Acute and Chronic Galanin Administration Decreases Hypothalamic Galanin Synthesis in Both Male and Female Adult Rats: Evidence for a Long-Loop Galanin Autofeedback

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The secretion of growth hormone (GH) in both male and female rats is controlled by two main neuropeptides, GH-releasing hormone (GHRH), which is stimulatory, and somatostatin, which is inhibitory. Recently, it has been shown that galanin (GAL) also stimulates GH secretion, although the underlying mechanism is still unknown. It was the aim of this study to begin to elucidate if and how GAL regulates its own production at the hypothalamic and pituitary level. Rats underwent the following experimental trials. In experiment 1, adult male and female rats had blood samples collected at −15 minutes, −7.5 minutes, and immediately preceding a subcutaneous (SC) injection of GAL at a dose of either 50 or 200 μg/kg. Blood samples were collected at 5, 10, 15, 30, and 60 minutes, and the GH concentration was measured using a radioimmunoassay. The tissues were collected and analyzed for mRNA levels of hypothalamic and pituitary GAL. In experiment 2, adult male and female rats were treated long-term with 200 μg/kg GAL for 7 days SC, and the pituitary and hypothalamus were analyzed for GAL mRNA. Serum GH concentrations were significantly increased in acutely dosed male and female rats regardless of the dosage level. For the male and female animals acutely dosed with both 50 and 200 μg/kg GAL, hypothalamic GAL mRNA was decreased, whereas pituitary GAL mRNA was affected by 200 μg/kg GAL only in females (increased). For the animals treated long-term with GAL, hypothalamic GAL mRNA was decreased while mRNA for pituitary GAL was increased. We conclude that regardless of the dosage and duration of treatment, administration of GAL negatively regulates hypothalamic GAL mRNA in a non–gender-specific way. Pituitary GAL synthesis appears to be stimulated particularly during chronic SCGAL administration.

GROWTH HORMONE (GH) secretion in vivo is controlled mainly by two neuropeptides, GH-releasing hormone (GHRH), which is stimulatory, and somatostatin, which is inhibitory. The effect of these hypothalamic peptides is to produce a pulsatile pattern of GH secretion in both male and female rats, although the pattern in females is somewhat less distinct.[1,2] A third potential neuropeptide involved in regulating GH secretion is galanin (GAL). It was first discovered and isolated from porcine intestine in 1983 by Tatemoto et al[3] during a systematic search for amides of peptides found in the intestinal regions. It is composed of 29 amino acids, and it has been shown that the first 2 *N*-terminal amino acids are essential for its function.[4,5] GAL has been localized to a number of regions within the body, with primary immunoreactivity found in the digestive and central nervous system.[6,7] It has also been localized to the neurohypophysis,[7] the median eminence,[8] and specific areas of the hypothalamus such as the arcuate nucleus, periventricular nucleus, and ventromedial hypothalamus.[9]

The biological activity of GAL is very diverse, presumably reflecting functions related to its location within the body. While there is evidence that GAL acts to regulate intestinal motility[6] and to modulate the endocrine pancreas,[10,11] most of what is known about GAL involves its control of the anterior pituitary gland. It is generally accepted that GAL can increase the secretion of GH in a dose- and time-dependent fashion both in the rat and in normal humans,[12,13] although the underlying mechanism is largely unknown at this point. It has been suggested that GAL works via GHRH[12,14] to produce the increase in GH, although it has been shown that GAL acts directly at the level of the pituitary using a mechanism distinct from that of the hypothalamic peptides GHRH and somatostatin.[15,16] It has also been suggested that GAL effects are mainly due to catecholaminergic pathways controlling somatostatin-containing neurons.[17] Concerning the regulation of GAL production at the molecular level, it has already been shown that in the human GHRH transgenic mouse, the GAL peptide and mRNA levels are increased in the anterior pituitary and hypothalamus with respect to nontransgenic mice.[18] In contrast, it has been shown that in female animals given high-dose GH, GAL mRNA levels were unchanged.[19] However, to date, the molecular mechanisms underlying the GAL regulation of its own secretion at the hypothalamic and pituitary level have not been investigated.

It was the purpose of this study to begin to determine the molecular effects of acute and chronic GAL administration in male and female rats on hypothalamic and pituitary GAL synthesis.

MATERIALS AND METHODS

Animals

Adult male and female Sprague-Dawley rats were housed in a temperature- and humidity-controlled environment and exposed to a 14-hour light/10-hour dark lighting schedule (lights on at 6 AM). The animals had free access to food and water throughout the experimental period. All animal procedures were approved by the University of Wisconsin-Milwaukee Animal Care and Use Committee. The female rats were studied in the follicular phase of their estrous cycle.

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Molecular Biology Procedures

For tissue collection, the animals were decapitated and the pituitary and hypothalamus, delineated anteriorly by the optic chiasm, posteriorly by the mammillary bodies, laterally by the sulci formed with the temporal lobes, and superiorly by a plane 3 mm dorsal to the ventral surface of the median eminence, were removed and stored at −80°C for 24 to 48 hours prior to RNA extraction. Total RNA was extracted using the method of Chomczynski and Sacchi[20] unless otherwise stated. After extraction, RNA was quantified using a spectrophotometer at absorbancies of 260 and 280 nm. One microgram of RNA was size-fractionated on a 1.2% agarose gel containing 2.2 mol/L formaldehyde as previously described,[21] to confirm the quality and evenness of loading of total RNA.

Radioactive sense and antisense riboprobes for the RNase protection assays were produced from cDNAs for GAL (a gift from Dr M. Vrontakis, Winnipeg, Manitoba, Canada). The cDNAs were linearized with the appropriate restriction endonuclease before production of the radioactive cRNA. The clones were transcribed with an in vitro transcription kit (Ambion, Houston, TX) according to the manufacturer's instructions. The riboprobes were hybridized overnight at 42°C with 5 μg total RNA from either control tissue or GAL-treated animals. The products of the reaction that included the mRNA:radioactive RNA probe hybrids were then subjected to digestion with RNase One (Promega, Madison, WI) for 1 hour at 30°C, incubated with proteinase K for 15 minutes at 37°C, phenol/chloroform-extracted, precipitated, and centrifuged.[22] After centrifugation, the hybrid pellets were resuspended and resolved on an 8% acrylamide denaturing gel and subjected to autoradiography for 15 hours at −80°C. A clone for the 18S subunit of ribosomal RNA was used as an internal loading control. α-Actin was not used as an internal control in the RNase protection assays, since manipulations that alter the endocrine system are known to modify α-actin levels.[23,24] Densitometric results for the RNase protection assays were determined using the Bioscan Optimas Digital Imaging System, Version 3.01 (Bioscan, Edmonds, WA). Densitometric results for the RNase protection assays are reported either as the mean ± SEM (acutely dosed rats) or as a percent of the value obtained in control-treated rats (control values were assigned a value of 100%).

Acute GAL Treatment

Adult male and female animals were prepared with intravenous catheters under ether anesthesia as previously described[25] 3 days before experimentation. At approximately 9 AM on the day of experimentation, a control blood sample (0.3 mL) was drawn at −15 minutes, −7.5 minutes, and immediately preceding (time 0) a subcutaneous (SC) injection of either 50 μg/kg (males, n = 6; females, n = 6) or 200 μg/kg (males, n = 7; females, n = 4) rat GAL in 2 mL saline (courtesy of Dr D. Coy, Tulane University, New Orleans, LA) or 2 mL saline (males, n = 6; females, n = 6). Blood samples were collected at 5, 10, 15, 30, and 60 minutes after GAL injection, and the animals were then killed. To confirm that GAL produced an increase in GH secretion, serum GH concentrations were measured by radioimmunoassay using a double-antibody method and reagents provided by the National Institutes of Health (Bethesda, MD). The within-assay coefficient of variation was approximately 8%, and the between-assay coefficient of variation was 10%. Serum GH peak data were analyzed using the Wilcoxon signed-rank test. A P level less than .05 was chosen to identify significant treatment effects.

The hypothalamus and pituitary from the animals were harvested, immediately frozen in dry ice, and stored at −80°C. RNA was extracted using Trizol (GIBCO Life Technologies, Grand Island, NY). All mRNA data were analyzed using ANOVA and the Bonferroni test.

Chronic GAL Treatment

To determine if the presence of an enhanced GAL tone over an extended period influenced GAL production, adult male (n = 10) and female (n = 6) rats were treated SC daily (9 AM) for 7 days, including the day of experimentation, with the highest doses of GAL used in the acute experiment, ie, the 200-μg/kg GAL dose in 2 mL saline. A group of 6 male and 6 female rats received SC saline 2 mL for 7 days. On the day of experimentation, tissue samples were collected as described in experiment 1.

RESULTS

Acute GAL Treatment

GAL SC injection acutely increased serum GH secretion dose-dependently in both male and female rats, without any sex differences. The data reported in Fig 1 are therefore pooled. The 200-μg/kg dose caused a more than 2-fold GH peak response as compared with the 50-μg/kg dose (Fig 1).

Male rats. The effects of 50 μg/kg SC GAL on the level of mRNA for hypothalamic and pituitary GAL of male rats are illustrated in Fig. 2. There was no significant change for pituitary GAL. Hypothalamic GAL was significantly decreased compared with control-treated tissue. The effect of the 200-μg/kg SC GAL dose on mRNA levels is also reported in Fig 2. Hypothalamic GAL mRNA was significantly reduced compared with control-treated tissue, but was slightly increased compared with the 50-μg/kg GAL dose. Pituitary GAL mRNA remained unchanged.

Female rats. The effects of an acute SC administration of a 50-μg/kg dose of GAL on the mRNA levels of hypothalamic and pituitary GAL in the adult female rat are depicted in Fig 3. Hypothalamic GAL mRNA was decreased (53%) compared with the control tissue mRNA levels. Pituitary GAL was increased 205% versus the control mRNA levels. Following the 200-μg/kg dose of GAL, hypothalamic GAL mRNA was decreased to 30% of control tissue mRNA. Pituitary GAL was increased over 3-fold compared with mRNA levels.

Chronic GAL Treatment

The effects of chronic SC administration of 200 μg/kg GAL on the mRNA levels of hypothalamic GAL and pituitary GAL are shown in Fig 4. As with the acute GAL administration, hypothalamic GAL mRNA was decreased (up to 27%) compared with control tissue mRNA levels. Pituitary GAL was increased to 149% of the control tissue mRNA levels in male rats and to 230% in female rats after GAL administration.

DISCUSSION

The mechanism for GAL regulation of its own synthesis and secretion is still unclear.[26] The data presented here describe the effects of acute and chronic administration of GAL on hypothalamic and pituitary GAL mRNA levels in adult male and female rats. Our data show that a single SC injection of GAL is able to acutely (60 minutes) and dramatically (85%) decrease GAL synthesis at the hypothalamic level in both male and female normal adult rats. This is suggestive of a negative-feedback situation in which circulating GAL autoregulates its own synthesis at the level of the hypothalamus. Interestingly, our

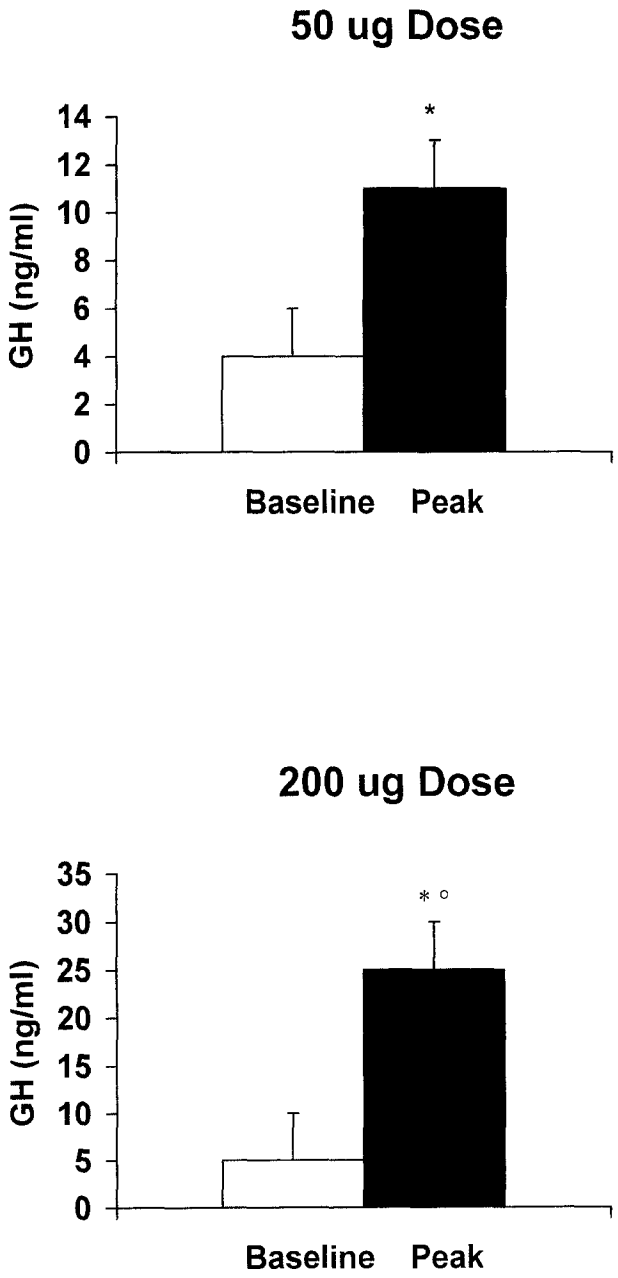

Fig 1. Serum GH peak (■) concentrations after SC administration of either 50 or 200 μg/kg GAL in male and female rats. Baseline (□) samples were taken at time 0 immediately preceding the injection of GAL. Peak GH levels represent the concentration of GH at the highest point after the injection. Data were analyzed using the Mann-Whitney *U* test for independent samples, and significance was assigned at $P < .05$. *$P < .05$ *v* baseline; °$P < .05$ *v* 50 μg.

data show that with the lower dose (50 μg/kg) of GAL, there is already maximal acute inhibition of hypothalamic GAL mRNA. Combined, these two findings suggest that the hypothalamic synthesis of GAL is very tightly regulated by circulating GAL, since changes in hypothalamic mRNA levels are pronounced and already maximal very early after increases in circulating GAL levels which are biologically effective but do not cause the maximal biological effect, at least in terms of a GH increment.[27]

The data presented here complement the studies of Lopez et al,[27] who demonstrated that in the arcuate nucleus of the hypothalamus, there are GAL-containing perikarya and proximal dendrites that are able to regulate their own activity via axodendritic synapses. Our study shows that in the presence of an anatomical basis for interactions between GAL immunoreactive neurons, a negative long-loop feedback mechanism is at work.[28] We did not measure plasma GAL after GAL administration, and therefore, the exact extent and duration of elevated GAL levels after dosing is not known. On the other hand, as mentioned before, we had a positive biological control, since

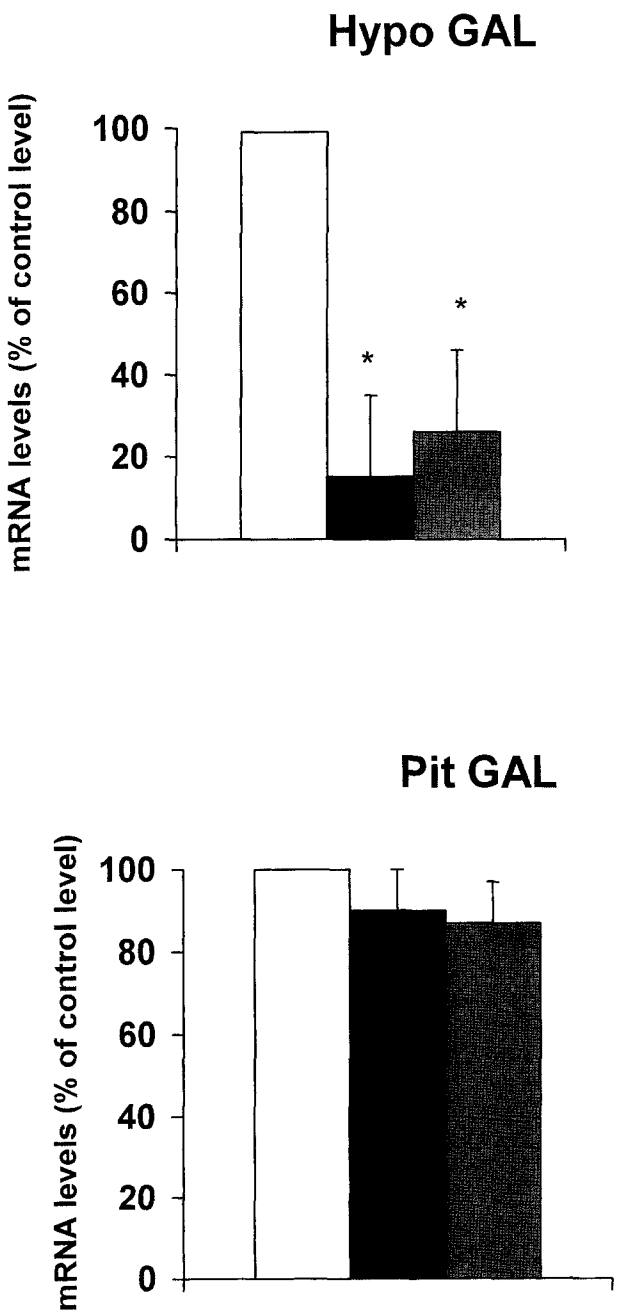

Fig 2. Hypothalamic (Hypo) and pituitary (Pit) GAL mRNA for control tissue (□), tissue from adult male animals treated with 50 μg/kg GAL (■), and tissue from adult male animals treated with 200 μg/kg GAL (▨). *$P < .05$ *v* control tissue.

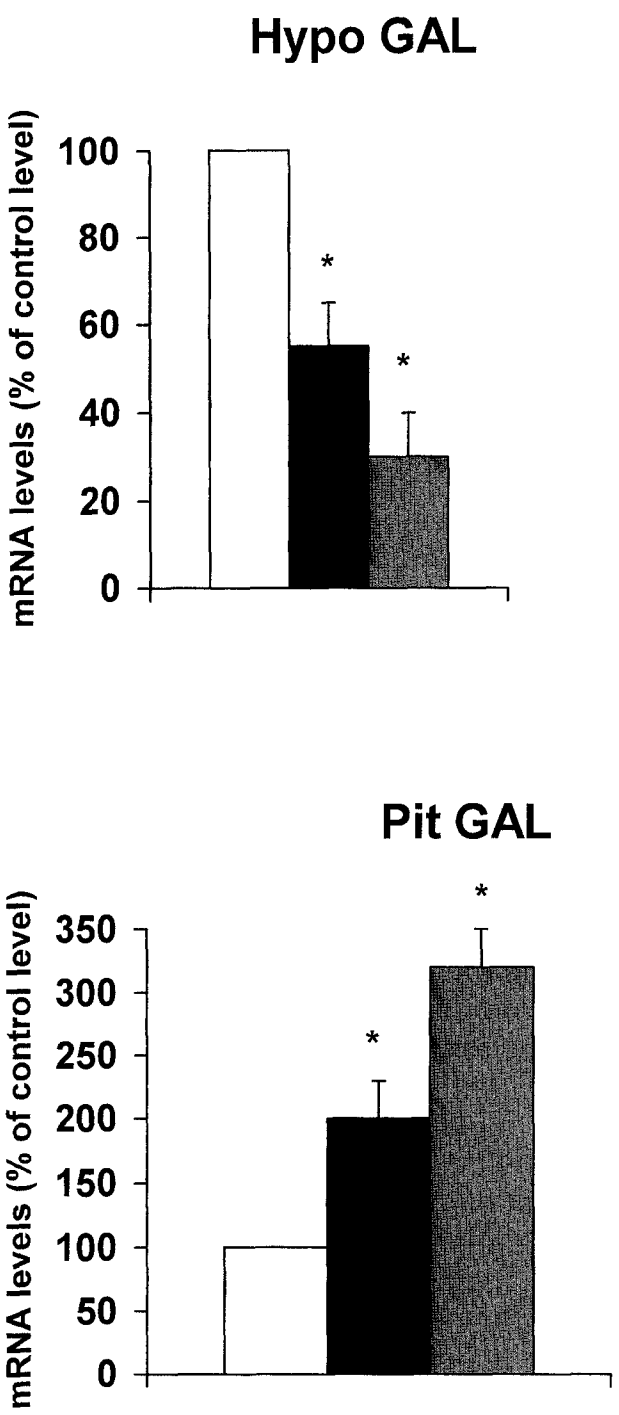

Fig 3. Hypothalamic (Hypo) and pituitary (Pit) mRNA for control tissue (□), and tissue from adult female animals treated with 50 μg/kg GAL (■) and 200 μg/kg GAL (▩). *$P < .05$ *v* control tissue.

the 2 doses of GAL were both able to significantly and acutely increase serum GH above baseline levels, with our highest dose of GAL (200 μg/kg) causing in both males and females the most significant GH response. However, the results presented in this study are not attributable to an increase in serum GH, as Selavais et al[19] demonstrated that the addition of GH to hypophysectomized female rats had no effect on hypothalamic GAL mRNA. Interestingly, and this is another finding supporting the existence of a tight and very sensitive GAL autofeedback independent of the GH-increasing effect of GAL, the acute decrease in hypothalamic GAL mRNA was similar with both doses of the peptide, whereas the 200-μg/kg dose caused a more pronounced effect on serum GH compared with the 50-μg/kg dose. Finally, the decrease in hypothalamic GAL mRNA caused by GAL administration was similar in both male and female rats. Interestingly, there are pronounced sex differences in the neuroregulation of GH secretion in the rat, due to the differential effect of estrogens and testosterone on the GH axis[1,2]; our findings suggest that GAL autofeedback occurs independently of the sex hormone milieu. If one considers that at least in the human, the GH-releasing effect of GAL is importantly regulated by estrogens,[29,30] it can be hypothesized that the GAL autofeedback is an independent phenomenon with respect to the biological effects of GAL. These findings suggest that in the rat, both circulating and central levels of the peptide need to be highly controlled, pointing toward a significant physiological role of GAL[31,32] in the regulation of primary functions such as food intake and sexual/reproductive behavior.[27]

Chronic GAL administration in both male and female rats consistently and persistently decreased hypothalamic GAL mRNA levels. These findings suggest that there is no central desensitization to the GAL negative-autofeedback mechanism, since the mRNA levels obtained after chronic and acute GAL administration are very similar. The findings of the chronic study showing the persistence of this control mechanism without receptor downregulation at the central level confirm our hypothesis that the rat requires a tight control of hypothalamic GAL synthesis depending on the level of circulating GAL and that GAL may play a significant physiological role in the rat. To hypothesize a direct-control effect of circulating GAL, it is necessary to postulate that the peptide can cross the blood-brain barrier. This has not been studied thus far, but it can be suggested as likely, as proven for longer peptides such as GHRH. Alternatively, it can be hypothesized that circulating GAL may indirectly influence hypothalamic GAL via its action on many peripheral target tissues or its action on GAL release at the median eminence level, or finally on GAL synthesis at the pituitary level. The first hypothesis is unlikely, since indirect effects are hard to foresee in acute administration studies, and pituitary effects of GAL do not seem to occur in the male rat. Finally, if circulating GAL exerts effects by inhibiting its own release at the median eminence level, one might expect an increase rather than a decrease in hypothalamic GAL synthesis after GAL administration. Our study does not give insight on the specific hypothalamic areas involved in the GAL autofeedback mechanism. In situ quantitative molecular approaches will be needed to designate the discrete anatomical areas (likely either the arcuate or periventricular nuclei) in which this effect of GAL occurs.

This same type of autoregulation of GAL would not be expected to occur at the level of the pituitary, as it has been shown in both male and female rats that pituitary GAL is estrogen-inducible.[28] In fact, the increase in circulating levels of GAL does not cause any change in pituitary GAL mRNA in the male rat. Conversely, in females, a trend for a dose-dependent increase in pituitary GAL mRNA after acute GAL administration was observed. In the chronically dosed male animals, as

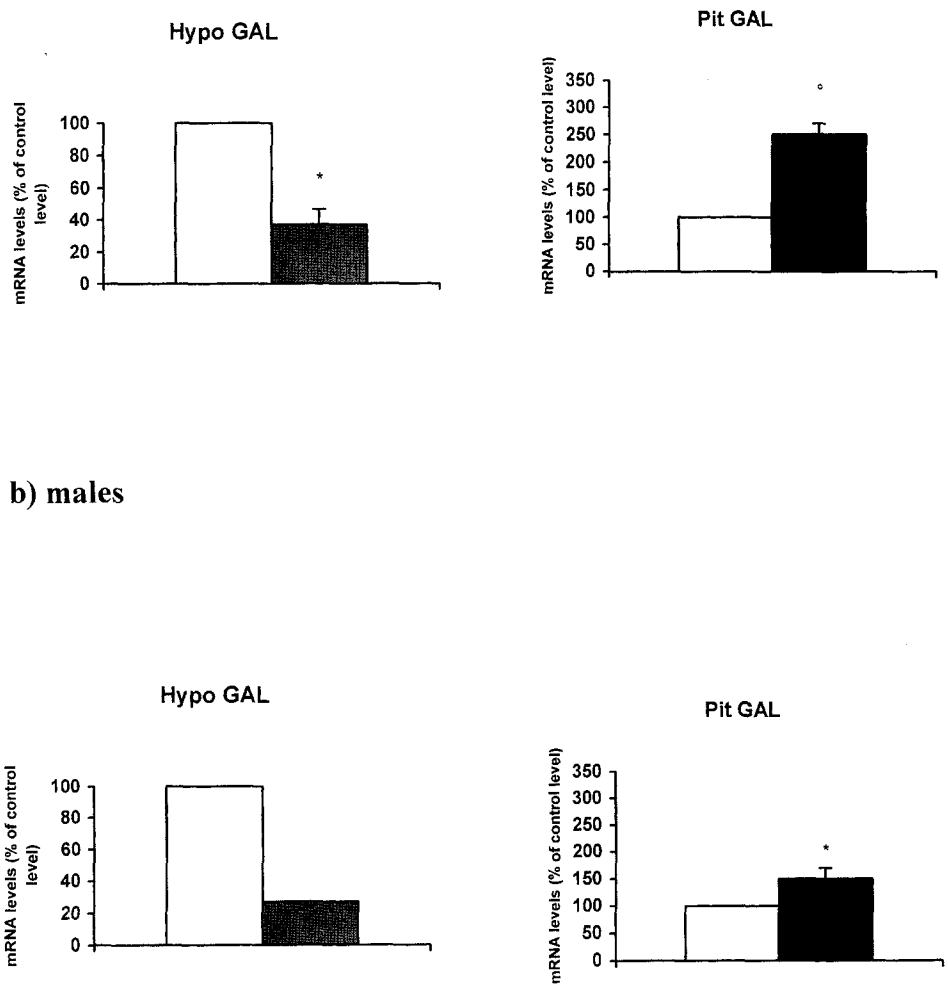

Fig 4. Hypothalamic (Hypo) and pituitary (Pit) mRNA for control tissue and treated tissue in (a) female and (b) male animals chronically treated with 200 μg/kg GAL as a percent of control (control assigned 100%). *$P < .05$ *v* control tissue; °$P < .05$ *v* males.

well as female animals, the level of pituitary GAL mRNA was increased. This may be due in part to effects of the other hypothalamic neuropeptides, such as GHRH, on the pituitary. In fact, a number of adaptative phenomena may occur in the GH axis following chronic GAL administration.[2] However, as mentioned earlier, hypothalamic GAL mRNA levels are decreased during chronic GAL administration, and there may be a generalized decrease in the amount of signal from the hypothalamus to the pituitary. These changes in the hypothalamus may be suggested to trigger the synthesis of pituitary-specific GAL synthesis if we assume that hypothalamic GAL may negatively regulate pituitary GAL synthesis. Alternatively, it can be suggested that circulating GAL may positively autoregulate its own synthesis at the pituitary level. This mechanism, in turn, may be implicated in the long-term maintenance of the negative long-loop autofeedback regulation of GAL (Fig 5). The finding in males that pituitary GAL is unaffected by acute GAL administration confirms that in the absence of estrogens it is difficult to induce pituitary GAL synthesis. In fact, only prolonged GAL administration triggers an increase of pituitary GAL mRNA in males. Alternatively, it may be hypothesized that time differences in the stimulatory effects on GHRH release mediated by GAL may exist between males and females.

It is interesting to note that although the growth axis in the rat model is somewhat different when comparing male and female animals,[1] every change in hypothalamic GAL with the administration of GAL, regardless of dose or gender, was in the same direction. In summary, the SC acute and chronic administration of GAL to both male and female rats indeed affects GAL synthesis at the molecular level. In fact, there is a generalized

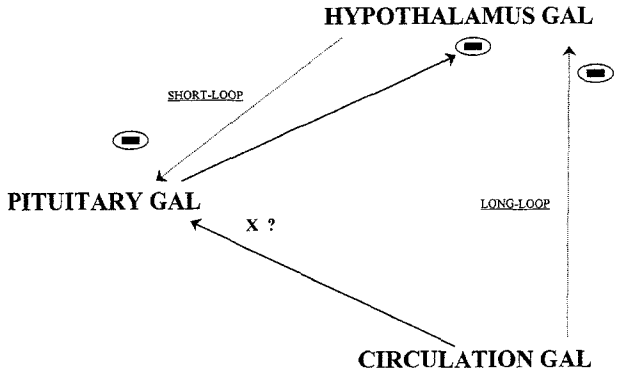

Fig 5. Schematic representation proposed for GAL autofeedback control mechanism.

decrease in hypothalamic GAL mRNA synthesis, indicating that GAL regulates its own hypothalamic mRNA synthesis in a negative fashion. Conversely, pituitary GAL synthesis is acutely increased only in females, whereas it is also increased in males after chronic treatment. This suggests that it is easier to induce an increase in pituitary GAL synthesis in females for the local endocrine conditions at the pituitary levels. However, this phenomenon, in both male and female rats, is likely an adaptative mechanism secondary to the decrease in hypothalamic GAL synthesis, suggesting, in turn, the existence of a short-loop feedback between hypothalamic and pituitary GAL (Fig 5).

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