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Effects of Galanin-Like Peptide on Food Intake and the Hypothalamo-Pituitary-Thyroid Axis

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Galanin · Galanin-like peptide · Neuropeptide Y · Food intake · Thyrotropin · Paraventricular nucleus · Thyrotropin-releasing hormone · Cocaine-and amphetamine-regulated transcript

Abstract
Galanin-like peptide (GALP) is a novel hypothalamic peptide synthesised in neurons in the arcuate nucleus which project to the paraventricular nucleus (PVN). GALP has recently been identified as an orexigenic peptide. In this study we aimed to further characterise the hypothalamic action of this peptide in energy homeostasis. Firstly, we investigated the orexigenic effect of GALP in the PVN and compared its effects with galanin and galanin 2–29. Secondly, we examined the effect of PVN administration of GALP and galanin on circulating thyroid-stimulating hormone (TSH). PVN administration of GALP significantly increased the food intake of satiated rats 1 h after administration at doses of 0.3, 1 and 3 nmol. In comparison with paraventricular administration of galanin, GALP was a more potent orexigen, whereas galanin 2–29, the relatively selective GAL R2 agonist, had no effect on food intake. Both GALP and galanin administration (1 nmol) into the PVN significantly decreased the level of circulating TSH. To investigate the mechanism of these effects, we examined the effect of GALP and galanin application on neuropeptide release from hypothalamic explants in vitro. GALP peptide (100 n*M*) stimulated the release of the orexigenic peptide neuropeptide Y from hypothalamic explants and decreased the release of the anorectic peptide cocaine-and-amphetamine-regulated transcript, whereas galanin (100 n*M*) peptide had no significant effect on the release of either peptide. Both GALP (100 n*M*) and galanin (100 n*M*) inhibited the release thyrotrophin-releasing hormone. These data suggest that in the PVN, GALP may play a role in energy homeostasis by stimulating food intake and suppressing TSH release.

Introduction

Galanin-like peptide (GALP) is a 60-amino-acid peptide recently isolated from the porcine hypothalamus [1]. Within the hypothalamus, GALP mRNA has been detected exclusively in the arcuate nucleus (ARC) where a proportion of GALP neurons express the leptin receptor but do not co-localise with neuropeptide Y (NPY), agouti-related protein (AGRP), α-melanocyte-stimulating hor-

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mone (α-MSH) or galanin peptide [2]. GALP-immunoreactive fibres project from the ARC to the anterior parvicellular part of the paraventricular nucleus (PVN), the medial preoptic area, the ventral part of the lateral septal nucleus and the bed nucleus of the stria terminalis [2]. GALP, as its name suggests, shows structural similarities to galanin and binds with high affinity to the galanin receptors. Recently it has been shown that GALP [3], like galanin [4], increases food intake after intracerebroventricular (ICV) administration and thus may be a regulator of feeding behaviour.

The receptor or receptors responsible for mediating the orexigenic effect of galanin and GALP have yet to be determined. To date, three galanin receptor subtypes (GAL R1–3) have been cloned [5–7]. All three receptors are expressed in the hypothalamus and galanin binds with high affinity to each. GALP was originally identified as a GAL R2 ligand. GALP and galanin show similar affinity and activity at GAL R2 but GALP has a 44-fold lower affinity and 180-fold lower activity at GAL R1 [1]. GALP's affinity and activity at GAL R3 are unknown. Highly selective galanin receptor antagonists are not available. However, the galanin receptor agonist, galanin 2–29, shows a degree of receptor selectivity. It binds to GAL R2 with similar affinity to galanin but with an approximately 50-fold lower affinity at GAL R1 and 10-fold lower at GAL R3 [8].

The distribution of GALP and its orexigenic properties suggest it may be involved in the hypothalamic circuits controlling energy balance. Such circuits regulate both energy intake and energy expenditure. Other orexigenic neuropeptides such as NPY and AGRP have been proposed to play a role in the regulation of energy expenditure in addition to their effects on food intake. A reduction in energy expenditure may be mediated, in part, by down-regulation of the hypothalamic-pituitary-thyroid (HPT) axis [9] and both NPY and AGRP have been shown to suppress circulating TSH levels when administered centrally [10, 11].

In these studies we aimed to further characterise the hypothalamic action of GALP in energy homeostasis. The distribution of GALP cell bodies and projections in the hypothalamus suggest GALP may mediate its orexigenic actions through the PVN. The PVN is associated with the control of ingestion and is also rich in neurons containing thyrotropin-releasing hormone (TRH) [12], the central hypothalamic regulator of the HPT axis. We have therefore examined the effect of GALP administration into the PVN in promoting positive energy balance; by measuring the effect of GALP on food intake and on circulating plasma thyroid-stimulating hormone (TSH). We have compared these effects with those of galanin. In addition, we have compared GALP's effect on food intake with galanin 2–29, a galanin fragment which has a similar pharmacological to profile to GALP at GAL R1 and GAL R2. Finally, we have used an in vitro hypothalamic explant system to examine if the actions of GALP on food intake and TSH release could be mediated by key hypothalamic neuropeptides associated with energy homeostasis.

Materials and Methods

Materials

Porcine GALP was purchased from Bachem (UK) Ltd (Merseyside, UK). Porcine galanin and galanin fragment 2–29 were synthesised using an automated peptide synthesiser (model 6 MPS, Advanced Chemotech, Louisville, Ky., USA). Peptides were purified to homogeneity by reverse-phase HPLC on a C_8 column and molecular weight was checked by mass spectroscopy [13]. Reagents for the hypothalamic explant experiments were supplied by BDH (Poole, Dorset, UK).

Animals

Adult male Wistar rats weighing 250–300 g were maintained in individual cages under controlled temperature (21–23°C) and light (12:12 h light-dark cycle lights on at 07:00 h) with ad libitum access to food (RM1 diet SDS, Witham, UK) and water. All animal procedures undertaken were approved by the British Home Office Animals (Scientific Procedures) Act 1986.

Intranuclear Cannulation and Injection

Rats were anaesthetised by intraperitoneal injection of a mixture of Ketalar (ketamine HCl 60 mg/kg; Parke-Davis, Pontypool, UK) and Rompun (xylazine 12 mg/kg; Bayer, Bury St. Edmunds, UK) and placed in a Kopf stereotaxic frame. Permanent 26-gauge stainless steel guide cannulae (Plastics One Roanoke, Va., USA) were stereotactically placed into the PVN as previously described [14] (co-ordinates; 1.8 mm posterior from bregma and 0.5 mm lateral from the midline, taken from the Paxinos and Watson rat brain atlas [15]). Rats were left for 1 week to recover from the surgical procedure. After this period the animals were handled daily for 1 week to acclimatise them to the study procedures and minimise stress. Three days prior to the beginning of the study, animals were acclimatised to the injection procedure by a sham injection. Substances were administered via a stainless steel injector placed in and projecting 1 mm below the tip of the guide cannula. All compounds were dissolved in 0.9% saline and injection of peptide or saline was in a volume of 1 μl over 1 min. A Hamilton gas-tight syringe was connected to an infusion pump to ensure an accurate and constant volume of delivery. Experiments were carried out during the early light phase (09:00–11:00 h). To prevent cumulative effects, there was a washout period of 3 days between injections. Histological sections of the hypothalami were examined at the end of the study and cannula position determined by two independent observers. Any animals with cannulae outside the PVN were excluded from the study (<10%).

Comparison of the Effect of GALP with Galanin on Food Intake

This study was of a cross-over design. The animals were divided into 5 groups (n = 16). On study day 1, half the animals received treatment 1 and half treatment 2. On study day 2, the animals which had received treatment 1 now received treatment 2 and vice versa. The treatments were as follows: saline/internal positive control, 0.03 nmol GALP/galanin, 0.3 nmol GALP/galanin, 1 nmol GALP/galanin and 3 nmol GALP/galanin. Therefore, each animal received one sham and two treatment injections. After injection, animals were returned to their cages, which contained a preweighed amount of chow. The remaining food was then weighed 1, 2, 4, 8 and 24 h post-injection.

Effect of Galanin Fragment 2–29 on Food Intake

Satiated rats received an injection of saline, 1 nmol galanin 2–29 or 1 nmol GALP into the PVN. The 1-nmol dose was chosen as this is the lowest dose at which both GALP and galanin significantly stimulate food intake. After injection, animals were returned to their cages, which contained a preweighed amount of chow. The remaining food was then weighed 1, 2, 4, 8 and 24 h post-injection.

Effect of PVN GALP Administration on Circulating TSH and Free Triiodothyronine (T_3)

Satiated rats received an injection of saline, 1 nmol GALP or 1 nmol galanin into the PVN. After injection, animals were returned to their cages with the food removed. Ten minutes post-injection, rats were decapitated and trunk blood collected into plastic tubes containing lithium heparin. Plasma was separated by centrifugation, frozen on dry ice and stored at –70°C until assay. The 1-nmol dose was chosen, as above, as this was the lowest dose at which both GALP and galanin significantly stimulate food intake.

Static Incubation of Hypothalamic Explants

The static incubation system was used as previously described [16]. A 1.7-mm slice was taken from the basal hypothalamus to include the PVN with a vibrating microtome (EnergyBeam Sciences Inc, Agawam, Mass., USA). The slice was incubated in artificial cerebrospinal fluid (aCSF) (20 n*M* $NaHCO_3$, 126 m*M* NaCl, 0.09 m*M* Na_2HPO_4, 6 m*M* KCl, 1.4 m*M* $CaCl_2$, 0.09 m*M* $MgSO_4$, 5 m*M* glucose, 0.18 mg/ml ascorbic acid and 100 μg/ml aprotinin), equilibrated with 95% O_2 and 5% CO_2 at 37°C. After an initial 2-hour equilibration period, the hypothalami were incubated for 45 min in aCSF (basal period) before being challenged with peptide for 45 min. Finally, the viability of the tissue was verified by a 45-min exposure to 56 m*M* KCl; isotonicity was maintained by substituting K^+ for Na^+. Explants were excluded from analysis if release during the K^+ exposure was less than basal release (<10%). The aCSF collected at the end of each period was frozen at –20°C until measurement of peptide immunoreactivity (IR) by radioimmunoassay (RIA).

Effect of GALP and Galanin on Release of Neuropeptides

Hypothalami were challenged with GALP or galanin (100 n*M*) during the peptide period. The aCSF was then assayed for NPY-IR, cocaine- and amphetamine-regulated transcript (CART)-IR, α-MSH-IR, AGRP-IR and TRH-IR.

Radioimmunoassays

Plasma TSH concentrations were assayed using reagents and methods kindly provided by the National Institute of Diabetes and Digestive and Kidney Diseases and the National Hormone and Pituitary Program (Dr. A. Parlow, Harbor University of California, Los Angeles Medical Centre) as previously described [11]. Plasma-free T_3 was measured in a commercial coat-a-count assay from Diagnostic Products Corp., Los Angeles, Calif., USA. NPY-IR, α-MSH-IR, CART-IR and AGRP-IR were measured using established RIAs developed in this laboratory [17–20]. TRH-IR levels were measured using reagents and methods kindly provided by H. M. Fraser, Medical Research Centre Reproductive Biology Unit, Edinburgh.

Statistical Analysis

Results are shown as mean values ± SEM. Data from the cross-over feeding study was analysed using a one-way ANOVA with repeated measures with a Dunnet's post-hoc test for comparisons against saline; direct comparison between GALP and galanin at equimolar doses was analysed by paired t test. Data from the hypothalamic explant experiments was analysed by paired t test between the basal and test periods. All other data was analysed using a one-way ANOVA with repeated measures with a Dunnet's post-hoc test. In all cases the level of statistical significance was set at $p < 0.05$.

Results

Comparison of the Effect of GALP with Galanin on Food Intake in Satiated Male Rats

PVN injection of GALP produced a dose-responsive increase in food intake at 1 h. GALP significantly increased food intake at doses 0.3, 1 and 3 nmol (1 h food intake: saline 0.7 ± 0.2 g, 0.3 nmol GALP 2.2 ± 0.6 g, $p < 0.05$, 1 nmol GALP 3.8 ± 0.5 g, $p < 0.001$, 3 nmol GALP 4.2 ± 0.6 g, $p < 0.001$, n = 16) (fig. 1). Food intake at all other time points was not significantly different from saline controls. Similarly, PVN injection of galanin also produced a dose-responsive increase in food intake at 1 h. Galanin significantly increased feeding at doses 1 and 3 nmol (1 h food intake: saline 0.7 ± 0.2 g, 1 nmol galanin 2.3 ± 0.3 g, $p < 0.05$, 3 nmol galanin 3.4 ± 0.3 g, $p < 0.001$, n = 16) (fig. 1). Food intake at all other time points was not significantly different from saline controls. Pairwise comparison of the feeding response to GALP and galanin demonstrated a significant difference in feeding response at 1 nmol (1 h food intake: GALP 1 nmol 3.8 ± 0.5 g, galanin 1 nmol 2.3 ± 0.3 g, $p < 0.05$, n = 16) (fig. 1).

Effect of Galanin Fragment 2–29 on Food Intake in Satiated Male Rats

PVN administration of 1 nmol galanin 2–29 had no significant effect on food intake at any time point. Whereas, as previously shown, GALP significantly increased the 1-hour food intake at this dose (1 h food intake: saline 0.4 ± 0.1 g, galanin 2–29 0.7 ± 0.3 g, p = n.s, GALP 2.6 ± 0.5 g vs. saline $p < 0.001$, n = 10).

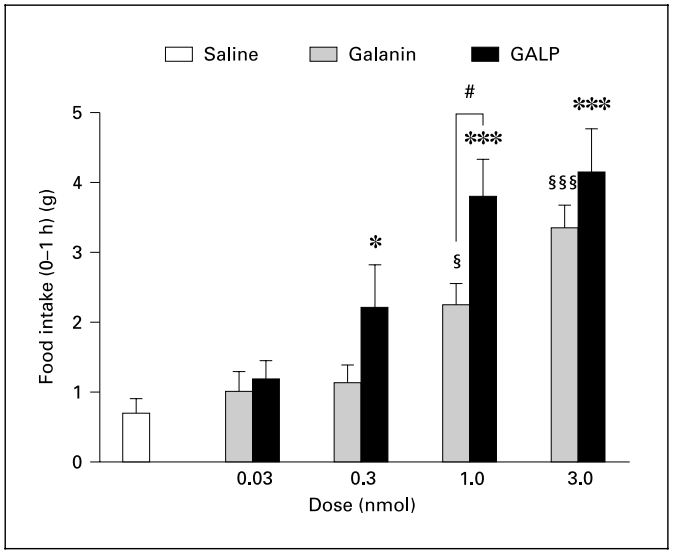

Fig. 1. Effect of paraventricular administration of GALP and galanin (0.03–3 nmol) on 1 h food intake. * $p < 0.05$; *** $p < 0.001$, GALP vs. saline; § $p < 0.05$, §§§ $p < 0.001$ galanin vs. saline; # $p < 0.05$, GALP vs. galanin.

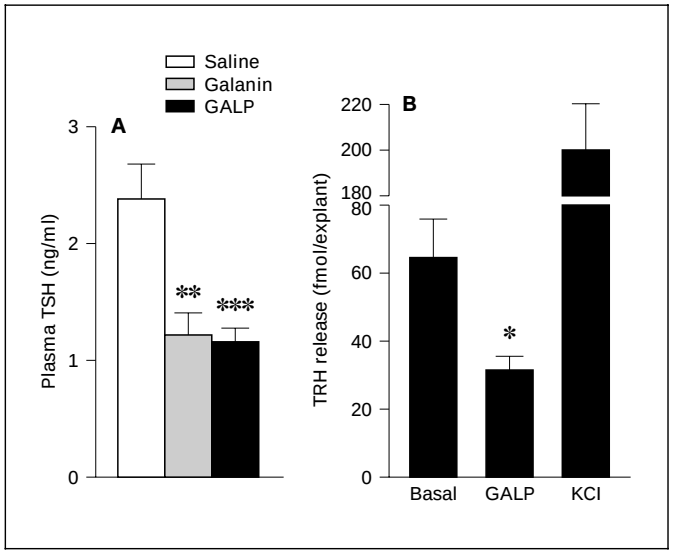

Fig. 2. A Effect of iPVN GALP and galanin (1 nmol) on plasma TSH after 10 min. ** $p < 0.01$; *** $p < 0.001$ vs. saline. B Effect of GALP on the release of TRH from hypothalamic explants. * $p < 0.05$, vs. basal release.

Effect of GALP and Galanin on Circulating TSH and Free T_3 Levels in Satiated Male Rats

PVN administration of both GALP and galanin into the PVN resulted in a significant decrease in plasma TSH 10 min post-injection when compared to the saline-injected group (plasma TSH (10 min): saline 2.38 ± 0.29 ng/ml, galanin 1.21 ± 0.20 ng/ml, $p < 0.01$, GALP 1.15 ± 0.11 ng/ml, $p < 0.001$, n = 9–10) (fig. 2A). However, there was no change in free T_3 between treatment groups (plasma T_3 (10 min): saline 1.12 ± 0.22 pg/ml, galanin 1.09 ± 0.14 pg/ml, GALP 1.00 ± 0.36 pg/ml, p = n.s., n = 9–10). This result was not unexpected as the half-life of T_3 is 24 h [21] and the sample was collected only 10 min after peptide administration, further long-term studies are required to determine the effect of GALP and galanin on thyroid hormones.

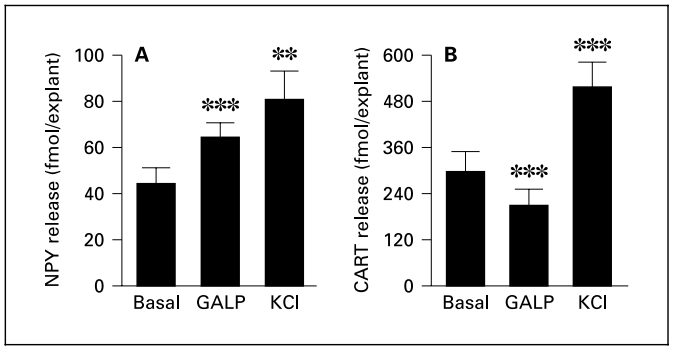

Fig. 3. A Effect of GALP on NPY release from hypothalamic explants. B Effect of GALP on CART release from hypothalamic explants. ** $p < 0.01$, *** $p < 0.001$ vs. basal release.

Effect of GALP and Galanin on Neuropeptide Release from Hypothalamic Explants

GALP (100 n*M*) significantly increased NPY release from static hypothalamic incubations when compared to basal release (basal 44.6 ± 3.2 fmol/explant, GALP 65.0 ± 6.1 fmol/explant, $p < 0.001$, n = 20) (fig. 3A). GALP (100 n*M*) also significantly reduced CART release (basal 298.2 ± 51.8 fmol/explant, GALP 207.3 ± 43.0 fmol/explant, $p < 0.001$, n = 22) (fig. 3B). However, GALP (100 n*M*) had no significant effect on α-MSH release (basal 31.1 ± 9.0 fmol/explant, GALP 35.7 ± 8.2 fmol/explant, p = n.s, n = 11.) or AGRP release (basal 1.5 ± 0.3 fmol/explant vs. GALP 1.0 ± 0.3 fmol/explant, p = n.s, n = 12). Galanin (100 n*M*) did not significantly alter NPY, CART, α-MSH or AGRP release from hypothalamic explants (NPY: basal 59.6 ± 9.2 fmol/explant, galanin 63.2 ± 8.3 fmol/explant, p = n.s, n = 22; CART: basal 273.5 ± 50.9 fmol/explant, galanin 227.4 ± 51.8 fmol/explant, p = n.s, n = 21; α-MSH: basal 24.2 ± 3.7 fmol/explant, galanin 32.8 ± 4.3 fmol/explant, p = n.s, n = 11;

AGRP: basal 1.6 ± 0.2 fmol/explant, galanin 1.6 ± 0.3 fmol/explant, p = n.s., n = 12).

GALP (100 n*M*) significantly inhibited the release of TRH from hypothalamic explants when compared to basal release (basal 64.3 ± 11.2 fmol/explant, GALP 31.5 ± 3.9 fmol/explant, $p < 0.05$, n = 10) (fig. 2B). Galanin (100 n*M*) also significantly inhibited the release of TRH from hypothalamic explants when compared to basal (basal 46.7 ± 15.4 fmol/explant, galanin 18.7 ± 3.5, $p < 0.05$, n = 10). A control peptide galanin 2–29 (100 n*M*) was applied to verify the specificity of the effect on TRH release. This peptide had no significant effect on the release of TRH from hypothalamic explants (basal 74.5 ± 18.3 fmol/explant vs. galanin 2–29, 83.8 ± 25.6 fmol/explant, p = n.s., n = 15).

Discussion

We have shown for the first time that GALP dose dependently increases food intake when injected into the PVN. In comparison with galanin, GALP was a significantly more potent stimulator of food intake at the 1-nmol dose and this was a trend which could be seen at all other doses although this failed to reach significance. These results confirm the findings from intracerebroventricular studies which demonstrate an orexigenic effect for GALP [3]. The distribution of GALP neurons and fibres suggest the PVN may be the critical site of action for this peptide in the control of food intake, as is the case with galanin [22], however, further investigation of the effect of GALP in other hypothalamic nuclei would be of interest. The more potent effect of GALP on food intake and its higher affinity for GAL R2 point towards a role for this receptor in mediating the orexigenic effect. However, galanin 2–29 which is also more selective for GAL R2 had no effect on food intake, as others have shown [8]. In addition, GAL R2 antisense oligomers have been reported to have no significant effect on food intake [23]. This evidence argues against GALP or galanin acting via a GAL R2 mechanism – suggesting GAL R3 or a novel receptor may mediate this action.

To examine whether GALP's effects on food intake are mediated through other known regulators of energy homeostasis, we investigated its action on the release of particular neuropeptides in vitro and compared this to the effect of galanin. GALP significantly increased the release of NPY, a potent orexigenic peptide [24]. GALP also significantly reduced the release of CART, a proposed satiety factor associated with decreased food intake after ICV administration [25]. In comparison, galanin had no significant effect on the release of either peptide. GALP and galanin had no effect on α-MSH or AGRP release. These data indicate the increase in food intake observed after GALP administration may be mediated through an increase in NPY release and a decrease in CART release. Our in vivo studies suggest GALP may be acting to stimulate food intake through the PVN. The PVN is an area rich in NPY- and CART-immunoreactive neurons [25] and it is also supplied by NPY and CART nerve terminals originating from the ARC [26]. Therefore, it is possible that GALP may be acting within this nucleus to effect changes in neuropeptide release, however in situ studies are required to further localise these effects. The differential effects of GALP and galanin on neuropeptide release suggests GALP may stimulate food intake through additional or even alternative pathways to galanin. That galanin was unable to elicit the same responses as GALP argues against a galanin receptor mediating these effects, supporting the hypothesis of a novel GALP receptor.

When administered into the PVN, GALP suppressed the levels of circulating TSH. In addition, GALP inhibited the release of TRH from hypothalamic explants. This data suggests GALP is acting through an inhibition of hypothalamic TRH to suppress circulating TSH levels. Consistent with this, previous studies have shown GALP has no effect on the release of TSH directly from dispersed rat pituitary cells in vitro [27]. The HPT axis is known to influence thermogenesis and basal metabolic rate [9] and other hypothalamic peptides which stimulate food intake and suppress TSH release, such as NPY, AGRP and MSH have also been implicated in the regulation of energy expenditure [28–30]. It is possible that GALP may also have similar metabolic effects and an investigation of the effect of chronic administration of GALP on energy expenditure and the thyroid axis would be of great interest.

A suppression of circulating TSH was also observed after administration of galanin into the PVN and we confirmed the finding that galanin inhibits TRH release from hypothalamic explants [J. Todd, pers. commun.]. Galanin has a well-established role in the HPT axis. ICV administration of galanin decreases plasma TSH, ICV administration of galanin anti-serum causes an increase in plasma TSH [31] and galanin administration into the PVN causes a reduction in energy expenditure [32]. These data suggest galanin may regulate the thyroid axis through the tonic inhibition of TRH release from neurons in the PVN. It is interesting to note that although GALP and galanin have significantly different effects on food intake at 1 nmol,

both have equivalent effects on circulating TSH at this dose. This suggests that different receptor systems may be mediating the effects on food intake and the HPT axis.

GALP has been found to be up-regulated by the anorectic adipose hormone leptin [33]. Therefore, GALP's effects on food intake and TSH may seem surprising. However, not all GALP neurons in the ARC express the leptin receptor [2] and this leptin-insensitive subpopulation of GALP neurons may be responsible for the effects observed on food intake and TRH release. These GALP neurons may be responsive to short-term metabolic demands rather than long-term signals relaying nutritional status such as leptin. Alternatively it is possible that GALP is physiologically important in circumstances where increased energy resources are required even in the presence of high circulating leptin – for instance during pregnancy. It is also possible that GALP functions as a negative modulator or terminator of leptin action on appetite. Clearly, further work is required to determine the significance of the interaction between GALP and leptin in relation to energy intake and expenditure.

In conclusion, this data suggests that GALP acts via the PVN to stimulate food intake and inhibit TSH release. These studies indicate GALP and galanin have differential effects on food intake and the release of hypothalamic appetite effectors; however, both have equivalent effects on the thyroid axis. A potential mechanism for GALP's orexigenic effect is through an increase in hypothalamic NPY release and a decrease in hypothalamic CART release. Our data also suggests that GALP mediates its effects on TSH levels through a suppression of hypothalamic TRH release. These findings confirm that the novel hypothalamic peptide GALP may be a regulator of food intake and suggest a further role for this peptide in the regulation of the thyroid axis.

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