Original Article

Galanin-Like Peptide Rescues Reproductive Function in the Diabetic Rat

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Galanin-like peptide (GALP) is expressed in the hypothalamic arcuate nucleus and is regulated by leptin and insulin. Centrally administered GALP stimulates gonadotropin secretion and sexual behavior in the rat. Type 1 diabetes is associated with reduced expression of GALP, as well as an overall decline in reproductive function. We postulated that tonic activity of GALP in the brain is required to sustain normal reproductive activity. To test this hypothesis, we examined whether central (intracerebroventricular) immunoblockade of GALP would reduce sexual behaviors and serum levels of luteinizing hormone (LH) in normal adult male rats. We found that GALP antibody reversibly reduced serum levels of LH and abolished male sexual behaviors ($P < 0.05$ and 0.001, respectively). Second, we tested whether intracerebroventricular GALP could restore normal plasma LH levels and sexual behavior in diabetic animals. We compared groups of diabetic rats that received intracerebroventricular GALP or vehicle and found that GALP increased serum levels of LH and sexual behavior. Third, we examined whether intracerebroventricular administration of affinity-purified GALP antibody could block the effect of insulin and leptin in reversing the effects of diabetes on LH and sexual behavior. We found that treatment of diabetic animals with insulin and leptin nearly normalized LH levels and sexual behaviors; however, this effect was attenuated by intracerebroventricular administration of GALP antibody ($P < 0.05$). These observations demonstrate that endogenous GALP provides trophic support to the neuroendocrine reproductive axis, including sexual behavior. *Diabetes* 54:2471–2476, 2005

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aCSF, artificial cerebrospinal fluid; GALP, galanin-like peptide; LH, luteinizing hormone; STZ, streptozotocin.

Deviations of normal metabolism and nutrition can disrupt reproduction. The activity of the reproductive axis may be altered by changes in circulating levels of metabolic hormones such as leptin and insulin, whose blood levels typically reflect an animal's metabolic status and fuel reserves (1). Reproductive dysfunction is common in metabolic diseases, such as type 1 and type 2 diabetes (2,3; rev. in 4). Sexual dysfunction in diabetes is thought to be attributable to a central neuropathy (5); however, the cellular and molecular etiology of this phenomenon is unknown. It seems plausible that the reproductive dysfunction associated with diabetes is caused by a disruption in the cellular and molecular targets in the brain for insulin and other metabolic factors that may be altered in diabetes. Neurons that produce galanin-like peptide (GALP) are prime candidates for mediating this phenomenon.

GALP is a neuropeptide that is expressed in the arcuate nucleus of the hypothalamus and has been implicated in the regulation of gonadotropin-releasing hormone. Central infusions of GALP stimulate the secretion of gonadotropin-releasing hormone as well as luteinizing hormone (LH) and induce all aspects of sexual behavior in the male rat (6–9). GALP has also been implicated in the neuroendocrine regulation of feeding and body weight (7,10–12). Fasting and uncontrolled diabetes reduce GALP gene expression, an effect that can be prevented by the administration of insulin, leptin, or a combination of these hormones (13). Hypothalamic neurons that produce GALP also express the leptin receptor and leptin regulates the expression of GALP mRNA (10,14). The distribution of GALP-containing neurons overlaps with cells that express the insulin receptor and insulin receptor substrate-1 (15,16); furthermore, insulin, like leptin, regulates the expression of GALP mRNA (13). Hence, GALP neurons are targets for the action of both leptin and insulin, and GALP is recognized to exert a potent stimulatory effect on the neuroendocrine reproductive axis. Based on our previous observations, we have postulated that GALP may serve as a molecular conduit that couples metabolism to reproduction (7,17). We suggest that reproductive dysfunction associated with diabetes may be attributable to a decay in the function of GALP neurons.

Our first objective was to test the hypothesis that GALP neuronal activity provides trophic support for the mainte-

TABLE 1
Summary of central and peripheral treatments in experiment 2

	n	ICV treatment	Peripheral insulin	Peripheral leptin	Peripheral saline
Normal	6	aCSF + control IgG	No	No	Yes
DbX + control	6	aCSF + control IgG	No	No	Yes
DbX + GALP	7	aCSF + 15 nmol/day GALP	No	No	Yes
DbC + control	6	aCSF + control IgG	Yes	Yes	No
DbC + anti-GALP	6	aCSF + 2.5 mg/day goat anti-GALP	Yes	Yes	No

Doses of administered substances: peripheral insulin, 1.0 unit twice daily; peripheral leptin, 2.5 mg/kg twice daily; peripheral saline 0.1 ml twice daily. ICV, intracerebroventricular.

nance of the hypothalamic-pituitary-gonadal axis and sexual behavior in the normal male rat. We examined this by studying the effects of an immunoblockade of GALP activity in the brain on reproductive function in normal adult male rats. The second objective was to determine whether the reproductive deficits and increased food intake observed in the diabetic animal might be caused by reduced GALP activity. This was tested by examining whether a sustained, centrally administered infusion of GALP could restore normal plasma LH levels and sexual behaviors in rats with streptozotocin (STZ)-induced diabetes. The third objective was to test the hypothesis that GALP neurons mediate the effects of insulin and leptin on reproductive function. To this end, we investigated whether an immunoblockade of central GALP activity could mitigate the ability of insulin and leptin to reverse the effects of diabetes on reproductive function. Our results suggest that GALP neurons mediate the effects of insulin and leptin on reproduction and that reduced activity of GALP neurons contributes to the reproductive dysfunction associated with diabetes.

RESEARCH DESIGN AND METHODS

Adult male Sprague Dawley rats (280–320 g) were purchased from Harlan (Indianapolis, IN). Animals were housed within the vivarium at Hope College (Holland, MI) in individual cages and given access to a standard rodent diet and water ad libitum. The animals were maintained on a 12:12-h light:dark cycle, with lights on at 0700. To minimize the effects of the "novelty" of the behavioral tests, all animals were given three bouts of sexual experience before the beginning of the experiment (6). All procedures were approved by the Hope College Animal Care and Use Committee, in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Lateral ventricle cannulation and osmotic minipump implantation. 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 (Brain Infusion Kit II; Alzet, Cupertino, CA) was inserted into the lateral ventricle (1.1 mm lateral to bregma, 1.1 mm posterior to bregma, 3.5 mm ventral to dura mater). The cannula placement was secured with skull screws and cranioplastic cement.

The osmotic minipumps (200 μl) were weighed then filled with appropriate solutions and reweighed as per the manufacturer's recommendation (Alzet). A 15.5-mm polyethylene tubing (internal diameter 0.69 mm, outer diameter 1.14 mm) was filled with vehicle and attached to the cannula and minipump. This length of tubing was calculated to allow for ~6.5 days of control treatments before minipump delivery of experimental treatments. The minipumps were implanted subcutaneously, and the incisions were closed with wound clips. After surgery, the animals were allowed to recover for 72 h, during which time they were handled daily. Proper cannula placement was determined histologically at the end of the experiment.

Radioimmunoassays. Serum concentrations of LH were measured at Northwestern University (Evanston, IL) with reagents from the National Institutes of Health. The LH antiserum was anti–rLH-S11, the standard was rLH-RP3, the assay sensitivity was 0.2 ng/ml, and the intra-assay coefficient of variation was 2.2%.

Experimental design

Experiment 1: effects of intracerebroventricular anti-GALP on reproductive physiology and behavior. Control injections consisted of vehicle with 2.5 mg/ml goat IgG (Chemicon, Santa Cruz, CA). The experimental treatments consisted of a combination of two separate, affinity-purified GALP antibodies (goat polyclonal antibodies against $GALP_{41–60}$ [CILDLWKAIDG LPYSRSPRMT] and $GALP_{23–42}$ [CLHLSSKANQGRKTDSALAIL]; Bethyl Labs, Montgomery, TX) combined and reconstituted at 2.5 mg/ml in artificial cerebrospinal fluid (aCSF). Both antibodies were shown by enzyme-linked immunosorbent assay, Western blot, and immunocytochemical analyses to be specific for full-length GALP and not to bind nonspecifically to other hypothalamic neuropeptides, such as galanin, oxytocin, neurophysin, vasopressin, or neuropeptide Y (data not shown).

Intact, sexually experienced male rats (n = 10) were given a 5-μl intracerebroventricular injection of aCSF or anti-GALP. The injection was repeated 5 min later. The dose of anti-GALP was chosen to be comparable with that used in other immunoblockade studies (18–20). Injections were performed so that each rat received all of the treatments. Subsequent injections were carried out at least 72 h apart. The order of treatments and the testing order were randomized. Rats were first tested for sexual behavior at 1000 (lights on) and then 1 week later at 2000 (lights off).

Within 5 min after the second injection, the rats were placed into a testing arena (polyethylene housing cage) with a sexually experienced steroid-primed female rat (10 μg estradiol benzoate in 0.1 ml safflower oil injected subcutaneously 48 h before testing and 500 μg progesterone in 0.1 ml safflower oil injected subcutaneously 2 h before testing). The males were evaluated for the number of mounts, intromissions, and ejaculations as well as their latency to these behaviors in a 30-min test. A mount was recorded if the male approached the female from the rear and placed both forelegs firmly on her flanks and locked his hips. An intromission was recorded if the foregoing behavior was noted with the addition of a clear pelvic thrust followed by genital grooming.

To augment plasma LH levels, all rats were castrated and implanted with a 2-mm Silastic capsule (internal diameter 1.57 mm, outer diameter 3.17 mm; VWR, Seattle, WA) containing crystalline testosterone propionate (Sigma, St. Louis, MO) (21). After 2 weeks, the injections were repeated (vehicle or anti-GALP; n = 5 per group). The animals were killed 15 min after the injections. Trunk blood was collected, and plasma was measured for LH levels by radioimmunoassay.

Experiment 2: GALP and GALP-mediated effects of insulin and leptin in diabetic male rats. Sexually experienced adult male rats were implanted with 26-gauge stainless steel cannulas (Brain Infusion Kit II; Alzet) into the lateral ventricle of the brain. The cannulas were attached to a 200-μl osmotic minipump implanted subcutaneously via a 15.5-mm length of PE 50 tubing containing aCSF. The length of tubing was calculated to deliver aCSF for 6.5 days before pump delivery of experimental solutions. The minipumps were filled with aCSF containing control antibody solution (as described for experiment 1) for three separate treatment groups: control group (normal), uncontrolled diabetic group (DbX), and controlled diabetic group (DbC). The minipumps for the experimental groups contained GALP (15 nmol/day delivery, DbX + GALP) or anti-GALP (prepared as in experiment 1; DbC + anti-GALP) preparation. This dosage of GALP was determined from pilot studies and is comparable with that used in other studies involving bolus injections (7,12,22,23). The animals were tested for sexual behavior 72 h after surgery to determine if the cannula or minipump implantation had had any adverse effects. After the test, diabetes was induced in the appropriate groups of rats (Table 1).

Diabetes was induced in animals by subjecting them to two subcutaneous injections of STZ (45 mg/kg in citrate buffer; Sigma) given 24 h apart. This dose of STZ has been shown to induce diabetes in at least 95% of animals without affecting the adrenal axis (13,24). Control rats were given two subcutaneous injections of citrate buffer. Rats were maintained on food and water ad libitum and body weights were measured on a daily basis. On alternate days, blood was collected from the tail veins and serum glucose levels were measured to ascertain diabetic status (serum glucose >450 mg/dl). On the morning of day

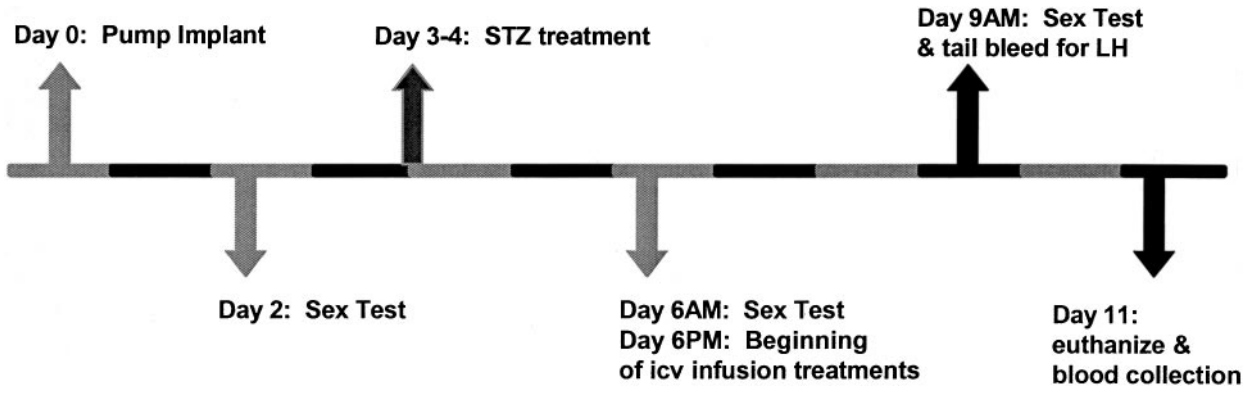

FIG. 1. Time line illustrating the course of treatments and tests for experiment 2. icv, intracerebroventricular.

6 (after implantation surgery), animals were again tested for sexual behavior and their blood glucose levels were measured. On the evening of day 6, hormone treatments to control diabetes (DbC groups) were begun by giving twice-daily subcutaneous injections (at 0700 and 1900) of combined insulin (1.0 units porcine insulin in 0.2 ml saline, 2 units/day total; Sigma) and leptin (2.5 mg/kg in 0.2 ml saline, 5.0 $mg \cdot kg^{-1} \cdot day^{-1}$), as previously described (13). Saline was given subcutaneously to the remaining groups (0.2 ml to normal, DbX, and DbX + GALP groups). Hormone and saline treatments began the evening of day 6 after the implantation surgeries and continued until the end of the experiment (day 11). Food intake and body weight were monitored daily. On day 9, rats were tested for sexual behavior and blood glucose and LH levels beginning at 1000. At 1000 on day 11, the animals were killed with an overdose of pentobarbital (400 mg/kg, Fatal-Plus; Vortech, Dearborn, MI). Before they died, their blood was collected via the heart and analyzed for plasma glucose and LH levels. At the end of the experiment, minipump activity was confirmed according to the manufacturer's recommendations; only data from animals whose minipumps were active were included in the final results. A summary of treatment groups is shown in Table 1, and the time line of treatments is illustrated in Fig. 1.

Statistical analyses. Analyses of behavioral data were performed with Wilcoxon's signed-rank analysis. All other data were analyzed by ANOVA with Fisher's protected least significant differences post hoc test. Where appropriate, a repeated-measures ANOVA was performed for comparison of pre- to posttreatment behavior data. All analyses were performed with GB-Stat Statistical Software (General Dynamics, Bethesda, MD) for the Macintosh. $P < 0.05$ was considered significant.

RESULTS

Experiment 1: effects of intracerebroventricular anti-GALP on reproductive physiology and behavior. All rats were able to ambulate, groom, feed, and drink; no abnormal motor effects were noted after the GALP antibody preparation was injected. The intracerebroventricular treatment with anti-GALP produced a nearly complete loss of sexual behavior in male rats during the 30-min test at both 1000 ($P < 0.001$; data not shown) and 2000 ($P < 0.001$) (Fig. 2*A*) compared with control injections, despite the fact that control-treated rats showed increased sexual behavior during the nocturnal test, as expected (25). The only sexual behaviors shown by anti-GALP–treated rats were mounts, and then only near the end of the test (last 5–7 min, both tests). All rats showed normal levels of sexual behavior 3 h after the injections (data not shown). After the final injections, anti-GALP elicited a significant reduction in plasma LH levels compared with levels in control-treated rats ($P < 0.05$) (Fig. 2*B*).

Experiment 2: effects of central GALP infusion on diabetic male rats. All animals showed typical levels of male sexual behavior 72 h after the cannula and minipump implantations. On the morning of day 6, plasma LH levels and all aspects of male sexual behavior were reduced in diabetic animals compared with in normal controls (data not shown). Central and peripheral treatments began on the afternoon of day 6. Analyses of male sexual behavior on the morning of day 9 showed that uncontrolled diabetes (DbX + aCSF) resulted in a complete loss of mounts, intromission, and ejaculations ($P < 0.001$) (Fig. 3*A–C*), thus confirming earlier reports (26–29). Only the insulin and leptin treatment (DbC + aCSF) were shown to restore male sexual behaviors to levels that were not significantly different from that of normal controls. However, central GALP infusion (DbX + GALP) significantly increased the number of mounts ($P < 0.01$) and intromissions ($P < 0.01$)

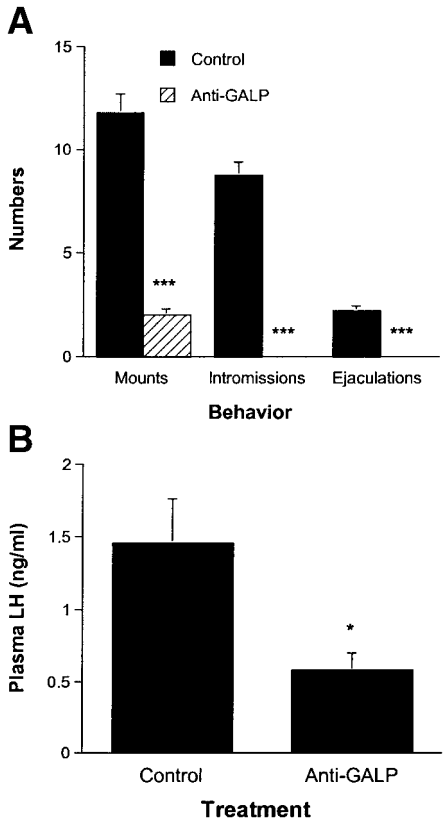

FIG. 2. Sexual behaviors and serum levels of LH from experiment 1. *A*: The central administration of anti-GALP virtually abolished sexual behaviors, as reflected by the number of mounts, intromissions, and ejaculations in a 30-min test (compared with control treatments; data shown for 2000 test). *B*: Compared with control treatment, anti-GALP antibodies reduced serum levels of LH. *$P < 0.05$; ***$P < 0.001$.

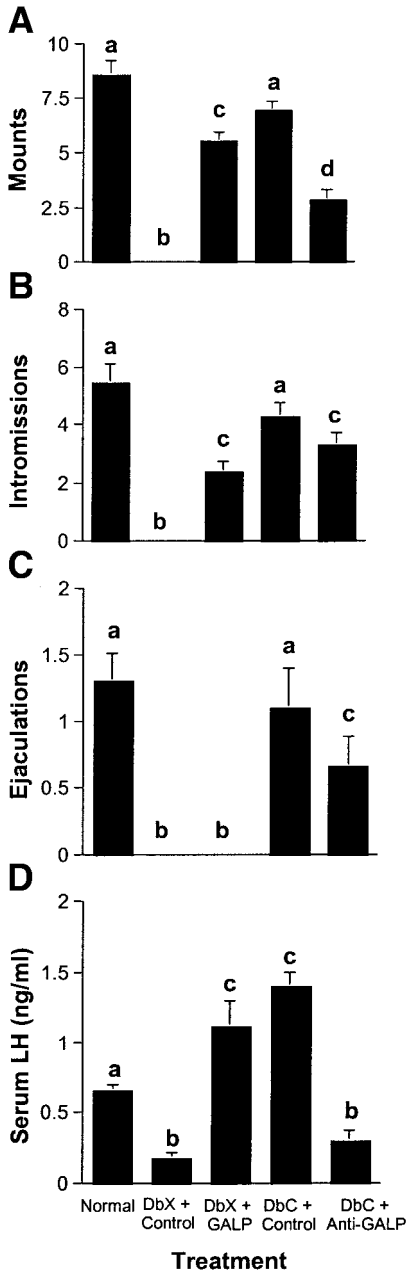

FIG. 3. Sexual behaviors and serum levels of LH from experiment 2. Treatment of diabetic animals with either insulin and leptin (DbC + Control) or GALP (DbX + GALP) restored sexual behaviors (*A–C*) and serum levels of LH (*D*) to near-normal levels. Treatment of diabetic animals with insulin and leptin plus anti-GALP significantly attenuated insulin and leptin's positive effects on these variables. Bars with different letters above differ significantly ($P < 0.05$) from one another.

compared with the uncontrolled diabetes group (DbX + aCSF), although no ejaculations were observed in the DbX + GALP group. In one interesting finding, anti-GALP treatment (DbC + anti-GALP) significantly reduced the ability of leptin and insulin to promote male sexual behavior (mounts, intromissions, and ejaculations; $P < 0.05$ vs. DbC + control) (Fig. 3*A–C*). The various treatments had similar effects on plasma LH levels. By day 9, the uncontrolled diabetic group (DbX + aCSF) had significantly lower LH levels compared with normal controls ($P < 0.001$) (Fig. 3*C*). This reduction in plasma LH was prevented by GALP (DbX + GALP) and insulin and leptin treatment (DBC + aCSF). Anti-GALP treatment (DbC + anti-GALP) significantly reduced plasma LH levels compared with both the DbC + aCSF groups and normal controls ($P < 0.05$).

TABLE 2
Summary of day 9 behavioral and physiological data from experiment 2

	Serum glucose (mg %)	Body weight (g)	Food intake (g)
Normal	126 ± 4.5*	330.5 ± 4.99*	25.1 ± 0.99*
DbX + Control	462 ± 21.0†	323.7 ± 10.93*	40.4 ± 2.43†
DbX + GALP	405 ± 18.0†	296.3 ± 8.85*	28.4 ± 2.78*
DbC + Control	162 ± 8.2*	279.9 ± 7.65†	22.2 ± 0.62*
DbC + Anti-GALP	173 ± 14.1*	299.4 ± 4.46*	29.0 ± 2.16‡

Data are means ± SE. *, †, and ‡ indicate significantly different treatment groups, $P < 0.05$. See RESULTS for details of significance.

Significant effects of treatments on food intake were observed by the afternoon of day 7 (1 day after the onset of central infusions and peripheral treatments) and continued until the end of the experiment. Data for food intake, body weight, and serum glucose at day 9 are summarized in Table 2. On day 9, food intake and blood glucose levels were elevated in the uncontrolled diabetes group (DbX + aCSF) compared with in normal controls ($P < 0.001$ for both). Both the DbC + aCSF and the DbX + GALP treatments reduced food intake in diabetic rats to levels that were not statistically different from those in normal controls. However, anti-GALP treatment (DbC + anti-GALP) resulted in significantly higher levels of food intake relative to that of DbC + aCSF controls ($P < 0.05$), although this newly increased value was still not significantly different from that of normal controls. On the morning of day 11, a slight but significant attenuation of the effects of brain infusion of both GALP and anti-GALP on food intake was noted. This may have been due to an earlier-than-calculated loss of minipump action, a time-dependent degradation of peptide/protein within the minipump, a clogged cannula, or habituation to the treatments; nevertheless, plasma LH levels on day 11 were similar to those seen on day 9 (data not shown). The final weights of the minipumps suggested a slight and earlier-than-predicted loss of function.

DISCUSSION

The two primary objectives of this study were to *1*) determine whether the tonic activity of GALP neurons supports the neuroendocrine reproductive axis and sexual behavior in the normal male rat and *2*) establish whether alterations in the activity of GALP neurons can be implicated in mediating the effects of diabetes on reproductive function. We demonstrated that immunoblockade of endogenous GALP reduced circulating levels of LH and diminished sexual behavior in the normal adult male rat. We also showed that central infusions of GALP reversed the reproductive deficits associated with diabetes in the rat, mirroring the effects of insulin treatment in the diabetic animal, and further that the effects of insulin and leptin given to diabetic animals were attenuated by immunosuppression of endogenous GALP. This study demon-

strates the importance of endogenous GALP in supporting the reproductive axis in the normal rat and provides evidence that the deleterious effect of diabetes on reproductive function is mediated by a decrease in the secretory activity of GALP neurons.

Because there are no specific GALP antagonists yet available, we used passive immunoneutralization to block the endogenous activity of GALP in vivo to reveal GALP's physiological significance. Although this approach can be instructive, the results from GALP immunosuppression studies should be interpreted with caution. It is conceivable that in our study the injection of antibodies to GALP into the brain produced nonspecific pharmacological effects that compromised the function of the neuroendocrine reproductive axis. However, we think this is unlikely. The studies were controlled with the administration of a vehicle that contained concentrations of IgG equal to that of the purified GALP antibodies, which had no discernable effect on any measured variable. Furthermore, the antibody preparation was affinity purified, which could be expected to reduce or eliminate potentially toxic constituents of serum. The dose of antibody used in both experiments was comparable with that used in prior immunosuppression studies, which again had vehicle controls that were comparable with those used here (18–20). Although GALP antibody treatment significantly attenuated male sexual behavior, it did not abolish it entirely. This suggests that the immunoblockade was either incomplete or that other neuroendocrine factors were involved in mediating the effects of metabolic hormones on sexual behavior.

In this study, a central GALP infusion reduced the hyperphagia that normally accompanies uncontrolled diabetes, suggesting an inhibitory effect of GALP on food intake. This apparent anorexigenic action of GALP would appear to be in conflict with several previous reports showing that a bolus injection of GALP elicits an acute increase in food intake in the rat (30,31). This short-term orexigenic effect of GALP in the rat (but not in mice) (7) appears to be similar in size and magnitude to that elicited by galanin and may be caused by GALP binding to galanin receptors (30,32), an action that may not occur under normal physiological circumstances. Thus, pharmacological interactions of GALP with the various galanin receptor subtypes complicate the interpretation of the results regarding the effects of GALP on feeding (rev. in 22,33). This caveat notwithstanding, a bolus injection of GALP (intracerebroventricularly) has been shown to cause a significant reduction of food intake in the rat over a 24-h period and does so to a greater degree than can be accounted for by compensation for the initial increase in feeding (7,8). Leptin's anorexigenic effects coupled with the observation that leptin and insulin stimulate GALP gene expression would support the argument that endogenous GALP partially mediates the effects of leptin and insulin on satiety (13,34). Here we offer further evidence for this argument by demonstrating that the effects of insulin and leptin on satiety in diabetic animals are attenuated by a central infusion of a GALP antibody, which presumably reduces the central actions of endogenous GALP.

Reproductive dysfunction is common in people with type 1 diabetes, even those on insulin therapy (5,35). Several studies have demonstrated a loss or reduction in the activity of the hypothalamic-pituitary-gonadal axis and sexual behavior in rats with insulin-dependent diabetes (23–36). Although some of these indexes of reproductive function can be improved with insulin treatment, others remain compromised despite treatment (27). Here we have shown that a combination of insulin and leptin treatment can reverse the effects of diabetes on serum levels of LH and reproductive behaviors. We have also shown that a sustained central infusion of GALP can mimic the action of insulin and leptin on LH secretion in the diabetic rat, although this treatment did not fully normalize sexual behavior. Nevertheless, the disruption of reproductive function observed in insulin- and leptin-treated diabetic rats that simultaneously received anti-GALP antibodies suggests that endogenous GALP mediates, at least partially, the effects of insulin and leptin on sexual function.

The results of this investigation demonstrate that exogenously administered GALP can rescue sexual behavior in the diabetic male rat, findings that corroborate our earlier report demonstrating that central injections of GALP stimulate sexual behavior in the male rat (6). However, this action would not appear to be applicable to all species, as GALP has the opposite effect on sexual behavior in the male mouse (37). It is also important to note that the administration of intracerebroventricular GALP produces motor deficits in the mouse (7,22,37), which does not appear to be the case in the rat. Species-specific differences in behavioral responses to neuropeptides have been reported (38). Thus caution must be exercised when drawing generalizations about the effects of GALP on sexual behavior across species.

In summary, we have presented evidence that the central activity of GALP neurons provide tonic support for reproductive function, including sexual behavior, in the normal male rat. We have also demonstrated that exogenously administered GALP can reverse some of the deleterious effects of diabetes on the reproductive axis and provided evidence for a role of endogenous GALP in mediating the effects of leptin and insulin reproductive function. These results underscore the importance of hypothalamic GALP in the integration of metabolism and reproduction.

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