

The Role of Galanin and Its Receptor in the Feedback Regulation of Growth Hormone Secretion*

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ABSTRACT

GH controls its own secretion through a mechanism involving short-loop feedback regulation of the synthesis and release of GH-releasing hormone (GHRH). GHRH neurons coexpress the peptide galanin, but the functional significance of this coexpression is unknown. In this study, we tested the hypotheses that 1) galanin gene expression in GHRH neurons is regulated by GH and 2) somatostatin (SS) or GHRH neurons are a target for the action of galanin in the hypothalamus. First, we compared levels of galanin messenger RNA (mRNA) in GHRH neurons between normal male rats and Lewis dwarf rats, which have markedly reduced blood levels of GH. The brains of normal and dwarf animals were processed for detection of galanin mRNA and GHRH mRNA by double-label *in situ* hybridization. We observed that Lewis dwarf rats had significantly reduced levels of galanin mRNA in their GHRH neurons ($P < 0.05$). Next, we tested the hypothesis that GH regulates galanin gene expression in GHRH neurons by experimentally altering circulating levels of GH. Three groups of adult male rats were used: 1) intact rats ($n = 7$); 2)

hypophysectomized (hypox) rats ($n = 7$); and 3) hypox rats treated with 1.5 mg of rat GH (rGH) over a 3-day period ($n = 6$). At the end of the treatment period, the animals were killed, and their brains were collected and processed for double-label *in situ* hybridization for GHRH mRNA and galanin mRNA. The signal level of galanin mRNA in GHRH neurons was reduced in hypox animals to less than 10% of that in intact controls ($P < 0.0001$); whereas, the levels of galanin mRNA signal in GHRH neurons did not differ significantly between the groups of intact and rGH-treated hypox rats. Finally, to determine whether SS or GHRH neurons are targets for galanin, we used double-label *in situ* hybridization to determine whether either of these populations of neurons express galanin receptor mRNA. A subset of SS neurons in the PeN appeared to express the galanin receptor mRNA, whereas few, if any, GHRH neurons appeared to do so. We conclude that galanin, like its cotransmitter GHRH, is a target for GH action, and we infer that galanin may play a role in the feedback control of GH secretion by exerting a direct effect on SS neurons. (*Endocrinology* 137: 5303-5310, 1996)

THE SECRETION OF GH is governed by the actions of two hypothalamic hormones, somatostatin (SS) and GH-releasing hormone (GHRH), which inhibit and stimulate the synthesis and secretion of GH, respectively (1). SS secreted from axon terminals in the median eminence is produced by neurons whose cell bodies reside primarily in the periventricular nucleus (PeN), and GHRH is produced by neurons with cell bodies in the arcuate nucleus (ARC). GH participates in the homeostatic control of its own secretion through a mechanism involving reciprocal regulation of the synthesis and secretion of SS and GHRH by GH (see 2 for review). While these relationships comprise the basic elements of the short-loop feedback regulation of GH secretion, an additional, potentially important, component of this control system may be the neuropeptide galanin which is coexpressed in GHRH neurons (3, 4).

In the brain, galanin is colocalized with a variety of classical neurotransmitter and neuropeptide systems (e.g. acetylcholine and vasopressin), where it is thought to act as a

neuromodulator (5, 6). A role for galanin in the regulation of GH secretion has been postulated based on the observations that galanin stimulates the secretion of GH (7-9), whereas galanin antiserum reduces GH pulse amplitude and increases the frequency of GH pulses (10). Although the source of endogenous galanin involved in the physiological regulation of GH secretion is unknown, several lines of evidence suggest that galanin expressed in GHRH neurons is important. First, as in the case with GHRH messenger RNA (mRNA), galanin mRNA levels in GHRH neurons increase over the course of development, in parallel with the amplification of pulsatile GH secretion (11-13). Second, just as the circulating patterns of GH are sexually differentiated and amplified by androgens in the rat (14), levels of galanin mRNA in GHRH neurons are also sexually differentiated and increased by androgens, in parallel with cellular levels of GHRH mRNA (12, 13). Whether the galanin expressed in GHRH neurons can be implicated in the short-loop feedback regulation of GH has not been addressed. One of the objectives of the present investigation was to test the hypothesis that the short-loop feedback control of GH secretion involves the regulation of galanin gene expression in GHRH neurons by GH. To test this hypothesis, we compared levels of galanin mRNA in GHRH neurons between Lewis strain dwarf and control rats that have different circulating levels of GH (15). Then, as a follow-up experiment, we compared levels of

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galanin mRNA in GHRH neurons among 3 other groups of male rats whose circulating GH levels were experimentally altered.

The target cells for the action of galanin in the regulation of GH secretion are unknown. It is conceivable that galanin acts directly on either SS- or GHRH-producing neurons to regulate their secretory activity, which could then modulate GH secretion (16, 10). There is anatomical evidence for synaptic contacts between galanin-containing axon terminals and the dendrites and cell bodies of SS-containing neurons in the anterior region of the PeN (17); however, it is unclear whether galanin acts either pre- or postsynaptically at this putative synapse. We argued that if galanin exerts an effect on SS or GHRH neurons, then the target neuron should express the galanin receptor. To test this hypothesis, we cloned a rat galanin receptor cDNA, and using double-label *in situ* hybridization, we determined whether SS and GHRH neurons express this galanin receptor mRNA.

Materials and Methods

Animal accommodations

For Exp 1, adult male Lewis control and dwarf dw-4/OLA/HSD rats (70 days old) were purchased from Harlan Sprague Dawley Inc. (Indianapolis, IN). For Exp 2, intact and hypophysectomized (hypox) adult male Sprague Dawley rats (70 days old) were purchased from Charles River Laboratories (Wilmington, MA). For Exp 3, adult male Sprague Dawley rats (89 days old) were born at the University of Washington facilities. All animals were housed in group cages (three to four animals/cage) in the animal-care facilities of the University of Washington Department of Comparative Medicine. The facilities were maintained at a constant temperature and a 14-h light, 10-h dark cycle with lights on at 0700 h and off at 2100 h. Rat chow and water were available *ad libitum*, and hypox animals were given water containing 35 mM NaCl, 1.1 mM KCl, 0.24 mM CaCl₂, and 0.18 mM MgCl₂. To allow the animals to acclimatize to their new environment, the experiments were performed 2 weeks after their arrival from the supplier. All animal procedures were approved by the Animal Care Committee of the School of Medicine at the University of Washington in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Experimental design

Exp 1. Age matched adult male dwarf rats ($n = 5$) and Lewis control rats ($n = 7$) were killed and brains were collected and sectioned. The tissue sections were processed by double-label *in situ* hybridization and analyzed for GHRH mRNA and galanin mRNA, and serum levels of rat insulin-like growth factor 1 (rIGF-1) were measured by RIA.

Exp 2. Rats were anesthetized by ether inhalation between 1100 h and 1200 h while osmotic minipumps (Model 1003D, Alzet, Palo Alto, CA) were implanted into the interscapular sc space. These minipumps dispensed a solution containing either vehicle (0.03 M NaHCO₃, 0.15 M NaCl, and 1% BSA) or rat GH (rGH 1.5 mg in the vehicle). A group of hypox rats were treated with rGH for 3 days ($n = 6$). Another group of hypox rats ($n = 7$) and a group of normal (intact) rats ($n = 7$) received vehicle for the same length of time. At the end of the treatment period, the rats were killed and their brains and trunk blood were collected. The brains were processed for GHRH mRNA and galanin mRNA by double-label *in situ* hybridization, and serum levels of rGH were measured by RIA.

Exp 3. Adult male rats ($n = 3$) were killed and brains were collected and sectioned. Tissue sections were processed by double-label *in situ* hybridization and analyzed for double-labeling of galanin receptor mRNA/GHRH mRNA and galanin receptor mRNA/SS mRNA.

Tissue preparation

Animals were killed between 1100 h and 1200 h by rapid decapitation in the first two experiments, and in the third experiment by carbon dioxide asphyxiation and subsequent decapitation. Brains were removed, frozen on dry ice, and stored at -80°C . Using the rat atlas of Paxinos and Watson as an anatomical guide (18), we collected coronal brain slices at 20 μm with a cryostat, beginning rostrally at the decussation of the anterior commissure and continuing caudally through the ARC. The sections were thaw-mounted onto four sets of Superfrost/Plus Microscope Slides (Fisher Scientific, Pittsburgh, PA), thereby placing every 4th slice into a given set. Slides were stored in air-tight boxes at -80°C until hybridization histochemistry was conducted.

cDNA templates for GHRH, galanin and SS cRNA probes

A 198 bp *EcoRI-HindIII* fragment, subcloned into the transcription vector pGEM4 (Promega Biotec, Madison, WI) from the original proGHRF-2 plasmid, contains the entire rat GHRH-43 coding sequence and a portion of coding sequence for the associated 3' peptide (19). A 340 bp *BglIII-SmaI* fragment, consisting of 44 bases of 5' untranslated sequence and the bases coding for the first 98 amino acids of rat pre-pro SS, was subcloned into the transcription vector pSP64 (Promega Biotec, Madison, WI) from the original plasmid vector EV142 (20). A 680 bp rat galanin cDNA, inserted into the *EcoRI* site of Bluescript M13(-) (Stratagene, San Diego, CA), contains 124 bases of the 5' untranslated sequence, the entire open reading frame, and a portion of the 3' untranslated sequence (21).

Cloning of the rat galanin receptor cDNA

A partial cDNA for the rat galanin receptor was amplified by PCR from a rat brain cDNA library (Stratagene, San Diego, CA), by using nested primers derived from the human Bowes melanoma receptor cDNA sequence and standard methods. The first round of amplification used the primers, 5'-GCCAGCAACCAGACCTTC-3' and 5'-ATTGGT-TGATGGTGGGGT-3' to produce a 700 bp product which served as a template for a second round of amplification using the primers, 5'-TGAATTCCTGCATCTGCTTCTGCTA-3' containing an *EcoRI* site and 5'-TACTAGTTGATGGTGGGGTGTCTATTC-3' containing a *SpeI* site. Two putative cDNA clones were sequenced and both showed 84% identity with the corresponding nucleotides 637-1030 of the human galanin receptor cDNA sequence (22), and were identical to the rat galanin receptor cDNA sequence previously reported (23). An approximately 400 bp PCR product was inserted into *EcoRI/SpeI* digested Bluescript KS (Stratagene, San Diego, CA).

Probe preparation

For *in vitro* antisense galanin cRNA probe and galanin receptor cRNA probe syntheses, the linearized plasmid DNA was transcribed with T7 RNA polymerase. A mixture of 25% [³⁵S] α -thio-UTP (New England Nuclear Research Products, Boston, MA) and 75% α -thio-UTP (cold) at a total concentration of 50 μM was used in the galanin probe synthesis; 10 pmol ³³P-UTP (DuPont-New England Nuclear, Wilmington, DE) was used in the synthesis of the galanin receptor probe. Residual DNA was removed by digestion with DNase (Promega Biotec, Madison, WI), and the cRNA probes were purified on a Nensorb column (New England Nuclear-DuPont, Wilmington, DE). The identity and integrity of both transcripts were verified by polyacrylamide gel electrophoresis against known standards. Digoxigenin-labeled cRNA probes for GHRH mRNA and SS mRNA were synthesized from linearized plasmid DNA, transcribed *in vitro* from a digoxigenin labeling mixture containing 3.5 mM digoxigenin UTP, 6.5 mM unlabeled UTP, and 10 mM GTP, ATP, and CTP at pH 6.5 (Boehringer Mannheim, Indianapolis, IN), and T7 RNA and SP6 RNA polymerase, respectively. Residual DNA was removed by digestion with DNase and the probes were purified on a G-50 Sephadex column (Boehringer Mannheim, Indianapolis, IN).

Control experiments

Three control experiments were performed to assess the specificity of binding of the galanin receptor riboprobe to the tissue. These control experiments have been performed in our laboratory previously for the

GHRH, galanin, and SS probes (24–26). First, the tissue was treated with RNase A before the placement of ^{33}P -labeled antisense probe. Second, excess unlabeled antisense probe in the presence of radiolabeled antisense probe was placed on the tissue. Third, a sense probe was applied to the tissue. The tissue was processed for single-label hybridization histochemistry as previously described (27). These experiments resulted in complete loss of photographic grain clusters over cells, suggesting that the ^{33}P -labeled probe binds to a specific sequence in the galanin receptor mRNA (data not shown). Further, the distribution of the rat galanin receptor mRNA in the rat brain by single-label *in situ* hybridization (data not shown) was similar to that previously described (23).

Double-label *in situ* hybridization

Using the ^{35}S -labeled riboprobe complementary to galanin mRNA, the ^{33}P -labeled riboprobe complementary to galanin receptor mRNA and the digoxigenin-labeled GHRH cRNA probe and SS cRNA probe, we performed double-label *in situ* hybridization to identify cells containing both GHRH mRNA and galanin mRNA (Exps 1 and 2) and cells containing both GHRH mRNA and galanin receptor mRNA, and SS mRNA and galanin receptor mRNA (Exp 3) following a protocol as previously described (26), with modification. In brief, after fixation in 4% paraformaldehyde, tissue sections were rinsed in phosphate buffer and pretreated with 0.25% acetic anhydride in 0.1 M triethanolamine (pH = 8) for 10 min. The slides were rinsed in $2 \times \text{SSC}$ ($1 \times \text{SSC} = 150 \text{ mM NaCl}$ and 15 mM Na citrate), dehydrated in ethanol, delipidated in chloroform, partially rehydrated in ethanol and air-dried. Before the hybridization procedure, both riboprobes were heat denatured and diluted to a concentration of approximately 2 pmol/ml in a hybridization buffer containing 52% formamide, 10% dextran sulfate, 0.3 M NaCl, 8 mM Tris, 0.8 mM EDTA, 0.02% BSA, 0.02% polyvinylpyrrolidone, 200 mM dithiothreitol, 0.02% Ficoll and 0.5 mg/ml yeast transfer RNA. Since the exact yield of the transcription reaction with digoxigenin-UTP for the GHRH cRNA probe and SS cRNA probe could not be derived, a test *in situ* hybridization was performed to determine empirically the optimal concentration for these probes. The slide was covered with a silanized coverslip, and the sections were incubated overnight in moist chambers at 60 C. On the following day, the coverslips were rinsed off in $2 \times \text{SSC}$. Following treatment with RNase A and a series of washes in SSC of increasing stringencies, the slides were placed in a blocking buffer containing $2 \times \text{SSC}$, 0.05% Triton X-100 and 2% normal sheep serum (NSS) for 60 min. The slides were washed in buffer 1 (100 mM Tris-HCl, pH 7.5, and 150 mM NaCl) for 30 min and then incubated for 3 h at 37 C with antidigoxigenin antibody conjugated to alkaline phosphatase (Boehringer Mannheim Biochemical, Indianapolis, IN; 1:1000 in buffer 1 containing 1% NSS and 0.3% Triton X-100). The slides were washed in buffer 1 again and rinsed in buffer 2 (100 mM Tris-HCl, 100 mM NaCl, 50 mM MgCl_2 , pH 9.5) for 10 min; they were then incubated in buffer 2 with 340 $\mu\text{g/ml}$ nitro-blue-tetrazolium-chloride (Sigma, St. Louis, MO) and 175 $\mu\text{g/ml}$ 5-bromo-4-chloro-3-indolyl-phosphate (Sigma, St. Louis, MO), and 240 $\mu\text{g/ml}$ levamisole in a light-tight box for 3 h at 37 C. The reaction was stopped by rinsing the slides in 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA. The slides were placed in 70% ethanol for 15 sec, air-dried, dipped in 3% parlodion (Fisher Scientific, Fair Lawn, NJ) dissolved in isoamyl acetate, and allowed to air-dry. The slides were then dipped in, for Exps 1 and 2, Kodak NTB-2 and, for Exp 3, Kodak NTB-3 emulsion (Eastman Kodak, Rochester, NY), heated to 45 C in a water bath and diluted 1:1 with 600 mM ammonium acetate and distilled water, respectively. They were air-dried for 45 min and placed in light-tight boxes containing small tubes of desiccant and were exposed at 4 C. Hybridization assays for GHRH mRNA/galanin mRNA required 2 weeks of exposure, whereas those involving the galanin receptor mRNA required 4 weeks. Slides were then developed in Kodak D-19 (diluted 1:1 with distilled water), fixed with Kodak fixer, rinsed in water, dehydrated through alcohols, and cleared in Hemo-De (Fisher Scientific, Springfield, NJ), and coverslips were applied.

Image analysis

Forty-five tissue sections per animal equally spaced from the PeN through the ARC were examined. For Exps 1 and 2, anatomically matched slides were assigned a random three letter code, alphabetized, and analyzed in random order with an automated image processing

system. This system consisted of a PixelGrabber video acquisition board (Perceptics, Knoxville, TN) attached to a Macintosh IIfx computer (Apple Computer Corporation, Cupertino, CA). The tissue sections were viewed under a Zeiss Axioskop microscope (Zeiss, New York, NY) equipped with a 40 X epi-illumination darkfield objective. Video images were obtained by a Dage model 65 camera (Dage-MTI, Inc., Michigan City, IN) attached to the microscope. For each section that was analyzed, silver grains over digoxigenin-labeled GHRH cells were counted and the number of grains per cell is referred to as mRNA signal level. For Exp 3, grain count analyses were performed with each section first being visualized under brightfield illumination and the boundaries of the cRNA/digoxigenin-containing cell bodies outlined. The illumination was then switched to darkfield and silver grains were counted over the outlined cells and over the area surrounding the cells (the background area). As previously described (28), silver grain centers were identified by local light intensity peaks above a threshold that is determined by the gray-level histogram over the background area. All GHRH mRNA- and SS mRNA-containing neurons were analyzed in this manner, and their anatomical locations were recorded. In addition, 5–10 randomly chosen SS neurons per tissue section were analyzed in the cortex. According to anatomical areas, signal to background ratios were calculated as previously described (29) to assess the percentage of GHRH and SS neurons that coexpress galanin receptor mRNA.

RIAs for rIGF-1 and rGH

Serum was collected from blood samples and frozen at -20 C until assayed. For Exp 1, serum rIGF-1 levels were determined by a double-antibody heterologous RIA after IGF binding proteins were physically separated with high-performance liquid chromatography (30) in the laboratory of Dr. Philip Zeitler of the Children's Hospital Medical Center at the University of Cincinnati School of Medicine. The antiserum, provided by Drs. Louis Underwood and Judson Van Wyk of the University of North Carolina, Chapel Hill, NC, was raised to human IGF-1 but recognized rat IGF-1. The ^{125}I -[Thr-59] IGF-1 was purchased from Amgen Biologicals (Thousand Oaks, CA). The intraassay coefficient of variation was $<7\%$. For Exp 2, serum levels of rGH were measured by a double-antibody RIA under the auspices of the RIA Core of the Population Center for Research in Reproduction at the University of Washington (Dr. William Bremner, Director) with reagents provided by National Pituitary Agency. The antibody used was antirat GH-RIA-5 from NIDDK, Bethesda, MD. The ^{125}I -rGH was purchased from Corning Hazelton, Inc. (Vienna, VA). Final values were expressed in terms of nanogram equivalents of NIDDK rat GH-RP-2 per ml of serum. The intraassay coefficient of variation was 6.6%, and the minimum amount of rGH detectable by the assay was 0.63 ng/ml.

Statistical analysis

Unless otherwise noted, all data are presented as mean \pm SEM. The "n" refers to the number of animals within a group, and this was the "n" used in the data analysis. For Exp 1, the difference between control and dwarf rats was determined by Student's *t* test. For Exp 2, the statistical comparison of signal levels among groups was performed by one-way ANOVA. Fisher's protected least significant difference test was used to identify specific differences between groups. For Exp 3, a one-sample

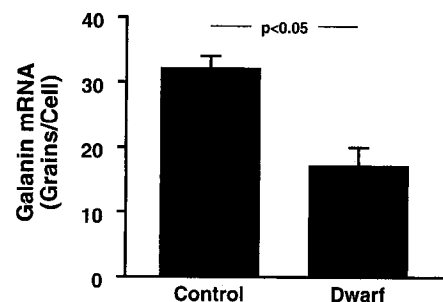


FIG. 1. Relative levels of galanin mRNA in the ARC GHRH neurons of Lewis control and dwarf animals. Values presented are the mean grains per cell \pm SEM.

sign test was used to determine whether galanin receptor signal was significantly greater than background (i.e., signal to background ratio > 1) and the Mann-Whitney *U* test was used to assess whether signal to background ratios differed significantly among various anatomical locations. A probability of 0.05 or less was considered significant for rejecting the null hypothesis that experimental and control values did not differ.

Results

Exp 1. Galanin mRNA levels in Lewis control and dwarf rats

As illustrated in Fig. 1, galanin mRNA levels in GHRH neurons, reflected as grains per cell, were reduced by 47% in the dwarfs, compared with controls ($P < 0.05$). Levels of IGF-1 in the serum of control Lewis strain rats were 434 ± 23 ng/ml, whereas levels were less than 261 ± 51 ng/ml in the dwarf rats, indirectly verifying the state of GH deficiency in the Lewis derived dwarf rats.

Exp 2. GH regulation of galanin mRNA levels

Galanin mRNA coexpression in ARC GHRH neurons varied as a function of GH state (Fig. 2). As shown in Fig. 3, galanin mRNA levels in GHRH neurons in the ARC were significantly less in hypox (vehicle-treated) animals, compared with the group of intact (vehicle-treated) animals and the group of hypox animals treated with rGH ($P < 0.0001$). There was no significant difference between the vehicle-treated intact group and the rGH-treated hypox group. Levels of GH in the serum of normal male rats were 51 ± 25 ng/ml, whereas levels were less than 1 ng/ml in hypox rats. Levels of GH were within the physiological range (94 ± 12 ng/ml) in 5 of the hypox rats that were treated with rGH. In the sixth rGH-treated hypox rat, the minipump was severed at the time of sacrifice, contaminating the trunk blood that was collected, and the GH level was not included in the analysis.

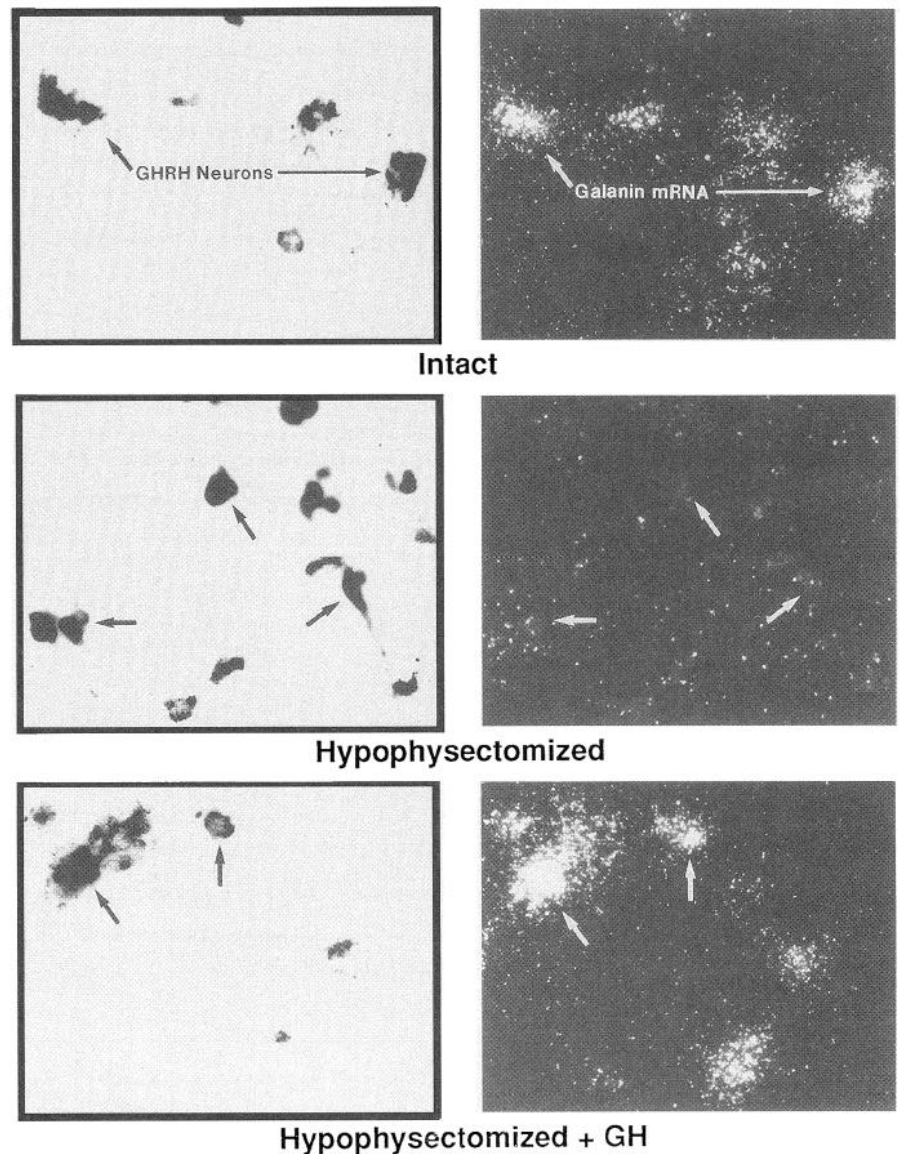


FIG. 2. Photomicrographs of neurons containing galanin mRNA and GHRH mRNA in the ARC of intact/vehicle (upper panel), hypox/vehicle (middle panel) and hypox/rGH (lower panel) rats. Black arrows in brightfield (left) point to examples of digoxigenin-labeled GHRH mRNA-containing neurons indicated by dark-stained cell bodies. White arrows in darkfield (right) point to the same GHRH mRNA-containing neurons with clusters of silver grains from the radiolabeled galanin probe.

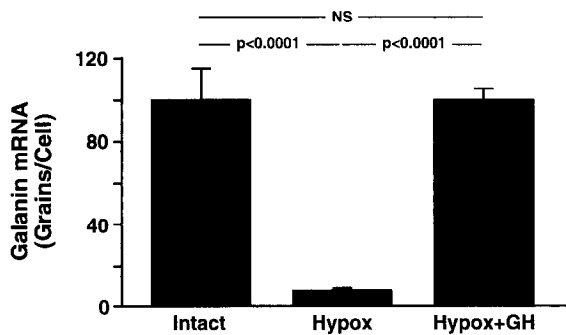


FIG. 3. Relative levels of galanin mRNA in the ARC GHRH neurons of intact/vehicle, hypox/vehicle and hypox/rGH groups. Values presented are the mean grains per cell \pm SEM.

Exp 3. Galanin receptor mRNA expression in SS and GHRH neurons

Photomicrographs showing galanin receptor mRNA expression in SS mRNA-containing neurons in the PeN and no apparent coexpression of galanin receptor mRNA in GHRH mRNA-containing neurons in the ARC are presented in Fig. 4. Galanin receptor mRNA levels were significantly higher than background levels in SS neurons in the PeN ($P < 0.0001$) but were indistinguishable from background in cortical SS neurons ($P > 0.5$) and GHRH neurons in the ventromedial nucleus of the hypothalamus (VMH) ($P > 0.8$). Although galanin receptor mRNA signal levels in ARC GHRH and SS neurons appeared to be slightly (but significantly) higher than background, these levels were not significantly different from galanin receptor mRNA levels in cortical SS neurons ($P > 0.15$ and $P > 0.45$, respectively). The percentage of SS and GHRH cells determined to coexpress galanin receptor mRNA is dependent upon the sensitivity of the *in situ* assay, the characteristics of the probe, and the acceptance criterion for calling a particular cell "double-labeled." For example, if all of the SS neurons in the PeN do, in fact, express galanin receptor mRNA, the percentage of SS cells identified as double-labeled will vary directly with the sensitivity of the assay for detecting the galanin receptor mRNA. To illustrate the uncertainty and difficulty of determining the actual percentage of neurons coexpressing galanin receptor mRNA, in Fig. 5 we show how the percentage of SS and GHRH neurons determined to express galanin receptor mRNA varies as a function of the signal to background acceptance criterion. A relatively low signal/background criterion of 3 would classify approximately 60% of the SS neurons in the PeN as containing galanin receptor mRNA and approximately 20–30% of the SS and GHRH neurons in the ARC, and SS neurons in the cortex as being double-labeled. The application of a more conservative criterion of 6 would result in approximately 35% of the SS neurons in the PeN and approximately 5% of the GHRH and SS neurons in the ARC being classified as double-labeled.

Discussion

The SS and GHRH genes are targets for GH regulation. Chomczynski and his colleagues have shown that GH inhibits hypothalamic GHRH gene expression (31), whereas our own work has demonstrated that GH stimulates the

expression of the SS gene in neurons of the hypothalamic PeN (24). We have also reported that mRNA levels of SS and GHRH vary inversely with one another as a function of the ultradian rhythm of GH secretion (32). The reciprocal regulation of SS and GHRH gene expression by GH identifies the fundamental elements governing the relationship between circulating levels of GH and the feedback control of SS and GHRH synthesis. The results of our present experiments refine this basic model by adding galanin as a target gene in GHRH neurons for the stimulatory action of GH in the hypothalamus of the rat.

Our studies were presaged by the earlier studies of Selvais *et al.* (33), who showed that hypophysectomy reduces hypothalamic content of galanin and galanin mRNA in the rat. In a follow-up study, this same group showed that replacement with GH alone did not reverse the effects of hypophysectomy but that only a combined replacement regimen of T_4 , cortisol, estradiol, and GH induced recovery of galanin mRNA in hypophysectomized animals (34). Our own results show that if the analysis is focused *only* on galanin mRNA expression in GHRH neurons, GH alone does reverse the inhibition of galanin expression caused by hypophysectomy—a result that would likely have been obscured in Northern blot analysis used by the Selvais group. Indeed, there are thousands of galanin-expressing neurons in the forebrain with many different phenotypes among them, some of which may be sensitive to estrogen or glucocorticoids with others being sensitive to GH, thyroid hormone, or some combination of these factors (see for example Ref. 35). The double-labeling technique permits focus on a single phenotype among the diverse array of these heterogeneous cells, and the results demonstrate that galanin expression in GHRH neurons is regulated by GH. It is also notable that since a constant infusion of GH produced by the minipumps completely reversed the effects of hypophysectomy on galanin expression in GHRH neurons, a pulsatile pattern of GH secretion does not appear to be essential for the maintenance of normal galanin expression in GHRH neurons.

The observation that GH induces galanin gene expression in GHRH neurons but suppresses the expression of the GHRH gene in the same cells suggests that GH acts on GHRH neurons via two different mechanisms, one stimulatory and the other inhibitory. We have previously shown that most GHRH neurons do not appear to respond directly to GH (36), so it seems likely that other GH-sensitive neurons relay the GH signal *indirectly* to GHRH neurons. Considerable evidence suggests that two neuronal systems may be involved in the relay function. The first is the population of SS neurons whose cell bodies reside in the PeN; these SS neurons express the GH receptor (28), and appear to make direct synaptic contact with GHRH neurons in the ARC (37). The second is the population of NPY neurons whose cell bodies are in the ARC. These NPY neurons express the GH receptor (29) and exhibit *c-fos* induction following acute exposure to GH (38). Hence, it is conceivable that one of these populations, either NPY neurons in the ARC or SS neurons in the PeN, mediates the stimulatory effects of GH on galanin gene expression in GHRH neurons and the other mediates the inhibitory effects of GH on GHRH gene expression.

The galanin synthesized in GHRH neurons is likely to be

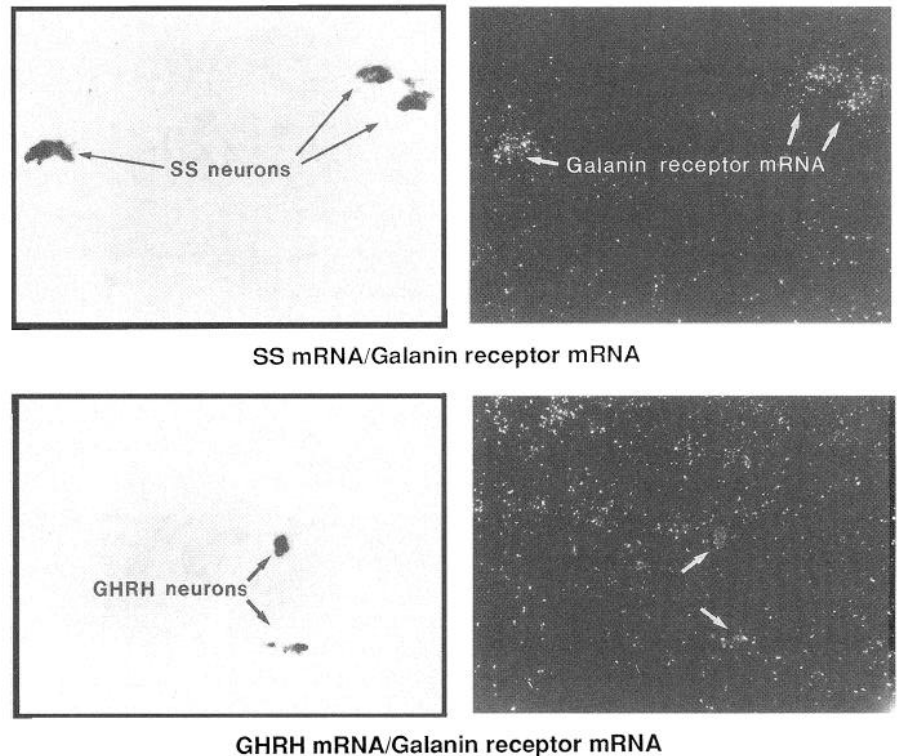


FIG. 4. Photomicrographs of SS neurons in the PeN expressing galanin receptor mRNA (upper panel) and GHRH neurons in the ARC lacking expression of galanin receptor mRNA (lower panel). In brightfield (left), black arrows point to SS mRNA- and GHRH mRNA-containing neurons that are filled with a dark digoxigenin reaction product. In darkfield (right), white arrows point to the corresponding cells; white silver grain clusters indicate galanin receptor mRNA.

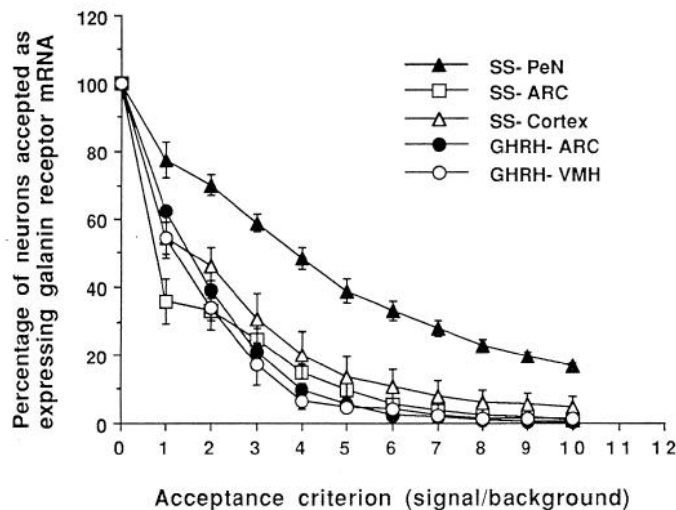


FIG. 5. Estimated percentage of GHRH and SS neurons in various anatomical areas accepted as coexpressing galanin receptor mRNA at different signal to background ratios. The vertical bars at each acceptance criterion indicate the SEM of the estimated percentage.

involved in the regulation of GH secretion, but the site of its action remains to be determined. Galanin from GHRH neurons may act either within the brain as a neurotransmitter or, following its release into the hypophysial portal system, at the anterior pituitary as a hypophysiotropic factor. Infusions of galanin, either by intracerebroventricular or peripheral routes, induce GH secretion (9, 39). Galanin binding sites have been identified in the anterior pituitary and galanin has been proposed to have direct post-synaptic effects at the pituitary (40-43); however, several *in vitro* studies have shown that galanin is ineffective in evoking GH secretion

from cultured pituitary cells (9, 44). Acting at the hypothalamic level, galanin could induce GH secretion either by facilitating the release of GHRH or inhibiting the release of SS. The observation that GHRH antiserum blocks galanin-induced GH secretion has been used as evidence to suggest that the effects of galanin are mediated by GHRH neurons (16). However, since somatostatin can influence the release of GH from somatotropes only in the presence of GHRH, this observation does not rule out an action of galanin on SS instead of, or in addition to, an action on GHRH. Furthermore, galanin potentiates the GH response to GHRH infusion (8) and pretreatment with SS antiserum diminishes galanin-induced GH secretion (45, 46), suggesting here that galanin may induce GH secretion by inhibiting the SS pathway. Therefore, the most parsimonious explanation for the collective results to date points to SS neurons being the primary hypothalamic target for the action of galanin on GH secretion. This possibility is further supported by evidence that galaninergic fibers synapse on SS neurons in the PeN (17).

The origin of galaninergic connections to SS neurons in the PeN has not yet been characterized. Nevertheless, both galanin and GHRH are neurotransmitter candidates at these synapses, because GHRH neurons express galanin mRNA and there is evidence for GHRH neurons making direct synaptic contact with SS neurons (37). It is conceivable that GHRH neurons use GHRH itself as a transmitter to communicate with other neurons. Certainly the primary site of GHRH action is at the anterior pituitary, where it acts to stimulate the secretion of GH from somatotropes (47); however, to date, there has been no clear evidence for expression of the GHRH receptor in the brain. Thus, it seems equally plausible that communication between GHRH neurons and

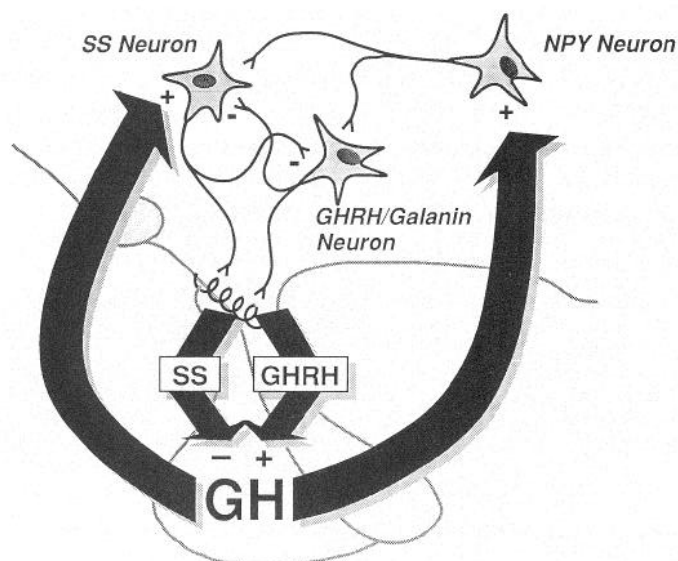


FIG. 6. A model illustrating the putative pathways involved in the short-loop feedback regulation of GH secretion in the rat.

their targets may involve its cotransmitter, galanin. Alternatively, and in addition to acting on SS neurons in the PeN, galanin coreleased with GHRH from the axon terminals in the median eminence could act presynaptically on receptors to enhance the release of GHRH. In this instance, galanin would modulate GHRH release in a manner analogous to that proposed for the action of galanin in the septo-hippocampal galanergic/cholinergic system (5, 48). If this were the case, the galanin receptor would be expressed in GHRH neurons.

To test the possibility that galanin can act directly on GHRH neurons in the ARC and SS neurons in the PeN, we examined galanin receptor mRNA expression in these neurons. Our finding that a subset of SS neurons in the PeN expresses galanin receptor mRNA extends the earlier report of galanin-immunoreactive fiber innervation of SS neurons in the PeN (17) and suggests that these SS neurons are the primary targets for galanin. The molecular mechanism of the presumptive action of galanin on SS neurons is unknown; however, based on *in vitro* studies of the action of galanin (see Ref. 49 for review), we may infer that galanin inhibits the expression of SS, thereby increasing GH secretion. Unlike SS neurons in the PeN, few, if any, GHRH neurons appear to express galanin receptor mRNA. This suggests that other galanin-sensitive neurons (perhaps SS neurons in the PeN) are involved in mediating any effects galanin may have on GHRH secretion (35). Still, our results do not rule out the possibilities that GHRH neurons express very low levels of galanin receptor mRNA or that GHRH neurons express mRNA of another galanin receptor subtype.

Based on the current state of knowledge on the expression of the GH receptor in SS and NPY neurons, the synaptic interactions among the various populations of neuropeptide-containing neurons, and the expression of the galanin receptor by SS neurons, we postulate a model for the short-loop feedback control of GH secretion and the putative role of SS, GHRH, galanin, and NPY in effecting this interaction (see Fig. 6). In this model, SS neurons in the PeN and NPY neu-

rons in the ARC are the primary targets for receiving feedback information about circulating levels of GH. GH stimulates the synthesis of both SS and NPY, and with some delay, enhances secretion of these neuropeptides. We postulate that both NPY and SS act on GHRH/galanin neurons, although the precise molecular basis of their actions on these neurons is unknown, since there are many different NPY and SS receptor subtypes. (This is an important area for future investigations.) Acting through either SS or NPY neurons, GH inhibits the expression of the GHRH gene and stimulates expression of the galanin gene. After some delay, GHRH/galanin neurons become activated and GHRH is released into the portal circulation to stimulate GH secretion, while at the same time, galanin is released at synapses on cell bodies or dendrites of SS neurons in the PeN. Galanin suppresses SS gene expression and diminishes the secretory activity of these SS neurons, for as long as the readily releasable stores of GHRH and galanin permit. Once these stores of GHRH and galanin are depleted, the process shifts to enhanced SS secretion, whose stores have been replenished by the action of GH on SS synthesis. These interactions allow for communication among the relevant populations of neurons, and given the time necessary for transcriptional events to be translated into proteins and for proteins to transport to axon terminals, the reciprocal events become expressed as the ultradian rhythm in GH secretion (32).

According to this model, galanin accentuates the pulsatile nature of GH secretion by inhibiting SS release, and subsequently reducing SS inhibition of GH and GHRH release during pulse generation. This is in agreement with evidence that the administration of galanin antiserum reduces pulse amplitude in male rats (10). It is also consistent with what is known about the relationship between galanin mRNA levels in GHRH neurons and pulsatile GH secretion. In the adult rat, GH pulse amplitude is much greater in the male than in the female (14) and the level of galanin mRNA expression in GHRH neurons is also considerably higher in males than females (13). Galanin mRNA levels in GHRH neurons increase across pubertal development in the male rat (13), in parallel with a dramatic increase in the amplitude of pulsatile GH secretion (11, 14). Finally, in the adult male, testosterone increases both the amplitude of GH pulses and the expression of galanin mRNA in GHRH neurons (14, 13). Therefore, even though the model we have proposed should be considered preliminary, it is consistent with the available evidence and should provide a useful framework for the design and interpretation of future studies.

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