same assay.

Statistical analysis

GAPDH was considered an inappropriate control, in that 72-h fasting significantly suppressed pituitary GAPDH mRNA levels in both normal and SDR (data not shown). In contrast, pituitary and hypothalamic β -actin expression levels were not altered by fasting and did not differ between strains; therefore, mRNA levels of each target gene were adjusted by mRNA levels of β -actin. All data were expressed as mean $\pm$ S.E.M. Comparisons between groups were made by Student's t -test, and P <0.05 was considered significant. All comparisons were made between samples electrophoresed on the same gel (for RPAs) or real-time PCR run.

Results

To test the sensitivity of the real-time PCR used in this study, standard curves were constructed from known amounts of purified PCR products (each diluted 100-fold from 10^7 to 10^1 copies). All standard curves showed correlation coefficients greater than 0.99. Each graph of fluorescence against cycle number demonstrates that as few as ten template copies could be distinguished from background levels. Figure 1 represents an example of a linear standard curve from 10^1 to 10^7 copies of β -actin (A) and GHS-R (C) template, and a plot of fluorescence against cycle number demonstrates discrimination down to ten copies of β -actin (B) and GHS-R (D) template. Standard curves for other transcripts were generated with similar results (data not shown).

Comparison of absolute levels of GHRH-R, GHS-R and SRIF receptor subtype mRNA levels between *ad libitum* fed normal rats and SDR using this quantitative system is shown in Fig. 2, and it confirms our previous reports using a semiquantitative PCR system (Kamegai *et al.* 1998a,b, Park *et al.* 2000). Specifically, GHRH-R, GHS-R, sst1 and sst2 mRNA levels of SDR were greater than those of normal rats, while sst5 mRNA levels were reduced.

As shown in Table 3, fasting resulted in a proportional weight loss in normal rats ($-19 \pm 1\%$) and SDR ($-17 \pm 1\%$). In normal rats, this weight reduction was associated with a significant suppression of circulating GH and IGF-I levels (Table 3). Despite the decline in circulating GH, GH mRNA levels did not differ between fed and fasted normal rats ($100 \pm 4\%$ vs $105 \pm 5\%$; Table 3). LaPaglia *et al.* (1998) also reported no effect of fasting on GH mRNA in the male rat, while others report that fasting decreases GH mRNA levels (Rodriguez *et al.* 1995, Ghigo *et al.* 1997). These variable responses may be related to the starting weight/age of the experimental animal. In the SDR, which lacks pituitary GH expression (Takeuchi *et al.* 1990), circulating IGF-I concentrations were also suppressed by fasting. Nogami *et al.* (1994) also reported that fasting decreased circulating IGF-I levels as

Table 3 Effects of 72-h fasting on body weight, circulating GH, IGF-I and corticosterone and pituitary GH mRNA in normal and spontaneous dwarf rats (SDR)*

	Normal		SDR	
	Fed	72-h fasted	Fed	72-h fasted
Mean body weights (g)	306 ± 4.0	230 ± 1.6	135.7 ± 2.1	106.0 ± 5.7
% Body weight Δ	+5.1 ± 0.8 [†]	-18.9 ± 0.9	-0.1 ± 0.7	-16.7 ± 1.1
Serum GH (ng/ml)	97.7 ± 60.5	7.6 ± 4.3	ND	ND
GH mRNA levels	7.3 ± 0.3 [‡]	7.7 ± 0.4	ND	ND
Serum IGF-I (ng/ml)	367.4 ± 35.1	152.8 ± 11.8	48.6 ± 2.0	30.0 ± 1.5
Serum corticosterone (ng/ml)	66.8 ± 6.8	166.6 ± 64.8	84.8 ± 20.0	197.8 ± 26.6

*Values are expressed as mean ± S.E.M.

[†]Body weight changes shown as percentage of starting body weight.[‡]GH mRNA signal intensity adjusted by β-actin mRNA signal intensity.

well as hepatic IGF-I mRNA in the SDR, suggesting that a component of fasting-induced suppression of IGF-I production is GH-independent. A possible inhibitor of IGF-I production is elevated glucocorticoids (Unterman *et al.* 1993); indeed, serum corticosterone concentrations were increased in response to fasting in both fasted normal (fed 66.8 ± 6.8 vs fasted 166.6 ± 64.8, $n=4$ /group) and SDR (fed, 84.8 ± 20.0 vs fasted, 197.8 ± 26.6, $n=6$ /group; Table 3).

Hypothalamic GHRH mRNA levels in fasted normal rats were 52% ($P<0.01$) of fed controls, whereas NPY mRNA levels increased in response to fasting to 138% ($P<0.01$). However, in SDR, despite a comparable fasting-induced rise in NPY expression (147% of fed controls, $P<0.05$), GHRH mRNA levels were significantly increased to 135% ($P<0.05$) of fed controls (Fig. 3).

Figure 4 represents the effect of fasting on pituitary GHRH-R and GHS-R mRNA levels in normal rats and SDR. GHRH-R mRNA levels were significantly increased in both fasted normal rats and SDR to 212 ± 43% ($P<0.05$) and 165 ± 22% ($P<0.05$) of fed controls respectively. Fasting also significantly increased pituitary GHS-R mRNA levels in normal rats and SDR to 243 ± 44% ($P<0.05$) and 149 ± 14% ($P<0.05$) of fed controls respectively.

Sst2 and sst5 mRNA levels were decreased in response to fasting in normal rats to 57 ± 3% ($P<0.05$) and 65 ± 6% ($P<0.05$) of fed controls respectively. In contrast, fasting did not significantly affect SRIF receptor expression in SDR (Fig. 5).

Discussion

In the present study, 72-h fasting clearly increased pituitary expression of GHRH-R and GHS-R independently of GH/IGF-I status. It has been previously reported that GHRH-R (Sugihara *et al.* 1996) expression is increased in response to fasting. In this report we also observed that fasting led to an increase in GHS-R mRNA. This

observation mirrors a recent report by Kim *et al.* (2003), in which a 48-h fast, in normal adult rats, resulted in a doubling of pituitary GHS-R mRNA levels. Given the fact that ghrelin, the endogenous ligand for GHS-R, also increases in response to fasting (Ariyasu *et al.* 2001, Cummings *et al.* 2001, Toshinai *et al.* 2001, Kim *et al.* 2003) and the fact that GHS (synthetic ghrelin agonists) and GHRH work synergistically to release GH (Cheng *et al.* 1989, Smith *et al.* 1997, Bowers 2001), we might speculate that increased pituitary sensitivity to ghrelin during states of nutrient deprivation may be yet another compensatory mechanism to help modulate pituitary sensitivity to altered hypothalamic input.

These results are inconsistent with our original hypothesis, that the decline in GH/IGF-I mediates the fasting-induced rise in GHRH-R mRNA. In fact, GHRH-R and GHS-R mRNA levels rose in the fasted SDR beyond their already elevated levels. Therefore, the question remains; what is responsible for fasting-induced upregulation of GHRH-R and GHS-R expression? Fasting leads to multiple changes in metabolic hormones that collectively serve to promote gluconeogenesis and breakdown of stored nutrients (glycogen and fat). A key player in fasting-mediated changes in nutrient partitioning is the rise in circulating glucocorticoids (Dallman *et al.* 1993). In this study, 72-h fasting resulted in a rise in corticosterone concentrations in both normal rats and SDR. Since synthetic and naturally occurring glucocorticoids are powerful stimulators of GHRH-R and GHS-R expression *in vivo* and *in vitro* (Tamaki *et al.* 1996, Miller & Mayo 1997, Nogami *et al.* 1999, Tamura *et al.* 2000), we might speculate that glucocorticoids are, at least in part, responsible for upregulation of pituitary GHRH-R and GHS-R expression during fasting.

In contrast to the GH-stimulatory receptors, fasting decreased expression of SRIF receptor subtypes, sst2 and sst5, in normal rats, but not in SDR. These observations suggest that GH and/or IGF-I play a key role in regulation of pituitary SRIF receptor synthesis in the fasted state. It should be noted that the fasting-induced changes in SRIF

Figure 3 Effect of 72-h fasting on hypothalamic GHRH, SRIF, and NPY mRNA levels in normal and spontaneous dwarf rats (SDR). Animals were fasted for 72 h or supplied food *ad libitum* (fed). Both groups were allowed free access to water. GHRH, SRIF, NPY and β -actin mRNA levels were measured by RPA. The upper panel represents an autoradiogram of RPA for hypothalamic neuropeptide mRNA. The lane marked P represents full-length riboprobes undigested by RNase. The lane marked y-RNA indicates a negative control, showing the absence of hybridization of riboprobes with yeast t-RNA. F, fed; S, 72-h fasted. The lower panel indicates quantitation of hypothalamic neuropeptide mRNA levels by phosphorimager. Hypothalamic neuropeptide mRNA levels were expressed as percentage of fed controls (mean $\pm$ S.E.M., n =five animals/group for normal rats and six animals/group for SDR). * P <0.05, ** P <0.01.

receptor subtypes in normal rats observed in this study differed from those reported by Bruno *et al.* (1994), who observed fasting-induced suppression of sst1, sst2 and sst3 pituitary mRNA levels, but no change in the levels of sst5. In both studies, a 72-h fast of adult male rats was employed; however, the methods utilized to assess SRIF receptor mRNA levels differed. In the present study, real-time quantitative RT-PCR was used, and receptor mRNA levels were adjusted by β -actin to control for the amount of RNA used in the RT reaction and the efficiency of the RT reaction. Bruno *et al.* (1994) used RPAs but did not say whether an endogenous control was used. Despite this discrepancy, both methods of analysis revealed a fasting-induced fall in sst2 mRNA, where sst2 mRNA represents over 50% of all the SRIF receptor transcripts. Given that sst2 was shown to mediate the inhibitory actions of SRIF on GH release (Rohrer *et al.* 1998), we might speculate that a decline in sst2 would lessen the impact of fasting-induced increases in peripheral

SRIF synthesis and release (Tannenbaum *et al.* 1978, Shapiro *et al.* 1979, Plotsky 1991, Ishikawa *et al.* 1997), and thus would help maintain GH release.

In the present study, we also examined the effects of fasting on hypothalamic neuropeptide expression in the SDR and found that, in the absence of GH, fasting increased NPY mRNA levels, as observed in normal rats, but fasting unexpectedly enhanced hypothalamic GHRH expression. This observation appears, at first glance, to be in direct opposition to the hypothesis that the fasting-induced rise in NPY is responsible for the decline in GHRH. However, such an interpretation may be simplistic, since we have previously reported that NPY mRNA levels are suppressed in the fed SDR to 70% of normal controls (Kamegai *et al.* 1998a). Therefore, a 38% rise in NPY mRNA levels in the fasted SDR, as observed in this report, would serve to raise NPY expression levels only to that of normal and thus may not be sufficient to suppress GHRH expression, as in the normal fasted rat. In

Figure 4 Effect of 72-h fasting on pituitary GHRH-R and GHS-R mRNA levels in normal and spontaneous dwarf rats (SDR). Animals were fasted for 72 h or supplied food *ad libitum* (fed). Both groups were allowed free access to water. GHRH-R and GHS-R mRNA levels were measured by real-time RT-PCR assay. Receptor mRNA levels were adjusted by β -actin and expressed as a percentage of fed controls (mean $\pm$ S.E.M., n =five animals/group for normal rats and five animals/group for SDR). * P <0.05.

fact, such a reduction in NPY inhibitory tone in the SDR may allow other factors to stimulate GHRH synthesis. A potential candidate for fasting-induced stimulation of GHRH synthesis is ghrelin, which is increased in response to fasting (Ariyasu *et al.* 2001, Cummings *et al.* 2001, Toshinai *et al.* 2001). Central sensitivity to ghrelin may also be enhanced in the fasted state, in that Kim *et al.* (2003) reported hypothalamic GHS-R mRNA levels are elevated after 48 h of fasting in the rat. Since GHSs have been shown to stimulate GHRH neuronal activity (Bluet-Pajot *et al.* 2001, Tannenbaum & Bowers 2001) and ghrelin stimulates the release of GHRH from hypothalamic explants (Wren *et al.* 2002), it is possible that in the

face of reduced NPY inhibitory tone, as occurs in the SDR, the fasting-induced rise in ghrelin may also act to stimulate GHRH synthesis. Taken together, these results suggest that there are both positive and negative regulators of GHRH expression in the fasted state, where under certain physiologic conditions the balance between these regulators can be skewed to favor GHRH synthesis. This logic is supported by the report of Henry *et al.* (2001), who observed that food restriction in the ovariectomized ewe, which increases NPY mRNA (McShane *et al.* 1993), also increased GHRH mRNA.

In conclusion, we have demonstrated that fasting-induced upregulation of pituitary GHRH-R and GHS-R

Figure 5 Effect of 72-h fasting on pituitary SRIF receptor subtype (sst1-sst5) mRNA levels in normal and spontaneous dwarf rats (SDR). Animals were fasted for 72 h or supplied food *ad libitum* (fed). Both groups were allowed free access to water. Sst1-sst5 mRNA levels were measured by real-time RT-PCR assay. Receptor mRNA levels were adjusted by β -actin and expressed as a percentage of fed controls (mean $\pm$ S.E.M., n =five animals/group for normal rats and six animals/group for SDR). * P <0.05.

is GH/IGF-I independent, while changes in pituitary SRIF receptor expression are GH/IGF-I dependent. In addition, the unexpected rise in hypothalamic GHRH in the absence of GH suggests that both positive and negative regulatory mechanisms of GHRH expression are present in the fasted state.

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