0013-7227/00/$03.00/0
Endocrinology

Vol. 141, No. 7
Printed in U.S.A.

Galanin-Like Peptide (GALP) is a Target for Regulation by Leptin in the Hypothalamus of the Rat

ANDERS JURÉUS, MATTHEW J. CUNNINGHAM, MOLLY E. MCCLAIN, DONALD K. CLIFTON and ROBERT A. STEINER*

Departments of Physiology and Biophysics (A.J., R.A.S.), Obstetrics and Gynecology (D.K.C., R.A.S), Zoology (R.A.S), and Graduate Program in Neurobiology and Behavior (M.J.C., M.E.M.), University of Washington, Seattle, WA 98195-7290, USA

*To whom correspondence should be addressed

ABSTRACT

Galanin-like peptide (GALP), which was recently isolated from the porcine hypothalamus, shares sequence homology with galanin and binds with high affinity to galanin receptors. To study the distribution and regulation of GALP-expressing cells in the brain, we cloned a 120 base-pair cDNA fragment of rat GALP and produced an antisense riboprobe. *In situ* hybridization for GALP mRNA was then performed on tissue sections throughout the forebrain of adult ovariectomized female rats. We found GALP mRNA-containing cells in the arcuate nucleus (Arc), caudal dorsomedial nucleus, median eminence and the pituitary. Because GALP mRNA in the Arc appeared to overlap with the known distribution of leptin receptor mRNA, we tested the hypothesis that GALP expression is regulated by leptin. Using *in situ* hybridization, we compared the number of GALP mRNA-containing cells among groups of rats that were fed *ad lib* or fasted for 48 h and treated with either leptin or vehicle. Fasting reduced the number of identifiable cells containing GALP mRNA in the Arc, whereas the treatment of fasted animals with leptin produced a 4-fold increase in the number of cells expressing GALP message. The presence of GALP mRNA in the hypothalamus and pituitary and its regulation by leptin suggests that GALP may have important neuroendocrine functions, including the physiological regulation of feeding, metabolism, and reproduction.

THE HYPOTHALAMUS contains many neuropeptides that are involved in the regulation of primitive behaviors and basic physiological functions. Galanin is a peptide that is found throughout the central nervous system, including the hypothalamus, where it has been implicated in the regulation of food intake and body weight, and in the neuroendocrine control of reproduction and growth (1-7). To date, three different galanin receptor subtypes (GalR1-3) have been cloned, and until recently, galanin was the only known endogenous ligand for these receptors (8). However, the molecular heterogeneity of galanin-like immunoreactivity and inconsistencies in the pharmacological action of chimeric galanin receptor ligands pointed towards the existence of additional galanin-like neuropeptides (9-11).

Recently, Ohtaki et al. isolated a novel galanin-like peptide (GALP) from porcine hypothalamus, and GALP cDNA was subsequently cloned from porcine, rat and human brain (12). GALP is a 60 amino acid peptide that, unlike galanin, has a non-amidated C-terminus. GALP amino acid residues (9-21) are identical to the biologically active N-terminal (1-13) portion of galanin. Rat GALP selectively recognizes GalR2 with high affinity (IC_{50}=0.24 nM) and GalR1 with lower affinity (IC_{50}=4.3 nM), whereas galanin is relatively non-selective for these receptors (12).

We had two primary objectives for this study. The first was to map the distribution of GALP mRNA-expressing cells in the hypothalamus of the rat. The second was to test the hypothesis that the expression of GALP is regulated by the metabolic stress of fasting, and if so, to determine whether leptin could reverse this putative effect.

Materials and methods

Animals. Female 90-day old Sprague-Dawley rats (≈ 250 g) were purchased from B&K Universal (Kent, WA). Animals were housed in groups of 3 and maintained on a 14:10 light/dark cycle with lights on at 0700 h. Ovaries were removed while rats were anesthetized with a ketamine (100 mg/ml)/xylazine (20 mg/ml) cocktail (5.0:1.6 ratio, respectively). Rats were allowed to recover for one week before the beginning of the experiment. During this time, rats were handled and given daily saline injections (sc) to acclimate them to the experimental protocol. All 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 Care and Use of Laboratory Animals.

Rats were divided into three experimental groups (n=5 or 6/group). Two groups had their food removed at 1500 h on Day 0. Fasted animals were given either (sc) injections of recombinant human leptin (5 μg/g body wt) or vehicle solution. This dose of leptin was sufficient to significantly suppress food intake over a 48 h period (data not shown). The third group was allowed to feed *ad lib* and injected with the vehicle alone. Injections were performed twice daily, between 0800 and 0900 h and again between 2000 and 2100 h. Animals were sacrificed starting at 1500 h on Day 2. Thus, during the 48 hours of fasting, the animals received 4 injections of either leptin or the vehicle. Animals were euthanized with CO_2, then quickly decapitated. Brains were removed and rapidly frozen on dry ice.

Received 05/08/00.

Cloning of a partial rat GALP cDNA. Total mRNA was extracted from rat hypothalamus with a total RNA isolation kit (Ambion, Austin, TX). Reverse transcription of mRNA was performed with M-MLV reverse transcriptase (Gibco, Gaithersburg, MD) and oligo-dT primers (Ambion) at 42 C for 1 h. PCR cloning was performed with the CloneAmp pAMP system (Gibco) and deoxy UMP-containing PCR primers, 5'-CAUCAUCAUCAUAGGCTGGACCCTCAATA GTGC-3' (forward primer) and 5'-CUACUACUACUATGG CCTTCCACAGGTCTAGGA-3' (reverse primer). A PCR program was designed with initial melting set at 95 C followed by 35 cycles at 94 C for 30 sec, 62 C for 30 sec, 72 C for 60 sec, and a final extension at 72 C for 10 min. PCR was performed with *Taq* DNA polymerase (Gibco). A single band was detected at the expected size of 120 bp on a 1 % agarose gel. The PCR product was purified with a QIAprep Spin Miniprep kit (Qiagen, Valencia, CA) and cloned into the pAMP 1 plasmid (Gibco). The insert was sequenced and confirmed to be a 120 bp GALP cDNA fragment corresponding to bases 218-337 of the rat GALP precursor mRNA.

In situ hybridization. To identify cells in the rat brain that express GALP mRNA, ^{33}P-labeled antisense riboprobes were transcribed from the linearized plasmid vector containing the GALP cDNA. Single label *in situ* hybridization was performed on coronal sections (20 μm) that were cut on a cryostat and thaw-mounted onto Superfrost Plus slides (VWR Scientific, West Chester, PA). Sections were collected in a 1:8 series from the diagonal band of Broca caudally through the mammillary bodies, as described previously (13). In brief, tissue sections were fixed, acetylated and delipidated. Hybridization solution containing denatured ^{33}P-labeled (1 pmol/ml) GALP cRNA probe and yeast tRNA (2 mg/ml) in hybridization buffer, was applied to the tissue (100 μl/slide). The slides were coverslipped and incubated in humid chambers overnight at 54 C. The next day slides were treated with RNase-A and washed under conditions of increasing stringency. The tissue was dehydrated in ethanol and finally dipped in Kodak NTB-3 emulsion. The slides were exposed for 5 days, developed, and counterstained with cresyl violet. Specific labeling was absent from control experiments, which included excess unlabeled antisense probe or radiolabeled sense probe, validating the specificity of the GALP cDNA probe.

Image analysis. All sections from the 3 treatment groups showing cell-specific clusters of silver grains in the arcuate nucleus (Arc) of the hypothalamus were read. The slides were analyzed blindly with an automated image processing system, as described earlier (14). The system consisted of a PixelGrabber video acquisition board (Perceptics Corp., Knoxville, TN) attached to a Power Macintosh G3 computer. Cells with a signal to background ratio of at least 2 were considered to express GALP mRNA.

Statistical analysis. The Kruskal-Wallis nonparametric analysis of variance was used to test for overall differences in the number of GALP mRNA-containing cells and number of grains/cell among groups. Because differences in the number of GALP mRNA-containing cells were found to be statistically significant, individual Mann-Whitney U tests were then used to determine whether fasting or leptin treatment significantly altered the number of cells that express GALP mRNA. The data are presented as means±SEM, and differences are considered significant when $p<0.05$.

Results

Distribution of GALP mRNA in the rat hypothalamus. Intensely labeled clusters of silver grains corresponding to GALP mRNA-expressing cells were found in the Arc, median eminence and the infundibular stalk (Fig. 1). A few GALP mRNA-expressing cells were also found in the caudal part of the dorsomedial nucleus. In addition, GALP mRNA-expressing cells were detected in the pituitary. No labeling was observed in the hippocampus, thalamus, amygdala or cortex. The majority of labeled cells were found in the caudal part of the Arc (Figs. 1 and 2), with a high number of cells appearing close to the third ventricle. The pattern of

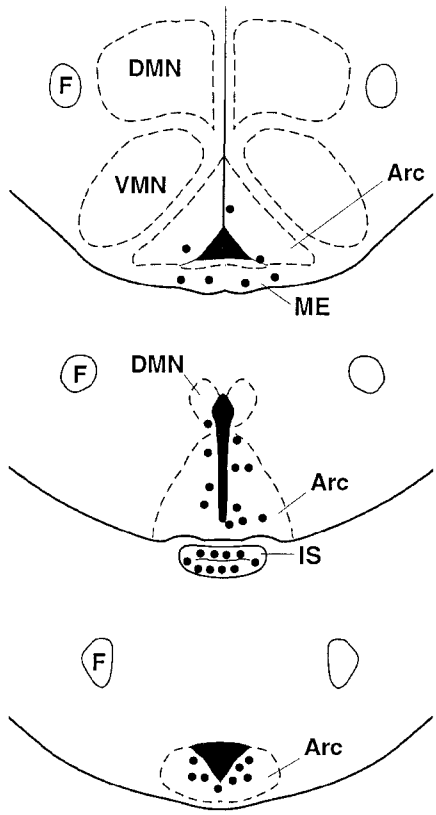

Fig. 1. Distribution of GALP mRNA containing cells (•) in the hypothalamus of the rat. Abbreviations: Arcuate nucleus, Arc; dorsomedial nucleus, DMN; Fornix, F; Infundibular stalk, IS; Median eminence, ME; Ventromedial nucleus, VMN.

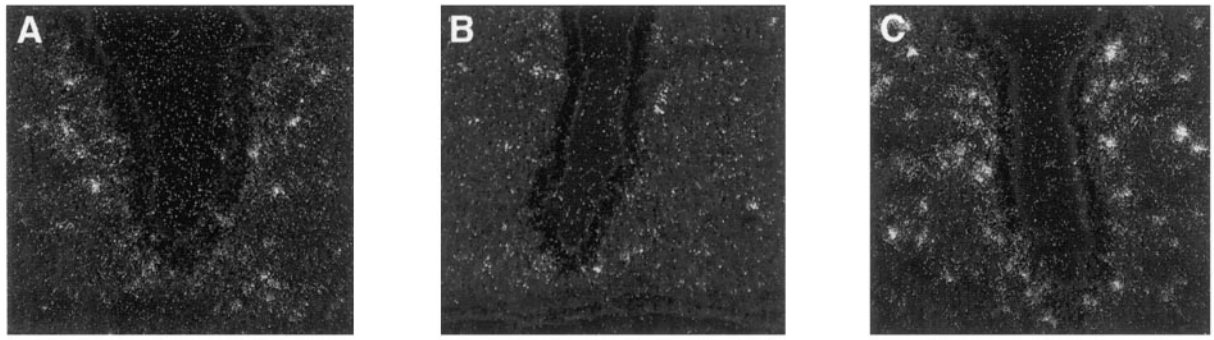

Fig.2. Photomicrographs of GALP mRNA-expressing cells in the caudal arcuate nucleus of A) *ad lib*-fed/vehicle-treated, B) fasted/vehicle-treated and C) fasted/leptin-treated rats. Clusters of silver grains indicate the presence of cells containing GALP mRNA. Fasting reduced the number of identifiable GALP mRNA-containing cells compared to *ad lib*-fed controls, whereas leptin treatment reversed this effect.

expression of GALP was observed to overlap with the known distribution of leptin receptor mRNA-expressing neurons in the hypothalamus.

Effects of fasting and leptin-treatment on GALP mRNA. Analysis of the number of identifiable GALP mRNA-expressing cells among the fed, fasted and fasted/leptin-treated animals revealed clear evidence of regulation by these metabolic manipulations (Fig. 3). Fasting reduced the number of GALP mRNA-containing cells by half in the Arc, although this difference was not statistically significant. Treatment with leptin reversed the fasting-induced inhibition of GALP expression and increased the number of detectable GALP mRNA-containing cells by 4-fold over that of the fasted/vehicle group (p=0.01 between fasted/vehicle and fasted/leptin). The experimental manipulations did not significantly alter GALP mRNA levels (as indicated by the number of grains per cell) in the cells that expressed GALP mRNA.

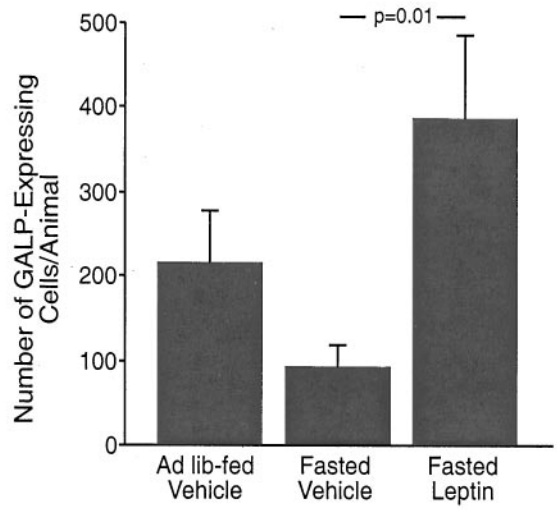

Fig. 3. The number of identifiable cells expressing GALP mRNA in the Arc of *ad lib*-fed and fasted rats treated with either vehicle or leptin. Bars represent means ± SEM.

Discussion

This study demonstrates that GALP mRNA is expressed in discrete nuclei in the hypothalamus of the rat. The dense labeling of cells in the Arc, median eminence, infundibular stalk and pituitary suggests that GALP may have a neuroendocrine function. Within the Arc, GALP mRNA-expressing cells were observed in the caudal portion of the nucleus, located medially near the third ventricle. The pattern of GALP mRNA distribution in the hypothalamus of the rat is distinct from that of any other commonly recognized neuropeptide. Although there may be some modest overlap, the anatomical localization of GALP mRNA-containing cells is different from that of proopiomelanocortin (POMC), neuropeptide Y, growth hormone-releasing hormone and galanin (15). This would argue that GALP cells have their own distinct organization and function in the larger family of neuropeptides involved in neuroendocrine function.

Leptin is a protein hormone produced by adipocytes, which acts as a satiety factor in the regulation of body weight and metabolism (16). The signaling form of the leptin receptor (Ob-Rb) is expressed in the caudal medial part of the Arc (17). The overlapping distribution of the pattern of GALP mRNA with the known distribution of Ob-Rb mRNA suggested that GALP might be a target for leptin regulation. We observed that fasting inhibits and leptin stimulates the expression of GALP mRNA in the hypothalamus. The effect of leptin on GALP expression is similar to its stimulatory effect upon POMC and cocaine- and amphetamine-regulated transcript (CART) mRNAs, suggesting that GALP may act along with POMC-derived peptides and CART to mediate leptin's inhibition of feeding and stimulation of metabolism (18-20). Leptin has also been implicated in the neuroendocrine regulation of the adrenal, thyroid, growth, and reproductive axes, and GALP may serve as an intermediary, coupling the action of leptin to these endocrine

control systems. It is also conceivable that the leptin-induced alteration in the activity of one or more of these hormonal axes (e.g., thyroid hormone, corticosterone, or sex steroids) underlies the effects of leptin on GALP expression (21, 22).

GALP is able to bind and activate both GalR1 and GalR2 *in vitro*, having a higher affinity for GalR2 (12). Both galanin receptor subtypes are expressed in the Arc, and GalR2 is expressed in the pituitary gland (23, 24). GALP may act on galanin receptors in the Arc and/or be released into the hypophyseal portal system, where it could activate GalR2 in the pituitary. GALP is a relatively large peptide, indicating that it may have a greater resistance to degradation by peptidases. Thus, it is plausible that GALP is secreted from the pituitary into the general circulation, where it is transported to peripheral target organs. Indeed, GalR2 is widely expressed in the periphery, including the testis, pancreas, spleen, kidney, and liver (9, 25). The fact that GALP shares homology with only 13 amino acids of the galanin sequence suggests that GALP might also interact with yet-to-be discovered specific GALP receptors.

Acknowledgments

This work was supported by NIH grants U54 HD12629 and R01 HD27142, NSF grant IBN 9720143, the Andrew W. Mellon foundation and the Pediatric Epilepsy Research Center (PERC) at the University of Washington. The authors are also grateful to Stephanie M. Krasnow and John G. Hohmann for their critical reading of the manuscript, and Thomas H. Teal for providing excellent technical assistance.

References

1. **Ottlecz A, Samson WK, McCann SM** 1986 Galanin: evidence for a hypothalamic site of action to release growth hormone. Peptides 7:51-53
2. **Sahu A, Crowley WR, Tatemoto K, Balasubramaniam A, Kalra SP** 1987 Effects of neuropeptide Y, NPY analog (norleucine4-NPY), galanin and neuropeptide K on LH release in ovariectomized (ovx) and ovx estrogen, progesterone-treated rats. Peptides 8:921-926
3. **Crawley JN, Austin MC, Fiske SM, Martin B, Consolo S, Berthold M, Langel Ü, Fisone G, Bartfai T** 1990 Activity of centrally administered galanin fragments on stimulation of feeding behavior and on galanin receptor binding in the rat hypothalamus. J Neurosci 10:3695-3700
4. **Gundlach AL, Wisden W, Morris BJ, Hunt SP** 1990 Localization of preprogalanin mRNA in rat brain: in situ hybridization study with a synthetic oligonucleotide probe. Neurosci Lett 114:241-247
5. **Bartfai T, Hökfelt T, Langel Ü** 1993 Galanin--a neuroendocrine peptide. Crit Rev Neurobiol 7:229-274
6. **Crawley JN, Robinson JK, Langel Ü, Bartfai T** 1993 Galanin receptor antagonists M40 and C7 block galanin-induced feeding. Brain Res 600:268-272
7. **Sahu A** 1998 Leptin decreases food intake induced by melanin-concentrating hormone (MCH), galanin (GAL) and neuropeptide Y (NPY) in the rat. Endocrinology 139:4739-4742
8. **Branchek TA, Smith KE, Gerald C, Walker MW** 2000 Galanin receptor subtypes. Trends Pharmacol Sci 21:109-116
9. **Smith KE, Forray C, Walker MW, Jones KA, Tamm JA, Bard J, Branchek TA, Linemeyer DL, Gerald C** 1997 Expression cloning of a rat hypothalamic galanin receptor coupled to phosphoinositide turnover. J Biol Chem 272:24612-24616
10. **Wang ZL, Kulkarni RN, Wang RM, Smith DM, Ghatei MA, Byfield PG, Bennet WM, Bloom SR** 1997 Possible evidence for endogenous production of a novel galanin-like peptide. J Clin Invest 100:189-196
11. **Smith KE, Walker MW, Artymyshyn R, Bard J, Borowsky B, Tamm JA, Yao WJ, Vaysse PJ, Branchek TA, Gerald C, Jones KA** 1998 Cloned human and rat galanin GALR3 receptors. Pharmacology and activation of G-protein inwardly rectifying K+ channels. J Biol Chem 273:23321-23326
12. **Ohtaki T, Kumano S, Ishibashi Y, Ogi K, Matsui H, Harada M, Kitada C, Kurokawa T, Onda H, Fujino M** 1999 Isolation and cDNA cloning of a novel galanin-like peptide (GALP) from porcine hypothalamus. J Biol Chem 274:37041-37045
13. **Marks DL, Wiemann JN, Burton KA, Lent KL, Clifton DK, Steiner RA** 1992 Simultaneous visualization of two cellular mRNA species by use of a new double *in situ* hybridization method. Mol Cell Neurosci 3:395-405
14. **Chowen JA, Steiner RA, Clifton DK** 1991 Semiquantitative analysis of cellular somatostatin mRNA levels by in situ hybridization histochemistry. Methods in Neurosci 5:137-158
15. **Tohyama M, Takatsuji K** 1998 Neuroactive substances and their receptors in the rat., Ed., Oxford University press, New York:182-318
16. **Friedman JM** 2000 Obesity in the new millennium. Nature 404:632-634
17. **Elmquist JK, Bjørbaek C, Ahima RS, Flier JF, Saper CB** 1998 Distribution of leptin receptor mRNA isoforms in the rat brain. J Comp Neurol 395:535-547
18. **Seeley RJ, Yagaloff KA, Fisher SL, Burn P, Thiele TE, van Dijk G, Baskin DG, Schwartz MW** 1997 Melanocortin receptors in leptin effects. Nature 390:349
19. **Thornton JE, Cheung CC, Clifton DK, Steiner RA** 1997 Regulation of hypothalamic proopiomelanocortin mRNA by leptin in *ob/ob* mice. Endocrinology 138:5063-5066
20. **Kristensen P, Judge ME, L. T, Ribel U, Christjansen KN, Wulff BS, Calusen JT, Jensen PB, Madsen OD, Vrang N, Larsen JP, Hastrup S** 1998 Hypothalamic CART is a new anorectic peptide regulated by leptin. Nature 393:72-76
21. **Friedman JM, Halaas J** 1998 Leptin and the regulation of body weight in mammals. Nature 395:763-770
22. **Casanueva FF, Dieguez C** 1999 Neuroendocrine regulation and actions of leptin. Front Neuroendocrinol 20:317-363
23. **O'Donnell D, Ahmad S, Wahlestedt C, Walker P** 1999 Expression of the novel galanin receptor subtype GalR2 in the adult rat CNS: distinct distribution from GalR1. J Comp Neurol 409:469-481
24. **Depczynski B, Nichol K, Fathi Z, Iismaa T, Shine J, Cunningham A** 1998 Distribution and characterization of the cell types expressing GALR2 mRNA in brain and pituitary gland. Ann N Y Acad Sci 863:120-128
25. **Waters SM, Krause JE** 2000 Distribution of galanin-1, -2, and -3 receptor messenger RNAs in central and peripheral rat tissues. Neuroscience 95:265-271