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Cloning and Distribution of Galanin-Like Peptide mRNA in the Hypothalamus and Pituitary of the Macaque

MATTHEW J. CUNNINGHAM, JARRAD M. SCARLETT, AND ROBERT A. STEINER*

Graduate Program in Neurobiology & Behavior (M.J.C.), Undergraduate Program in Neurobiology (J.M.S.), Department of Obstetrics & Gynecology (R.A.S.), Department of Physiology & Biophysics (R.A.S.), Department of Zoology (R.A.S.), University of Washington, Seattle, Washington 98195

Galanin-like peptide (GALP) is a newly discovered hypothalamic neuropeptide, which is regulated by leptin and implicated in the regulation of GnRH secretion in the rodent. We searched the human genome database and determined that the human GALP gene comprises six exons, as has been shown for human galanin. We used rapid amplification of cDNA ends to clone a full-length cDNA (802 bp) of the macaque homologue of GALP and found it to be highly conserved between human and macaque at both the nucleotide (93%) and peptide (94%) levels. Mature GALP is predicted to be 60 amino acids in the macaque as in other species, and the region of GALP (9–21) that shows homology to the N-terminal 13 amino acids of galanin is perfectly conserved. We mapped the distribution of GALP mRNA in the hypothalamus and pituitary of the macaque by *in situ* hybridization and observed that, as in rodent species, the expression of GALP mRNA is confined to the arcuate nucleus, median eminence, and neurohypophysis. Using double-label *in situ* hybridization, we found that nearly all (98%) GALP mRNA-expressing cells in the arcuate nucleus also express mRNA for the long form of the leptin receptor. These findings suggest that a leptin-GALP signaling pathway exists in a primate species. (*Endocrinology* 143: 755–763, 2002)

THE NEUROPEPTIDE GALANIN and its mRNA are found throughout the central nervous system of the primate, most notably in both the basal forebrain and hypothalamus (1, 2). In humans, iv galanin infusion has been shown to increase basal as well as GH-releasing hormone-stimulated release of GH (3, 4), whereas in the rhesus macaque, galanin infusion has been shown to increase LH secretion (5). In addition to its involvement in anterior pituitary hormone secretion, galanin in rodents has been implicated in, among other things, the regulation of reproduction, cognitive function, and feeding behavior (6–10). There are three known galanin receptor subtypes (GalR1-GalR3) that have been cloned and characterized in the human, all of which appear to be expressed within the brain (11–14). Galanin is a 30-amino acid peptide in humans (a 29-amino acid C-terminally amidated peptide in other species), of which the highly conserved N-terminal 13 amino acids confer biological activity at galanin receptors (15–17).

Galanin-like peptide (GALP) is a 60-amino acid peptide, which was originally isolated and cloned after screening porcine hypothalamic extracts for activation of galanin receptors (18). Indeed, it was found that amino acids 9–21 of GALP are identical to amino acids 1–13 of galanin. GALP cDNAs were subsequently cloned from human and rat (18), and in the rat, GALP mRNA was shown to be expressed in the hypothalamic arcuate nucleus (Arc) and median eminence (ME), as well as within the posterior pituitary (19–21). In concert with the distribution of GALP mRNA, Takatsu *et al.* (22) have reported that cell bodies containing GALP immunoreactivity are limited to the Arc/ME in the brain of the rat. Immunoreactive GALP fibers were reported in several regions of the forebrain, including the parvicellular region of the hypothalamic paraventricular nucleus, the medial preoptic area, the bed nucleus of the stria terminalis, and the lateral septum (22).

Evidence suggests that GALP is responsible for mediating some of the actions of the satiety hormone leptin within the brain. The number of GALP mRNA-expressing cells is reduced in the Arc of rats fasted for 48 h (a state of leptin deficiency), and this suppression is prevented in rats supplemented with leptin injections during the fast (19). We have also demonstrated leptin's ability to regulate GALP mRNA in another model of leptin deficiency, the obese (*ob/ob*) mouse. *Ob/ob* mice have reduced numbers of GALP mRNA-expressing cells in the Arc compared with wild-type mice; giving *ob/ob* mice intracerebroventricular injections of leptin restores GALP mRNA to levels seen in wild-type mice (23). Leptin may act directly on GALP neurons, as these cells have been shown to coexpress leptin receptor (Ob-R) by double-label immunocytochemistry (22).

The present studies attempt to establish a primate model for future studies designed to explore the physiological significance of GALP in this group of animals. Our objectives were: 1) to determine the genomic structure of human GALP by searching the public human genome database; 2) to clone the full-length cDNA for GALP in the macaque and compare its sequence to that of the human; 3) to map the distribution of GALP mRNA in the macaque hypothalamus and pituitary gland via *in situ* hybridization (ISH); and 4) to determine if GALP mRNA-expressing cells also express Ob-R mRNA by double-label ISH. As part of our comparison of genes among primate species, we also cloned the portion of the macaque preprogalanin cDNA corresponding to the coding region for

Abbreviations: AGRP, Agouti-related protein; Arc, arcuate nucleus; dT, deoxythymidine; GALP, galanin-like peptide; ISH, in situ hybridization; ME, median eminence; Ob-Rb, long form of the leptin receptor; RACE, rapid amplification of DNA ends; SBRs, signal to background ratios; SSC, sodium saline citrate; UTR, untranslated region.

galanin (1–30) and present a comparison of its amino acid sequence between the human and macaque.

Materials and Methods

Genomic analysis of human GALP

The Web-based NCBI BLAST program (http://www.ncbi.nlm.nih.gov/BLAST) was used to search the human genome for sequence homology to the published human GALP cDNA sequence. Fragments of the cDNA sequence that produced search hits were then aligned against the human genomic sequence using Sequencher software (Gene Codes, Ann Arbor, MI).

Experimental animals

Tissue was obtained from either adult male or ovariectomized adult female pigtailed macaques (*Macaca nemestrina*) through the Tissue Distribution Program at the Regional Primate Research Center at the University of Washington. Housing and procedures were approved by the Institutional Animal Care and Use Committee at the University of Washington, in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Tissue preparation

Animals were sedated with ketamine (10 mg/kg) and anesthetized with sodium pentobarbital (25 mg/kg). After removal of tissues for other projects, animals were killed by severing the spinal cord. The brain and pituitary were removed from the skull, the brain was dissected, and both tissues were frozen on dry ice. For ISH experiments, a hypothalamic block was dissected as follows: coronal cuts were made approximately at the rostral extent of the diagonal band of Broca, and caudal to the mammillary bodies; lateral cuts were made on one side at the medial aspect of the globus pallidus, and on the opposite side at the globus pallidus/putamen junction. For RNA extraction, the hypothalamic block was further dissected into a smaller block containing the infundibular stalk, Arc, and ventromedial nucleus. Frozen tissues were stored at −80 C after dissection. The large hypothalamic blocks and pituitaries were cryostat sectioned at 20 μm, thaw-mounted onto SuperFrost Plus slides (VWR Scientific, West Chester, PA), and stored at −80 C.

Cloning of partial cDNA for GALP and galanin

Total RNA was extracted from hypothalamus and pituitary by the acid phenol-guanidinium thiocyanate-chloroform method as described by Sambrook and Russell (24). RNA was reverse transcribed with the use of a RetroScript kit (Ambion, Inc., Austin, TX), primed with oligo deoxythymidine $(dT)_{18}$, and then used for subsequent PCR. Primers were designed based on the published sequence of the human GALP gene (GenBank accession no. AF188492) with forward primers corresponding to bases 114–131 and reverse primers corresponding to bases 407–427 (short GALP forward/reverse; see Fig. 2B) and custom synthesized (Life Technologies, Inc., Gaithersburg, MD) with modified 5′ ends containing deoxy-UMP for use with the CloneAmp pAMP1 rapid cloning system (Life Technologies, Inc.). This portion of the gene corresponds to the majority of the coding region of human GALP. PCRs (50 μl) contained the following: 5 μl of reverse transcriptase reaction product, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each NTP, 0.6 μM each primer, and 3 U *Taq* DNA polymerase (Life Technologies, Inc.). Reactions were performed in a PTC-100 thermal cycler (MJ Research, Inc., Watertown, MA) using a touchdown protocol: cDNA was denatured for 5 min at 95 C, then 30 cycles were carried out at 94 C for 30 sec, 60 C for 30 sec, and 72 C for 60 sec, where the annealing temperature was lowered by 0.5 C per cycle. Then 10 cycles were carried out at 94 C for 30 sec, 50 C for 30 sec, and 72 C for 60 sec, and the program ended with a 5-min extension at 72 C. After electrophoresis in a 1% agarose (wt/vol) gel, a single DNA fragment was obtained of approximately the expected size (314 bp) from both hypothalamic and pituitary reverse transcriptase template, and gel purified with a QIAQuick gel extraction kit (QIAGEN, Valencia, CA). This fragment was then ligated into the pAMP1 vector by incubating fragment, vector, and uracil DNA glycosylase for 30 min at 37 C. JM109 cells (Promega Corp., Madison, WI) were transformed by heat shock and recombinant clones (ampicillin resistant) were chosen for sequence analysis.

A partial cDNA of macaque preprogalanin was cloned from the hypothalamus as above with the following changes. Primers were designed based on the published sequence of the human galanin gene (GenBank accession number M77140) with forward primers corresponding to bases 91–108 and reverse primers corresponding to bases 274–297 and synthesized by Life Technologies, Inc. PCRs were performed as described above. A band of expected size (207 bp) was gel purified and sequenced in both directions with the original PCR primers.

Rapid amplification of cDNA ends (RACE)

5′ and 3′ RACE were performed with a GeneRacer kit (Invitrogen, Carlsbad, CA). For 3′ RACE, first-strand synthesis was performed on total RNA using the RetroScript kit (as described above) with exception of the use of a modified oligo dT_{18} primer with known 5′ sequence (GeneRacer). A macaque GALP-specific primer (3′ RACE forward; see Fig. 2B) was designed based on the 314-bp macaque GALP fragment cloned previously and used in PCR with a primer targeting the GeneRacer oligo dT primer. The PCR protocol was as follows: cDNA was denatured for 2 min at 94 C, then 5 cycles were carried out at 94 C for 30 sec and 72 C for 60 sec, then 5 cycles were carried out at 94 C for 30 sec, 70 C for 30 sec, and 72 C for 60 sec, then 20 cycles were carried out at 94 C for 30 sec, 68 C for 30 sec, and 72 C for 60 sec, ending with a 10-min extension at 72 C. This produced an approximately 700-bp band that was gel purified, cloned into the pCR4-TOPO vector (Invitrogen), and transformed into JM109 cells. Three colonies were cultured for miniprep (QIAprep Spin Miniprep Kit, QIAGEN), and the cDNA insert was sequenced in both directions.

For 5′ RACE, total RNA was processed according to GeneRacer kit instructions, culminating in the ligation of a RNA oligo of known sequence to the 5′ end of full-length mRNAs. Modified mRNA was reverse transcribed as described above except priming was performed with random decamers (Ambion, Inc.). An initial round of PCR was performed with a macaque GALP-specific primer (outer 5′ RACE reverse; see Fig. 2B) and a primer against the GeneRacer oligo sequence under the following protocol: cDNA was denatured for 3 min at 94 C, then 45 cycles were carried out at 94 C for 30 sec, 60 C for 30 sec, and 72 C for 30 sec, ending with a 7-min extension at 72 C. A subsequent round of PCR was performed under the same protocol using the previous PCR reaction as template, with a nested primer against the GeneRacer oligo sequence as well as a nested macaque GALP-specific primer (inner 5′ RACE reverse; see Fig. 2B). This produced an approximately 500-bp band that was gel purified, cloned into the pCR4-TOPO vector (Invitrogen), and transformed into JM109 cells. Nine colonies were cultured for miniprep and were sequenced using primers against the T7 polymerase promoter sequence.

Sequences from individual 5′ and 3′ RACE colonies were assembled into consensus sequences using Sequencher and 5′ and 3′ RACE consensus sequences were aligned with CLUSTALW.

Riboprobe preparation

^{33}P-labeled GALP cRNA riboprobe. The partial GALP cDNA (314 bp) described above was used as a template for *in vitro* transcription. The cDNA was linearized with *Hin*dIII, and the radiolabeled, antisense cRNA probe was synthesized *in vitro* by inclusion of the following ingredients in a volume of 20 μl: 250 μCi ^{33}P-UTP (NEN Life Science Products, Boston, MA); 1 μg linearized DNA; 0.5 mM each ATP, CTP, GTP; 40 U of RNase inhibitor (Roche Molecular Biochemicals, Indianapolis, IN); 20 U of T7 RNA polymerase (Roche). Residual DNA was digested with 10 U of DNase (Roche), and the DNase reaction was terminated by addition of 2 μl of 0.5 M EDTA (pH 8.0). Yeast tRNA was added as carrier (10 μg), and then the riboprobe was separated from unincorporated nucleotides with a Quick Spin Sephadex G-50 column (Roche Molecular Biochemicals).

^{33}P-labeled leptin receptor long form (Ob-Rb) cRNA riboprobe. A 422-bp portion of the macaque Ob-Rb cDNA sequence coding for the cytoplasmic tail of the receptor (corresponding to bases 2880–3301 of rhesus macaque Ob-Rb; GenBank accession number AF222960) was synthesized by PCR and cloned into pAMP1. The cDNA was linearized with

*Bam*HI, and the radiolabeled, antisense cRNA was synthesized with T7 RNA polymerase and purified as described above.

Digoxigenin-labeled GALP cRNA riboprobe. A 629-bp portion of macaque GALP sequence was cloned using a composite GALP cDNA sequence to design primers (long GALP forward/reverse; see Fig. 2B). This fragment was synthesized by PCR and cloned into pAMP1. The cDNA was linearized with *Hin*dIII, and digoxigenin-labeled, antisense cRNA was synthesized using T7 RNA polymerase and DIG RNA labeling mix (Roche) according to the manufacturer's protocol. After synthesis, digoxigenin-labeled riboprobe was treated with DNase and purified as described above.

Single-label ISH

Slides with either hypothalamic (adult males; n = 3) or pituitary (ovariectomized females; n = 3) sections were processed before hybridization as follows (all steps were performed at room temperature): sections were fixed for 5 min in 4% paraformaldehyde in 0.1 M phosphate buffer, acetylated for 10 min in 0.25% acetic anhydride in 0.1 M triethanolamine, and delipidated in chloroform. After this pretreatment, sections were stored at room temperature until used for hybridization. Radiolabeled, antisense GALP riboprobe was denatured, dissolved in hybridization solution [62.5% formamide (vol/vol), 12.5% dextran sulfate (wt/vol), 0.375 M NaCl, 10 mM Tris (pH 8.0), 1 mM EDTA, 0.02% BSA (wt/vol), 0.02% ficoll (wt/vol), 0.02% polyvinylpyrrolidone (wt/vol)] at a concentration of 0.2 pmol/ml along with tRNA (1.9 mg/ml), and applied to slides. Two negative controls were used to demonstrate specificity of the GALP riboprobe: slides were incubated with radiolabeled antisense probe in the presence of excess (400×) unlabeled antisense probe, or an equivalent concentration of radiolabeled sense GALP riboprobe. Slides were covered with silane-coated glass coverslips, placed in a humid chamber, and incubated overnight at 55 C. The following day, slides were treated with RNase A (29 μg/ml; Sigma, St. Louis, MO) and washed under conditions of increasing stringency, including two 30-min washes in 0.1× SSC (sodium saline citrate; 1× SSC is 150 mM NaCl, 15 mM sodium citrate) at 60 C. Slides were then dehydrated, air-dried, and then dipped in NTB-3 liquid emulsion (Eastman Kodak Co., Rochester, NY). Slides were developed 5 d later and coverslipped.

Double-label ISH

Slides with hypothalamic sections from ovariectomized females (n = 3) were processed before hybridization as described above. Radiolabeled, antisense Ob-Rb riboprobe (0.4 pmol/ml) and digoxigenin-labeled GALP riboprobe (concentration determined empirically) were denatured, dissolved in hybridization solution along with tRNA (1.7 mg/ml), and applied to slides. Controls used to establish specificity of the Ob-Rb riboprobe include slides incubated with radiolabeled antisense probe in the presence of excess (1,000×) unlabeled antisense probe, or an equivalent concentration of radiolabeled sense Ob-Rb riboprobe. Slides were covered with silane-coated glass coverslips, placed in a humid chamber, and incubated overnight at 55 C. The following day, slides were treated with RNase A and washed twice in 0.1× SSC at 60 C. Sections were then incubated in blocking buffer [2× SSC, 0.05% Triton X-100 (vol/vol), 2% normal sheep serum (vol/vol; Pel-Freeze Biologicals, Rogers, AZ)] for 60 min, rinsed in Buffer 1 (100 mM Tris, 150 mM NaCl, pH 7.4), and then incubated in Buffer 1 containing antidigoxigenin fragments conjugated to alkaline phosphatase (Roche Molecular Biochemicals) diluted 1:200, 0.3% Triton X-100, and 1% normal sheep serum for 3 h at 37 C. Next, sections were rinsed in Buffer 1 and Buffer 2 (100 mM Tris, 50 mM $MgCl_2$, 100 mM NaCl, pH 9.5) and then incubated in Buffer 2 containing 4-nitro blue tetrazolium-chloride (340 μg/ml; Roche Molecular Biochemicals), 5-bromo-4-chloro-3-indolyl phosphate (175 μg/ml; Roche Molecular Biochemicals), and levamisole (240 μg/ml; Sigma). When cells containing purple/black precipitate were clearly visible at the light microscope level, the reaction was stopped by rinsing the sections in TE (10 mM Tris, 1 mM EDTA, pH 8.0). Slides were then dipped in 70% ethanol, air-dried, and coated in 3% parlodion (wt/vol; Fisher Scientific, Pittsburgh, PA) dissolved in isoamyl acetate. After air-drying, slides were dipped in NTB-3 emulsion. Slides were developed 9 d later and coverslipped.

Data analysis

For the single-label ISH assay, tissue was viewed with a Carl Zeiss Axioskop microscope (Carl Zeiss, Thornwood, NY), allowing identification of silver grain clusters corresponding to GALP mRNA-expressing cells. The distribution of these clusters was transferred to representative atlas plates modified from Martin *et al.* (25). Coexpression of GALP and Ob-Rb mRNAs within the same cells was analyzed as previously described (26). Briefly, sections with GALP mRNA-containing cells were viewed under a microscope, and images were captured with a Cohu 4910 camera (San Diego, CA) attached to a Power Macintosh G3 (Apple, Cupertino, CA). Using custom-designed software, we identified GALP mRNA-containing cells and then counted the number of silver grains (corresponding to Ob-Rb mRNA) over each cell. Signal to background ratios (SBRs) for individual cells were then calculated. For each individual animal, the number of counted cells with a SBR greater than a particular integer value was calculated, and then converted to a percentage of the total counted cells (cumulative frequency curve). The percentages at each SBR interval were averaged across animals to produce means and SEM values.

Results

Partial cDNA cloning of galanin

PCR, with primers based on the published human galanin sequence, produced a 207-bp fragment, of which 165 bases (minus primer regions) were unique to macaque preprogalanin (GenBank accession no. AF459740; Fig. 1A). These bases correspond to amino acids 1–30 of galanin and the first 25 amino acids of galanin message-associated peptide. This region of macaque preprogalanin has 95% homology to the human sequence at the nucleotide level, and within the region encoding for galanin (1–30), there is only one base substitution. Protein translation of the cDNA sequence (Fig. 1B) revealed that in the macaque, as in the human, the C-terminal contains a serine residue, which predicts that ma-

A

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GGCTGGACCC TGAACAGCGC GGGCTACCTG CTGGGCCCAC ATGCTGTTGG CAACCACAGG  60

TCATTCAGCG ACAAGAATGG CCTCACCAGC AAGCGGGAGC TGCAGCCCCA AGACGACGTG 120

AAACCAGGAA GCTTTGACAG GTCCATGCCT GAGAACAATA TCATG                 165
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B

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mGal  GWTLNSAGYL LGPHAVGNHR SFSDKNGLTS KRELQPQDDV KPGSFDRSMP ENNIM 55
hGal  GWTLNSAGYL LGPHAVGNHR SFSDKNGLTS KRELRPEDDM KPGSFDRSIP ENNIM 55
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FIG. 1. Partial cDNA (A) and translated peptide sequence (B) of macaque preprogalanin (mGal) compared with cloned sequence of human galanin (hGal; GenBank accession no. CAA01907). Residues in *bold* represent those comprising galanin (1–30), and *asterisks* indicate amino acids that are identical between macaque and human.

caque galanin comprises 30 amino acids like its human counterpart. Indeed, the peptide sequence of macaque galanin (1–30) is 100% conserved compared with human galanin. Over the whole cloned fragment (55 residues), there are predicted to be four amino acid substitutions [84% homology for galanin message-associated peptide (1–25)].

Genomic analysis of human GALP

Six continuous fragments of the human GALP cDNA sequence produced search engine hits against the human genome. All six fragments were contained in an approximately 11-kb span of chromosome 19 (Fig. 2A). All five intronic sequences were between 0.9 and 3.4 kb in length (Table 1) and followed the GT-AG rule, starting with a 5′ GT and ending with a 3′ AG. The start codon for preproGALP is contained in exon 2, and the stop codon is contained in exon 6. The region coding mature GALP (1–60) is contained in exons 2–5, and the region with homology to galanin [GALP (9–21)] is contained within exon 3.

Cloning of full-length macaque GALP

Using primers based on the published human GALP sequence, we cloned a 314-bp fragment of macaque GALP (short GALP; Fig. 2B), which was found to be present both in hypothalamus and pituitary. This sequence was then used to make gene-specific primers to perform 5′ and 3′ RACE. RACE fragments were then sequenced and aligned to produce a putative full-length GALP cDNA of 802 bp (GenBank accession no. AF459739). The 5′ untranslated region (UTR) of macaque GALP is slightly longer (98 bp) than that of human GALP (82 bp), but the 3′ UTR is shorter in the macaque (356 bp *vs.* 506 bp). Overall (excluding the nonoverlapping UTRs), macaque GALP has 93% homology to human GALP at the nucleotide level; in the coding region for GALP (1–60), nucleotide homology is 96%. Translation of the cDNA and analysis of the 5′ end revealed an in-frame stop codon and a single downstream ATG codon. Macaque GALP is predicted to be a preprohormone of 115 amino acids (one less than human GALP due to an in-frame triplet deletion) that has 7 mismatches (94% homology) with human GALP (Fig. 2C). Within the region coding GALP (1–60), there are three mismatches (95% homology), whereas GALP (9–21), the region that shares homology with galanin (1–13), is 100% conserved at both the peptide and cDNA levels between macaque and human.

Distribution of GALP mRNA in hypothalamus and pituitary

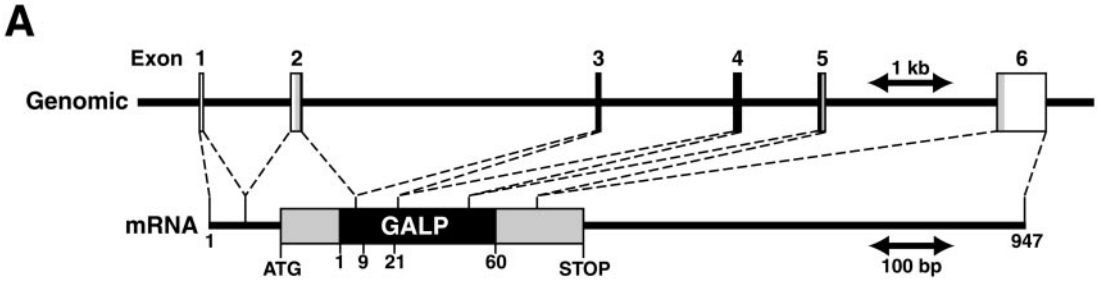
Sections of brain from the diagonal band of Broca to the mammillary bodies were inspected for evidence of GALP mRNA expression. Silver grain clusters indicative of cells expressing GALP mRNA were found exclusively in the Arc and ME (Fig. 3). GALP mRNA was also expressed in the posterior lobe of the pituitary gland (data not shown). Including excess unlabeled antisense probe with radiolabeled antisense probe abolished all specific signal, and no signal was seen with application of radiolabeled sense probe. Within the rostral portions of the Arc, GALP mRNA-expressing cells were mostly found dorsally in the periventricular region, whereas in the caudal Arc, cells were concentrated more ventrolaterally.

Ob-Rb mRNA expression in GALP mRNA-expressing cells

Using digoxigenin-labeled antisense riboprobe, we observed GALP mRNA-expressing cells in the Arc with a similar pattern to that seen with a radiolabeled probe, although the number of cells detected with digoxigenin-labeling was less than that detected with ^{33}P-labeled probe. The pattern of Ob-Rb mRNA expression in the hypothalamus was similar to that reported previously by us for a probe that detected all forms of Ob-R (27). Separate experiments with either excess unlabeled antisense probe or radiolabeled sense probe verified the specificity of the Ob-Rb riboprobe. Nearly all cells expressing GALP mRNA had clusters of silver grains (representing expression of Ob-Rb mRNA) overlying them (Fig. 4). We used computer image analysis to determine SBRs for GALP mRNA-expressing cells to create a cumulative density function (Fig. 5). The ordinate value of each point represents the percentage of cells with a SBR greater than a particular value (abscissa). The graph reveals the percentage of cells considered to be double-labeled at any arbitrarily determined threshold, or SBR level. For example, with a threshold of SBR greater than three, approximately 98% of GALP mRNA-expressing cells would be considered to coexpress Ob-Rb mRNA.

Discussion

GALP was discovered in a search for additional endogenous ligands capable of activating galanin receptors (18). Thus, the discovery of peptide sequence homology between GALP and galanin was not unexpected; however, analysis of the human genomic structure of GALP has revealed extensive similarity between the two genes as well. The human galanin gene is located on chromosome 11 and is comprised of six exons (28), the first of which is noncoding. Preprogalanin is encoded by exons 2–6, and the first 13 amino acids of galanin are contained in exon 3. Human GALP is located on chromosome 19 and is comprised of six exons, the first of which is noncoding. PreproGALP is encoded by exons 2–6, and the portion of galanin homology [GALP (9–21)] is contained in exon 3. Sequence alignment shows that the region of homology between galanin and GALP both begin at amino acid 33 of their respective prepro-hormones. The relationship between the genomic structure of galanin and GALP is reminiscent of that between human agouti and agouti-related protein (AGRP). Both agouti and AGRP are melanocortin receptor antagonists that are comprised of the same number of coding exons (29, 30). The gene families agouti/AGRP and galanin/GALP have similar amino acid identities within pairs (25% *vs.* 28%, respectively); however, galanin/GALP have higher amino acid similarity across the whole sequence (67% *vs.* 48% for agouti/AGRP). It seems likely that galanin and GALP are paralogous genes, *i.e.* created by a duplication event (31). Galanin has been characterized in a wide variety of vertebrate species, including mammals, birds, reptiles, and fish (32). Currently, GALP has only been examined in mammals, but determining the extent to which GALP is



B

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GAAAAGGCAC TGGAGCCCAG AGCCAGAGAG AGCTGCAGAG AGCTGCCAGC TGCACCGAGC  60

GTGTTCCGCA GCTGTAGGCA TCCGTCGTCC TGCCTTCGAT GGCTCCCTCC GTCCCCCTGG 120

TCCTCCTCCT CGTCCTCTTG CTGAGCCTGG CAGAGACTCC AGCATCCGCG CCTGCCCACC 180
          short GALP forward          long GALP forward
AGGGACGAGG AGGCTGGACC CTCAATAGTG CTGGCTACCT TCTGGGTCCC GTCCTCCACC 240
          3' RACE forward
TTCCCCAAAT GGGTGACCAA GACCGGAAGA GGGAGACAGC CCTTGAGATC CTAGACCTGT 300

GGAAGGCCAT CGATGGGCTC CCCTATTCCC ACCCTCTGCA GCCCTCCAAG AGGAACGTGA 360

TGGAGGCGTT TGCCAAACCG GAGATTGGAG ATCTGGACGT GCTCAGCAAG AAAATTCCCA 420

AGGAGGAAGA TGTCCTGAAG TCATAGATGT CTCTGAATCC CTATTCATAT CCTTCTTCTC 480
short GALP reverse                                  inner 5' RACE reverse
CAGCTCCTTC TGTTCCCTCT GAAACCCTTT CTAGGTACCC TGTGTTGAGA CTAAGATCCT 540

GAAGTTAAAT ACCCAGGTCT CATGAAGACA TTGTAAAGCA TTTGAATTAT TCTATACAAC 600
          outer 5' RACE reverse
TTCTGGAGTG TCTTAGGTTT TGTTGATTCG TCGTTGGAGT CAGTATAACG TGTCGTTAAT 660

GAAACTAATA GTAATTCGAG GACTAGTTGG AAGGTTCTGG GGTAATCAGG GAATATTCCT 720

GCAACACATT TAAAGTAATT GTGTTCTTTT GGAATCGTCC CAATTATCAC ACGAGCTATT 780
                                   long GALP reverse
GAATAATACA AATAACCCTC TTAAAAAAAA AAAAAAAAAA                       802
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C

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                                        ▼
mGALP  MAP-SVPLVL LLVLLLSLAE TPASAPAHQG RGGWTLNSAG YLLGPVLHLP
hGALP  MAPPSVPLVL LLVLLLSLAE TPASAPAHRG RGGWTLNSAG YLLGPVLHLP  50
       ********** ********** ******** * ********** **********

                                        ▼
mGALP  QMGDQDRKRE TALEILDLWK AIDGLPYSHP LQPSKRNVME AFAKPEIGDL
hGALP  QMGDQDGKRE TALEILDLWK AIDGLPYSHP PQPSKRNVME TFAKPEIGDL 100
       ****** *** ********** **********  *********  *********

mGALP  DVLSKKIPKE EDVLKS
hGALP  GMLSMKIPKE EDVLKS                                      116
         ** ***** ******
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FIG. 2. Analysis of primate GALP sequences. The cDNA of human GALP comprises 6 exons (A) and is located on chromosome 19. The entire preproGALP molecule is encoded by exons 2–6, whereas the mature peptide GALP (1–60) is contained within exons 2–5. Full-length macaque GALP cDNA was found to be 802 bp in length (B). Sequences used as primers for cloning and RACE are *underlined*, and start/stop codons are displayed in *bold* text. Protein translation shows a predicted length of 115 amino acids for macaque GALP (C), of which 7 are mismatched (94% homology) with human GALP. Residues in *bold* represent those comprising the region of GALP that is homologous to galanin (1–13), *asterisks* indicate amino acids that are identical between macaque and human, and *triangles* indicate cleavage sites producing mature GALP (1–60).

present across taxa could shed light on when gene duplication may have occurred.

In the macaque, the partially cloned sequence of prepro-galanin predicts that the galanin peptide is identical to human galanin; thus, it is 30 amino acids long, ends with a serine residue, and is nonamidated at its C terminus. Exam-

ination of several vertebrate species has revealed that these two primate species are the only ones in which galanin is not a 29-amino acid, C-terminally amidated peptide (6, 7). It has been reported that there is a short form of galanin consisting of amino acids 1–19 in the large intestine of the human (33); whether this molecular form is also present in macaques is currently unknown. The biological significance of primate galanin lacking amidation at the C terminus is also unclear. Porcine galanin is capable of producing physiological effects in humans (3, 34). Human, rat, and porcine galanin all have equal affinities for human GalR1 and GalR2, whereas human galanin has lower affinity for human GalR3 than rat or porcine galanin (12, 35). It would be interesting to investigate the pharmacokinetics of GALP at GalR3 and learn whether GALP is the preferred endogenous ligand for that receptor.

TABLE 1. Exon/intron boundaries of the human GALP gene

Exon	Length (bp)	Location	Intron	Length (kb)
1	43	1–43	1	1.0
2	126	44–169	2	3.4
3	49	170–218	3	1.5
4	81	219–299	4	0.9
5	78	300–377	5	2.0
6	562	378–939		

We have identified the macaque homologue of GALP and found that it shares extensive nucleotide and amino acid identity with the previously identified human GALP sequence. The mature macaque GALP peptide is predicted to be similar to human GALP, with conservation of amino acids surrounding the cleavage sites proposed by Ohtaki *et al.* (18). Macaque GALP (1–60) has the highest amino acid identity to human GALP (1–60) at 95%, followed by identity to rat GALP (73%) and porcine GALP (68%). Two major regions of 100% cross-species homology existing in GALP (1–60) are residues 9–21 (the galanin homology region) and residues 41–53; these regions are perfectly conserved in the macaque as well. Presumably, the N-terminal homology is responsible for the activity of GALP at galanin receptors (18), but whether or not the C-terminal homologous region represents a motif with biological activity of its own has yet to be determined.

The distribution of GALP mRNA-expressing cells in the

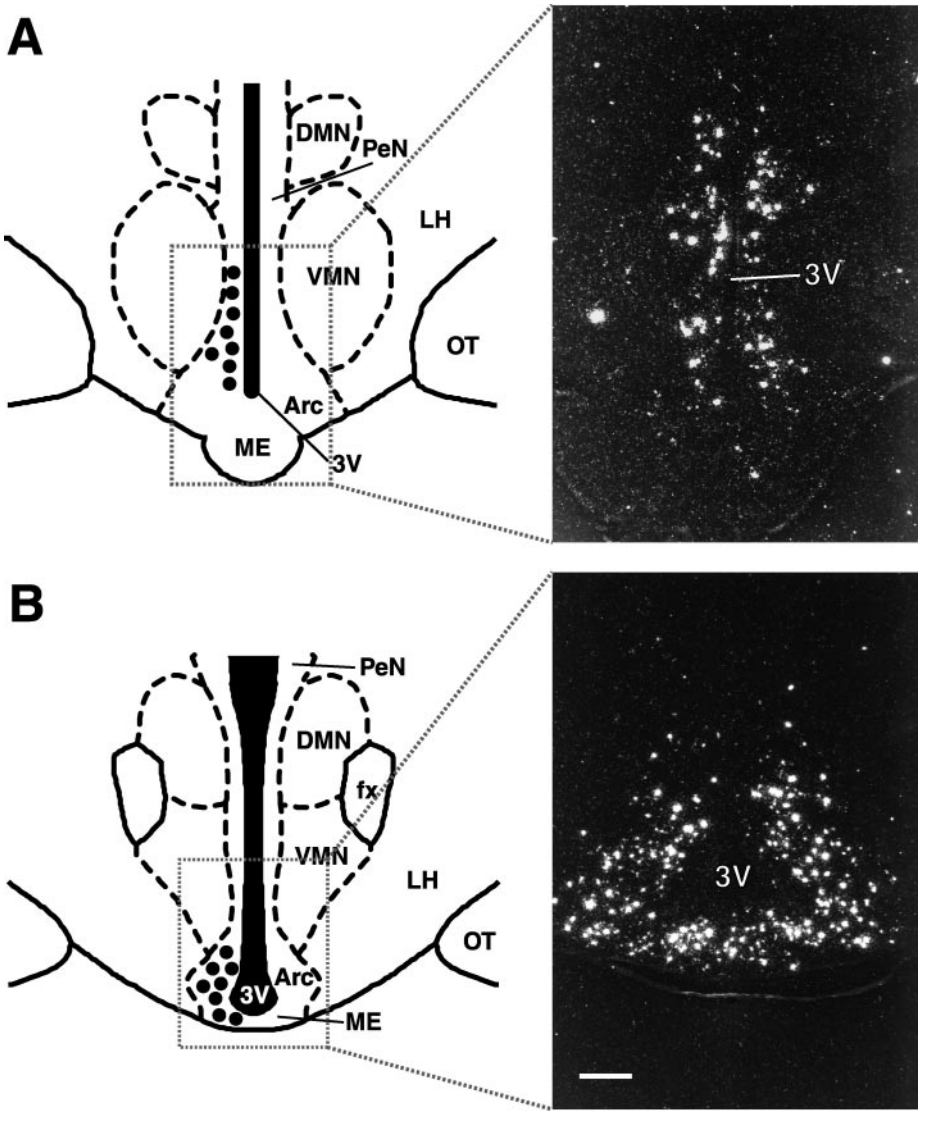

FIG. 3. Distribution of GALP mRNA-expressing cells in the rostral (A) and caudal (B) hypothalamus. GALP mRNA-expressing cells are represented by *black dots* in atlas plates (*left*) and *white dots* (corresponding to silver grain clusters) in photomicrographs (*right*). 3V, Third ventricle; Arc, arcuate nucleus of hypothalamus; DMN, dorsomedial nucleus of hypothalamus; fx, fornix; LH, lateral hypothalamus; ME, median eminence; OT, optic tract; PeN, periventricular nucleus of hypothalamus; VMN, ventromedial nucleus of hypothalamus. *Scale bar*, 500 μm.

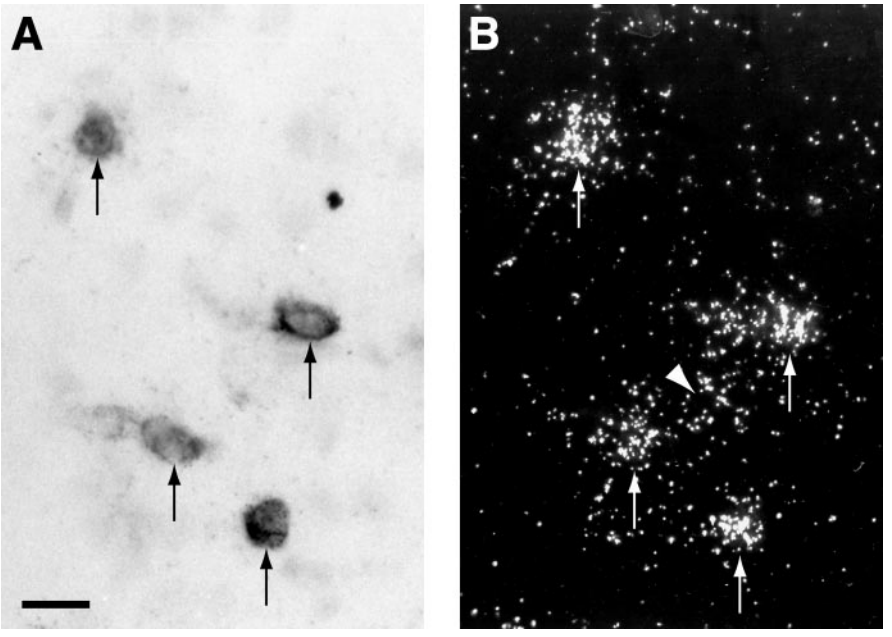

FIG. 4. Coexpression of GALP mRNA and Ob-Rb mRNA. Brightfield photomicrograph (A) shows GALP mRNA-expressing cells filled with dark precipitate (*arrows*). Darkfield photomicrograph (B) shows clusters of silver grains representing Ob-Rb mRNA-expressing cells overlying GALP mRNA-expressing cells (*arrows*). *Arrowhead* represents a silver grain cluster not overlying a GALP mRNA-expressing cell. *Scale bar*, 20 μm.

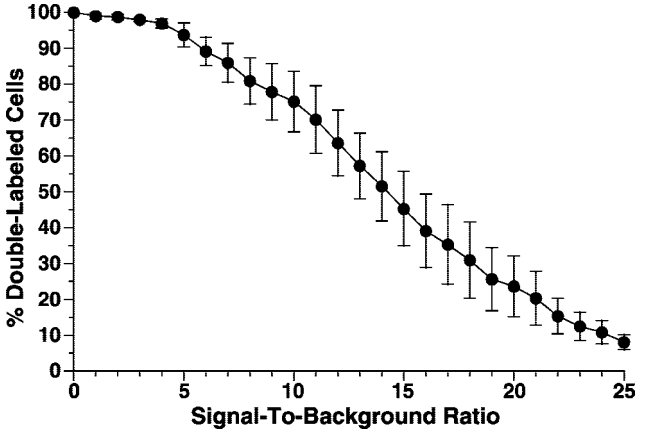

FIG. 5. Percentage of cells considered to coexpress both GALP and Ob-Rb mRNA as a function of SBR. The ordinate value represents the percentage of cells having SBR greater than the value of the abcissa. Data are means and SEMs from three animals.

macaque hypothalamus is similar to that described previously in the rat (19–21) and the mouse (23). In the forebrain regions examined, cells with specific signal were found only in the Arc and ME. GALP mRNA-expressing cells even within the Arc have a similar distribution in both rat and macaque, medially along the third ventricle in the rostral Arc, with the majority of cells being found in the caudal portion of the Arc. The distribution of GALP seems to be more conserved across species than does galanin (1, 36). This difference could be due to the fact that galanin is more widely expressed in the brain than GALP and is thus more likely to show minor species differences, or it may reflect a more specific, conserved physiological role for GALP.

In addition, GALP mRNA was detected by RT-PCR in the pituitary gland, and then localized with ISH to the neurohypophysis as has been reported by our lab and others in the rat (19, 21, 37). Shen *et al.* (37) assert that the cells expressing GALP mRNA in the rat neurohypophysis are pituicytes, but we did not determine the identity of these cells in the current experiments. GALP mRNA is dramatically up-regulated in the neurohypophysis by both dehydration and salt loading of rats, whereas these treatments had no effect on levels of GALP mRNA in the hypothalamus (37). This suggests that GALP expressed in the pituitary may play a role in fluid/salt balance in rats; whether the same is true in macaques is unknown.

We have found that GALP mRNA-expressing cells in the macaque Arc also express Ob-Rb mRNA. This finding corroborates that of Takatsu *et al.* (22), who found GALP and Ob-Rb to be coexpressed in the rat. Takatsu *et al.* report a high level of GALP/Ob-Rb coexpression (85%); indeed, our own data indicate that virtually all GALP mRNA-expressing cells coexpress Ob-Rb mRNA (98%). Qualitative estimates of the converse relationship (*i.e.* the percentage of Ob-Rb mRNA cells that coexpress GALP mRNA), based upon the previously described distribution of Ob-R mRNA (27), would suggest that GALP/Ob-Rb cells constitute a relatively small fraction of the total number of hypothalamic cells expressing Ob-Rb. Contrasting the current observations with previous quantifications of Ob-R coexpression in the macaque, we have reported previously that (at a SBR of 3) 33% of GnRH neurons, 55% of NPY neurons, 70% of 5-HT neurons (expressing serotonin transporter), and 75% of POMC neurons express Ob-R mRNA (26, 27). Among other studies providing quantitative data regarding Ob-R mRNA coexpression with neuropeptides in the hypothalamus (38–40), GALP cells represent the population of cells with the highest level of Ob-R coexpression.

Little is known thus far about the physiological significance of GALP cells in the hypothalamus. We have found previously that GALP mRNA is reduced in two different models of leptin deficiency (fasted rat, *ob/ob* mouse) and that leptin injections, either peripherally or centrally, are able to restore GALP mRNA levels to normal in these animals (19, 23). This would appear to be a direct effect of leptin on GALP

cells, but this has not been shown conclusively. What role GALP might play as an executor of leptin's actions is still unknown. Leptin is primarily recognized for its role in regulating food intake and metabolism; indeed, other Arc cells that express Ob-R, such as NPY and POMC neurons, have been implicated in mediating these effects of leptin (41). However, there have been no published reports implicating GALP in the regulation of appetite or adiposity to date.

There is evidence that GALP is involved in the regulation of GnRH release in the rat; GALP fibers make contact with GnRH cell bodies in the medial preoptic area (22), and intracerebroventricular injection of GALP stimulates both c-Fos in GnRH neurons and LH secretion (42). GALP-stimulated LH secretion is blocked by concurrent treatment with the GnRH receptor antagonist Cetrorelix (42), implying that GALP is affecting LH secretion at the level of the hypothalamus rather than the pituitary. There is evidence that leptin is capable of stimulating GnRH secretion (43–45), as well as evidence for at least some GnRH neurons expressing Ob-R (27, 46). Thus, it is possible that leptin acts both directly on GnRH neurons and indirectly through activation of GALP signaling to stimulate GnRH release.

In summary, we have cloned a full-length cDNA corresponding to the macaque homologue of GALP that is highly conserved at the genomic and protein level between primate species. In the hypothalamus and pituitary, we have found GALP mRNA expression exclusively in the Arc, ME, and neurohypophysis. In addition, virtually all GALP mRNA-expressing cells in the Arc coexpress Ob-Rb mRNA. Hopefully, further studies will elucidate the physiological significance of these observations in primates.

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Address all correspondence and requests for reprints to: Robert A. Steiner, Department of Physiology & Biophysics, Box 357290, University of Washington, Seattle, Washington 98195-7290. E-mail: steiner@u.washington.edu.

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References

1. **Evans HF, Huntley GW, Morrison JH, Shine J** 1993 Localisation of mRNA encoding the protein precursor of galanin in the monkey hypothalamus and basal forebrain. J Comp Neurol 328:203–212
2. **Kordower JH, Le HK, Mufson EJ** 1992 Galanin immunoreactivity in the primate central nervous system. J Comp Neurol 319:479–500
3. **Giustina A, Gastaldi C, Bugari G, Chiesa L, Loda G, Tironi C, Negro-Vilar A** 1995 Role of galanin in the regulation of somatotrope and gonadotrope function in young ovulatory women. Metabolism 44:1028–1032
4. **Todd JF, Edwards CMB, Ghatei MA, Bloom SR** 2000 The differential effects of galanin-(1–30) and -(3–30) on anterior pituitary hormone secretion *in vivo* in humans. Am J Physiol Endocrinol Metab 278:E1060–E1066
5. **Finn PD, Pau K-YF, Spies HG, Cunningham MJ, Clifton DK, Steiner RA** 2000 Galanin's functional significance in the regulation of the neuroendocrine reproductive axis of the monkey. Neuroendocrinology 71:16–26
6. **Bartfai T, Hökfelt T, Langel Ü** 1993 Galanin–a neuroendocrine peptide. Crit Rev Neurobiol 7:229–274
7. **Merchenthaler I, López FJ, Negro-Vilar A** 1993 Anatomy and physiology of central galanin-containing pathways. Prog Neurobiol 40:711–769
8. **McDonald MP, Crawley JN** 1997 Galanin-acetylcholine interactions in rodent memory tasks and Alzheimer's disease. J Psychiatry Neurosci 22:303–317
9. **Finn PD, Clifton DK, Steiner RA** 1998 The regulation of galanin gene expression in gonadotropin-releasing hormone neurons. Mol Cell Endocrinol 140:137–142
10. **Crawley JN** 1999 The role of galanin in feeding behavior. Neuropeptides 33:369–375
11. **Habert-Ortoli E, Amiranoff B, Loquet I, Laburthe M, Mayaux J-F** 1994 Molecular cloning of a functional human galanin receptor. Proc Natl Acad Sci USA 91:9780–9783
12. **Fathi Z, Battaglino PM, Iben LG, Li H, Baker E, Zhang D, McGovern R, Mahle CD, Sutherland GR, Iismaa TP, Dickinson KE, Zimanyi IA** 1998 Molecular characterization, pharmacological properties and chromosomal localization of the human GALR2 galanin receptor. Brain Res Mol Brain Res 58:156–169
13. **Kolakowski Jr LF, 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, Chateauneuf 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
14. **Borowsky B, Walker MW, Huang L-Y, Jones KA, Smith KE, Bard J, Branchek TA, Gerald C** 1998 Cloning and characterization of the human galanin GALR2 receptor. Peptides 19:1771–1781
15. **Evans HF, Shine J** 1991 Human galanin: molecular cloning reveals a unique structure. Endocrinology 129:1682–1684
16. **Schmidt WE, Kratzin H, Eckart K, Drevs D, Mundkowski G, Clemens A, Katsoulis S, Schafer H, Gallwitz B, Creutzfeldt W** 1991 Isolation and primary structure of pituitary human galanin, a 30-residue nonamidated neuropeptide. Proc Natl Acad Sci USA 88:11435–11439
17. **Branchek TA, Smith KE, Gerald C, Walker MW** 2000 Galanin receptor subtypes. Trends Pharmacol Sci 21:109–117
18. **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
19. **Juréus 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
20. **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
21. **Kerr NCH, Holmes FE, Wynick D** 2000 Galanin-like peptide (GALP) is expressed in rat hypothalamus and pituitary, but not in DRG. Neuroreport 11:3909–3913
22. **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
23. **Juréus 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
24. **Sambrook J, Russell DW** 2001 Molecular cloning: a laboratory manual. Cold Spring Harbor: Cold Spring Harbor Laboratory Press
25. **Martin RF, Bowden DM** 2000 Primate brain maps: structure of the macaque brain. Amsterdam: Elsevier Science
26. **Finn PD, Cunningham MJ, Rickard DG, Clifton DK, Steiner RA** 2001 Serotonergic neurons are targets for leptin in the monkey. J Clin Endocrinol Metab 86:422–426
27. **Finn PD, Cunningham MJ, Pau K-YF, Spies HG, Clifton DK, Steiner RA** 1998 The stimulatory effect of leptin on the neuroendocrine reproductive axis of the monkey. Endocrinology 139:4652–4662
28. **Evans H, Baumgartner M, Shine J, Herzog H** 1993 Genomic organization and localization of the gene encoding human preprogalanin. Genomics 18:473–477
29. **Shutter JR, Graham M, Kinsey AC, Scully S, Lüthy R, Stark KL** 1997 Hypothalamic expression of ART, a novel gene related to agouti, is up-regulated in *obese* and *diabetic* mutant mice. Genes Dev 11:593–602
30. **Dinulescu DM, Cone RD** 2000 Agouti and agouti-related protein: analogies and contrasts. J Biol Chem 275:6695–6698
31. **Gogarten JP, Olendzenski L** 1999 Orthologs, paralogs and genome comparisons. Curr Opin Genet Dev 9:630–636
32. **Crawley JN** 1995 Biological actions of galanin. Regul Pept 59:1–16
33. **Bersani M, Johnsen AH, Højrup P, Dunning BE, Andreasen JJ, Holst JJ** 1991 Human galanin: primary structure and identification of two molecular forms. FEBS Lett 283:189–194
34. **Arvat E, Gianotti L, Ramunni J, Grottoli S, Brossa PC, Bertagna A, Camanni F, Ghigo E** 1995 Effect of galanin on basal and stimulated secretion of prolactin, gonadotropins, thyrotropin, adrenocorticotropin and cortisol in humans. Eur J Endocrinol 133:300–304

35. **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
36. **Cheung CC, Hohmann JG, Clifton DK, Steiner RA** 2001 Distribution of galanin messenger RNA-expressing cells in murine brain and their regulation by leptin in regions of the hypothalamus. Neuroscience 103:423–432
37. **Shen J, Larm JA, Gundlach AL** 2001 Galanin-like peptide mRNA in neural lobe of rat pituitary. Increased expression after osmotic stimulation suggests a role for galanin-like peptide in neuron-glial interactions and/or neurosecretion. Neuroendocrinology 73:2–11
38. **Cheung CC, Clifton DK, Steiner RA** 1997 Proopiomelanocortin neurons are direct targets for leptin in the hypothalamus. Endocrinology 138:4489–4492
39. **Baskin DG, Breininger JF, Schwartz MW** 1999 Leptin receptor mRNA identifies a subpopulation of neuropeptide Y neurons activated by fasting in rat hypothalamus. Diabetes 48:828–833
40. **Elias CF, Lee CE, Kelly JF, Ahima RS, Kuhar M, Saper CB, Elmquist JK** 2001 Characterization of CART neurons in the rat and human hypothalamus. J Comp Neurol 432:1–19
41. **Ahima RS, Saper CB, Flier JS, Elmquist JK** 2000 Leptin regulation of neuroendocrine systems. Front Neuroendocrinol 21:263–307
42. **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
43. **Yu WH, Kimura M, Walczewska A, Karanth S, McCann SM** 1997 Role of leptin in hypothalamic-pituitary function. Proc Natl Acad Sci USA 94:1023–1028
44. **Lebrethon M-C, Vandersmissen E, Gérard A, Parent AS, Bourguignon JP** 2000 Cocaine and amphetamine-regulated-transcript peptide mediation of leptin stimulatory effect on the rat gonadotropin-releasing hormone pulse generator *in vitro.* J Neuroendocrinol 12:383–385
45. **Woller M, Tessmer S, Neff D, Nguema AA, Roo BV, Waechter-Brulla D** 2001 Leptin stimulates gonadotropin releasing hormone release from cultured intact hemihypothalami and enzymatically dispersed neurons. Exp Biol Med (Maywood) 226:591–596
46. **Magni P, Vettor R, Pagano C, Calcagno A, Beretta E, Messi E, Zanisi M, Martini L, Motta M** 1999 Expression of a leptin receptor in immortalized gonadotropin-releasing hormone-secreting neurons. Endocrinology 140:1581–1585

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