Intracerebroventricular Galanin-Like Peptide Induces Different Brain Activation Compared with Galanin

CATHERINE B. LAWRENCE, TORRIE WILLIAMS, AND SIMON M. LUCKMAN

School of Biological Sciences, University of Manchester, Oxford Road, Manchester M13 9PT, United Kingdom

Like galanin, the 60-amino-acid peptide, galanin-like peptide (GALP), has orexigenic actions, demonstrated by an acute increase in feeding after central injection in rodents. However, in contrast to galanin, GALP causes a prolonged rise in core body temperature and a reduction in body weight over 24 h. In an attempt to identify potential explanations for the observed differences between GALP and galanin, this study examined which brain areas were activated by these peptides. Intracerebroventricular injection of GALP into conscious rats significantly stimulated feeding over 0–1 h, increased core body temperature, but reduced body weight gain over 24 h. Immunohistochemistry to detect c-fos demonstrated that intracerebroventricular injection of GALP or galanin activated several brain regions in common, including the dorsomedial nucleus of the hypothalamus, lateral hypothalamus, and nucleus tractus solitarius of the brainstem. However, GALP also induced c-fos expression in the periventricular hypothalamic region and supraoptic hypothalamic nucleus. Cell activation induced by GALP in the supraoptic hypothalamic nucleus and nucleus tractus solitarius was dependent on food intake but independent of food consumption in all other brain regions. Double immunohistochemistry indicated that small cells expressing c-fos in the periventricular hypothalamic region after GALP were astrocytes and not microglia. (Endocrinology 144: 3977–3984, 2003)

GALANIN-LIKE PEPTIDE (GALP) is a 60-amino-acid peptide, initially isolated from porcine hypothalamus (1) and subsequently cloned from the rat, human, mouse, and macaque (1–3). GALP is related to the 30-amino-acid peptide galanin, and in all species to date, amino acids 9–21 of GALP are identical with the biologically active N-terminal (amino acids 1–13) of galanin. However, in contrast to galanin, which displays similar affinities in vitro for the galanin receptor subtypes 1 and 2 (GalR1 and 2), GALP exhibits a higher affinity for GalR2 (1). In addition, the distribution of GALP mRNA and protein in the central nervous system is limited, being detected only in the hypothalamic arcuate nucleus, median eminence, and posterior pituitary (2–6), but galanin is found in several hypothalamic nuclei (7).

Galanin is an orexigenic peptide in rodents that causes an acute (within the first 30 min) increase in food intake after central injection (8) and its expression is regulated negatively by the adipocyte-derived hormone, leptin, which reduces food intake and body weight (9, 10). Recent data have demonstrated that GALP also stimulates feeding in satiated rats over an acute period (11–13). However, in contrast to actions on galanin, leptin causes an increase in GALP mRNA in the arcuate nucleus in both fasted rats (14) and ob/ob mice (2). Furthermore, this effect may be direct because the majority (>85%) of GALP-containing arcuate nucleus neurons express the leptin receptor (3, 6). GALP is also present in the blood of mice, in which its levels decrease with food deprivation (15). Thus, these latter observations on GALP are more akin to those of several well-characterized anorectic peptides (e.g. α-MSH) that decrease food intake and body weight. In support of a complex action of GALP, we recently demonstrated that GALP has both orexigenic and anorectic actions in rats (12). Intracerebroventricular (icv) injection of GALP causes an initial increase in feeding (1 h), but at 24 h post injection, both food intake and body weight are reduced. In addition, GALP causes a significant rise in core body temperature over an 8-h period that is mediated by prostaglandins, an effect not observed with galanin (12). The longer-term anorectic effects of GALP have also been recently reported in mice by Krasnow et al. (13). However, these authors failed to observe the acute orexigenic effect of GALP reported in rats, although this may have been because the mice were anesthetized during injection.

To understand the differences between galanin and GALP, and where they act in the brain, we compared the effects of central injection of these peptides in rats on brain activation, using c-fos as a marker. The activation of brain regions secondary to food consumption was distinguished in a separate experiment by denying animals access to food following injection. Finally, a nonneuronal population of cells activated by GALP was identified by double immunohistochemistry using an astrocytic marker.

Materials and Methods

Animals

Male Sprague Dawley rats (Charles River Laboratories Inc., Sandwich, UK) weighing 250–300 g were used in all studies and were housed at a constant ambient temperature of 21 ± 2°C on a 12-h light, 12-h dark cycle (lights on at 0800 h). Rat chow (Beekay International, Hull, UK) and tap water were provided ad libitum unless stated. All procedures con-
formed to the requirements of the United Kingdom Animals (Scientific Procedures) Act, 1986.

Intracerebroventricular cannulation and injections

Rats were anesthetized with 2.5% halothane (AstraZeneca, Macclesfield, UK) and stereotaxically implanted with guide cannulae into the lateral ventricle [posterior 0.8 mm, lateral 1.5 mm, and ventral 3.5 mm (1 mm above injection site) from bregma according to the atlas of Paxinos and Watson (16)]. In some experiments core body temperature was monitored in undisturbed animals by remote radiotelemetry using radio transmitters (TA10TA-F40, Data Sciences, Minneapolis, MN) implanted into the peritoneum at the same time as cannulation. All animals were allowed to recover from surgery for a minimum of 5–7 d and then housed individually 24 h before injections, which were carried out in conscious, unrestrained animals commencing 2 h after lights on (1000 h).

Experiment 1: effect of GALP on food intake, body weight, and core body temperature

Rats (n = 5–6) were given a single icv injection of either rat GALP (10 μg in 1 μl; Phoenix Pharmaceuticals Inc., Belmont, CA) or vehicle (1 μl sterile saline). Food and water intake were measured 1, 2, and 8 h after injection, and core body temperature was measured continuously for 24 h. Changes from baseline temperature at 30-min intervals over 8 h were plotted for presentation.

GALP peptide was tested for endotoxin contamination in a limulus amebocyte lysate gelation test by the National Institute for Biological Standards and Control (NIBSC) and was found to contain negligible amounts (0.3 pg/mg), which do not induce fever (12).

Experiment 2: effect of galanin and GALP on c-fos expression

Rats (n = 7–8 per group) were injected icv with equimolar (1.6 mmol) doses of either rat GALP (10 μg in 1 μl) or rat galanin (5 μg in 1 μl; Bachem Ltd., Saffron Walden, UK) or 1 μl saline vehicle. After injections animals were given a preweighed amount of food, which was reweighed 90 min later when animals were anesthetized with sodium pentobarbital (90 mg/kg ip; Sagatal, Rhône-Mérieux, Harlow, UK). Rats were then perfused transcardially with heparinized saline followed by 4% paraformaldehyde in PBS. Brains were removed, postfixed in 4% paraformaldehyde for 2 h, and transferred to 30% sucrose solution until the tissue sank. The forebrain and brainstem were sectioned coronally at 30 μm on a freezing sledge microtome. Immunohistochemistry for c-fos was then performed on free-floating sections (90 μm apart) that were incubated in biotinylated anti-αB-crystallin polyclonal antibody (1:1000; Ab5, Oncogene Research Products, Cambridge, MA) followed by 2 h in a peroxidase-labeled goat antirabbit IgG antibody (1:500; Vector Laboratories, Burlingame, CA). Nuclear c-fos was visualized using a nickel-intensified diaminobenzidine reaction to produce a black precipitate. A region close to and surrounding the third ventricle (within 100 μm; bregma −0.30 to −4.16 mm) was defined as the perithird ventricular region for analysis (note this is not the same as, but will include the periventricular nucleus). Otherwise, nuclei were defined by the atlas of Paxinos and Watson (16): preoptic area bregma −0.30 to −0.92; supraoptic nucleus (SON) −0.80 to −1.80; Paraventricular hypothalamic nucleus (PVN) −1.30 to −2.12; arcuate nucleus (ARC) −2.56 to −4.30; dorsomedial nucleus (DMH) −2.56 to −3.60; ventromedial nucleus (VMH) −2.56 to −3.60; lateral hypothalamic area (LHA) −2.56 to −3.60; nucleus of the tractus solitarius (NTS) −12.8 to −14.60; area postrema −13.68 to −14.08. The number of immunopositive cells per section for each area (7–20 sections, depending on the region analyzed) was assessed bilaterally for each animal. Counting was carried out manually and blinded to the investigator. These values were then averaged to determine a group mean for each area of the brain.

Experiment 3: effect of food intake on GALP-induced c-fos expression

Rats (n = 5–6 per group) were given an icv injection of either vehicle (1 μl saline) or rat GALP (10 μg). After injections animals were either allowed access to food or had food withheld. Ninety minutes later food intake was measured (where appropriate), and transcendial perfusion followed by immunohistochemistry for c-fos protein was carried out as above (see experiment 2).

Experiment 4: effect of GALP on activation of astrocytes and microglia

Immunohistochemistry for c-fos was performed (as in experiment 2) on separate sets of forebrain sections from rats that had been injected icv with GALP (10 μg, n = 6) or vehicle (1 μl saline, n = 5–6). Sections were then incubated in either a mouse monoclonal antibody against glial fibrillary acidic protein (GFAP) (1:128K; Sigma-Aldrich Co. Ltd., Poole, UK) to detect astrocytes or OX-42 (1:50; Serotec, Oxford, UK) for microglia (and macrophages). After 24 h, sections were sequentially incubated in biotinylated antimouse IgG (1:200; Vector Laboratories) and streptavidin–horseradish peroxidase-labeled goat antirabbit IgG antibody (1:500; Vector Laboratories). After washing in Tris-buffered saline the sections were incubated in either nickel-intensified diaminobenzidine to produce a black precipitate or in diaminobenzidine with a nickel sulfate precipitate. Sections were counterstained with cresyl violet.
and then streptavidin-biotin-peroxidase complex (1:200; Amersham Pharmacia Biotech, Little Chalfont, UK). Astrocytic or microglia staining was then visualized by a normal diaminobenzidine reaction to yield a brown precipitate. The number of astrocytes that were c-fos positive or negative, and the number of Fos-positive cells were counted bilaterally in the perithird ventricular hypothalamic area. It was difficult to confidently identify the boundaries of individual microglial cells. However, because c-fos was not expressed in microglia, no counts were made.

Data and statistical analyses

All data are presented as mean ± SEM. Body temperatures were plotted as the mean change from the point of injection (time zero). The integrated temperature response between 0 and 8 h [area under the curve (AUC), C h] was calculated for each animal by the trapezoidal method. Average AUC values were then determined for each treatment group.

Statistical comparisons between two groups were performed using a nonparametric Mann-Whitney U test. Four-group comparisons involved parametric ANOVA followed by Tukey-Kramer post hoc multiple comparisons test or the nonparametric Kruskal-Wallis followed by Dunn’s multiple comparisons test. Nonparametric analyses were used if differences were recorded in group sds. Statistical significance was taken when P < 0.05.

Results

Experiment 1: effect of GALP on food intake, body weight, and core body temperature

The icv injection of 10 μg GALP caused a significant stimulation in food intake over a 1-h period (P < 0.01, Fig. 1A). Initiation of feeding was rapid, starting immediately after injection, and appeared to be complete after 30 min. Although in this experiment there was no change in food intake 24 h after GALP injection, there was a significant reduction in gain in body weight in this group when compared with vehicle-treated animals (P < 0.05, Fig. 1B). The icv administration of GALP also caused a significant increase in core body temperature that began to rise directly after injection and remained elevated over control values until approximately 8 h after injection (AUC for 0–8 h: vehicle, 2.2 ± 0.7 C h vs. GALP, 6.5 ± 1.2 C h, P < 0.05, Fig. 1C). There were no differences in water intake between the groups (results not shown).

Experiment 2: effect of galanin and GALP on c-fos expression

The icv injection of either galanin or GALP significantly increased food intake over the 90-min test period, compared with vehicle injection (vehicle, 0.6 ± 0.2 g; galanin, 2.0 ± 0.3 g; GALP, 3.1 ± 0.6 g; P < 0.01 and P < 0.01 vs. vehicle for galanin and GALP, respectively). When compared with vehicle injection, central administration of galanin or GALP induced a significant increase in c-fos expression in several identical brain regions; the hypothalamic DMH and LHA, and the NTS of the brainstem (Figs. 2 and 3). However, in contrast to animals treated with galanin, injection of GALP also caused expression of c-fos in two additional brain regions. These areas were the hypothalamic SON and a region surrounding the whole of the hypothalamic third ventricle (here termed the perithird ventricular hypothalamic region). Marked Fos protein was also detected after GALP in other regions surrounding the majority of the ventricular system.
(e.g. lateral ventricle), but no quantification of Fos-positive cells was performed in these areas. GALP also induced significant c-fos expression in the medial region of the hypothalamic ARC. However, the c-fos-positive cells detected in the ARC formed a continuum with cells in the perithird ventricular hypothalamic region. Finally, exten-
sive staining of cells expressing c-fos protein was observed in the ependymal lining of the ventricles of animals treated with GALP (not quantified).

**Experiment 3: effect of food intake on GALP-induced c-fos**

In the group of animals allowed access to food, icv injection of GALP stimulated food intake over 90 min, compared with control animals (vehicle plus food, 0.1 ± 0.1 g vs. GALP plus food, 3.1 ± 1.4 g; P < 0.05).

The pattern of c-fos-positive cells induced by icv injection of GALP in animals allowed access to food was comparable with that reported in experiment 2, with significant increases in c-fos detected in the SON, DMH, NTS, and the perithird ventricular hypothalamic region, which extended into the medial ARC (Fig. 4). In addition, there was an equivalent number of cells expressing c-fos in GALP-treated animals that had food withheld in the DMH, ARC, and the perithird ventricular hypothalamic region. However, when food was unavailable after GALP injection, there was no significant induction of Fos in the SON and NTS when compared with vehicle-treated animals.

**Experiment 4: c-fos and astrocyte or microglia immunohistochemistry**

In the perithird ventricular hypothalamic region, the c-fos immunoreactive nuclei appeared smaller than elsewhere and prompted us to identify the cell type. Double immunohistochemistry revealed that the majority of GALP-induced c-fos expressing cells were astrocytes (GFAP positive; Fig. 5, A and B). The percentage of Fos-expressing cells that were GFAP positive was 63 ± 5% vs. 12 ± 4% in the vehicle-treated animals (P < 0.001). In contrast, GALP did not induce c-fos in cells that were identified as microglia (positive with OX-42 antibody; Fig. 5, E and F). The c-fos-positive cells detected in the DMH (Fig 5, C and D), adjacent to the perithird ventricular hypothalamic region, and in the LHA did not label with either GFAP or OX-42, and were presumably neuronal.

**Discussion**

We have reported previously that central administration of GALP in rats increases food intake acutely (within 1 h) but causes a reduction in body weight over a 24-h period and a prolonged rise in core body temperature over 8 h (12). These latter responses are in contrast to those observed after icv injection of an equimolar dose of galanin. Although a single injection of galanin also causes an acute orexigenic effect, there are no longer-term effects (measured up to 24 h) on feeding, body weight, or body temperature (12). The acute orexigenic action observed after icv injection of GALP in rats has been reported by others (11, 13) and is also seen after intrahypothalamic injections (17). Although 24 h food intake was not statistically different in the present experiment, both the food intake and body weight changes after 24 h that we originally described in the rat (12) have been confirmed in a more recent study (13).

To study which areas of the brain might be involved in the actions of GALP and galanin, the distribution and extent of c-fos expression was assessed after central injection of either peptide. When GALP or galanin were centrally administered to rats in equimolar doses (1.6 nmol), a distinct pattern of cellular activation was observed. Both peptides caused a significant induction of c-fos in the DMH, LHA, and NTS, all regions involved in the control of feeding behavior. However, GALP also caused activation of cells in the SON and in the medial ARC and other areas surrounding the ventricles (including the perithird ventricular hypothalamic region).
The icv administration of GALP induces the immediate and rapid intake of a large meal, a response that is complete within 30 min (12). Because ingestion of a large, satiating meal can itself cause activation of neurons, e.g., in the NTS (18, 19), it is possible that the c-fos expression observed in the present study is secondary to food intake. Indeed, c-fos was not induced in the NTS by GALP in rats not allowed access to food. Likewise, c-fos induction is substantially reduced in the SON, a region involved in water balance. It is possible that some of the cell activation observed in the SON is secondary to the ingestion of a large meal, leading to an osmotic load, and part is due to a direct action (see below).

GALP did activate cells independently of food ingestion in the majority of brain areas studied (DMH, LHA, ARC, and perithird ventricular hypothalamic region) because the same extent of c-fos expression was found in animals not allowed access to food. GALP has been found previously to activate cells in the preoptic region of the hypothalamus, including neurons containing LHRH (20, 21). However, the present study illustrates that a large proportion of the GALP-activated cells in perithird ventricular hypothalamic regions were astrocytes rather than neurons. This is probably also the case for other areas of the brain close to the ventricular system, including the ARC and preoptic area, which have

**Fig. 5.** Effect of GALP on the activity (indicated by c-fos) of astrocytes or microglia in the hypothalamus. Photomicrographs represent equivalent sections through the perithird ventricular hypothalamic region (A, B, C, E, and F) and DMH (C and D) after icv injection of 10 μg GALP. A-D, Astrocytes using GFAP staining. E and F, Microglia using OX-42 staining. Solid arrows represent the localization of c-fos-positive nuclear profiles (dark back spots) in astrocytes (but not microglia) located in the perithird ventricular hypothalamic region (but not the DMH) after GALP injection. Open arrows indicate examples of astrocytes or microglia that did not contain c-fos, and arrowheads illustrate c-fos-positive only cells. The dashed box in A, C, and E is the magnified area represented in B, D, and F. Scale bars, 50 μm.
been analyzed as distinct anatomical regions here and elsewhere. The figures presented by Matsumoto et al. (20) and Fraley et al. (21) also appear to indicate a general activation of cells around the third ventricle. In contrast to the periventricular region, the cells that were stimulated by GALP in adjacent areas, the DMH (Fig. 5), and the LHA were neither astrocytes nor microglia and were presumably neurons. The action of GALP on astrocytes may be a direct effect because galanin receptors have been located on cultured astrocytic cells (22, 23). The relevance of this GALP-mediated activation of astrocytes is unclear. However, astrocytes do contain the enzyme cyclooxygenase, which under stimulation mediates the synthesis of prostaglandins that are involved in the production of fever (rise in core body temperature; for reviews see Refs. 24 and 25). We have demonstrated previously that central injection of GALP induces a rise in core body temperature that is mediated by prostaglandins (12). Thus, the hyperthermia observed here and previously (12) may be due to GALP-induced release of prostaglandins from astrocytes in periventricular regions.

To date, three galanin receptors have been cloned, GalR1–3 (see Ref. 26 for review), each with a distinct pattern of expression in the brain. Specifically, mRNA for all three receptors has been detected in several hypothalamic nuclei, including the PVN, DMH, LHA, and periventricular hypothalamic nucleus (27–30), and in addition GalR1 is expressed in the SON (27, 29) and NTS (31). It is reported that in vitro GALP displays a higher affinity for GALR2, compared with GalR1, whereas galanin binds to both receptors with equal affinities (1). It has yet to be determined whether GALP, like galanin, can act through GalR3. It is likely, though not certain, that activation of the DMH and LHA in response to both galanin and GALP is due to a direct effect on these peptides on either one or all of the three galanin receptors that are expressed in these areas and that this activation is involved in the acute orexigenic effect. However, c-fos is usually expressed only in cells that are activated by a stimulus, and any cells that are inhibited by either galanin or GALP are unlikely to express this immediate-early gene. Thus, it is possible that the activity noted in different brain regions may partially represent an indirect response because of disinhibition by other neurons that are upstream targets of galanin or GALP. GALP-induced c-fos expression in two areas of the brain in which galanin had no effect: the SON and the perithird ventricular hypothalamic region. Because GALP shows a higher affinity for GalR2, compared with GalR1, activation in these regions therefore may be due predominantly to GalR2, although this is not necessarily a direct effect. Discrepancies and differences between the effects of GALP and galanin may instead indicate the existence of an unknown galanin receptor and/or a novel GALP-specific receptor.

In summary, central administration of GALP and galanin caused differential brain activation. The response to GALP was not secondary to food ingestion in most brain regions. However, a large proportion of the cells that responded in periventricular regions were astrocytes rather than neurons. This is important because much of the c-fos reported by others in the preoptic area, ARC, and median eminence following central injection of GALP (20, 21) does not appear to be neuronal or in distinct anatomical nuclei but instead in a population of astrocytes completely surrounding the third ventricle. A smaller proportion of cells in these regions are still likely to be neurons, as shown by double staining for LHRH (20), and further such identification will be required for a fuller interpretation of all the available data. We should like to hypothesize that the acute orexigenic effects of both galanin and GALP administered centrally are mediated at least in part through a common pathway involving the hypothalamic DMH and LHA. In addition, GALP induces a hyperthermic response, perhaps by causing astrocytic release of prostaglandins. The anorectic and body weight-reducing effects of GALP at later time points are independent of this hyperthermia (12). The physiological relevance of the complex responses to central administration of GALP and the receptors involved remain to be determined.

Acknowledgments

Received March 27, 2003. Accepted June 5, 2003.

Address all correspondence and requests for reprints to: Dr. Simon Luckman, 1.124 Stopford Building, School of Biological Sciences, University of Manchester, Oxford Road, Manchester M13 9PT, United Kingdom. E-mail: simon.luckman@man.ac.uk.

This work was supported by the Biotechnology and Biological Sciences Research Council and AstraZeneca.

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