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The effects of galanin-like peptide on energy balance, body temperature and brain activity in the mouse and rat are independent of the GALR2/3 receptor

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Keywords

GALP; Fos; food intake; body weight; AR-M1986

Galanin-like peptide (GALP) is a neuropeptide that is thought to act on the galanin receptors GALR1, GALR2 and GALR3. In rats intracerebroventricular (i.c.v.) injection of GALP has dichotomous actions on energy balance, stimulating feeding over the first hour, but reducing food intake and body weight at 24 h, as well as causing an increase in core body temperature. In mice GALP only induces an anorexic action, and its effects on core body temperature are unknown. One aim of this study was to determine the effects of GALP on core body temperature in mice. I.c.v. injection of GALP into conscious mice had no effect on feeding over 1 h, but caused a significant reduction in food intake and body weight at 24 h. It also caused an immediate drop in core body temperature that was followed by an increase in body temperature. To understand these different effects of GALP on energy balance in mice compared to rats, and to determine the involvement of GALR2 and GALR3, immunohistochemistry was performed to localise c-Fos, a marker of cell activation. I.c.v. injection of GALP induced c-Fos expression in the parenchyma surrounding the ventricles, the ventricular ependymal cells, and the meninges in mice and rats. GALP also induced c-Fos expression in the supraoptic nucleus, dorsomedial hypothalamic nucleus, lateral hypothalamus, and nucleus tractus solitarius in rats but not mice. Central administration of a GALR2/3 agonist in rats did not induce c-Fos in any of the brain regions that expressed this protein after GALP injection, and had no effect on food intake, body weight, and body temperature in rats or mice.

These data suggest that GALP induces differential effects on energy balance and brain activity in mice compared to rats, which are unlikely to be due to activation of the GALR2 or GALR3 receptor.

Galanin-like peptide (GALP) is a 60-amino-acid neuropeptide, originally isolated from porcine hypothalamus, that shares partial sequence homology with galanin (1). Amino acids 9-21 of GALP are identical to the first 13 amino acids of galanin, which are essential for galanin receptor activation. Whereas galanin is widely distributed in the central nervous system (CNS), GALP is found only in neurones of the hypothalamic arcuate nucleus, the median eminence, and pituicytes of the posterior pituitary in the rat (2-5), mouse (6) and primate (7).

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GALP has been described as a central mediator of feeding and metabolism and, like galanin, intracerebroventricular (i.c.v.) injection of GALP acutely increases feeding (over 30 min – 1 h) in rats (8,9). However 24 h after GALP administration, a decrease in food intake is usually observed in rats, which is accompanied by a reduction in body weight (9,10). These longer-term anorectic actions of GALP are also observed after i.c.v. injection of this neuropeptide into mice (11–13). However, the acute orexigenic effects of GALP observed in the rat are not reported in mice (11). In this latter study, injections were performed under brief anaesthesia, which raises the possibility that a rapid orexigenic action of GALP in mice may be masked by any lasting effects of the anaesthesia. GALP also causes a significant rise in core body temperature in rats that lasts for approximately 6 h and is mediated by prostaglandins (9,10). The effect of GALP on core body temperature has not yet been fully assessed in mice, and further studies are required to elucidate whether severe changes in temperature may affect the ability of GALP to stimulate feeding.

Central administration of GALP induces a specific pattern of cell activation in the rat brain, as indicated by c-Fos protein expression in the ependymal cells of the ventricles, the periventricular regions, and the supraoptic nucleus (SON) of the hypothalamus, a response not seen with galanin (10). GALP also induces expression of c-Fos in the same areas as those observed after i.c.v. injection of galanin, such as the dorsomedial hypothalamus (DMH), lateral hypothalamus, the preoptic area (POA) of the hypothalamus, and the nucleus tractus solitarius (NTS) of the brainstem (10,14,15). The DMH and lateral hypothalamus are hypothalamic areas known to be involved in the regulation of food intake and recent data suggest that GALP promotes feeding in the rat via activation of known orexigenic neurones, including neuropeptide Y (NPY) neurones located in the DMH, and orexin neurones of the lateral hypothalamus (16,17).

The receptor involved in the effect of GALP on energy balance is currently unknown. Originally the actions of GALP were thought to be mediated by the three known galanin receptors, GALR1, GALR2 and GALR3. *In vitro* galanin shows high affinity for all three receptor subtypes, while GALP is reported to act predominantly via GALR2 and GALR3 (1,18). However, several reports now suggest that GALP may act via a novel GALP receptor, since this neuropeptide has differential effects to galanin, such as its actions on feeding and body temperature. Furthermore, the anorexic actions of GALP are observed in GALR1- and GALR2-deficient mice (12). Although this latter study provides further evidence for the presence of a novel GALP receptor, it does not exclude the possibility that GALP signals via GALR3 to affect energy balance. Moreover, GALP only produces anorexic actions in the mouse, so the galanin receptor responsible for the orexigenic effects in the rat still remains to be determined.

The aim of the present study therefore was to monitor feeding, body weight and core body temperature after central administration of GALP to conscious mice. Furthermore, to try to understand the potential differences between rats and mice, the effect of i.c.v. injection of GALP on brain activation, using c-fos as a marker, was assessed in mice. Finally, to determine the galanin receptors involved in the actions of GALP, the effects of the GALR2/3 agonist AR-M1896 on feeding and brain activity were compared to those of GALP. AR-M1896 was originally thought to be a specific GALR2 agonist (19) but has subsequently been shown to bind to GALR3 with equal affinity to GALR2 (20).

Materials and methods

Animals

Male Sprague-Dawley rats (230–300 g; Charles River Laboratories Inc., Sandwich, UK) or C57BL/6 mice (25–30 g; Harlan UK, Oxon, UK) were used in all studies. All animals were

housed at a constant ambient temperature of $21 \pm 2^\circ\text{C}$ on a 12: 12 h light/dark cycle (lights on 0800 h). Rodent chow (Beekay International, Hull, UK) and tap water were provided *ad libitum*. All procedures conformed to the requirements of the UK Animals (Scientific Procedures) Act, 1986.

Surgical procedures

Rats or mice were anaesthetised with isoflurane (1.5-3% in O_2) and stereotactically implanted with guide cannulae into the lateral cerebral ventricle. Injection co-ordinates relative to bregma were posterior 0.8mm, lateral 1.5mm, and ventral 4mm for rats, according to the atlas of Paxinos and Watson (21), and posterior 0.2mm, lateral 1mm and ventral 2.5mm for mice, according to the atlas of Franklin and Paxinos (22). Core body temperature was monitored in mice by remote radiotelemetry using radiotransmitters (TA10TA-F20, Data Sciences, Minneapolis, USA) that were implanted abdominally into the peritoneum at the same time as cannulation. After surgery, mice were housed individually and allowed to recover for at least 7 days. Rats were also left to recover for the same period but were housed individually 24-48 h before experimental injections.

Intracerebroventricular injections

I.c.v. injections were performed in lightly restrained conscious mice or unrestrained rats, commencing 2h after lights on (1000h). Rat GALP (0.15-3 nmol; Bachem, UK) was dissolved in sterile saline and the GALR2/3 agonist, AR-M1896 (1.5-4.5 nmol; AstraZeneca, Montreal, Canada) was dissolved in sterile water and diluted with sterile saline. The doses of GALP were based on previous studies in the rat that showed $1\mu\text{g}$ (0.15 nmol) and $10\mu\text{g}$ (1.5 nmol) effect food intake, body weight, and core body temperature, and $10\mu\text{g}$ (1.5 nmol) induces Fos expression in the brain (9,10). A total volume of $1\mu\text{l}$ was administered in both rats and mice. After injections, animals were given a pre-weighed amount of food that was re-weighed at varying time points. In all mice core body temperature was measured continuously in undisturbed animals. At the end of all studies placement of the guide cannulae was checked by a positive drinking response to angiotensin or by histological examination.

c-Fos immunohistochemistry

At 2 h post-injection, animals were anaesthetised using an overdose of sodium pentobarbitone (intraperitoneal; Sagatal, Rhône-Mérieux, Harlow, UK) and transcardially-perfused with 0.9% heparinised saline followed by 4% paraformaldehyde (in 0.1 M phosphate buffer, PB). Brains were then removed, post-fixed overnight in the same fixative, and cryopreserved in 30% sucrose. Coronal $30\mu\text{m}$ brain sections were cut throughout the level of the hypothalamus and brainstem on a freezing sledge microtome.

Immunohistochemistry for c-Fos protein was performed on free floating sections. After removal of endogenous peroxidase and treatment with blocking solution (2% normal goat serum in PB/0.3% triton), sections were incubated overnight at 4°C in a rabbit polyclonal anti-c-Fos antibody (1:1000, Ab5, Oncogene Research Products, UK), washed in PB/0.3% triton, and incubated for 2 h in a peroxidase-labelled goat anti-rabbit IgG antibody (1:500; Vector Laboratories Inc, UK). Following further washes (in 0.1 M PB), nuclear c-Fos was detected by incubation in a nickel-diaminobenzidine solution (Sigma-Aldrich, UK) that produced a blue-black precipitate.

The number of immunopositive cells expressing c-Fos protein per section (2-12 sections depending on the region analysed) were counted bilaterally, using a light microscope, in nuclei defined by Paxinos and Watson (21) for the rat: POA -0.30 to -0.92 ; SON -0.80 to -1.80 ; DMH -3.14 to -3.60 ; lateral hypothalamus -2.56 to -3.60 ; NTS -13.24 to -14.30 , or Franklin and Paxinos (22) for the mouse: POA 0.14 to -0.34 ; SON -0.58 to -0.94 ; DMH

–1.58 to –2.06; lateral hypothalamus –1.58 to –2.06; NTS –6.96 to –7.48. For the periventricular regions, only the parenchyma immediately surrounding the hypothalamic third ventricle was analysed (rat, –1.30 to –3.60; mouse, –0.34 to –2.06), and is termed the perithird ventricular hypothalamic (Pe3V) region here. The average number of cells per section was then calculated, and the group mean determined for each brain region. The brain regions analysed for c-Fos expression were those that have been previously reported to significantly express c-Fos after i.c.v. injection of GALP in the rat with the exception of the arcuate nucleus (10). As the expression of c-Fos in the parenchyma of the Pe3V region was extensive, and extended laterally into the medial arcuate nucleus, the arcuate nucleus was not analysed as a distinct anatomical region.

Cell culture and western blot

Rat neuronal co-cultures were prepared from post-natal brains (day 0-2) of male Sprague-Dawley rats according to a previously described protocol (23). Cells were treated with either normal culture medium (control) or rat galanin (0.01-10 μ M; Bachem, UK), rat GALP (0.01-10 μ M), or AR-M1896 (0.01-10 μ M) at 37 °C (5% CO₂/95% O₂) for 20 min. Cells were then washed in 1x phosphate buffered saline (PBS) and prepared in lysis buffer at 4 °C. Protein concentration in the cell lysate was determined by the Bradford method (Bio-Rad, Germany). Samples (20 μ g protein/lane) were loaded on a 10% SDS acrylamide gel, and resolved by gel electrophoresis. Proteins were then transferred onto a PVDF membrane (Bio-Rad, Germany). To reduce non-specific binding, membranes were washed in 10% milk (Marvel, UK; 10% in 0.25% PBS-Tween; Gibco BRL) for 1 h. Membranes were then incubated for 1 h in either a rabbit polyclonal anti-rat antibody to detect total extracellular signal-regulated kinase 1/2 (ERK1/2; 1:2000 in 1% BSA; Santa Cruz SC-93, Santa Cruz Biotechnology, CA), or phosphorylated ERK1/2 (1:2000 in 1% BSA; Phospho-p44/42 MAP kinase, Cell Signaling Technology, UK), followed by an 1 h incubation with a horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (1:2000 in 10% milk; Dako, UK). Protein bands were detected by chemiluminescence (ECL kit; GE Healthcare). Densitometry was used to calculate changes in phospho-ERK1 and ERK2 band intensity relative to total ERK1 and 2.

Statistical analysis

All data are presented as mean $\pm$ standard error of the mean (SEM). Core body temperature in mice is expressed as the change from the average baseline values taken at time 0. The integrated temperature response (between 2-10 h or 2-6 h), stated as area under the curve (AUC, °C/h), was calculated for each animal by the trapezoidal method. Average AUC values were then determined for each group.

Statistical comparisons between two groups were analysed using a non-parametric Mann-Whitney U-test. Three or four group comparisons were performed using a parametric analysis of variance (ANOVA) followed by a Tukey-Kramer post-hoc multiple comparisons test or, if the data were non-parametric, the non-parametric Kruskal-Wallis followed by Dunn's multiple comparisons test. Non-parametric tests were used if the sample standard deviations were significantly different. Statistical significance was taken when $P < 0.05$.

Results

The effect of GALP on feeding, body weight and body temperature in the mouse

I.c.v. injection of GALP (0.15, 1.5, or 3 nmol) or vehicle ($n = 5-9$ per group) in conscious C57BL/6 mice had no effect on food intake 1 h after injection. However, 24 h after treatment, GALP caused a dose-related reduction in food intake, although the effect was significant only for 3 nmol GALP (vehicle: 4.7 ± 0.4 g; 0.15 nmol: 3.4 ± 0.4 g; 1.5 nmol: 3.4

± 0.2 ; 3 nmol: 2.8 ± 0.5 g, $P < 0.05$ for 3 nmol versus vehicle; Fig. 1A). Central administration of GALP also caused a reduction in 24 h change in body weight when compared to vehicle-treated mice (vehicle: 0.3 ± 0.2 g; 0.15 nmol: -0.4 ± 0.3 g; 1.5 nmol: -1.1 ± 0.4 g; 3 nmol: -1.6 ± 0.6 g, $P < 0.05$ for 3 nmol versus vehicle; Fig. 1B).

Core body temperature was monitored in the same group of mice (Fig. 2). Although there was a slight rise in core body temperature between 2–10 h after i.c.v. injection of 0.15 nmol GALP, this was not significantly different from that of vehicle-treated mice (Fig. 2A; Table 1A and B). However, both 1.5 nmol and 3 nmol GALP caused a rapid and transient drop in temperature that reached a nadir at 30 min for 1.5 nmol and 1 h for 3 nmol GALP (Fig. 2B and C; Table 1A). During this period of hypothermia the mice were inactive, which was especially apparent in mice that had the greatest drop in temperature. Although no behavioural analysis was performed, mice did not appear to be asleep during this period of inactivity as classical sleep behaviour was not observed, and they recovered within 1 h. At 2 h after injection of 1.5 or 3 nmol GALP, core body temperature was greater than that of vehicle-treated mice, which was significant between 2–10 h (Table 1B). There was no difference in temperature between vehicle- and GALP-treated mice (at all doses) over the 10–24 h period (data not shown).

The effect of GALP on c-Fos expression in the mouse

As GALP did not increase food intake at 1 h in mice, core body temperature was monitored as a measure of a successful response to GALP. As before, i.c.v. injection of 1.5 nmol GALP in mice resulted in a significant increase in core body temperature at 2 h post injection, compared to vehicle-treated mice (vehicle, 36.5 ± 0.2 °C, $n = 6$; GALP, 37.8 ± 0.2 °C, $n = 5$; $P < 0.01$).

At 2 h after i.c.v. injection of 1.5 nmol GALP in mice, c-Fos expression was observed in the parenchyma that surrounds the ventricles (termed peri-ventricular), which included the lateral, dorsal third, third and fourth ventricles. The ependymal cells that line the ventricles and the meninges were also induced to express c-Fos protein (Fig. 3). In some animals the intensity and quantity of Fos-positive cells were greater in the ventral part of the Pe3V region compared to dorsal regions (Fig. 3D). Quantification of the number of cells expressing c-Fos for the Pe3V region, revealed a significant increase in the number of c-Fos positive cells in this area when compared to control mice (Fig. 4). The vehicle-treated mice rarely expressed c-Fos in the peri-ventricular regions and the ependymal layers. No significant increase in c-Fos was observed after GALP treatment in all other mouse brain regions analysed: POA, SON, DMH, lateral hypothalamus and the NTS of the brainstem.

The effect of the GALR2/3 agonist AR-M1896 on ERK activity in neuronal cultures

Activation of GALR2 stimulates ERK phosphorylation *in vitro*. In order to test the biological activity of AR-M1896, and compare its actions to GALP, we tested the effect of AR-M1896 and GALP on ERK phosphorylation in cultured neurones. Galanin has been shown to phosphorylate ERK *in vitro* (24) thus, as a positive control, we also tested the effect of this peptide on ERK activity.

The highest dose of AR-M1896 (10 μ M) induced a significant increase in ERK1 and ERK2 phosphorylation 20 min after treatment (Figure 5). In contrast, all doses of galanin and GALP had no significant effect on the phosphorylation of ERK1 or ERK2, although there was a trend for an increase in both.

The effect of the GALR2/3 agonist AR-M1896 on feeding and body weight in the rat and mouse

I.c.v. administration of the GALR2/3 agonist, AR-M1896 (1.5 or 3 nmol, $n = 4-5$ per group) in rats had no effect on food intake at 1 or 24 h after injection when compared to vehicle-treated rats (Table 2). In a separate experiment, higher doses of AR-M1896 (3 and 4.5 nmol) had no significant effect on food intake, body weight and core body temperature over 24 h after i.c.v. injection in the rat (data not shown). In mice i.c.v. injection of 3 nmol AR-M1896 also had no effect on feeding, body weight and core body temperature over 6 h (Figure 6A and B), which was in contrast to the significant reduction in food intake and body weight observed after GALP administration. Core body temperature was also monitored in the same group of animals, and as expected, i.c.v. injection of 3 nmol GALP caused a hypothermic response that was followed by a period of hyperthermia (Figure 6C). However, i.c.v. administration of 3 nmol AR-M1896 had no effect on core body temperature when compared to vehicle-treated mice (AUC, °C/h: Vehicle, 3.0 ± 0.6 ; GALP, 11.3 ± 1.2 ; AR-M1896, 4.8 ± 2.0 ; $P < 0.01$, $n = 4$ /group).

The effect of the GALR2/3 agonist AR-M1896 and GALP on c-Fos expression in the rat

As GALP induces c-Fos in several brain regions in rats (10), but only in the Pe3V in mice, the effect of AR-M1896 on c-Fos expression was initially tested in the rat. GALP significantly increased food intake at 2 h after i.c.v. injection of 1.5 nmol in the rat compared to vehicle-treated rats (vehicle, 0.4 ± 0.4 g; GALP, 4.3 ± 0.5 g; $P < 0.01$; $n = 5$ per group). An equimolar dose (1.5 nmol) of AR-M1896 had no effect on feeding behaviour, and food intake was not significantly different from vehicle-treated rats (vehicle, 0.4 ± 0.4 g versus AR-M1896, 0.5 ± 0.5 g; $n = 5$ per group).

As previously reported (10), i.c.v. injection of GALP caused a significant induction of c-Fos expression in the SON, DMH, lateral hypothalamus and NTS at 2 h after treatment (Fig. 6). In addition, expression of c-Fos protein was observed in the peri-ventricular regions after i.c.v. GALP, which included the parenchyma around the lateral, dorsal third, hypothalamic third and fourth ventricles. Fos-positive cells were also seen in the ependymal cells lining the ventricles and the meninges of the rat brain after i.c.v. GALP. Central administration of AR-M1896 did not significantly induce expression of c-Fos protein, when compared to vehicle-treated rats in any of the brain regions analysed (Fig 7).

As central administration of AR-M1896 did not induce expression of Fos in the rat, and also had no effect on food intake, body weight, body temperature in either rats or mice, the effect of this GALR2/3 agonist on expression of Fos in mice was not tested.

Discussion

Central injection of GALP in the rat increases food intake over 1 h, but causes a reduction in feeding and body weight over 24 h (8,9,11). The results of the present study demonstrate that central administration of GALP in mice also caused a reduction in feeding and body weight at 24 h, but had no effect on food intake at 1 h after injection. These observations are in agreement with previous studies in the mouse, and further confirm the lack of orexigenic effect of GALP in mice (11-13). In addition, the present study is the first to administer central injections of GALP to conscious mice, thus eliminating any confounding effects of anaesthesia. Continuous measurements of core body temperature in the mouse revealed that i.c.v. injection of GALP caused a dose-related hypothermia, during which mice appeared subdued and inactive. The drop in core body temperature occurred immediately, but was transient, as body temperature returned to control levels between 1-2 h post-injection. This phase of hypothermia was followed by a prolonged period of hyperthermia that lasted for

approximately 8 h. I.c.v. injections of GALP do not cause hypothermia in the rat but lead to a rapid and prolonged increase in core body temperature that is prostaglandin-dependent (9). This difference in the effect of GALP on core body temperature between the two species may explain why GALP does not acutely stimulate eating in the mouse. The initial hypothermia and inactivity induced by GALP in the mouse may be a non-specific effect that could directly or indirectly interfere with the ability of GALP to stimulate food intake. Other studies have also shown that GALP reduces spontaneous locomotor activity after central administration (11). The reason for these effects on locomotion are unknown, but it is possible that GALP may be inducing sickness behaviour in mice as central injection of this peptide causes taste aversion in mice (11,13).

To study the mechanisms underlying the different actions of GALP in mice compared to rats, the pattern of c-Fos protein expression was assessed in the brain after central injections of GALP. As previously published (10), GALP caused a significant induction of c-Fos in the SON, DMH, lateral hypothalamus, NTS, peri-ventricular regions (e.g. Pe3V region), ventricular ependymal cells, and meninges of the rat brain. However, GALP failed to stimulate the expression of c-Fos in the SON, DMH, lateral hypothalamus and NTS of the mouse, but a significant increase was detected in the peri-ventricular regions, ependymal cells and meninges. GALP also failed to stimulate c-Fos expression in the POA of the hypothalamus in mice, which is in agreement with previously published work in the rat (10), and data from the present study. Other groups have demonstrated the induction of c-Fos in the POA of the rat after injection of 5 nmol GALP (14,15), however, the difference in dose in those studies compared to here (5 nmol vs 1.5 nmol) may explain this discrepancy.

GALP-induced cell activation in the SON and NTS of the rat brain is dependent on food consumption, as GALP does not stimulate c-Fos expression in these brain regions when food is withheld after injection (10). Thus, the absence of c-Fos in the SON and NTS of the mouse after GALP injection is likely to be due to the lack of food consumption in this species. The inability of GALP to activate cells within the DMH and lateral hypothalamus of the mouse may explain why an orexigenic effect of GALP is not observed in this species. The DMH and the lateral hypothalamus both play a crucial role in feeding, and recent data suggest both these areas to be targets for GALP. Kuramochi and colleagues have demonstrated that GALP stimulates food intake (over 2 h) when injected directly into the DMH of rats, and have proposed that this effect is due to the action of NPY neurones (17). These authors show that i.c.v. injection of GALP increases c-Fos expression in NPY-containing neurones in the DMH, and inhibition of endogenous NPY, or NPY receptors, inhibits the acute orexigenic effect of GALP in rats. The involvement of orexin in the acute orexigenic actions of GALP has also been established, as central administration of GALP activates orexin neurones in the lateral hypothalamus, and blocking the effects of orexin with an anti-orexin antibody inhibits GALP-induced hyperphagia (16). These studies therefore suggest that the orexigenic effect of GALP in rats is due to the action of NPY and orexin in the DMH and lateral hypothalamus, respectively. Thus, the lack of an orexigenic response to GALP in the mouse in the present study may be explained by an inability of GALP to activate cells in the DMH and lateral hypothalamus in this species.

Central injections of GALP induced a robust expression of c-Fos in the Pe3V region in both the rat and the mouse. Cells that are activated in this region in rats have been shown to be astrocytes, rather than neurones or microglia (10), and it is likely that this is also true in mice. Activated astrocytes are involved in inflammatory responses to infection and injury in the brain, and release key mediators of inflammation, such as cytokines (25). Analogous to actions of GALP, inflammation and infection are associated with negative energy balance, characterised by weight loss, reduced food intake and fever (rise in core body temperature).

Hence, it is possible that the longer-term anorectic actions of GALP in both the rat and mouse are due to an astrocyte-mediated immune response.

The receptors involved in the actions of GALP on energy balance are unknown, but to date, *in vitro* studies reveal that GALP displays activity only at galanin receptors. Three galanin receptors, GALR1-3, have been cloned in the rat and mouse (26). *In vitro* GALP is an agonist for all three galanin receptors (GALR1-3) that displays slight preference for GALR2 and GALR3 (1,18). However, the effects of GALP on feeding and body weight in the mouse are unlikely to be mediated via GALR2, as GALP reduces food intake and body weight in GALR2-deficient mice (GALR2 $-/-$) to a similar degree as in wild-type mice (12), and GALR2-deficient mice display no abnormalities in feeding behaviour (27). In addition, galanin 2-29, a GALR2 selective agonist, has no effect on food intake at 1 h after injection in rats (28,29), although later time points were not assessed in these studies. The actions of galanin at GALR2 (and GALR1) have been shown to be mediated via mitogen-activated protein kinase (MAPK) pathways (30), and studies have shown that galanin stimulates the phosphorylation of MAPK/ERK1 in hippocampal organotypic cultures (24). The current study shows that the GALR2/3 specific agonist, AR-M1896 (19,20), stimulated ERK1 and ERK2 phosphorylation in rat neurones *in vitro* whereas galanin and GALP had no significant effect on ERK1 or ERK2 activation. It is unclear as to why galanin and GALP did not affect ERK activation in the present study, but this may be due to the difference in culture system employed (organotypic versus cell), and the time point of analysis (2-10 min versus 20 min) (24). Although our data demonstrate that AR-M1896 affects MAPK signalling pathways more potently than galanin or GALP, the GALR2/3 agonist had no effect on food intake, body weight or core body temperature in the rat and mouse. These data further support that GALR2 is not involved in the anorexic or temperature response of GALP in the mouse and rat. Furthermore, these data demonstrate that the orexigenic effect of GALP in rats is independent of the GALR2 receptor, and that GALR3 is not responsible for any actions of GALP in the rat or mouse. It is possible, therefore, that the orexigenic effect of GALP is mediated via the GALR1 receptor, and studies by Wang and colleagues have shown that activation of GALR1 stimulates feeding acutely (28). However, the anorexic actions of GALP are unlikely to be due to GALR1, as mice deficient in this receptor still eat less and lose body weight in response to central administration of GALP (12). Further work is therefore required to determine the receptor involved in the long-term anorectic actions of GALP, and the development of fully selective GALR agonists/antagonists is essential. It is also possible that GALP mediates some of its actions through a novel GALP-specific receptor that remains to be identified.

Central administration of GALP induced c-Fos in the SON, DMH, lateral hypothalamus and NTS of the rat brain, an effect not observed with the GALR2/3 agonist, AR-M1896. These findings indirectly suggest that GALR1 (or a novel receptor) may be responsible for the GALP-induced cell activation in these brain regions in the rat, although further evidence is needed, such as the effect of a GALR1 agonist on brain activity.

The reason for the different effects of GALP on feeding, body temperature and brain activity between rats and mice is unknown, but may be related to galanin receptor distribution. The distribution of GALR1-3 has been studied extensively in the rat, and each receptor displays a specific expression pattern in the brain. All three receptors have been detected in the DMH, lateral hypothalamus and peri-ventricular hypothalamic nucleus, and GALR1 is also expressed in the SON and NTS of the rat brain (31-35). Although GALR1-3 have been detected in the mouse brain (see (26)), there is limited anatomical data available on the distribution of these receptors (e.g. in the hypothalamus) in this species. GALR1 mRNA is located in the SON, DMH, lateral hypothalamus and periventricular hypothalamic nucleus of the mouse brain (36) and therefore, differences in galanin receptor distribution are

unlikely to explain the results reported in the present study. However, discrepancies between the actions of GALP in the mouse and the rat could be due to any differences in the binding properties of GALP on the known galanin receptors or a novel GALP receptor.

In summary, central administration of GALP produces different effects on feeding, core body temperature and brain activity in the mouse compared to the rat. In the mouse, GALP caused a transient hypothermia, and failed to stimulate key brain regions (DMH and lateral hypothalamus) that are involved in energy balance, effects that are in direct contrast to the rat. These actions may therefore explain why GALP is unable to acutely stimulate feeding in the mouse. Furthermore, activation of the GALR2 or GALR3 receptor is unlikely to be responsible for the effects of GALP on energy balance in the rat or mouse. Additional work is required to confirm if GALP plays a physiological role in energy homeostasis and to confirm the receptor that mediates its actions.

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Fig. 1. Effect of i.c.v. 0.15 nmol, 1.5 nmol or 3 nmol galanin-like peptide (GALP) on food intake (A) and change in body weight (B) in mice. Injections were performed 2 h after lights on. Food intake was measured at 1 and 24 h, and body weight at 24 h after injections and data for core body temperature are shown in Figure 2. Data are mean $\pm$ SEM for $n = 5-9$ mice per group. * $P < 0.05$, versus vehicle-treated animals.

Fig. 2.

Effect of i.c.v. 0.15 nmol (A), 1.5 nmol (B) or 3 nmol (C) galanin-like peptide (GALP) on the change in core body temperature in mice. Injections were performed 2 h after lights on. Body temperature was monitored continuously by remote radiotelemetry for 24 h, but data are shown only for 0-12 h (see Table 1 for analysis). For clarity, data for each dose of GALP are plotted against those for vehicle-treated mice. Data for food intake and body weight are shown in Figure 1. Data are mean $\pm$ SEM for $n = 5-9$ mice per group.

Fig. 3.

Representative photomicrographs illustrating c-Fos expression after i.c.v. injection of vehicle (1 μ l saline; A-C) or galanin-like peptide (GALP; 1.5 nmol; D-F) in the mouse brain. Injections were performed 2 h after lights on. Significant increases in c-Fos expression were observed in the peri-ventricular regions of the ventricles including the peri-third ventricular hypothalamic region (A and D, see Fig. 4 for analysis), the dorsal third ventricle (B and E), and in the meninges (C and F). Robust c-Fos protein expression was also observed in the ependymal lining of the ventricles (see arrows). Scale bars, 100 μ m. D3V, dorsal third ventricle; 3V, third ventricle.

Fig. 4.

Effect of i.c.v. injection of galanin-like peptide (GALP) on c-Fos protein expression in the mouse brain. The bar graph illustrates the number of c-Fos positive nuclei per section in several brain regions 2 h after i.c.v. injection of GALP (1.5 nmol, $n = 5$) or vehicle (1 μ l saline, $n = 6$). Injections were performed 2 h after lights on. All data are mean $\pm$ SEM. * $P < 0.05$ versus vehicle. DMH, dorsomedial hypothalamic nucleus; LH, lateral hypothalamus; NTS, nucleus of the tractus solitarius; Pe3V, peri-third ventricular hypothalamic region; POA, preoptic area of the hypothalamus; SON, supraoptic nucleus.

Fig. 5.

Effect of the GALR2/3 agonist, AR-M1896, on extracellular signal-regulated kinase (ERK1/2) phosphorylation in rat neuronal cultures. Neurones were treated with galanin, GALP or AR-M1896 (all at 0.01, 0.1, 1 and 10 μ M). After 20 min treatment, cells were lysed and ERK1 (A) and ERK2 (B) phosphorylation was analysed by immunoblotting equal amounts of protein. Representative blots from 3 independent experiments on separate cultures are shown for phosphorylated ERK1 and ERK2 and total ERK1 and ERK2. Densitometry was used to calculate changes in phospho-ERK1 and ERK2 band intensity relative to total ERK1 and ERK2. All data are mean $\pm$ SEM. * $P < 0.05$ versus control (C).

Fig. 6.

Effect of i.c.v. injection of 3 nmol galanin-like peptide (GALP) or 3 nmol AR-M1896 on food intake (A), change in body weight (B), and change in core body temperature in mice. Injections were performed 2 h after lights on. Food intake and body weight were measured at 6 h after injections. Core body temperature was monitored continuously by remote radiotelemetry for 6 h (see text for analysis by AUC). Data are mean $\pm$ SEM for $n = 4$ mice per group. * $P < 0.05$, *** $P < 0.001$ versus vehicle-treated animals.

Fig. 7.

Effect of i.c.v. injection of galanin-like peptide (GALP) or the GALR2/3 agonist, AR-M1896 on c-Fos expression in the rat brain. The bar graph illustrates the number of c-Fos positive nuclei per section in several brain regions 2 h after i.c.v. injection of GALP (1.5 nmol, $n = 5$), AR-M1896 (1.5 nmol, $n = 5$) or vehicle (1 μ l saline, $n = 5$). Injections were performed 2 h after lights on. All data are mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$ versus vehicle. DMH, dorsomedial hypothalamic nucleus; LH, lateral hypothalamus; NTS, nucleus of the tractus solitarius; Pe3V, peri-third ventricular hypothalamic region; POA, preoptic area of the hypothalamus; SON, supraoptic nucleus.

Table 1

The temperature response after i.c.v. injection of galanin-like peptide (GALP) in mice

A) The change in temperature at 30 min and 1 h

Treatment	$\Delta^{\circ}\text{C}$ at 30 min	$\Delta^{\circ}\text{C}$ at 1 h
Vehicle	0.5 $\pm$ 0.3	1.3 $\pm$ 0.2
0.15 nmol GALP	0.6 $\pm$ 0.5	1.8 $\pm$ 0.2
1.5 nmol GALP	-2.0 $\pm$ 0.6 ^a	-0.2 $\pm$ 1.5
3 nmol GALP	-1.2 $\pm$ 0.4	-2.9 $\pm$ 0.8 ^a

Data are mean $\pm$ SEM for n = 5-9 per group.

^aP < 0.01 versus vehicle and 0.15 nmol GALP

B) The integrated temperature response (AUC, °C h) for 2-10 h

Treatment	AUC (°C h)
Vehicle	7.1±1.2
0.15 nmol GALP	15.1±3.5
1.5 nmol GALP	19.4±2.2 ^a
3 nmol GALP	20.5±1.3 ^a

Data are mean± SEM for n = 5-9 per group.

^aP < 0.01 versus vehicle

Table 2

The effect of AR-M1896 on food intake after i.c.v. injection in rats

Treatment	1 h food intake (g)	24 h food intake (g)
Vehicle	0.0±0.0	27.4±3.1
1.5 nmol AR-M1896	0.0±0.0	26.6±1.6
3 nmol AR-M1896	0.0±0.0	27.4±2.4

Injections were performed in satiated rats (2h after lights on, 1000h).

Data are mean±SEM for n = 4-5 per group.