0021-972X/04/$15.00/0
Printed in U.S.A.

The Journal of Clinical Endocrinology & Metabolism 89(4):1760–1766

doi: 10.1210/jc.2003-031628

Galanin-Like Peptide as a Possible Link between Metabolism and Reproduction in the Macaque

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Galanin-like peptide (GALP) is a hypothalamic neuropeptide that has been implicated in the control of feeding, metabolism, and reproduction. The goal of this study was to examine the effects of central infusions of GALP on GnRH and LH secretion and to identify physiological factors that influence the expression of GALP mRNA in the brain of a primate species. Infusions of GALP into the lateral cerebroventricle of the macaque caused a significant increase in LH secretion, which was blocked by administration of the GnRH receptor antagonist acyline. However, the expression of GALP mRNA in the arcuate nucleus, as determined by *in situ* hybridization, was not regulated by either estradiol or progesterone. Compared with feeding *ad libitum*, fasting for 48 h produced a significant reduction in the hypothalamic expression of GALP mRNA. GALP neurons were found to express both neuropeptide Y Y1 receptor and serotonin 2C receptor by double-label *in situ* hybridization. Taken together, these results suggest that GALP neurons play a role of integrating metabolic signals, which are relayed to circuits controlling GnRH release in the macaque. (*J Clin Endocrinol Metab* 89: 1760–1766, 2004)

SINCE ITS DISCOVERY as an endogenous ligand for galanin receptors, galanin-like peptide (GALP) and its mRNA have been localized to cells of the hypothalamic arcuate nucleus (Arc), median eminence, and neurohypophysis (1–6). GALP cDNA has been cloned from several mammalian species, including humans and macaques (1, 6–8). The human GALP gene is located on chromosome 19q13.43 and is comprised of six exons (6). GALP mRNA expression is highly regulated by the adipocyte-derived hormone leptin, being increased by exogenous leptin injection to fasted rats and decreased in several rodent models of insufficient leptin signaling (*ob/ob* and *db/db* mice and *fa/fa* rats) (2, 7, 8). The responsiveness of GALP neurons to leptin is likely to reflect the expression of the long form of the leptin receptor (Ob-Rb) in nearly all GALP neurons (5, 6). The injection of GALP directly into the cerebral ventricles causes changes in food intake and body weight as well as stimulates the release of LH in rodents (9–13). Little is known about the functional significance of GALP in the primate.

The reproductive system of the macaque, like that in other mammals, is sensitive to changes in energy availability (14, 15). Leptin is believed to be part of the signaling mechanism that leads to this sensitivity; however, the means by which this signal (and others) is transmitted to GnRH neurons are largely unknown (16). The observations that GALP is regulated by leptin and has the ability to stimulate LH secretion have led to the hypothesis that GALP may serve as an intermediary between energy reserves and reproductive neuroendocrine output. The primary focus of these studies was to provide evidence for a similar relationship in the nonhuman primate. To this end, we tested several hypotheses concerning the function of GALP in macaques: 1) that intracranial injections of GALP would elicit LH secretion; 2) that GALP mRNA would be regulated by gonadal steroids; 3) that GALP mRNA would be regulated by fasting; and 4) that GALP neurons would be receptive to signals in the brain (other than leptin) that are known to be involved in regulating food intake.

Abbreviations: aCSF, Artificial cerebrospinal fluid; Arc, arcuate nucleus; BW, body weight; E, estradiol; GALP, galanin-like peptide; 5-HT2C, serotonin 2C; ICV, intracerebroventricular; ISH, *in situ* hybridization; NPY, neuropeptide Y; P, progesterone; POMC, proopiomelanocortin; RNase, ribonuclease; SBR, signal to background ratio.

JCEM is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.

Materials and Methods

Experimental animals

All procedures were approved by the institutional animal care and use committees of University of Pittsburgh (experiment 1), Oregon National Primate Research Center (ONPRC; experiments 2 and 3), or University of Washington (experiment 4) and were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Reagents

Artificial cerebrospinal fluid (aCSF) was custom-prepared by Life Technologies (Grand Island, NY) as described previously (17). Rat GALP-(1–60) was synthesized as previously described (9) and has 75% amino acid identity with the cloned sequence of macaque GALP (1, 6). GALP was dissolved in aCSF and filtered through a 0.22-μm pore size syringe filter, and 200-μl aliquots containing either 500 or 1428 μg were stored at −20 C. GnRH and acyline were synthesized at The Salk Institute (La Jolla, CA) and Bioqual (Rockville, MD), respectively and were made available by the Contraception and Reproductive Health Branch of Center for Population Research, National Institute of Child Health

and Human Development (NICHD). GnRH was diluted in saline, and acyline was dissolved in 5% aqueous mannitol.

Experimental design

Experiment 1: lateral ventricle infusion of GALP. Three adult (62–79 months of age) agonadal male rhesus monkeys (*Macaca mulatta*) were used in the study. The monkeys were housed in individual cages and maintained under a controlled photoperiod (lights on from 0700–1900 h) and temperature (20 C). Monkeys were fed daily at approximately 1100 h with a high protein monkey diet (no. 5045, PMI Nutrition International, Richmond, IN), supplemented with fruit in the afternoon. Drinking water was available *ad libitum*.

Surgical procedures were conducted under aseptic conditions after sedation with ketamine hydrochloride [20 mg/kg body weight (BW), im/iv; Vetlar, Parke-Davis, Morris Plains, NJ] and anesthesia with isoflurane inhalation (1.5–2.5% with oxygen). The monkeys were implanted with an indwelling jugular and/or femoral catheter, then fitted with a jacket and tether system for obtaining sequential blood samples (18). Intravenous catheters were kept patent by infusion of heparinized saline (4 U/ml; ~4 ml/h). Monkeys were castrated 2–8 months before initiation of the present experiment for studies of testicular function. Three weeks to 4 months before the start of the present experiment, monkeys were implanted sc with three 4-cm SILASTIC brand [3.35 mm (inside diameter) × 4.65 mm (outside diameter); Dow Corning, Midland, MI] implants filled with crystalline testosterone to suppress LH secretion.

To administer GALP centrally, monkeys were implanted with a 22-gauge stainless steel cannula in the lateral cerebroventricle using an approach similar to that described previously (19). The intracerebroventricular (ICV) catheter was led with the venous catheter through the tether to a swivel device on top of the cage that allowed infusions to be given without sedating the monkeys. The initial dose of GALP (500 μg; ~50 μg/kg BW) was chosen to be comparable to that used previously in similar studies in the rat (10). A 200-μl aliquot of GALP or aCSF (described above) was infused through the ICV catheter (~200 μl/min) between 1030 and 1130 h. Animals received both treatments on separate occasions in a cross-over design. Sequential blood samples (1.5 ml) were obtained from the iv catheter into sterile glass tubes. Blood samples were collected at 20-min intervals for 3 h pre- and postinfusion periods. Immediately thereafter, some monkeys were administered an injection of GnRH (0.3 μg, iv), and a blood sample was collected 10 min later to confirm the responsivity of the gonadotropes. After plasma was harvested, red blood cells were resuspended in heparinized saline and returned to the monkeys. To assess the role of GnRH in mediating any action of GALP on LH release, two monkeys were injected with the GnRH receptor antagonist, acyline (60 μg/kg BW in the morning and 120 μg/kg BW in the evening, sc) under ketamine sedation (50 mg, iv) 1 d before GALP administration (20).

Experiment 2: regulation of GALP mRNA by ovarian steroids. Brain tissue for this experiment was provided by the Nonhuman Primate Reproductive Tissue Bank of NICHD Specialized Cooperative Centers Program in Reproductive Research. Details of the experimental procedure have been reported previously (21). Briefly, adult female rhesus monkeys exhibiting normal menstrual cyclicity were ovariectomized and hysterectomized (spayed) at the ONPRC 3 months before steroid capsule implantation. Spayed rhesus macaques were treated with an empty SILASTIC brand capsule (Spay group; n = 5), with a capsule containing estradiol (E) for 28 d (E group; n = 5), or with an empty capsule for 14 d, then with a capsule containing progesterone (P) for 14 d (P group; n = 5), or treated with a capsule containing E for 28 d and then supplemented with a capsule containing P for the final 14 of the 28 d (EP group; n = 5). At the end of the steroid treatment period, animals were euthanized, and the heads were perfused with 4% paraformaldehyde. All solutions were made with diethylpyrocarbonate-treated water. Brains were removed, postfixed, cryoprotected, then frozen and stored at −80 C until processed for *in situ* hybridization (ISH).

Experiment 3: regulation of GALP mRNA by fasting. Eight juvenile male Japanese macaques (*Macaca fuscata*; 1–2 yr old) at ONPRC were housed individually for 2 wk before being killed and were fed two meals a day at 0900 and 1500 h. Macaques were either allowed to feed *ad libitum* (n = 4) or were fasted for 48 h (n = 4) starting at 1300 h, missing four regularly scheduled meals (1500 and 0900 h for 2 d) before being killed at 1300 h 2 d later. On the afternoon of the second day of fasting, macaques were perfused as previously described (22, 23). The brains were removed, and hypothalamic tissue blocks were cut and immersed in the same fixative for 2 h at 4 C. The tissue blocks were immersed in 10% glycerol solution for 24 h at 4 C, then saturated in 20% glycerol. The tissue was frozen on dry ice and stored at −80 C until processed for ISH.

Experiment 4: GALP mRNA colocalization studies. Three ovariectomized adult female pigtailed macaques (*Macaca nemestrina*) were obtained through the Tissue Distribution Program at the Washington National Primate Research Center. All animals were sedated with ketamine (10 mg/kg BW, im) and anesthetized with sodium pentobarbital (25 mg/kg BW, iv). Brains were removed from the skull, and a hypothalamic block of tissue was removed, frozen on dry ice, and stored at −80 C until processed for ISH.

RIA

Plasma concentrations of LH for experiment 1 were measured with a double-antibody RIA system that employs recombinant cynomolgus LH (AFP6936) as standard and radioiodinated tracer, and a rabbit polyclonal antiserum to recombinant LH (AFP342994) as first antibody (24). Reagents were provided by the National Hormone and Peptide Program. The sensitivity of the assay varied from 0.05–0.2 ng/ml, and the intra- and interassay coefficients of variation were less than 9% and less than 12%, respectively. Details of ovarian steroid assays performed for experiment 2 have been reported previously (21).

Histology

For experiments with perfused tissue from ONPRC (experiments 2 and 3), sections were cut on a sliding microtome at 25 μm in the coronal plane through the rostral-caudal extent of the Arc and collected in cryoprotectant. A set comprised of every 10th section was washed three times in sodium phosphate buffer made with diethylpyrocarbonate-water, mounted on SuperFrost Plus slides (VWR Scientific, West Chester, PA), allowed to dry completely, and then stored at −80 C. For the experiment with nonperfused tissue (experiment 4), brains were cryostat-sectioned at 20 μm in the coronal plane and thaw-mounted onto SuperFrost Plus slides. Sections were collected into sets of every 10th section through the rostral-caudal extent of the Arc. Slides were then stored at −80 C.

Plasmid information

All of the cDNA constructs that were not provided by others [short/long GALP and neuropeptide Y (NPY) Y1] were synthesized by PCR and cloned into the pAMP1 plasmid by methods previously described (6).

Short and long GALP

Two different GALP cDNA constructs were used as templates for riboprobes for ISH, as described previously (6). The pAMP1 plasmid (Invitrogen, Carlsbad, CA) containing either a 314-bp fragment (short GALP) or a 629-bp fragment (long GALP) of the macaque GALP gene was used for *in vitro* transcription of antisense riboprobes. The short GALP construct was used for experiment 2, and the long GALP was used for experiments 3 and 4.

NPY Y1 receptor

A 763-bp portion of macaque Y1 cDNA sequence [corresponding to bases 159–921 of rhesus macaque Y1 (25); GenBank accession no. AF303089] was synthesized by PCR and cloned into pAMP1.

Serotonin 2C (5-HT2C) receptor

This plasmid was provided by Dr. Cynthia Bethea, ONPRC, and experiments using this plasmid have been reported previously (26). A fragment containing 294 bp of the macaque 5-HT2C receptor cDNA was cloned by PCR into the pGEM-T plasmid (Promega Corp., Madison, WI) with primers designed to target bases 36–329 of the human ortholog (GenBank accession no. AF498983).

NPY

This probe was provided by Carolyn Worby (University of Michigan, Ann Arbor, MI). A 521-bp fragment of human NPY cDNA was subcloned into Bluescript II KS (Stratagene, La Jolla, CA) as described previously (27).

Riboprobe preparation

Plasmids were linearized by restriction enzyme digest and were purified by phenol/chloroform extraction. Typically, radioactive antisense or sense riboprobe was synthesized *in vitro* by inclusion of the following ingredients in a volume of 20 μl: 250 μCi [^{33}P]UTP (PerkinElmer Life Sciences, Boston, MA); 1 μg linearized plasmid DNA; 0.5 mM each of ATP, CTP, and GTP; and 40 U of the appropriate RNA polymerase (T7, T3, or SP6 RNA Polymerase Plus, Ambion, Inc., Austin, TX). After 2 h, template DNA was digested with ribonuclease (RNase)-free deoxyribonuclease (Roche, Indianapolis, IN). Riboprobe was separated from unincorporated nucleotides with a NucAway Spin Column (Ambion, Inc.) reconstituted with 10 mM Tris (pH 8.0), 1 mM EDTA, and 100 mM NaCl.

For nonradioactive ISH, antisense riboprobes were synthesized with DIG RNA labeling mix (Roche) and the appropriate RNA polymerase according to the manufacturer's protocol. After synthesis, the reaction was digested with deoxyribonuclease and purified as described above.

In situ hybridization

Controls. Two types of negative controls were used to demonstrate specificity of the Y1 riboprobe: slides were incubated with either radiolabeled antisense probe in the presence of an excess (>100-fold) of unlabeled antisense probe or an equivalent concentration of radiolabeled sense riboprobe. The specificity of all other probes has been determined previously (6, 26, 27).

Single-label ISH. Tissue from experiments 2 and 3 were subjected to single-label ISH as described previously with slight modifications (6). Slides were removed from −80 C, briefly fixed, and then subjected to proteinase K treatment (10 μg/ml) for 30 min at 37 C. Sections were then acetylated, delipidated, and dehydrated. After this pretreatment, slides were stored at room temperature until used for hybridization. Radiolabeled, antisense GALP riboprobe (short GALP for experiment 2, long GALP for experiment 3) was denatured, dissolved in hybridization solution at a concentration of 0.2 pmol/ml along with transfer RNA (1.9 mg/ml), and applied to slides. 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 and washed under conditions of increasing stringency. Slides were then dehydrated, air-dried, and dipped in NTB-3 liquid emulsion (Eastman Kodak Co., Rochester, NY). Slides were developed after optimum exposure (several days), and coverslips were applied.

Double-label ISH. Tissue from experiment 4 was subjected to double-label ISH as described previously with slight modifications (6). Slides were processed before hybridization as described above, except proteinase K treatment was omitted. One set of tissue spanning the Arc was used for each of three separate double-label ISH experiments in which GALP mRNA was colocalized with Y1, 5-HT2C, or NPY mRNA. For these experiments, the long GALP construct was used to make digoxigenin-labeled riboprobe, whereas the other probes (Y1, 5-HT2C, and NPY) were radiolabeled. Radiolabeled antisense riboprobe and digoxigenin-labeled riboprobe (concentration determined empirically) were denatured, dissolved in hybridization solution along with transfer RNA (1.7 mg/ml), and applied to slides. 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 and washed under conditions of increasing stringency. Sections were incubated in blocking buffer and then in Tris buffer containing antidigoxigenin fragments conjugated to alkaline phosphatase (Roche) diluted 1:200 overnight at room temperature. Vector Red (Vector Laboratories, Inc., Burlingame, CA) was used as substrate for alkaline phosphatase according to the manufacturer's directions. Slides were dipped in 70% ethanol and air-dried. After air-drying, slides were dipped in NTB-3 emulsion. Slides were developed several days later, and coverslips were applied.

Image analysis

Single-label ISH. For determination of GALP mRNA regulation in experiments 2 and 3, slides were analyzed as previously described (28). Custom-designed software was used (by an experimenter who was blind to the treatment groups) to count silver grains from all clusters that were judged to be GALP mRNA-expressing cells in the Arc of all sections processed. Thus, each animal generated data points for the number of grains per cell and the total number of cells. Readings from individual slides were averaged within animals, and these numbers were used as individual n values for analysis of treatment group means.

Double-label ISH. The coexpression of GALP mRNA with various other mRNAs within the same cells was analyzed as previously described (27). GALP mRNA-containing cells were identified under fluorescent illumination, and custom-designed software was used to count the number of silver grains (corresponding to radiolabeled mRNA) over each cell. Signal to background ratios (SBRs) for individual cells were calculated; an individual cell was considered to be double-labeled if it had an SBR of 3 or more. For each animal, the amount of double-labeling was calculated as a percentage of the total number of GALP mRNA-expressing cells and then averaged across animals to produce the mean and SEM.

Statistical analysis

For experiments 2 and 3, group means were compared by ANOVA and *t* test, respectively. Differences were considered significant at $P < 0.05$.

Results

Experiment 1: lateral ventricle infusion of GALP

All monkeys showed increased LH secretion in response to infusions of GALP (Fig. 1). Monkeys 2692 and 2651 were infused with 500 μg GALP (Fig. 1, A and B), whereas monkey 2649 was infused with 1428 μg GALP (Fig. 1C). On a separate occasion, monkey 2649 received an infusion of 500 μg GALP (data not shown); however, these data are not presented here due to incomplete suppression of basal LH release by the testosterone implant at the time of that particular infusion trial. Within 40 min of ICV GALP infusion, all three monkeys examined demonstrated a marked rise in LH secretion that peaked by 60 min after infusion and lasted nearly the entire postinfusion sampling period (Fig. 1). No evidence of increased LH secretion was observed after aCSF infusion. Two monkeys were pretreated with the GnRH receptor antagonist acyline before receiving GALP infusion. Administration of acyline abolished the effect of GALP infusion on LH secretion in both animals tested (Fig. 1, A and B).

Experiment 2: regulation of GALP mRNA by ovarian steroids

The results of hormone assays from these animals have been reported previously (21). The mean serum E concentration for both the E and EP groups is consistent with levels during the mid- to late follicular phases of the menstrual cycle, and the mean serum P concentration for the P and EP groups is consistent with levels during the mid-luteal phase. No differences in GALP mRNA expression were detected across the four treatment groups, as measured by both grains per cell (Fig. 2A) and total number of cells counted (Fig. 2B).

Experiment 3: regulation of GALP mRNA by fasting

Monkeys who had been fasted 48 h before being killed had significantly fewer grains per cell corresponding to GALP

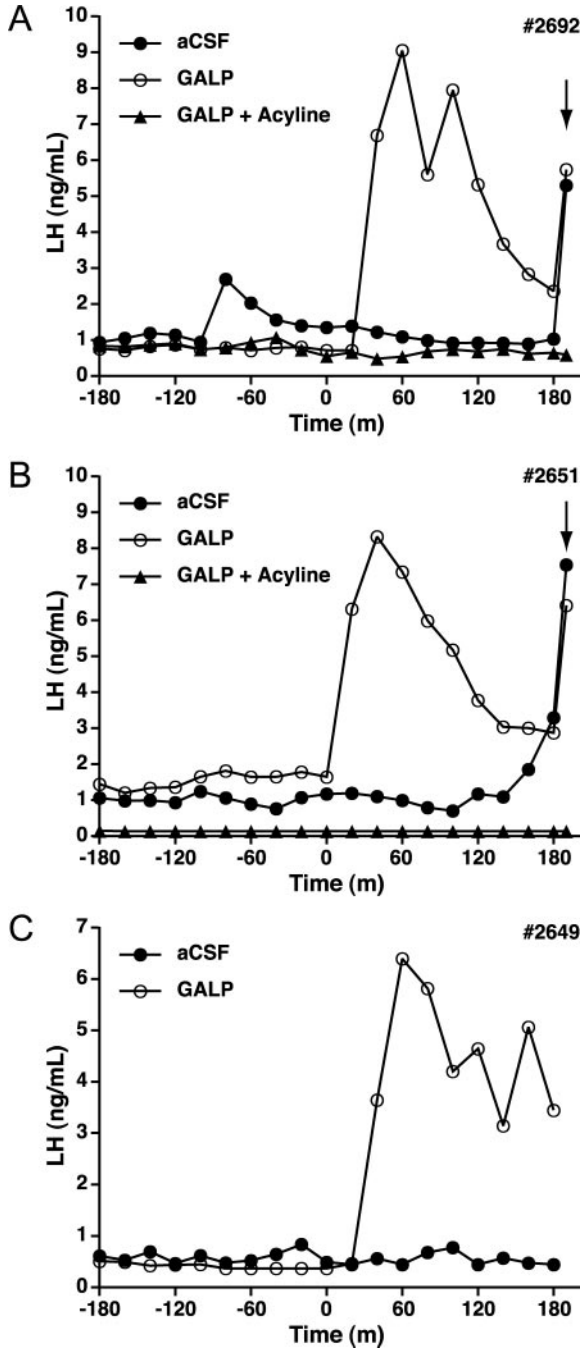

FIG. 1. Plasma LH concentration in agonadal male macaques. LH profiles are from three different animals: 2692 (A), 2651 (B), and 2649 (C). Samples were taken every 20 min for 3 h both pre- and postinfusion (infusion = time zero). On separate occasions, either aCSF (●) or GALP (500 μg in A and B; 1428 μg in C; ○) was infused into the lateral cerebral ventricle. On another occasion, two of the three macaques (A and B) received GALP infusions 1 d after receiving two sc injections of the GnRH receptor antagonist acyline (▲). Ten minutes after the end of the postinfusion sampling period, two of the three macaques (A and B) were given a GnRH bolus (0.3 μg, iv; *arrow*) to confirm the responsiveness of the gonadotropes.

mRNA than *ad libitum* fed monkeys (76 ± 4 *vs.* 90 ± 1, respectively; $P < 0.05$; Fig. 3A). However, no difference in the counted number of GALP mRNA-expressing cells was detected between the two treatment groups (Fig. 3B).

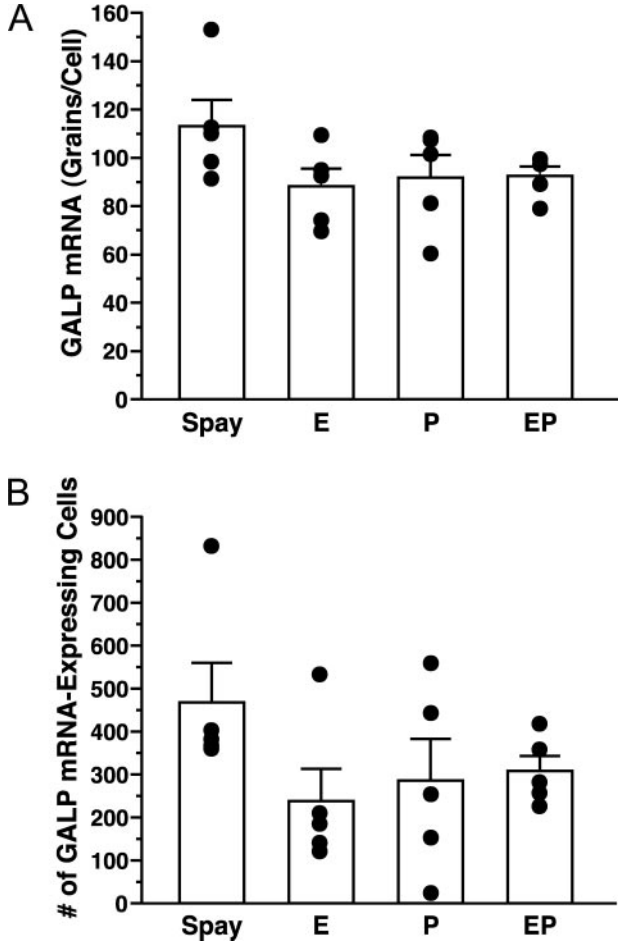

FIG. 2. Effects of ovarian steroids on GALP mRNA. Female rhesus macaques were spayed and treated for 28 d with an empty SILASTIC brand capsule (Spay; n = 5) or E (n = 5), were treated for 14 d with an empty capsule before 14 d of treatment with P (n = 5), or were treated with a combination of E for 28 d and P for 14 d (EP; n = 5). GALP mRNA was quantified by recording the number of silver grains per cell (A) and the total number of GALP mRNA-expressing cells counted (B). ●, Individual data points from each treatment group. □, Mean ± SEM.

Experiment 4: GALP mRNA colocalization studies

NPY Y1 receptor. Y1 receptor mRNA was expressed in the Arc, dorsomedial ventromedial nucleus, paraventricular nucleus, bed nucleus of the stria terminalis, and amygdala of the pigtailed macaque. Many cells expressing GALP mRNA had clusters of silver grains (representing the expression of Y1 mRNA) overlying them (Fig. 4, A and B). Quantitative analysis of SBR, with a threshold of signal three times greater than background, showed that 42 ± 5% of the GALP mRNA-expressing cells counted also expressed Y1 mRNA.

5-HT2C receptor. The expression pattern seen for 5-HT2C mRNA was similar to that described previously (26), with silver grain clusters formed in the Arc, ventrolateral ventromedial nucleus, lateral septum, preoptic area, and amygdala. Some cells expressing GALP mRNA had clusters of silver grains (representing the expression of 5-HT2C mRNA) overlying them (Fig. 4, C and D). Quantitative analysis of SBR, with a threshold of signal three times greater than back-

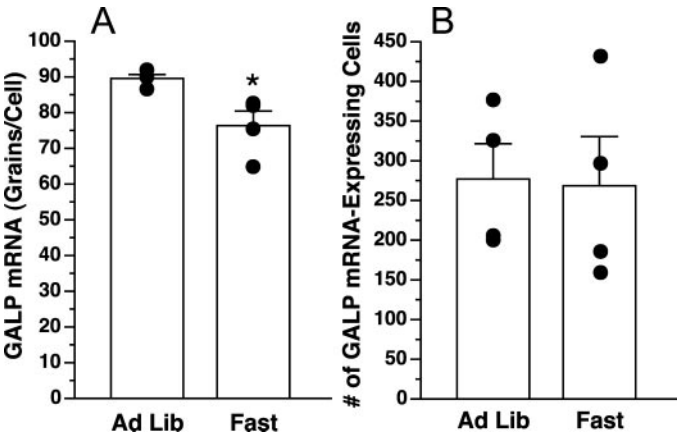

FIG. 3. Effect of fasting on GALP mRNA. Male Japanese macaques either were allowed to feed *ad libitum* (n = 4) or were fasted for 48 h (n = 4) before quantification of GALP mRNA, as measured by silver grains per cell (A) and total number of GALP mRNA-expressing cells counted (B). ●, Individual data points from each treatment group. □, Mean ± SEM. *, $P < 0.05$ *vs. ad libitum*-fed group.

ground, showed that 24 ± 3% of the GALP mRNA-expressing cells counted also expressed 5-HT2C mRNA.

NPY. The expression of NPY mRNA in the Arc of the pigtailed macaque tended to be in the ventromedial portions of the rostral Arc, whereas the expression of GALP mRNA was highest in the dorsomedial Arc. In the caudal Arc, where the majority of GALP mRNA-expressing cells were found, few NPY mRNA expressing cells were observed. Although NPY and GALP mRNA-expressing cells were found in close proximity to one another, extremely low levels of coexpression of the two mRNA species were observed (data not shown). During the analysis of GALP/NPY coexpression, it was noted that several GALP mRNA-expressing cells had a SBR of 3 or more, but this was not attributable to NPY silver grain clusters directly over these cells. In these cases, NPY-associated clusters were adjacent to GALP cells, but the expression of NPY was so great that silver grains from these clusters spilled over onto GALP cells. These cells were excluded from the analysis. Quantitative analysis of SBR, with a threshold of signal three times greater than background, showed that approximately 3% of the GALP mRNA-expressing cells counted also expressed NPY mRNA.

Discussion

Infusions of GALP into the brain stimulate LH secretion in rats and mice (9, 10); in this study we have extended this observation to a nonhuman primate species. We have also provided evidence that GALP elicits LH secretion through a GnRH-dependent mechanism, as the ability of GALP to increase LH secretion is blocked by the GnRH receptor antagonist acyline. This is consistent with the previous observation that GALP-stimulated LH release in the rat is blocked by coadministration of a GnRH receptor antagonist (10). Matsumoto *et al.* (10) also provide evidence that the actions of GALP are distinct from those of galanin in this context, as galanin does not stimulate LH secretion when tested under the same conditions as GALP. Although we did not directly compare the actions of GALP and galanin on LH secretion in this experiment, we have previously found that galanin infused directly into the Arc/median eminence of the male rhesus macaque does not elicit either GnRH or LH release (29). As a note of caution, there is at least some evidence that the location of neuropeptide infusion (ICV *vs.* intranuclear) can affect the outcome of such experiments (30).

Female rhesus macaques that were spayed had GALP mRNA expression not demonstrably different from that of spayed macaques who received replacement E, P, or both hormones, indicating that the expression of GALP mRNA is not influenced by these ovarian steroids. This also implies that GALP does not play a significant role in the feedback control of gonadotropin secretion by ovarian steroids during the menstrual cycle. We are confident that our treatment regimen was sufficient to detect a difference had one existed, as previous experiments on tissue from the same monkeys found regulation of other mRNA species between steroid treatment groups (21). This result is consistent with our previous finding that GALP mRNA is not regulated by estrogen treatment of ovariectomized rats (28). Notwithstanding, this negative finding does not rule out the possibility that GALP is regulated by steroids in a nontranscriptional manner, or that GALP contributes to the physiological release of GnRH secretion in the intact female macaque.

The expression of GALP mRNA in the Arc of the Japanese macaque is reduced by 48 h of fasting. In the fasted rat, GALP mRNA expression is significantly up-regulated by exogenous leptin injections (2). We did not measure leptin levels in the current experiment, but Lado-Abeal *et al.* (31) reported that within 24 h of fasting, the serum leptin concentration fell below detectable levels in pubertal male rhesus macaques. Taking into account that the majority of GALP mRNA-expressing cells in pigtailed macaques express leptin receptor mRNA (6), these observations are consistent with the hypothesis that leptin tonically stimulates GALP mRNA expression in the macaque, and that a fasting-induced reduction of leptin receptor signaling at GALP neurons leads to down-regulation of GALP gene expression. It should be noted, however, that many metabolic changes (including changes in glucose, insulin, and cortisol) have been documented in the fasted male rhesus macaque (14), and the design of the current study would not rule out the possible influence of any of these metabolic factors on the regulation of GALP mRNA during fasting.

We found that a portion of GALP mRNA-expressing cells also express mRNA for the Y1 receptor. Of the six known NPY receptor subtypes, the Y1 receptor is among those implicated in mediating the effects of NPY on energy homeostasis (32, 33). NPY signaling through the Y1 receptor has also been implicated in several roles related to reproductive hormone release, notably as a restraint on the reproductive system before pubertal onset (19, 34, 35) and as a contributing mechanism in the fasting-induced suppression of LH secretion (36, 37). Although GALP is a possible downstream effector of the Y1-mediated actions of NPY, a functional relationship between GALP and NPY has yet to be established in the macaque.

We detected a modest number of GALP mRNA-expressing cells that coexpress mRNA for the 5-HT2C receptor. 5-HT's anorectic properties are believed to be mediated in part by activating proopiomelanocortin (POMC)-expressing

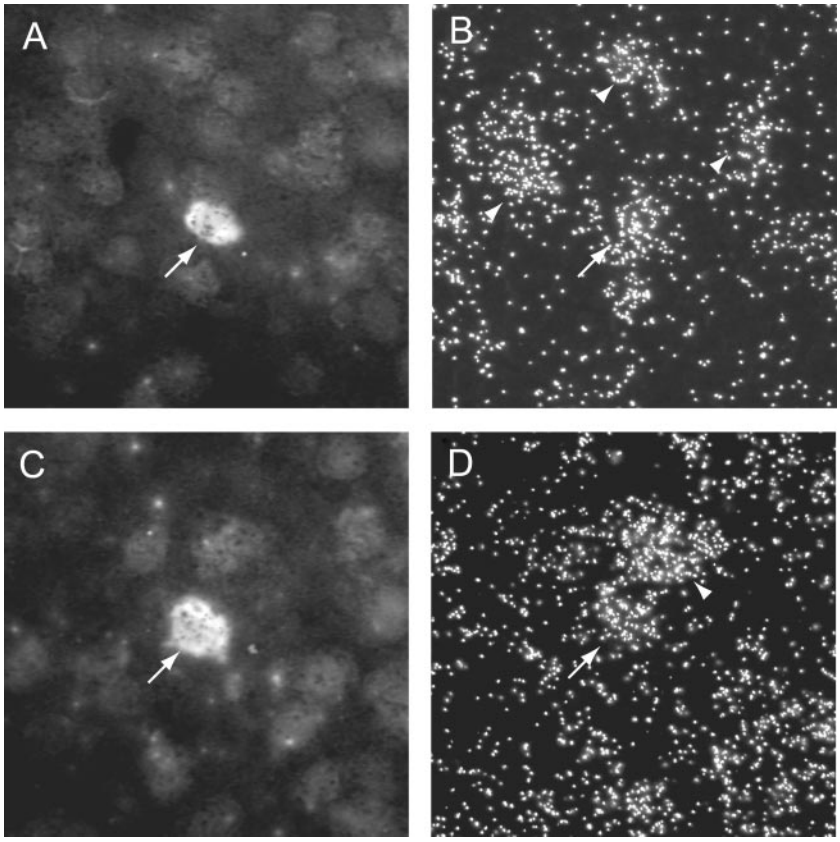

FIG. 4. Coexpression of GALP mRNA with Y1 mRNA or 5-HT2C mRNA. Representative photomicrographs from the brains of female pigtailed macaques. Fluorescent photomicrographs (A and C) show GALP mRNA-expressing cells filled with Vector Red substrate (*arrow*). Darkfield photomicrographs show clusters of silver grains representing a Y1 mRNA-expressing cell (B) or a 5-HT2C mRNA-expressing cell (D) overlying a GALP mRNA-expressing cell (*arrow*). *Arrowheads* represent silver grain clusters not overlying GALP mRNA-expressing cells. Approximately 42 ± 5% of GALP cells coexpress Y1 mRNA, and 24 ± 3% of GALP cells coexpress 5-HT2C mRNA (n = 3 for each experiment).

neurons in the Arc via the 5-HT2C receptor (38), and mice lacking the 5-HT2C receptor share an obesity phenotype with mice lacking leptin (39). Again, it remains to be seen whether GALP neurons contribute to any of the functions of serotonin attributed to the 5-HT2C receptor.

GALP mRNA was rarely coexpressed with NPY mRNA in the Arc. The current results are consistent with the findings of Takenoya *et al.* (40), who reported finding no colocalization between GALP and NPY immunoreactivity in the rat. We have also observed that a small number of POMC neurons coexpress GALP mRNA in the macaque (unpublished observations), consistent with the observations of Takenoya *et al.* in the rat (40). Both NPY and POMC neurons have been shown previously to express Ob-R in the macaque brain (27), and these two cell types are thought to be the major effectors of leptin's effects on body weight and food intake in the hypothalamus (41). GALP cells appear to represent a largely novel population of cells expressing the Ob-R in the Arc. In fact, GALP cells appear to also be distinct from several other neuropeptide-containing cell groups found in the Arc, such as somatostatin, AGRP, and galanin (5).

In summary, these studies suggest that GALP neurons are positioned to integrate information regarding nutritional status from varied sources and relay that information to the neuroendocrine reproductive axis. We have presented evidence that GALP mRNA is down-regulated by 48 h of fasting in the male Japanese macaque. The down-regulation of GALP mRNA during fasting is consistent with the hypothesis that the primary role of GALP is as an anorexigenic factor in the context of feeding control mechanisms. Although this may be an effect of reduced leptin signaling in the hypothalamus, it may also be attributable in part to fasting-induced changes in NPY and serotonin signaling at GALP neurons, via the Y1 and 5-HT2C receptors, respectively. We have also demonstrated that GALP is capable of eliciting LH release in the male rhesus macaque through a GnRH-dependent mechanism. Although the macaque species employed in the current study (*M. mulatta*, *M. fuscata*, and *M. nemestrina*) are quite similar in size and reproductive biology (42, 43), *M. nemestrina* is believed to be phylogenetically distinct from the other two *Macaca* species (44). Therefore, due caution should be used when generalizing individual results to all *Macaca* species.

Acknowledgments

We thank Dr. Anne Lichtenwalner and Dawit Teklemichael for their contributions to these experiments. Thanks to Amgen, Inc., for providing GALP, and to Dr. Jamie Baumgartner for his assistance with this project. We are also grateful to Dr. Cynthia Bethea for her contribution of tissue from the Nonhuman Primate Reproductive Tissue Bank of the National Institute of Child Health and Human Development (NICHD) Specialized Cooperative Centers Program in Reproductive Research.

Received September 17, 2003. Accepted January 13, 2004.

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This work was supported by the NICHD, National Institutes of Health, through cooperative agreement (U54-HD-012629, U54-HD-18185, and U54-HD-08610) as part of the Specialized Cooperative Centers Program in Reproductive Research as well as by Grants R01-DK-061517, R01-HD-027142, R01-HD-13254, NSF 0110686, P01-DK-055819, P51-RR-000163, and R01-MH-062677.

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