

Differential Patterns of Fos Induction in the Hypothalamus of the Rat Following Central Injections of Galanin-Like Peptide and Galanin

GREGORY S. FRALEY, ISSEI SHIMADA, JAMES W. BAUMGARTNER, DONALD K. CLIFTON, AND ROBERT A. STEINER

Departments of Physiology and Biophysics (G.S.F., I.S., R.A.S.), Obstetrics and Gynecology (D.K.C., R.A.S.), and Zoology (R.A.S.), University of Washington, Seattle, Washington 98195-7290; and Amgen, Inc. (J.W.B.), Thousand Oaks, California 91320

Galanin and its newly discovered relative galanin-like peptide (GALP) are neuropeptides that are implicated in the neuroendocrine regulation of body weight and reproduction. GALP has been shown to bind *in vitro* to galanin receptor subtypes 1 and 2, but whether it has its own specific receptor(s) is unknown. We reasoned that if GALP acts through a receptor that is distinct from galanin receptors, then GALP should activate central pathways that are different from those activated by galanin. The purpose of this study was to determine whether galanin and GALP produce different patterns of neuronal activation within the hypothalamus. Quantitative analysis of Fos immunoreactivity showed that galanin in-

duced a significantly greater number of Fos-positive nuclei in the paraventricular nucleus compared with GALP ($P < 0.001$); however, compared with galanin, GALP induced significantly more Fos-positive cells in the horizontal limb of the diagonal band of Broca, caudal preoptic area, arcuate nucleus, and median eminence ($P < 0.05$). These observations suggest that GALP and galanin act through different receptor-mediated pathways to exert their effects on the regulation of body weight and reproduction and identify target cells for GALP's specific actions in the hypothalamus, including the preoptic area, paraventricular and arcuate nuclei, and the median eminence. (*Endocrinology* 144: 1143–1146, 2003)

GALANIN-LIKE PEPTIDE (GALP) is a 60-amino-acid molecule that shares a partial amino acid sequence identity with galanin but is derived from a separate gene (1). Galanin is expressed widely throughout the central nervous system (2–6), whereas the expression of GALP in the forebrain is confined to the hypothalamic arcuate nucleus (ARC) (7–9). GALP binds *in vitro* to at least two of the galanin receptor subtypes, GALR1 and GALR2, and GALP shows a higher affinity for GALR2 than galanin (1). Whether GALP is an endogenous ligand for one or more of these galanin receptors or GALP has its own unique and specific receptor(s) is unknown.

Galanin has been implicated in many physiological functions. These include the hypothalamic regulation of anterior pituitary function (most notably LH, GH, and prolactin secretion), nociception, body weight regulation, and learning and memory (10–13). To date, GALP has also been implicated for a role in the regulation of body weight and the hypothalamic secretion of GnRH (8, 14–16). Although it's conceivable that the actions of endogenous GALP are mediated by one or more of the galanin receptors, some evidence suggests that GALP may have its own specific receptor. This supposition is based on the observations that centrally administered GALP produces different physiological responses than galanin. For example, GALP, but not

galanin, stimulates GnRH and LH secretion in the male rat (16). The different effects of exogenously administered galanin and GALP suggest that, *in vivo*, the two neuropeptides act through different mechanisms to target separate populations of cells in the brain. We used the protein product of the immediate early gene, Fos, as a marker of neuronal activation and compared the Fos induction patterns 120 min after central (third cerebral ventricle) injections of equimolar concentrations of GALP and galanin, or artificial cerebrospinal fluid (aCSF) as a control (10, 17).

Materials and Methods

Adult male Sprague Dawley rats (280–320 g) were purchased from B&K Universal (Kent, WA). Animals were housed in individual cages and given access to standard rodent chow and water *ad libitum*. The animals were maintained on a 12-h light, 12-h dark cycle with lights on at 0600 h. 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 anesthetized with a ketamine cocktail (100 mg/ml ketamine, 20 mg/ml xylazine, and 10 mg/ml acepromazine) and placed in a stereotaxic frame. A stainless steel 26-gauge cannula (Plastics One, Roanoke, VA) was inserted into the third ventricle (midline; 2.2 mm posterior to bregma, 7.5 mm ventral to dura mater). The cannula was secured to the skull with dental acrylic and screws. A 33-gauge stainless steel stylus (Plastics One) was inserted to occlude the guide cannula when not in use. After surgery, rats were allowed to recover for 1 wk, during which time they were handled daily. Cannula patency was assessed by injection of 1 μ g neuropeptide Y (NPY; American Peptide Co., Sunnyvale, CA). All compounds were administered in a volume of 3 μ l with a 10- μ l Hamilton syringe (Hamilton, Reno, NV) attached to polyethylene tubing (inner diameter, 0.58 mm; outer diameter, 0.96 mm) and a 33-gauge stainless steel injection cannula (Plastics One). Only the rats that consumed at least 4 g of food more than baseline within 1 h of

Abbreviations: aCSF, Artificial cerebrospinal fluid; ARC, arcuate nucleus; AVPV, anteroventral periventricular nucleus; GALP, galanin-like peptide; hDBB, horizontal limb of the diagonal band of Broca; ICV, intracerebroventricular; ME, median eminence; MPN, medial preoptic nucleus; NHS, normal horse serum; NPY, neuropeptide Y; PVN, paraventricular nucleus; POA, caudal preoptic area; RT, room temperature.

injection of NPY were considered to have correct cannula placement and were included in the study.

One week after the NPY injection, rats were injected with either 3 μ l of aCSF vehicle, 3 μ l of a 5.0-nmol galanin (American Peptide Co.) solution, or 3 μ l of a 5.0-nmol GALP (Amgen, Inc., Denver, CO) solution ($n = 5$ /group). The 5.0-nmol GALP dose was determined previously as the median dose to alter food intake in the rat (15). Galanin was injected as an equimolar dose to GALP. All animals were allowed full access to food and water, and their food intake was measured during the 2-h interval following the injection. After 2 h, rats were deeply anesthetized with Nembutal (150 mg/kg; Abbott Laboratories, Cincinnati, OH) and perfused transcardially with physiologic saline followed by 4% paraformaldehyde. The brains were then dissected and postfixed for 5 h in paraformaldehyde at 4 C, then placed into cryoprotectant (15% sucrose in 0.1 M PBS) overnight. Four sets of 40- μ m hypothalamic sections were collected on a sliding microtome and washed in PBS.

Immunocytochemistry for Fos was performed on one set of hypothalamic sections by a standard ABC (avidin/biotin complex) reaction, as previously described (18–20). Briefly, sections were washed in PBS and preincubated in 0.1 M PBS with 10% normal horse serum (NHS) for 1 h at room temperature (RT). Sections were then transferred to 0.1 M PBS with 1% NHS containing goat anti-Fos polyclonal antibody (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA) and incubated for 48 h at RT with agitation. After washing three times in PBS, sections were incubated in a biotinylated antigoat secondary antibody solution (1:500 in PBS with 1% NHS; Vector Laboratories, Burlingame, CA) for 2 h at RT with agitation. After three PBS washes, Fos was visualized with the standard ABC reaction with Ni-3,3',5,5'-diaminobenzidine as the chromagen to produce a blue-black reaction product (DAB Chromagen Kit No. PK6100, Vector Laboratories). Sections were mounted on Superfrost Plus slides (VWR Inc., Seattle, WA), air-dried, dehydrated, and cleared, after which coverslips were applied.

Analysis of Fos was performed under light microscopy. A qualitative analysis was conducted to determine gross areas of Fos labeling, which were subsequently analyzed quantitatively. Fos-positive cells were counted if dark blue-black nuclei were observed. Specific bilateral counts of Fos were made on individual, anatomically matched sections across treatment groups within the horizontal limb of the diagonal band of Broca (hDBB), caudal preoptic area (POA), paraventricular nucleus (PVN), ARC, and median eminence (ME).

Counts of Fos-positive cells were analyzed by repeated-measures ANOVA followed by a one-way ANOVA and a Tukey-Kramer *post hoc* test using GB-Stat for the Macintosh (General Dynamics, Bethesda, MD). $P < 0.05$ was considered significant.

Results

Both galanin and GALP similarly produced a modest, but significant, increase in feeding (compared with vehicle-treated controls) within minutes of their injection, as previously reported ($P < 0.05$; data not shown; and Refs. 15, 21, and 22). Qualitative assessment suggested considerably more dense Fos-positive staining in the galanin- and GALP-treated groups (compared with controls), in the hDBB, POA, PVN, ARC, and ME (Fig. 1). Quantitative analysis revealed a significant effect of treatment on the number of Fos-positive cells ($P < 0.01$). In addition, there was a significant interaction between treatment and anatomical area, indicating that the response to GALP and galanin is dependent on location ($P < 0.05$). Further analyses among treatments within individual hypothalamic nuclei revealed differential Fos expression among the three treatment groups (Fig. 2). Galanin administration led to a significant increase in Fos induction compared with controls in the POA ($P < 0.05$), PVN ($P < 0.001$), and ARC ($P < 0.05$) without changing the number of Fos-containing cells in the hDBB or ME. GALP significantly increased Fos expression in all areas studied except the PVN (hDBB, $P < 0.05$; POA, $P < 0.001$; ARC, $P < 0.001$; ME $P <$

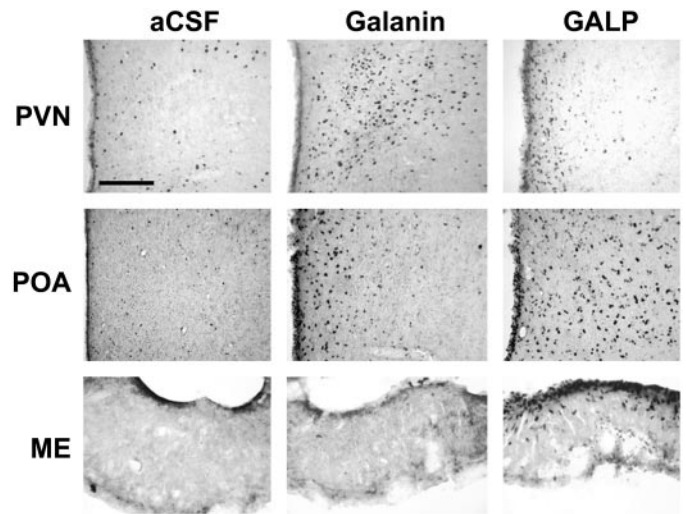


FIG. 1. Representative photomicrographs illustrating Fos immunoreactivity within the PVN, POA, and ME following ICV aCSF, galanin, or GALP injection. Bar, 100 μ m.

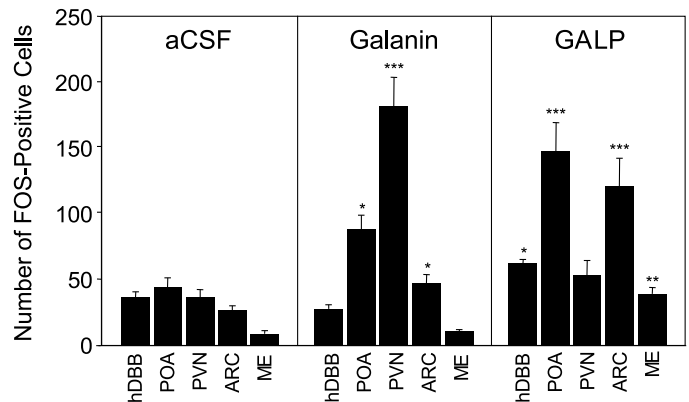


FIG. 2. Significant differences were seen in the number of Fos-positive cells following either ICV galanin or GALP compared with aCSF. *, Significance at $P < 0.05$; **, significance at $P < 0.01$; ***, significance at $P < 0.001$ compared with aCSF controls.

0.01). In the PVN, galanin induced significantly more Fos-positive cells than GALP ($P < 0.001$); however, in all other areas GALP was significantly more effective than galanin in inducing Fos expression (hDBB $P < 0.05$; POA, $P < 0.001$; ARC, $P < 0.001$; ME, $P < 0.01$). In the POA, galanin-induced Fos staining was restricted to the anteroventral periventricular nucleus (AVPV), whereas GALP-induced Fos expression included not only the AVPV but extended more laterally within the medial preoptic nucleus (MPN), to include the sexually dimorphic nucleus-POA.

Discussion

These results demonstrate that centrally administered GALP and galanin differentially activate Fos expression among different hypothalamic nuclei. This differential neuronal activation most likely underlies the different physiological and behavioral effects that these two neuropeptides have been shown to evoke when given centrally. For example, in the male rat, intracerebroventricular (ICV) GALP, but not galanin, stimulates GnRH and LH release (16). This is

consistent with the present observations showing that GALP, but not galanin, induces Fos in the hDBB and MPN, where many GnRH cell bodies are known to reside. In the context of feeding and body weight regulation, centrally administered galanin and GALP both produce an immediate, but transient, stimulatory effect on feeding in the rat; however, at 24 h following ICV injection, only GALP, and not galanin, produces an inhibition of feeding and body weight in both the rat and mouse, again testifying to the dissimilar neural targets of the two related neuropeptides (11, 15, 21, 22).

Several possibilities may explain the differential actions of GALP and galanin on Fos induction within the hypothalamus. First, it's conceivable that the distribution of the galanin receptor subtypes combined with the differential binding and molecular action between GALP and galanin at these receptors might explain the disparity in their activation of Fos. Galanin and GALP bind to both GALR1 and GALR2; however, GALP interacts with the galanin receptor subtypes GALR1 and GALR2 (*in vitro*), with a higher affinity for GALR2 ($IC_{50} = 0.24$ nM) than for GALR1 ($IC_{50} = 4.3$ nM). Galanin's affinity for these two receptors is relatively non-discriminating (1). The relative affinities of galanin and GALP for GALR3 (the other identified galanin receptor subtype) are unknown. *In situ* hybridization mapping studies have shown that galanin receptor transcripts are present throughout the hypothalamus, although each subtype exhibits a unique abundance and distribution. GALR1 mRNA is widely and robustly expressed throughout the hypothalamus, whereas GALR2 mRNA is expressed at lower levels, and GALR3 mRNA has a very limited and discrete hypothalamic expression pattern (23–26).

If the centrally administered galanin and GALP had equal access to GALR1 and GALR2 throughout the diencephalon and if Fos is induced by activation of both receptors, we would have predicted that regions where GALR1 expression is high would have shown a greater induction of Fos in response to galanin than GALP; likewise, we would have thought that areas where GALR2 expression is high would be likely to demonstrate greater Fos induction in response to GALP than galanin. However, this was not the case. In the PVN, for example, where there is considerable expression of GALR2, we observed little induction of Fos in response to GALP (although GALP binds GALR2 with higher affinity than does galanin). It's worth noting that, because neither receptor subtype-specific antibodies nor ligands are available, we don't know precisely where in the brain the actual receptors are located, which may be different from the locus of the cell bodies where they are synthesized and the mRNA is concentrated. Thus, differential activation of the various galanin receptor subtypes remains a plausible, but tenuous, explanation for the different patterns of Fos induction produced by galanin and GALP.

A second possible explanation of the differential Fos induction by galanin and GALP is that there might be a difference in the diffusion of the two molecules from the ventricular site of injection into the brain substrate itself. Although galanin and GALP were injected in equal molar concentrations, they may either have had unequal access to sites within the diencephalon or have been differentially cleared. Because GALP is approximately twice the size of

galanin (60 *vs.* 29 amino acids), it's conceivable that the larger molecule could require a longer time to diffuse further from the point of injection. If this were the case, one might expect that because all of the known galanin receptors are widely distributed throughout the forebrain, galanin (the smaller molecule), would penetrate the brain further laterally and produce greater and more widespread effects on Fos expression than GALP. However, this was not the case. First, galanin induced considerably more Fos expression than GALP in the PVN, which is immediately juxtaposed to the third ventricle. Second, in the POA, galanin-induced Fos staining was concentrated in the medial AVPV, whereas GALP-induced Fos expression included the AVPV but also extended much more laterally to comprise the MPN and sexually dimorphic nucleus-POA. Thus, it would appear that a simple limitation in GALP's diffusion and access to sites distant from the site of injections cannot account for discrepancies in the pattern of Fos induction produced by galanin and GALP.

A third plausible explanation of these results is that GALP has its own receptor(s), distinct from the known galanin receptors. Although galanin and GALP share a partial sequence identity, there are two highly conserved and unique amino acid sequences in GALP not shared by galanin (GALP 1–8 and 38–54) that may dictate its interaction with a unique GALP receptor and mediate its physiological effects. This explanation is attractive, considering that GALP induces Fos expression in the internal zone of the ME, where no galanin receptors have been reported to exist. This possibility is supported by earlier studies showing that the galanin receptor antagonist, galantide, inhibits GnRH release when it is injected into the vicinity of the ME-ARC of female rats (27). Because this region appears to be lacking in galanin receptors and shows Fos induction in response to GALP but not galanin, it is conceivable the effect of galantide is attributable to an inhibition of GALP-specific receptors.

In summary, centrally administered galanin and GALP produce different patterns of Fos expression in the forebrain of the male rat, reflecting the disparate actions of the peptides on the neuroendocrine axes regulating feeding and reproduction. The differential effects of galanin and GALP on Fos induction may be attributable either to the unique binding characteristics of the two peptides to the galanin receptors or reflect GALP's activation of an unidentified GALP-specific receptor.

Acknowledgments

The authors thank Scott Dodson and Mark Jarosinski for synthesizing and Amgen, Inc. for supplying the GALP peptide. We are also grateful to Drs. Susan Fraley, Robert Speth, John Hohmann, and Anders Juréus, and to Matthew Cunningham, Stephanie Krasnow, and Molly McClain for their critical review and comments on earlier drafts of this manuscript.

Received December 9, 2002. Accepted January 14, 2003.

Address all correspondence and requests for reprints to: Dr. Gregory S. Fraley, University of Washington, Department of Physiology and Biophysics, Box 357290, Seattle, Washington 98195-7290.

This work was supported by grants from the National Science Foundation (IBN-0110686) and the U.S. Public Health Service NIH (R01-HD-27142) and the Specialized Cooperative Centers Program for Reproductive Research (U54-HD-12629).

References

- 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
- Gundlach AL, Burazin TC, Larm JA 2001 Distribution, regulation and role of hypothalamic galanin systems: renewed interest in a pleiotropic peptide family. *Clin Exp Pharmacol Physiol* 28:100–105
- Ch'ng JL, Christofides ND, Anand P, Gibson SJ, Allen YS, Su HC, Tatemoto K, Morrison JF, Polak JM, Bloom SR 1985 Distribution of galanin immunoreactivity in the central nervous system and the responses of galanin-containing neuronal pathways to injury. *Neuroscience* 16:343–354
- Newton BW 1992 Galanin-like immunoreactivity in autonomic regions of the rat lumbosacral spinal cord is sexually dimorphic and varies with the estrous cycle. *Brain Res* 589:69–83
- Michener SR, Aimone LD, Yaksh TL, Go VL 1990 Distribution of galanin-like immunoreactivity in the pig, rat and human central nervous system. *Peptides* 11:1217–1223
- Skofitsch G, Jacobowitz DM 1986 Quantitative distribution of galanin-like immunoreactivity in the rat central nervous system. *Peptides* 7:609–613
- Larm JA, Gundlach AL 2000 Galanin-like peptide (GALP) mRNA expression is restricted to arcuate nucleus of hypothalamus in adult male rat brain. *Neuroendocrinology* 72:67–71
- Jur us A, Cunningham MJ, Li D, Johnson LL, Krasnow SM, Teklemichael DN, Clifton DK, Steiner RA 2001 Distribution and regulation of galanin-like peptide (GALP) in the hypothalamus of the mouse. *Endocrinology* 142:5140–5144
- Takatsu Y, Matsumoto H, Ohtaki T, Kumano S, Kitada C, Onda H, Nishimura O, Fujino M 2001 Distribution of galanin-like peptide in the rat brain. *Endocrinology* 142:1626–1634
- Veening JG, Coolen LM 1998 Neural activation following sexual behavior in the male and female rat brain. *Behav Brain Res* 92:181–193
- Gundlach AL 2002 Galanin/GALP and galanin receptors: role in central control of feeding, body weight/obesity and reproduction? *Eur J Pharmacol* 440:255–268
- Ottlecz A, Snyder GD, McCann SM 1988 Regulatory role of galanin in control of hypothalamic-anterior pituitary function. *Proc Natl Acad Sci USA* 85:9861–9865
- Zeng N, Kang T, Wen Y, Wong H, Walsh J, Sachs G 1998 Galanin inhibition of enterochromaffin-like cell function. *Gastroenterology* 115:330–339
- Jur us A, Cunningham MJ, McClain ME, Clifton DK, Steiner RA 2000 Galanin-like peptide (GALP) is a target for regulation by leptin in the hypothalamus of the rat. *Endocrinology* 141:2703–2706
- Krasnow S, Fraley GS, Schuh SM, Baumgartner JW, Clifton DK, Steiner RA 2003 A role for galanin-like peptide in the integration of feeding, body weight regulation, and reproduction in the mouse. *Endocrinology* 144:813–822
- Matsumoto H, Noguchi J, Takatsu Y, Horikoshi Y, Kumano S, Ohtaki T, Kitada C, Itoh T, Onda H, Nishimura O, Fujino M 2001 Stimulation effect of galanin-like peptide (GALP) on luteinizing hormone-releasing hormone-mediated luteinizing hormone (LH) secretion in male rats. *Endocrinology* 142:3693–3696
- Bialy M, Kaczmarek L 1996 c-Fos expression as a tool to search for the neurobiological base of the sexual behaviour of males. *Acta Neurobiol Exp (Warsz)* 56:567–577
- Fraleley GS, Kuenzel WJ 1993 Immunocytochemical and histochemical analyses of gonadotrophin releasing hormone, tyrosine hydroxylase, and cytochrome oxidase reactivity within the hypothalamus of chicks showing early sexual maturation. *Histochemistry* 99:221–229
- Fraleley GS 2002 Immunolesion of hindbrain catecholaminergic projections to the medial hypothalamus attenuates penile reflexive erections and alters hypothalamic peptide mRNA. *J Neuroendocrinol* 14:345–348
- Horowitz JM, Vernace VA, Myers J, Stachowiak MK, Hanlon DW, Fraley GS, Torres G 2001 Immunodetection of Parkin protein in vertebrate and invertebrate brains: a comparative study using specific antibodies. *J Chem Neuroanat* 21:75–93
- Matsumoto Y, Watanabe T, Adachi Y, Itoh T, Ohtaki T, Onda H, Kurokawa T, Nishimura O, Fujino M 2002 Galanin-like peptide stimulates food intake in the rat. *Neurosci Lett* 322:67–69
- Lawrence CB, Baudoin FM, Luckman SM 2002 Centrally administered galanin-like Peptide modifies food intake in the rat: a comparison with galanin. *J Neuroendocrinol* 14:853–860
- Mennicken F, Hoffert C, Pelletier M, Ahmad S, O'Donnell D 2002 Restricted distribution of galanin receptor 3 (GalR3) mRNA in the adult rat central nervous system. *J Chem Neuroanat* 24:257–268
- Kolakowski Jr LE, O'Neill GP, Howard AD, Broussard SR, Sullivan KA, Feighner SD, Sawzdargo M, Nguyen T, Kargman S, Shiao LL, Hreniuk DL, Tan CP, Evans J, Abramovitz M, Chateaufneuf A, Coulombe N, Ng G, Johnson MP, Tharian A, Khoshbouei H, George SR, Smith RG, O'Dowd BF 1998 Molecular characterization and expression of cloned human galanin receptors GALR2 and GALR3. *J Neurochem* 71:2239–2251
- 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
- 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
- Sahu A, Xu B, Kalra SP 1994 Role of galanin in stimulation of pituitary luteinizing hormone secretion as revealed by a specific receptor antagonist, galantide. *Endocrinology* 134:529–536