Galanin-like peptide (GALP) is produced in a small population of neurons in the arcuate nucleus of the hypothalamus, and leptin stimulates the hypothalamic expression of GALP mRNA. Because insulin and leptin share common signaling pathways in the brain, we reasoned that GALP neurons might also be responsive to changes in circulating concentrations of insulin. To test this hypothesis, we first studied the effect of insulin deficiency on the expression of GALP by comparing levels of GALP mRNA between normal and diabetic animals. Streptozotocin-induced diabetes was associated with a significant reduction in the expression of GALP mRNA, which was reversed by treatment with either insulin or leptin. Second, we examined the effect of insulin administered directly into the brain on the expression of GALP mRNA in fasted rats. Hypothalamic levels of GALP mRNA were lower in animals after a 48-h fast, and central treatment with insulin reversed this effect. These results suggest that GALP neurons are direct targets for regulation by insulin and implicate these cells for a role in the metabolic and behavioral sequelae of type 1 diabetes. Diabetes 53:1237–1242, 2004

In addition to its primary role in regulating glucose homeostasis and utilization, insulin serves an important function in the regulation of feeding and metabolism (1,2). Insulin acts directly on the brain to inhibit food consumption (3), presumably by binding to insulin receptors located within specific regions of the hypothalamus, including the arcuate nucleus (ARC) (4–6); however, the specific neuronal targets of insulin’s action as a satiety factor remain to be identified. Various populations of peptidergic neurons in the ARC are likely targets for insulin’s action (2), and among these are neurons that express galanin-like peptide (GALP). GALP shares a partial amino acid sequence identity with galanin and binds to several galanin receptor subtypes; however, GALP is derived from its own unique gene, distinct from galanin (7). GALP is a neuropeptide, comprising 60 amino acids, that is expressed in the ARC and has been implicated in the neuroendocrine regulation of body weight and feeding (8–11). Fasting reduces GALP gene expression, and this effect can be prevented by the administration of leptin (10,12,13). The distribution of cells that contain GALP mRNA and those that contain insulin receptor mRNA overlap in the ARC of the rat (8,13); thus, we postulated that GALP neurons are targets for the action of insulin.

We had two experimental objectives. The first was to test the hypothesis that insulin regulates the expression of GALP mRNA in the hypothalamus. This was accomplished by comparing intracerebroventricular GALP mRNA levels in the ARC of streptozotocin (STZ)-induced diabetic rats with diabetic rats given either insulin or leptin replacement. The second objective was to determine whether the effect of insulin on GALP mRNA expression (observed in the first experiment) was attributable to a central action of insulin. We tested this hypothesis by examining the effect of insulin, administered twice daily into the third cerebral ventricle, on the expression of GALP mRNA at the end of a 48-h fast.

RESEARCH DESIGN AND METHODS
Adult male Sprague-Dawley rats (280–320 g) were purchased from B&K Universal (Kent, WA). Animals were housed in individual cages and given access to standard rodent diet and water ad libitum. The animals were maintained on a 12:12 light/dark cycle, with lights on at 0600. All procedures were approved by the Animal Care Committee of the School of Medicine at the University of Washington, in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

In situ hybridization. Plasmids containing a partial cDNA for rat GALP were cloned as previously described (13). Linearized GALP cDNA was transcribed for antisense riboprobes with SP6 RNA polymerase (New England Biologicals, Beverly, MA) in the presence of 33P-UTP (Perkin-Elmer, Indianapolis, IN). Sense transcriptions were carried out with T7 RNA polymerase (Roche Biochemicals, Indianapolis, IN). Appropriate riboprobe length was determined with formaldehyde gel electrophoresis. After purification with Nuc-Away Spin Columns (Ambion, Austin, TX), riboprobes were quantified in a scintillation counter.

Two hours after the final treatment (see below), all rats were anesthetized with isoflurane and killed by decapitation. The brains were immediately removed from the calvaria and frozen on dry ice. Brains were stored at −80°C until sectioned. Five sets of 20-μm sections spanning the hypothalamus were collected and stored at −80°C until used. Hypothalamic sections were removed from the freezer, pretreated with acetic anhydride (0.1 mol/L in triethanolamine buffer [pH 8.0]), rinsed in 2× sodium chloride–sodium citrate (SSC; Ambion), depilated in chloroform, dehydrated in graded ethanols, then allowed to air-dry before the hybridization procedure. The volume of probe was calculated (0.1 pmol/μL per slide) and allowed to thaw on ice. The probe was combined with a 2.0 volume yeast RNA (Roche Biochemicals) in TE (0.1 mol/L Tris/0.01 mol/L EDTA [pH 8.0]) to produce the probe mix. Probe mix was heat denatured by placing into boiling water for 3 min, then returned to ice for 5 min. The denatured probe mix was added to prewarmed hybridization buffer.
(60% deionized formamide, 5% hybridization salts, 0.1% Denhardt’s buffer, 0.2% SDS), at a ratio of 1:4. The final volume of probe mix plus hybridization buffer was equal to 100 μl/slide. After adding the hybridization mix to the slides, sections were covered with glass coverslips. Slides were then placed in humidity chambers, which were then put into an oven at 55°C for 16 h.

After hybridization, humidity chambers were removed from the oven and allowed to return to room temperature. Coverslips were removed, and the slides were returned to slide racks, then washed twice in 0.1% SSC for 15 min each at room temperature. Slides were then placed into RNAse (37 mg/ml RNAse [Roche Biochemicals] in 0.15 mol/l sodium chloride, 10 mmol/l Tris, 1 mmol/l EDTA [pH 8.0]) for 30 min at 37°C. Then in RNAse buffer without RNAse at 37°C for another 30 min. After a 3-min wash in 2× SSC at room temperature, slides were washed twice with agitation in 0.1× SSC at 62°C for 30 min each. After a 3-min room temperature wash in 0.1× SSC, sections were dehydrated in graded ethanol (3 min each) and allowed to air-dry. The slides were dipped in Kodak NTB3 emulsion (VWR, West Chester, PA), air-dried, and stored at 4°C for approximately 1 week. They were then developed, dehydrated in graded ethanol, cleared in Citrasol (VWR), and coverslips were applied with DPX (Sigma Biologicals, St. Louis, MO).

GALP mRNA quantification and analysis. All GALP mRNA-containing ARC sections were analyzed unilaterally (~16–18 sections per rat). Slides from all of the animals were assigned a random three-letter code, alphabetized, and read by an operator who was unaware of the animals’ experimental group with an automated image processing system under darkfield illumination. The image processing system consisted of a Cohu 4915 CCD camera (Cohu, San Diego, CA) mounted on a Zeiss Axioskop microscope (Carl Zeiss Microimag ing, Thornwood, NY). The camera was attached to a Scion VG-5 Frame Grabber (Scion, Frederick, MD) mounted on a Macintosh G3 computer (Apple Computer, Cupertino, CA) running UW Grains Software (14). Briefly, for identifying the centers of individual silver grains, each pixel in the video field with a value greater than a low threshold level was examined to determine whether it represented a local intensity peak. When a pixel had a value greater than all eight adjacent pixels, it was flagged as a grain center and counted. Cells were counted as GALP mRNA-positive when the number of silver grains in a cluster seemed to exceed that of background. Data were analyzed by ANOVA with a Tukey/Kramer post hoc test with the GB-Stat Statistical Software (General Dynamics, Bethesda, MD) for the Macintosh. P < 0.05 was considered significant.

Radioimmunoassays for hormones and chemical analysis of free fatty acids and glucose. Analyses to determine serum levels of free fatty acids (FFAs), insulin, and leptin were performed by the Diabetes Endocrinology Research Center Immunoassay Core at the University of Washington, with the use of immunoassay kits purchased from Linco Research (Insulin Kit #R1-13K, Leptin Kit #R2-13K, St. Charles, MI) and Wako Chemicals USA (FFA kit #NEFAC, ACS-ACOD Method; Richmond, VA). Analysis of glucose concentrations in serum was performed with the glucose hexokinase method, described by Bergmeyer (15). Serum levels of corticosterone were determined by radioimmunoassay by Ms. Brigitte Mann at Northwestern University (Evanston, IL).

Third ventricular cannulation. Rats were anesthetized with a ketamine cocktail (100 mg/ml ketamine, 20 mg/ml xylazine, 10 mg/ml acepromazine; 5.0:2.5:1.0 ratio, respectively) and placed in a stereotaxic frame. A stainless steel 26-gauge cannula (Plastics One, Roanoke, VA) was inserted into the third ventricle (midline, 2.2 mm posterior to bregma, 7.5 mm ventral to dura mater). After surgery, the animals were allowed to recover for 1 week, during which time they were handled daily. All compounds were administered in a volume of 1 μl by a 10-μl Hamilton syringe (Hamilton, Reno, NV) attached to polyethylene tubing (internal diameter, 0.58 mm; outer diameter, 0.96 mm) and a 33-gauge stainless steel injection cannula. Cannula placement was verified at the end of the experiment by injecting 1 μl of blue food-coloring dye before the animals were killed and inspecting coronal brain slices for proper location of the dye.

Experimental design

Experiment 1: effects of diabetes, leptin, and insulin on the expression of GALP mRNA. Diabetes was induced in animals by subjecting them to two subcutaneous injections of STZ (45 mg/kg in citrate buffer; Sigma Biologicals) given 24 h apart. This dose of STZ has been shown to induce diabetes in at least 80% of rats without affecting the adrenal axis (16). Control rats were given two subcutaneous injections of citrate buffer. Rats were maintained on ad libitum food and water diet, and body weights were measured on a daily basis. On alternate days, blood was collected from the tail vein and serum glucose levels were measured to ascertain diabetic status (serum glucose >450 mg%). After confirmation of diabetic status (5 days), rats were given twice-daily intracerebroventricular injections of saline [STZ + saline, n = 8, 2.5 mg/kg in 0.2 ml saline/injection; Sigma Biologicals], leptin (STZ + leptin, n = 8, 8.0 unit porcine insulin in 0.2 ml saline/injection; Sigma Biologicals), or the combination of insulin and leptin treatments (STZ + insulin + leptin, n = 9). Citrate buffer control animals were given twice-daily intracerebroventricular injections of saline (citrate + saline, n = 8). Hormone treatments ensued for 5 days, and food intake was measured daily, then the animals were killed by decapitation and the hypothalami were processed for GALP mRNA with in situ hybridization. Trunk blood was collected and analyzed for glucose levels. In addition, serum was analyzed for FFAs, insulin, leptin, and corticosterone levels.

Experiment 2: effects of 48-h fast and intracerebroventricular insulin on the expression of GALP mRNA. Rats were divided into three experimental groups (n = 7 or 8/group). Two groups had their food removed at 0600 on day 1. Fasted animals were given a 3.0-μl intracerebroventricular (ICV) injection of either artificial cerebrospinal fluid (aCSF) or 4 μl of insulin in aCSF vehicle (iletin regular porcine insulin; Lilly, Indianapolis, IN). The third group was allowed to feed ad libitum and received an injection of aCSF. Five total injections over a 48-h period were made between 0800 and 0900 and again between 1700 and 1800. Body weight was measured each day after the morning injection. Beginning at 1000 on day 3, animals were killed by decapitation. Brains were rapidly removed, frozen on dry ice, sectioned at 20 μm, and then stored at −80°C until processed by in situ hybridization for GALP mRNA. Trunk blood was collected for serum glucose, leptin, and insulin assays.

RESULTS

Experiment 1: effect of diabetes, insulin, and leptin on the expression of GALP mRNA. STZ-induced diabetes was associated with a significant reduction in the expression of GALP mRNA in the ARC, as reflected both by a decline in the number of identifiable GALP neurons (P < 0.001) and a decrease in the per-cell content of GALP mRNA (P < 0.001). Treatment of the diabetic animals with insulin, leptin alone, or the combination of the two hormones restored the per-cell content of GALP mRNA to that of normal (nondiabetic) controls. Likewise, treatment with either insulin or leptin fully restored the number of identifiable GALP neurons present in the ARC, and the combined treatment of insulin and leptin increased the apparent number of GALP neurons in excess of nondiabetic controls (P < 0.05). Figure 1 summarizes the results from the in situ hybridization analysis, and Fig. 2 shows representative photomicrographs illustrating grain clusters that reflect the expression of GALP mRNA by cells in the ARC.

As expected, STZ-treated diabetic animals showed a significant decrease in body weight (P < 0.05; Table 1), which was not altered by any of the hormone treatments. Furthermore, the diabetic rats exhibited a significant increase in food intake compared with controls (P < 0.05), which was normalized by insulin, leptin, or the combination of the two hormones (Table 1). STZ-induced diabetic animals showed significantly increased serum levels of glucose compared with controls (P < 0.001), and insulin treatment, alone or in combination with leptin, reduced serum glucose levels to those of nondiabetic controls (Table 1). STZ-induced diabetes was associated with a significant increase in serum levels of FFA, and only the combined treatment of insulin and leptin resulted in a full reversal to levels equal that of nondiabetic controls (Table 1).
alone did not reverse the decline in circulating leptin associated with STZ-induced diabetes. None of the treatments had a significant effect on serum levels of corticosterone.

**Experiment 2: effects of 48-h fasting and ICV insulin on the expression of GALP mRNA.** There was a significant reduction in the number of GALP mRNA-expressing cells in the fast + aCSF group compared with the ad libitum–fed group \((P < 0.01)\). Similarly, there were significantly fewer grains per cell in the fast + aCSF group than in the ad libitum–fed group \((P < 0.05)\). ICV administration of insulin during the fast (fast + insulin) prevented the fasting-induced decline in the number of identifiable GALP mRNA-containing cells (fast + aCSF versus fast + insulin groups, \(P < 0.05\)). Figure 3 summarizes these data.

At the start of the experiment, there were no significant differences in body weights among the treatment groups. At the end of the treatment period, body weights in the two fasted groups were significantly reduced compared with ad libitum–fed controls (Table 2). Fasting produced a significant decline in serum levels of both insulin and leptin (compared with the ad libitum–fed group, \(P < 0.001\)). With respect to the ad libitum–fed group, there was a significant decrease in serum levels of leptin in the fast + insulin group \((P < 0.001)\), comparable to that seen in the fast + aCSF group \((P < 0.001)\). Serum levels of insulin were slightly but significantly higher in the fast + insulin group compared with the fast + aCSF group \((P < 0.05)\), although serum levels of insulin in the fast + insulin group were still significantly below that of the ad libitum–fed group \((P < 0.001; \text{Table 2})\). The 48-h fast elicited a significant reduction in serum glucose levels \((P < 0.001)\).
reduction in GALP mRNA, suggesting that insulin directly into the brain reversed the fasting-induced GALP mRNA, and this decline was reversed by the peripher-

able to changes in these metabolic fuels. Furthermore, the lack of change in circulating levels of glucose in response to the small increase in serum insulin after the ICV insulin suggests that this increase was probably physiologically irrelevant (see, e.g., 18,19). Taken together, these observations support the hypothesis that insulin acts directly on the brain to regulate GALP gene expression and argues against the proposition that the effects of ICV insulin are mediated by changes in peripheral concentrations of either insulin or other metabolic signals, such as leptin, glucose, or FFAs.

Insulin and leptin exhibit some common physiological effects and modes of action in the brain. Both insulin and leptin inhibit food intake (2,20,21), alter metabolic fuel availability (22–25), and support the neuroendocrine reproductive axis (26–29). Insulin and leptin both activate the sympathetic nervous system through direct effects on the brain, which in turn mediates some of the peripheral actions of these hormones (25,30,31). GALP is a plausible candidate for mediating the common effects of leptin and insulin, because ICV administration of GALP produces similar effects on food intake, reproduction, and the sympathetic nervous system (11,32–36). Furthermore, the observation that both leptin and insulin stimulate the expression of GALP mRNA provides additional evidence that GALP neurons are involved in transducing some of

Table 2) that was not affected by central insulin administra-

DISCUSSION

The results of these experiments demonstrate that insulin regulates the expression of GALP mRNA in the hypothal-amus. STZ-induced diabetes and its insulin-deficient state were associated with a decrease in the expression of GALP mRNA, and this decline was reversed by the peripheral administration of insulin. Moreover, the delivery of insulin directly into the brain reversed the fasting-induced reduction in GALP mRNA, suggesting that insulin’s effects on GALP gene expression are centrally mediated.

Although the results of the ICV insulin infusion experiment suggest that insulin acts directly on the brain to increase GALP mRNA expression, the interpretation of these results is confounded by the fact that the ICV insulin also produced a slight (albeit significant) increase in circulating insulin levels, comparable to that previously described by others using a similar experimental approach (17). It is plausible that even this modest increase in circulating insulin could alter other circulating factors, such as leptin or metabolic fuels, that could have indirectly mediated the putative actions of insulin. However, it seems unlikely that leptin mediates the effects of insulin treatment on GALP mRNA levels in these experiments, because serum levels of leptin remained unchanged after the ICV and peripherally administered insulin injections. Likewise, circulating levels of neither glucose nor FFAs were significantly altered by the central insulin injections. Hence, the observed increase in the expression of GALP mRNA after ICV insulin is unlikely to be attributable to changes in these metabolic fuels. Furthermore, the lack of change in circulating levels of glucose in response to the small increase in serum insulin after the ICV insulin suggests that this increase was probably physiologically irrelevant (see, e.g., 18,19). Taken together, these observations support the hypothesis that insulin acts directly on the brain to regulate GALP gene expression and argue against the proposition that the effects of ICV insulin are mediated by changes in peripheral concentrations of either insulin or other metabolic signals, such as leptin, glucose, or FFAs.

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![FIG. 3. Effect of ICV insulin on GALP mRNA expression in 48-h-fasted rats. Fasting is associated with a reduction in the number of identifiable cells expressing GALP mRNA and ICV insulin administration reverses this effect (A). Fasting is also associated with a significant reduction in the cellular content of GALP mRNA as reflected by the per-cell grain counts, which was reversed with ICV insulin treatment (B) (*P < 0.05, **P < 0.001 vs. ad libitum-fed controls).](image)
the shared physiological effects produced by these two hormones.

Leptin and insulin may activate common intracellular elements within GALP neurons. Both insulin and leptin activate phosphatidylinositol-3-OH kinase (PI3K)/STAT3 signaling pathways (37–40). Insulin can also influence cellular activity through an IRS-2/PI3K pathway, and some of the effects of leptin seem to be mediated by this same IRS-2/PI3K intracellular pathway (37,41–44). The regulation of GALP mRNA by both leptin and insulin suggest that the two hormones could utilize a common signaling pathway in GALP neurons (10,13).

The results of the present study, together with earlier work, suggest a possible role for GALP in the hyperphagia associated with type 1 diabetes. Because GALP has been shown to inhibit food intake, it is conceivable that the tonic activity of GALP neurons exerts some level of chronic restraint on feeding in the normal well-fed animal, which has normal circulating levels of insulin and leptin. Conversely, animals with type 1 diabetes have much reduced circulating levels of insulin and leptin. This would presumably cause a reduction in tonic activity of GALP neurons, thus removing GALP’s chronic restraint on food intake and producing the hyperphagia associated with type 1 diabetes. Although this model is consistent with our current understanding of the effects of leptin, insulin, and diabetes on appetite, the presence of a tonic inhibition of food intake by GALP in normal animals remains to be demonstrated. Moreover, it is likely that other hypothalamic neurotransmitters, such as NPY and the melanocortins, are also involved in diabetic hyperphagia (2), as part of a highly redundant circuitry. Notwithstanding, the involvement of GALP in some aspects of the pathological consequences of diabetes, such as hyperphagia, is worthy of further attention.

In summary, we have shown that STZ-induced diabetes, which suppresses leptin and insulin levels, results in a reduction of intracellular levels of GALP mRNA in the ARC of the rat. As is the case with leptin, insulin administered either peripherally or centrally induces the expression of GALP mRNA. These observations suggest that GALP neurons are an important component of the neural circuitry that mediates the central effects of insulin and implicate these neurons for a role in the metabolic and behavioral sequelae of diabetes.

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TABLE 2

Physiological data from experiment 2: effects of fasting and ICV insulin on the expression of GALP mRNA

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Final body weight (g)</th>
<th>Insulin (ng/ml)</th>
<th>Leptin (ng/ml)</th>
<th>Serum glucose (mg %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad libitum fed + aCSF</td>
<td>361.6 ± 5.4</td>
<td>1.9 ± 0.10</td>
<td>2.62 ± 0.50</td>
<td>147 ± 3.4</td>
</tr>
<tr>
<td>Fast + aCSF</td>
<td>311.6 ± 6.6</td>
<td>0.3 ± 0.06†</td>
<td>0.21 ± 0.07†</td>
<td>87 ± 5.1</td>
</tr>
<tr>
<td>Fast + insulin</td>
<td>311.8 ± 7.9†</td>
<td>0.7 ± 0.10†</td>
<td>0.43 ± 0.10†</td>
<td>82 ± 9.8</td>
</tr>
</tbody>
</table>

Data are means ± SD. *P < 0.01 compared with ad libitum-fed + aCSF group. †P < 0.001 compared with ad libitum-fed + aCSF group.
INSULIN REGULATES THE EXPRESSION OF GALP mRNA