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Distribution and Regulation of Galanin-Like Peptide (GALP) in the Hypothalamus of the Mouse

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Galanin-like peptide (GALP) is a newly discovered molecule whose expression in the brain is confined to the arcuate nucleus and median eminence. In the rat, cellular levels of GALP mRNA are reduced by fasting and reversed by peripheral administration of leptin. The purpose of this investigation was 1) to clone and map the distribution of GALP mRNA in the brain of the mouse; 2) to compare the pattern and magnitude of GALP mRNA expression in the leptin-deficient obese (*ob/ob*) mouse with that of wild-type controls; and 3) to examine the effects of leptin delivered into the brain on the expression of GALP mRNA in the *ob/ob* mouse. We report the sequence of a mouse GALP cDNA and show that GALP mRNA is expressed in the arcuate nucleus, median eminence, infundibular stalk, and the neurohypophysis of this species. The expression of GALP mRNA in the brain was markedly reduced in the *ob/ob* mice, compared with wild-type animals. Intracerebroventricular infusion of leptin to *ob/ob* mice increased both the number of GALP mRNA-expressing neurons and their content of GALP mRNA, compared with vehicle-treated controls. These observations demonstrate that GALP mRNA is induced by leptin through a direct action on the brain. (*Endocrinology* 142: 5140–5144, 2001)

GALANIN IS A neuropeptide comprising 29/30 amino acids, which is expressed in many regions of the central nervous system as well as in peripheral tissues (1). Three subtypes of galanin receptors have been cloned (GalR1-GalR3), all of which are coupled to G proteins (2). Galanin is coreleased with classical neurotransmitters and is thought to play a neuromodulatory role in higher cognitive brain function, including learning and memory (3–5). In the hypothalamus and pituitary, galanin has been implicated in the regulation of GH, PRL, and gonadotropin secretion as well as in the control of energy homeostasis (6–11). Until recently, galanin was the only known member of this neuropeptide family.

Galanin-like peptide (GALP) was recently isolated from pig hypothalamus, and its cDNA was cloned from the brain of the pig, rat, and human (12). GALP is a 60 amino acid peptide that is structurally related to galanin. Thirteen of GALP's amino acid residues (9–21) are identical to the N-terminal portion (1–13) of galanin, which is required to activate galanin receptors. GALP recognizes both the GalR1 (IC_{50} = 4.3 nM) and GalR2 (IC_{50} = 0.24 nM) receptors with high affinity, with a modest preference for the GalR2 receptor subtype (12). In contrast to galanin's wide distribution in the brain, GALP mRNA-expressing cells in the rat are restricted to the arcuate nucleus (Arc) of the hypothalamus, most notably in its medial and caudal aspects, plus the median eminence and infundibular stalk (13, 14). In the rat, GALP mRNA is also found in the posterior pituitary (13, 15) but not in dorsal root ganglion (16). Recently, Takatsu *et al.* (17) used antibodies directed to the N-terminal (1–10) part of GALP to map the distribution of GALP-containing nerve fibers in the rat brain. GALP immunostaining was observed in the parvocellular division of the paraventricular hypothalamic nucleus, preoptic area, bed nucleus of stria terminalis, and lateral septum (17). In the posterior pituitary, GALP mRNA has been shown to be dramatically up-regulated by salt loading and dehydration in pituicytes as well as in nerve terminals, suggesting that GALP may be involved in the regulation of vasopressin (and perhaps oxytocin) release (15).

Leptin is a satiety hormone secreted from adipocytes, which acts on the brain to regulate feeding behavior, metabolism and pituitary function (18). The obese (*ob/ob*) mouse has a mutation in the leptin gene that prevents production of functional leptin protein (19). The phenotype of the *ob/ob* mouse includes severe obesity, infertility, and insulin resistance, and administration of recombinant leptin to these animals quickly ameliorates these disorders (20–24). In the rat, GALP gene expression in the Arc is reduced by fasting and reversed by systemic administration of leptin, suggesting that GALP may be involved in mediating leptin's effects on the regulation of energy balance or in the regulation of pituitary function (13). The finding that the vast majority of the GALP cells in the Arc coexpress the leptin receptor, Ob-Rb (17), lends further credence to the idea that the GALP gene is involved in these processes. The purpose of the present investigation was first to clone and map the distribution of GALP mRNA in the brain and pituitary of the mouse; second, to compare the pattern and magnitude of its expression in the leptin-deficient *ob/ob* mouse with that of wild-type controls; and third, to examine the effects of leptin delivered centrally on the expression of GALP mRNA in the mouse brain. Here, we report the sequence of a mouse GALP cDNA and dem-

Abbreviations: Arc, Arcuate nucleus; CART, cocaine- and amphetamine-regulated transcript; GALP, galanin-like peptide; ICV, intracerebroventricular; *ob/ob,* obese; WT, wild-type.

onstrate that leptin acts centrally to induce the expression of GALP mRNA in the Arc of the *ob/ob* mouse.

Materials and Methods

Animals

Two separate experiments were performed. For the leptin infusion study, 60-d-old male *ob/ob* C57BL/6 (45–55 g) and wild-type (WT) C57BL/6 (25–30 g) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). WT and *ob/ob* mice were housed in individual cages with access to standard rodent chow and water *ad libitum* and maintained on a 14:10 h light/dark cycle with lights on at 0700 h. For the GALP distribution study, 5 adult male WT C57BL/6 mice were housed together and maintained under the same conditions as described above. 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.

Intracerebroventricular (ICV) injections

Male age-matched *ob/ob* (n = 12) and WT (n = 6) mice were housed in individual cages. Body weight and food intake were monitored for 1 wk before the start of the experiments. Animals were anesthetized for injection with isoflurane delivered by a vaporizer (Veterinary Anesthesia Systems, Bend, OR). For freehand ICV injections, a small hole was bored in the cranium at 1 mm posterior to bregma and 0.5 mm lateral to bregma. Afterward, the animals were allowed to recover for 4 d during which time the animals were handled daily. Three experimental groups of animals (n = 6 each) were given daily freehand ICV injections into the lateral ventricle at 1600 h, as previously described (25). One group comprised vehicle-treated WT mice, and the other two groups comprised *ob/ob* mice that received either vehicle or human recombinant leptin (0.02 nmol/d). Body weight and food intake were measured each day after the injection. The animals were killed after 7 d, 1 h after the last injection. The mice were rapidly decapitated, and the brains were quickly removed and frozen on dry ice.

Cloning of a partial mouse GALP cDNA

Four adult C57BL/6 mice were injected for 2 d, twice daily (0900 and 1600 h) with 6 nmol recombinant human leptin (sc), to up-regulate GALP gene expression. The animals were killed 1 h after the last injection, and the hypothalami were removed and immediately frozen on dry ice. Total RNA was extracted with a Totally RNA kit (Ambion, Inc., Austin, TX). Reverse transcription of mRNA was performed with M-MLV reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD), and oligo-dT primers at 42 C for 1 h. The enzyme was inactivated by heating at 92 C for 10 min.

PCR cloning was performed with the CloneAMP pAMP system (Life Technologies, Inc.) and deoxy UMP-containing primers designed from the rat GALP sequence, (forward primer: 5′-CAUCAUCAUCAUCCAAGCATCTGGTCCTCTTC-3′; reverse primer: 5′-CUACUACUACUATCTATGGCCTTCCACAGGTC-3′). A low-stringency PCR program with an initial melting temperature set at 95 C followed by 35 cycles at 94 C for 30 sec, 45 C for 30 sec, 72 C for 60 sec, and a final extension at 72 C for 10 min was used to obtain PCR product. PCR was performed with *Taq* DNA polymerase (Life Technologies, Inc.) in a total reaction volume of 50 μl. A single band was detected at the expected size of 205 bp on a 1% agarose gel. The PCR product was purified with a QIAquick gel extraction kit (QIAGEN, Valencia, CA). The purified cDNA fragment was then used as template in a second round of PCR under the same conditions. After gel purification, the amplified PCR product was cloned into the pAMP1 plasmid (Life Technologies, Inc.), which was transformed into JM-109 cells (Promega Corp., Madison, WI) and cultured overnight. Plasmids were isolated with a Plasmid Maxiprep kit (QIAGEN). The insert was sequenced and shown to be a 205-bp cDNA fragment sharing 95% sequence identity to rat GALP cDNA.[1]

[1] The portion of the sequence data that is specific to the mouse (165 bp) has been submitted to GenBank under accession number AF426450.

In situ hybridization

Antisense and sense mouse GALP ^{33}P-labeled cRNA probes were transcribed from linearized pAMP plasmid containing the mouse GALP cDNA insert with SP6 and T7 RNA polymerases (Roche, Indianapolis, IN), respectively. Two separate *in situ* hybridization assays for GALP mRNA were performed, one for the analysis of GALP mRNA distribution in the brain of WT animals and another for assessing the regulation of GALP mRNA by leptin in the *ob/ob* mouse. The assays were performed on separate sets of coronal sections (20 μm) of mouse brain, cut through the hypothalamus on a cryostat and thaw mounted onto Superfrost Plus slides (VWR Scientific, West Chester, PA). Sections were collected in a 1:4 series from the diagonal band of Broca, caudally through the mammillary bodies as described previously (26). In brief, tissue sections were fixed, acetylated, and delipidated. A hybridization solution containing denatured, radiolabeled GALP cRNA (0.3 pmol/ml) and yeast tRNA (2 mg/ml) in hybridization buffer was applied to the tissue (100 μl/slide). The slides were coverslipped, placed in horizontal slide racks, and incubated overnight in humid chambers at 55 C. The next day slides were treated with RNase A (Sigma, St. Louis, MO) and washed under conditions of increasing stringency with two final hot washes at 60 C. The tissue was dehydrated in ethanol and finally dipped in NTB-3 emulsion (Kodak, Rochester, NY). The slides were exposed for 6 d, developed, and counterstained with cresyl violet (Sigma).

Quantitative analysis

Slides were assigned a random three-letter code and read in alphabetical order, with an automated image analysis system by an operator unaware of the experimental group to which the animal belonged. The total number of cells and silver grains/cell were determined on nine anatomically matched sections taken through the rostrocaudal extent of the Arc with a grain-counting program, as previously described (27). Differences among groups were assessed by ANOVA. When the ANOVA indicated significant differences, Fisher's post hoc test was used to determine whether individual groups were significantly different from one another. Differences were considered significant when $P < 0.05$.

Results

Cloning of a partial mouse GALP cDNA

To identify cells expressing GALP mRNA in the mouse, we cloned a mouse GALP cDNA from total RNA isolated from the hypothalami of leptin-treated adult C57BL/6 mice. PCR primers were designed with the rat mRNA sequence as a template in areas that showed high degree of sequence identity among the GALP sequences cloned from rat, pig, and human (12). The resulting 205-bp PCR product showed 95% sequence identity to rat GALP mRNA (GenBank accession no. AF188491). The 39 base sequence coding for GALP (9–21), which shows sequence identity with galanin (1–13), was 100% conserved in the mouse. Within the cloned 205-bp fragment, nine mismatches, resulting in four substitutions at the amino acid level, are found between rat and mouse.

Distribution of GALP mRNA in the brain of the mouse

The 205-bp GALP cDNA was used to transcribe sense and antisense riboprobes for *in situ* hybridization. In the brain of the mouse, as in the rat, GALP mRNA-expressing cells were restricted to the Arc and median eminence (Fig. 1); however, the anatomical distribution of GALP mRNA-containing cells within the Arc of the mouse was different from that of the rat. In the rat, GALP cells are located medially and close to the third ventricle, whereas in the mouse, GALP cells are found more laterally and ventrally within this nucleus. Moreover, the rostral-caudal distribution of GALP mRNA-containing

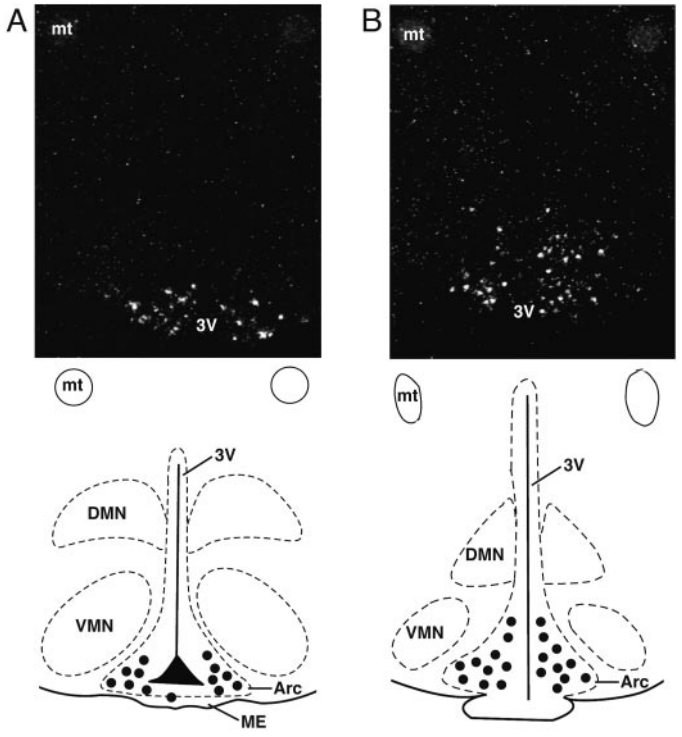

FIG. 1. Photomicrographs and distribution of GALP mRNA-expressing cells in the rostral (A) and caudal (B) aspects of the Arc in the mouse brain. In the photomicrographs, clusters of silver grains *(white clusters)* indicate the presence of GALP mRNA-expressing cells. The *large dots* in the diagrams depict the relative distribution of cells expressing GALP mRNA. Arc, Arcuate nucleus; DMN, dorsomedial nucleus; mt, mamillothalamic tract; VMN, ventromedial nucleus; ME, median eminence; 3V, third ventricle.

cells is different between the two rodent species. In the rat, the majority of the GALP mRNA-containing cells are found in the caudal part of the Arc (13), and this is also true in the mouse. However, a larger fraction of the total number of GALP mRNA-expressing cells are found in the rostral portion of the Arc in the mouse, compared with the rat. No significant signal was detected with the sense control probe.

Regulation of GALP mRNA in the brain of the ob/ob mouse

Ob/ob mice receiving leptin injections ate significantly less and were significantly lighter at the end of the treatment period, compared with those who received vehicle alone (Fig. 2). Food intake in WT animals was not significantly different from the *ob/ob* + leptin group; however, the WT animals ate significantly less than the *ob/ob* + vehicle group (data not shown). Compared with WT mice, leptin-deficient *ob/ob* mice expressed very low levels of GALP mRNA in the hypothalamus, with the number of identifiable cells expressing GALP mRNA being reduced by 70% in *ob/ob* mice, compared with WT mice ($P < 0.01$) (Fig. 3). In the few identifiable GALP mRNA-containing cells present in the *ob/ob* animals, the number of grains/cell (reflecting the amount of mRNA expressed by each individual cell) was 20% lower in the *ob/ob* mice, compared with WT controls, although this difference was not statistically significant (Fig. 3). The group of *ob/ob* mice injected with leptin showed a nearly 5-fold induction in

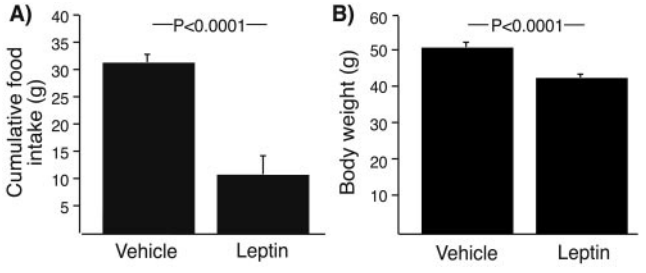

FIG. 2. A, Cumulative food intake of leptin-treated (0.02 nmol/d, delivered centrally) and vehicle-treated *ob/ob* mice (n = 6) after 7 d of injections ($P < 0.0001$). B, Final body weight of leptin- and vehicle-injected *ob/ob* animals after 7 d of injections ($P < 0.0001$). *Bars* represent means ± SEM.

the number of identifiable GALP mRNA-containing neurons in the Arc ($P < 0.0003$), with 40% higher levels of GALP mRNA signal (grains/cell), compared with vehicle-treated *ob/ob* mice (Figs. 3 and 4).

Discussion

This study describes the cloning of a partial cDNA for mouse GALP and the distribution of cells expressing GALP mRNA in the brain and pituitary of the mouse. The mouse GALP cDNA (205 bp) cloned in this study corresponds to amino acid residues (4–71) of the rat GALP preprohormone; the unique mouse sequence (excluding the rat primer sequence) corresponds to amino acids 11–65. The overall sequence homology between the rat and mouse in this region is 95% at the bp level. Within this sequence, nine mismatches, resulting in four substitutions at the amino acid level, are found between the rat and mouse. The part of the GALP sequence (9–21) that shares sequence homology with galanin (1–13) in the mouse is identical in all species studied to date (12), testifying to the likely biological significance of this amino acid motif. A similar 94% base pair sequence identity is found between the full-length rat and mouse galanin mRNA sequences (28, 29).

The cloned mouse GALP cDNA was used for generation of radiolabeled cRNA probes for *in situ* hybridization to map mRNA-expressing cells in the mouse hypothalamus. GALP mRNA-containing cells were found throughout the rostrocaudal extent of the Arc of the hypothalamus similar to the distribution observed in the rat (13, 14, 17). GALP mRNA-expressing cells were also found in the median eminence of the mouse; however, in this region there were fewer cells observed in the mouse, compared with the rat. Cells expressing GALP mRNA within the Arc of the mouse appeared to have a more lateral distribution than the rat. In this regard, the pattern of GALP mRNA distribution in the mouse Arc resembled that of galanin (30). In the rat, the location of GALP-expressing cells in the Arc is highly shifted toward the caudal part of the nucleus. GALP cells in the Arc of the mouse are spread more uniformly throughout the full extent of the nucleus, with the exception of the most rostral part, where very few GALP cells are found. These discrepancies could be attributed to differential organization of the arcuate nucleus between the rat and mouse or to GALP serving different physiological functions in the two species.

Our second objective was to examine whether GALP

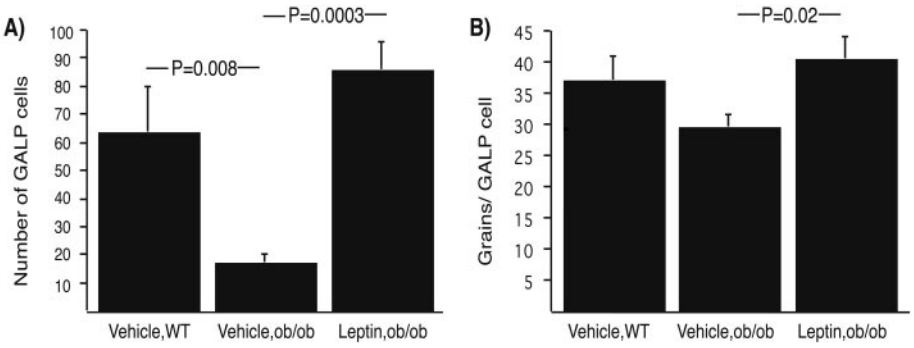

FIG. 3. Regulation of GALP mRNA in the Arc of *ob/ob* and WT mice. A, Number of identifiable GALP mRNA-expressing cells in the *ob/ob* mouse (vehicle, delivered centrally) is significantly reduced, compared with WT controls (vehicle, delivered centrally; $P = 0.008$). Leptin treatment (0.02 nmol/day, delivered centrally for 7 d) induced GALP mRNA expression and fully restored the number of identifiable GALP mRNA-expressing cells to that of WT mice. B, Number of grains/cell, reflecting the amount of GALP mRNA expressed in each individual cell, was reduced in the *ob/ob* mouse, compared with WT controls (NS) and was significantly increased in the leptin-treated mice, compared with the vehicle-treated group ($P = 0.02$). *Bars* represent means ± SEM.

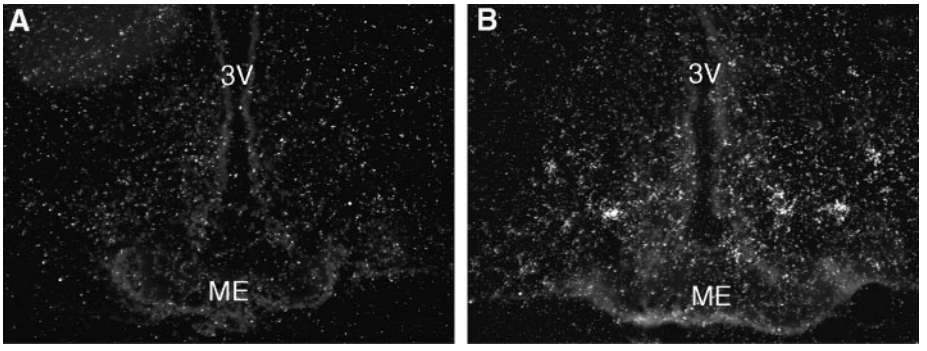

FIG. 4. Photomicrograph showing GALP mRNA-expressing cells in the Arc of *ob/ob* mice treated with vehicle (A) and leptin (B). *Clusters of silver grains* indicate the presence of cells expressing GALP mRNA. ME, median eminence; 3V, third ventricle.

mRNA was regulated by leptin in the mouse and, if so, to determine whether the effect of leptin was attributable to its central (*vs.* peripheral) action. We have previously shown in the rat that GALP gene expression in the Arc is reduced by fasting and up-regulated in fasted animals given peripheral (sc) administration of leptin (13). This experiment left unanswered the question of whether the induction of GALP mRNA was owing to a direct effect of leptin in the brain or to secondary mechanisms triggered by leptin acting on peripheral targets. We hypothesized that, compared with WT mice, leptin-deficient *ob/ob* mice would have relatively reduced levels of GALP mRNA in the hypothalamus, and this indeed proved to be the case. We also postulated that if GALP cells in the Arc were direct targets for leptin in the brain, infusion of leptin into the lateral ventricles should induce GALP gene expression in *ob/ob* mice, which again proved to be the case, both in terms of cell number and message content per cell.

Although our results demonstrate that leptin acts on the mouse brain to induce GALP gene expression, we cannot be absolutely certain that leptin directly targets GALP neurons to mediate this effect. In the rat, it is clear that most GALP neurons (> 85%) in the Arc express the leptin receptor, lending some credence to the inference that in the mouse, leptin acts directly on GALP neurons to induce GALP gene expression (17); however, this remains to be proven. In this regard, it would also be of interest to determine whether leptin activates signaling mechanisms downstream of the leptin receptor in GALP neurons, such as Fos and SOCS-3, as has been demonstrated in POMC neurons (31, 32).

The inductive effect of leptin on GALP mRNA in the Arc of the *ob/ob* mouse is similar to leptin's effect on the mRNAs coding for POMC and cocaine- and amphetamine-regulated transcript (CART) (33, 34) and opposite to its effect on NPY and agouti-related protein (35, 36). On the basis of these observations, we might infer that the induction of GALP may play a parallel role with POMC or CART in the mediation of leptin's effect on satiety and metabolism or in the regulation of pituitary function. In the rat, GALP does not appear to be colocalized with α-MSH, NPY, agouti-related protein, somatostatin, or galanin, indicating that GALP neurons in the Arc might represent a novel population of leptin-responsive neurons, distinct from those previously characterized (17). However, because GALP neurons in the mouse appear not to be restricted to the periventricular region of the Arc (unlike the rat), the possibility of GALP being coexpressed with one or more of these peptides in the mouse cannot be excluded. Galanin neurons located in the lateral part of the Arc, in contrast to GALP neurons, are neither regulated by leptin in the *ob/ob* mouse nor do they express the signaling form of the leptin receptor Ob-Rb, which would argue that galanin neurons in the Arc do not directly participate in leptin-mediated events in the brain (30).

In summary, we report the cloning of a partial cDNA for mouse GALP and the anatomical distribution of GALP mRNA-containing cells in the brain of the mouse. We have

also demonstrated that GALP mRNA in the Arc is a target for central regulation by leptin in the *ob/ob* mouse. These findings suggest that GALP neurons in the Arc of the mouse represent a novel target for leptin signaling and mark this newly discovered peptide as a possible molecular link coupling leptin and its effects on satiety, metabolism, and pituitary function.

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