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Reproductive Endocrinopathy in Acute Streptozotocin-Induced Diabetic Male Rats

Studies on LHRH

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Streptozotocin-(STZ) treated diabetic male rats have significant reproductive endocrinopathy. To determine the functional responsiveness of luteinizing hormone-releasing hormone (LHRH) neurons in STZ-treated diabetic male rats, stimulated LHRH release was assessed using hypothalami from short-term STZ-treated, STZ-treated insulin-replaced, and control male rats. LHRH release from control, STZ-treated, and STZ-treated, insulin-replaced explants in response to an initial and second 30-min pulse of phenylephrine were not different. A terminal pulse, containing 45 m*M* KCl, a general secretogogue, also revealed no differences between groups in stimulated LHRH release. Glucose and testosterone levels in the controls and the diabetic rats were significantly different. Cell counts on serial brain sections processed for LHRH immunohistochemistry suggested that the number of LHRH neurons in the preoptic arear (POA) and septal areas were not different between control and STZ-treated rats. Thus, the short-term STZ-treated rats of this study were diabetic, and they displayed associated endocrinopathy; however, explants obtained from control and STZ-treated rats exhibited a typical LHRH responsiveness to both phenylephrine and KCl, and appeared similar in LHRH neuron number. Therefore, these findings suggest that reproductive endocrinopathy accompanying short-term STZ-induced diabetes in male rats does not result from deficiency in LHRH neurons *per se*.

Key Words: STZ; diabetes; rat; gonadotrophin-releasing hormone.

Introduction

A frequent finding in diabetic men is dysfunction of the reproductive organs *(1–5)*. Erectile impotence due to vascular and peripheral autonomic nervous system changes are common findings in diabetes; however, as reported by Fairburn *(6)* and Ficher et al. *(7)*, changes in endocrine functions and central nervous system (CNS) control of sexual arousal also may be involved in sexual dysfunction. A useful and relevant model for the study of diabetes-related sexual dysfunction is the streptozotocin- (STZ) induced diabetic male rat *(8)*. Several reproductive problems accompany STZ-induced diabetes in male rats, including depressed gonadotrophin and testosterone (T) levels, altered feedback of T, deficits in sex behavior, and decreased accessory sex organ weights *(8–12)*. The observation of normal pituitary gland responsiveness to exogenous luteinizing hormone-releasing hormone (LHRH) in diabetic rats *(13)* suggests that a deficiency may be localized either to LHRH neurons *per se* or to systems that regulate LHRH neuron activity. Interestingly, King and Kang *(14)* showed that long-term STZ-induced diabetes results in attenuation of potassium chloride (KCl) and noradrenergic stimulation of LHRH release from hypothalamic slices in vitro. Bestetti, et al. *(15)* also have described altered LHRH release from mediobasal hypothalamic (MBH) fragments in vitro. Thus, it is a tenable hypothesis that endocrinopathy found in diabetic rats may result from direct perturbation of LHRH neurons as a result of STZ treatment. Alternatively, reproductive dysfunction in STZ-treated male rats may be secondary to depression of norepinephrine (NE) turnover in the brain of STZ-treated rats as reported by Chandrashekar et al. *(16)*, which becomes manifest by deficient regulation of LHRH neuron activity. The present study was designed to evaluate further the LHRH system by examination of (1) LHRH neuron numbers in the preoptic and septal areas of short-term diabetic and control male rats, and (2) the responsiveness of LHRH neurons to phenylephrine (an α-adrenergic-selective stimulatory agent to LHRH release) *(17–21)* and to KCl, a general depolarizing secretogogue.

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Results

Shown in Fig. 1, short-term STZ-treated rats had significantly elevated plasma glucose (377% increase) compared to control rats. Rats of the insulin-replaced group also had significantly elevated plasma glucose (308% increase); however, this was owing to the interval between insulin replacement and sacrifice (16 h). Previous studies in this lab *(22)* have demonstrated that plasma glucose in STZ-treated rats is effectively lowered by insulin replacement therapy (i.e., they are at control levels at 10 h post-insulin).

Experiment 1

The number of LHRH-immunopositive neurons in the preoptic area and the septal area of representative control and STZ-treated rats are shown in Table 1. In two representative animals analyzed per group (each animal provided approx 45 brain sections for analysis), no differences in total LHRH neuron number were observed with this quantitation in either brain region, nor were there differences in the average number of neurons per brain section through these areas. Additionally, qualitative observations found no apparent differences in staining intensity in the median eminence between control and STZ-treated rats.

Experiment 2

In vitro secretion of LHRH in control, STZ-treated, and STZ-insulin-replaced rats is shown in Fig. 2. Basal release rates (assessed by the mean release rate over the first seven fractions) were slightly; but significantly lower in control rat explants compared to STZ and STZ-insulin replaced rat explants (ANOVA, $F[2/18]:7.26, p<0.005$). LHRH release in response to two sequential pulses of phenylephrine and a terminal pulse of KCl was assessed *(a priori)* by two-way ANOVA across the entire data set. This analysis revealed a profound (approx 130% at peak) increase of LHRH in response to the KCl challenge after approx 8 h in culture ($p < 0.05$). The response to KCl was not different between the treatment groups. The apparent one-fraction delay in LHRH release in response to KCl of the STZ-treated insulin-replaced group (Panel C) is not meaningful, since it relates to slight alterations in perifusion-tubing dead space and time to reach peak concentration of drug in the microchambers. LHRH release in response to two pulses of phenylephrine were consistent and modest (approx 20–30% over baseline), but were not statistically significant in the overall two-way ANOVA. However, although not performed *a priori*, one-way ANOVAs with repeated measures across each of the stimulation intervals and surrounding two basal levels within each treatment group did reveal significantly increased LHRH release to each challenge, except Phen-1, (Panel A, Fig. 2).

Shown in Fig. 3, serum hormone assays in control, STZ-treated, and STZ-treated insulin-replaced rats revealed that LH and FSH levels were not significantly different between the control and the treatment groups. However, serum FSH was different between the STZ-treated and the STZ-treated insulin-replaced animals. Paradoxical to the LH levels, serum T was significantly lower in STZ-treated rats compared to controls and insulin-replaced,STZ-treated rats ($p < 0.05$), and was restored to normal by insulin replacement.

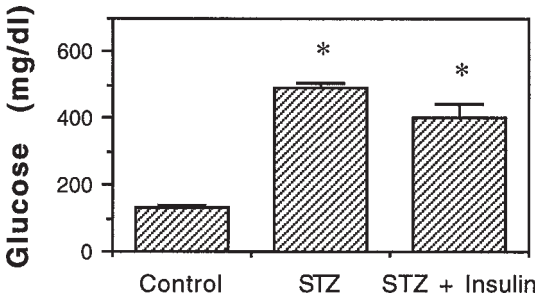

Fig. 1. Serum glucose in control, STZ-treated, and STZ-treated insulin-replaced male rats ($n = 10$/group). The vertical lines of this and subsequent figures indicate the SEM. STZ and STZ-treated insulin-replaced rats had significantly elevated plasma glucose compared to controls ($*p < 0.05$). Plasma was taken 16 h after insulin injection in the insulin-replaced group.

Table 1
Number of LHRH ImmunoreactiveNeurons in the POA and Septal (SEP) Areas of STZ-Treated and Control Rats

	Control, $n = 2$	STZ-treated, $n = 2$
POA		
Total #	393.0 ± 178.0	371.0 ± 81.0
#/Section	8.6 ± 2.0	6.7 ± 0.3
SEP		
Total #	187.0 ± 125.0	197.0 ± 49.0
#/Section	3.9 ± 1.9	3.5 ± 0.2

Experiment 3

LHRH contents in the median eminence (ME), MBH, anterior hypothalamus, and olfactory bulbs of control, STZ-treated, and STZ-treated with continuous or delayed insulin replacement are shown in Fig. 4. STZ treatment did not alter the concentration of LHRH in any brain region analyzed in gonad-intact rats. Continuous or delayed insulin therapy to STZ-treated rats also did not alter the profile of LHRH content in any brain area analyzed with the exception of the ME. In the ME, insulin replacement (both continuous and delayed) resulted in elevated ME LHRH contents, which were greater than those observed in control and STZ-treated brains.

Experiment 4

LHRH content in the ME of castrated and castrated T-replaced rats is shown in Fig. 5. LHRH content in the ME increased significantly following T treatment in castrated control rats and STZ-treated rats.

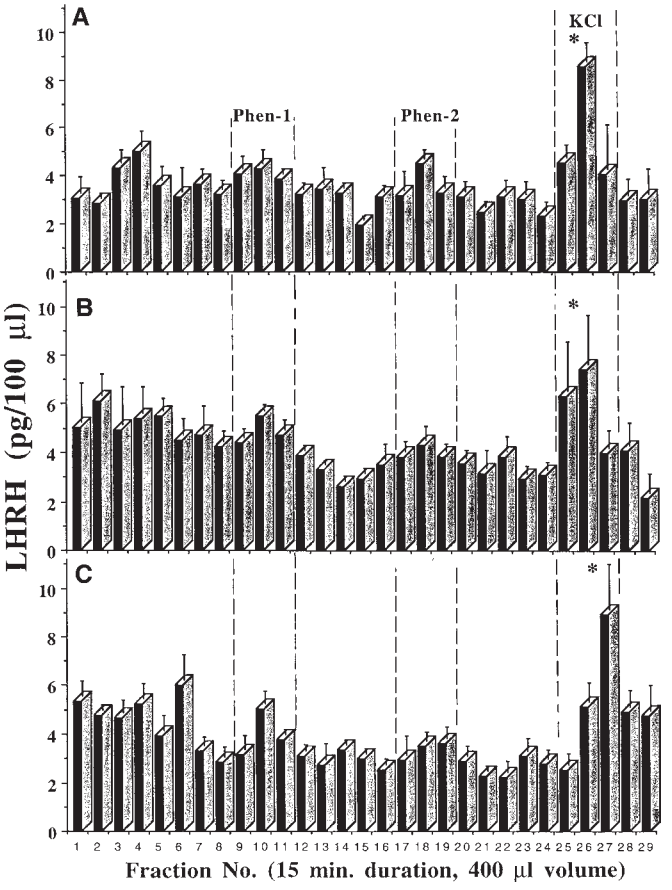

Fig. 2. Mean ± SEM. LHRH secretion from explants obtained from control (panel **A**), STZ-treated (panel **B**), and STZ-treated insulin-replaced (panel **C**) rats. Expected stimulation response intervals are indicated by the verticle dashed lines. Exposure of explants to sequential pulses of phenylephrine ($2 \times 10^{-4}M$), resulted in a slight (i.e., fractions 10 and 18), but not statistically significant, increases in LHRH release ($P > 0.05$, *a priori* two-way ANOVA over entire data set). However, when comparisons were made between the stimulation intervals and the two preceding and two following basal levels, fractions 10 and 18 had significantly higher LHRH concentrations. KCl (45 m*M*) induced a significant increase in LHRH release (*$p < 0.05$, two-way ANOVA). No detectable differences were observed between treatment groups with the exception of a slightly, but significantly lower initial basal release rate (i.e., first five fractions) from the control explants.

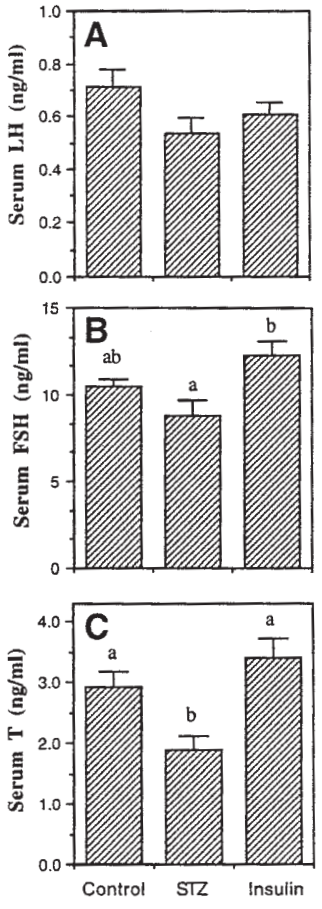

Fig. 3. Mean ± SEM. Serum LH (**A**), FSH (**B**), and T (**C**) in control, STZ-treated, and STZ-treated, insulin-replaced rats. Serum LH appeared lower in diabetic animals. However, this was not significant ($P > 0.05$). Serum FSH also appeared slightly lower in diabetic rats. However, this also was not significant. Insulin-replaced, STZ-treated rats had significantly elevated serum FSH in comparison to the STZ-treated rats ($P < 0.05$). Serum was lower in the STZ-treated rats compared to the other two groups ($P < 0.05$). Different letters denote significant differences.

Discussion

The present study has examined certain features of the LHRH neuronal system in an animal model of diabetes, the STZ-treated male rat. The results of these studies suggest that the LHRH neuroendocrine system remains intact in male, short-term, STZ-treated rats, and STZ-treated rats given insulin replacement. LHRH neuron number in serial sections through a limited number of representative animals suggests that there are a similar number of LHRH immunopositive neurons in the septal and preoptic areas between control and diabetic rats, and that their distribution is unaltered by STZ. Thus, it appears that STZ treatment is not detrimental to LHRH neuron number. Additionally, staining intensity of LHRH immunoreactivity and profile of distribution in the lateral intermediate and palisade regions of the ME also was not qualitatively different between STZ-treated and control rats. Moreover, there were no differences in the content of LHRH in ME, mediobasal hypothalamus (MBH), anterior hypothalamus, or olfactory bulbs between the STZ and control group. Although insulin therapy was associated with an increase in LHRH content in the ME, this was not associated with an increase in serum LH. Additionally, castration led to an increase in LHRH concentration in the ME, which appears to be complementary to castration-induced LH release in control rats *(16)*. The smaller castration-related increase in LHRH content in the median eminence of STZ-treated rats, although not statistically significant, also may contribute to the attenuated LH response to castration in diabetic rats *(16)*. In general, these findings suggest that the feedback effect of testoster-

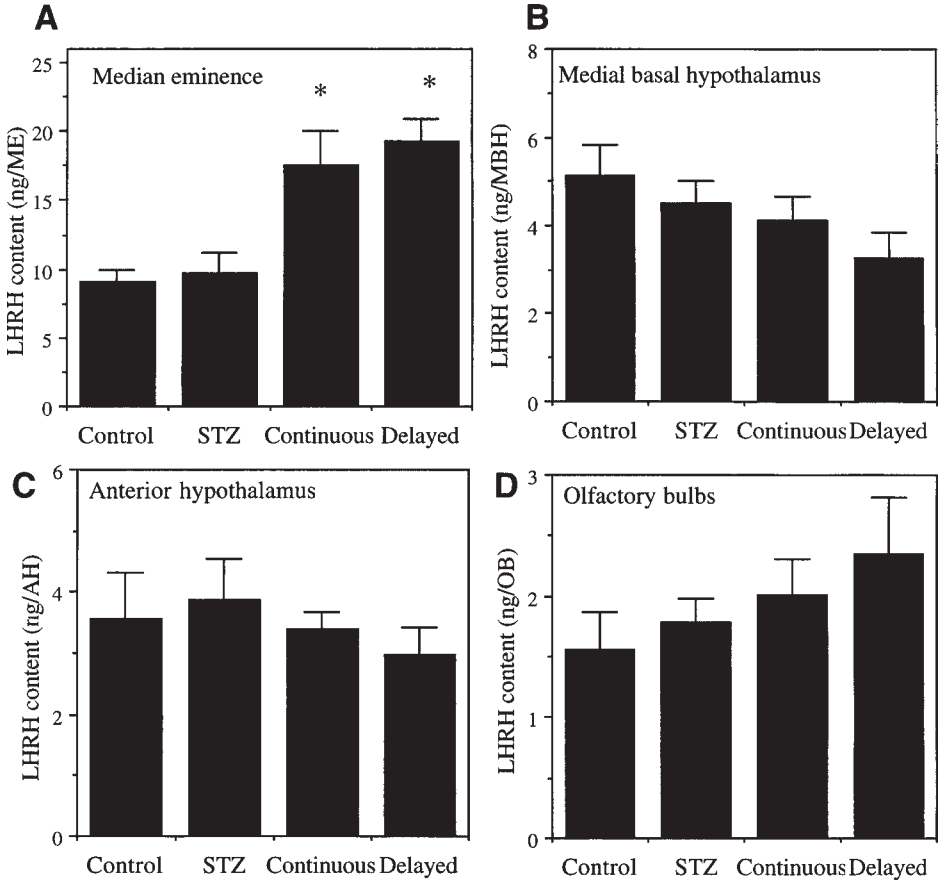

Fig. 4. Mean ± SEM. LHRH content in the ME, MBH, anterior hypothalamus, and olfactory bulbs of control, STZ-treated, and STZ-treated, insulin-replaced (continuous or delayed onset of treatment) intact male rats. No detectable differences in LHRH content were found between treatment groups in any brain region analyzed except the ME. In the ME, insulin-replaced rats had significantly elevated LHRH content compared to controls and STZ-treated rats (*$p < 0.05$).

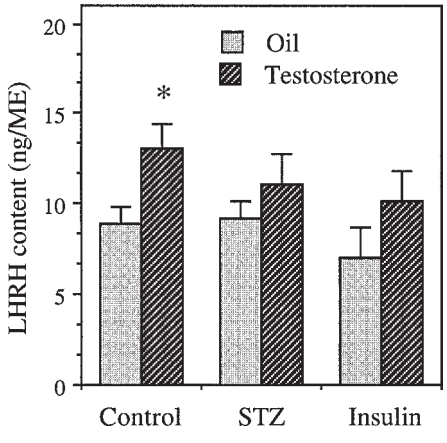

Fig. 5. LHRH content in the ME of castrated control and STZ-treated rats and castrated, STZ-treated and insulin-replaced rats with or without T replacement. LHRH content in the ME of castrated rats was not different between treatment groups. T replacement resulted in a significant increase in ME LHRH content in control rats (*$p < 0.05$) and a slight, but not significant increase in ME LHRH content of STZ-treated, and STZ-treated, insulin-replaced rats ($p > 0.05$).

one on LHRH synthesis is not altered in STZ-induced diabetic rats.

The LHRH-release responses of male hypothalamic explants to catecholamine stimulation were slight, but consistent, as we have previously found (unpublished observations). In rats treated with STZ with or without insulin therapy, the α-adrenergic agonist, phenylephrine, induced a typically modest stimulation of LHRH release from explanted hypothalami, which was similar to that observed in control rats. Furthermore, the degree of stimulation of LHRH release from male rat explants in response to phenylephrine, although not statistically significant, appears lower than female rat peoptic are-(POA), MBH-ME explant responses using this perifusion paradigm *(18,19)*. These finding suggest that the ability of norepinephrine to stimulate LHRH, though typically marginal, is operational in STZ-treated rats and that LHRH neurons in these animals respond normally to α adrenergic stimulation. In the present study, there also were no differences between groups in the ability of KCl to induce LHRH release. This finding appears to be in contrast to other published reports *(14,15)* wherein the LHRH release response to KCl was described as diminished in STZ-treated rats. There are several significant differences between the present study and that by Bestetti, et al. *(15)*. First, the route of administration of STZ was dif-

ferent (ip in the present study vs iv). Second, the explants used to assess LHRH release were significantly different. Explants of the present study contained the POA and the LHRH neuron somata, whereas the MBH explants of the other study *(15)* contained only LHRH terminals in the ME and severed axons of passage through the hypothalamus. Third, the incubation paradigms are not comparable in that the present study used a flowthrough perifusion system, whereas the other study *(15)* used a static incubation system in which the possibility of LHRH autofeedback may be increased. Finally, the KCl challenges in the present study were preceded by two challenges with phenylephrine and the responses to KCl may be altered by prior exposure to this agent. The explants of the other study *(15)* were not exposed to catecholamines prior to KCl-induced release. Therefore, the present study is not directly comparable to that of Bestetti et al. *(15)*. The study by King and Kang *(14)* also described an attenuated release of LHRH in response to KCl, which appears to be in contrast to the present findings. However, in that study, the STZ-treated rats showed what appears to be a generalized lower basal release rate (although not statistically significant) of LHRH, and, the observed stimulation of LHRH by KCl may actually be similar in magnitude in the STZ group to that of controls if correction is made for lowered basal LHRH release. Their findings with NE stimulation *(14)*, showed that slice explants obtained from long-term STZ-treated rats were less responsive to NE than were control explants. This is an interesting finding, which may appear to be in contrast to the findings of the present study regarding phenylephrine. However, phenylephrine is a more specific α-adrenergic receptor agonist, whereas NE will stimulate all types of adrenergic receptors in these explants. Moreover, the STZ-treated animals of the King and Kang study *(14)* were diabetic for at least 8 mo, whereas those of the present study were diabetic for only 4 wk. Therefore, these two studies are also not directly comparable. Nonetheless, it is an interesting notion that LHRH neurons within these explants may show deficiencies in noradrenergic stimulation apart from α-1 receptors, or perhaps after long-term STZ-induced diabetes.

It is known that there exists a significant diminution of NE turnover in the hypothalamus of STZ-treated rats and that this deficiency parallels alterations in androgen feedback *(16)*. In that the present study found a normal responsiveness of LHRH neurons to phenylephrine, and Chandrashekar et al. *(16)* found altered catecholamine profiles in short-term STZ-treated rats, these studies, taken together, suggest that the endocrine perturbations associated with short-term STZ-induced diabetes may result from altered catecholamine regulation of LHRH secretion, and not LHRH neuron demise *per se*. Long-term STZ-induced diabetes, such as that described by Kang and King *(14)*, may be associated with the development of LHRH neuron deficiency.

This study corroborates and extends previous findings that demonstrate that STZ-induced diabetes, as evidenced by dramatically increased plasma glucose, is associated with altered profiles of testosterone and testosterone feedback on the gonadotropins. The finding of no significant change in LH and FSH in the diabetic rats of the present study, despite significantly decreased circulating testosterone, indicates that the gonadotrophin response to decreasing T is dysfunctional. Previously, we reported that sensitivity of LH to testosterone negative feedback is greatly increased in intact STZ-treated rats *(16);* however, the present study suggests no alteration in the feedback of T on LHRH levels in the ME of castrated STZ-treated rats. We found that the content of LHRH in the ME of castrated control rats increased significantly following T replacement therapy and that there was a tendency toward a similar increase in castrated, T-replaced STZ-treated rats. However, this was not statistically significant. Furthermore, STZ-induced diabetes in rats is not associated with an altered pituitary gland response to LHRH *(13)*. These findings suggests that dysfunctional feedback of T on LH secretion may be owing to malfunction of LHRH neuron control, and not synthesis, storage, or potential for release of LHRH in the ME. We have previously reported alterations in catecholamine profiles in STZ-treated rats *(8)*, and thus, it would appear that catecholamine control of LHRH secretion may malfunction as a result of STZ treatment. Therefore, the lack of increase in LH levels despite lowered T levels in the STZ-treated rat may be secondary to reduced NE turnover in the ME and not owing to an altered responsiveness of LHRH neurons to NE. Several studies have shown that NE is a positive stimulus to LHRH and LH release *(17,20,21)*, and, whereas reductions in serum testosterone levels have been shown to increase norepinephrine turnover leading to increased LH release *(23)*, this does not occur in diabetic animals *(8)*. Alternatively, many other neurotransmitter are involved in LHRH secretion, including, but not limited to, the opiates *(19,24,25)* and GABA *(26)*, and, the effect of STZ treatment on these neurotransmitter systems are unknown. Thus, although the "upstream" regulation of LHRH release appears to malfunction in diabetic male rats and this malfunction parallels dysfunctional testosterone feedback on hypothalamic norepinephrine, it is presently unknown which particular systems "upstream" to the LHRH neuron are involved in the malfunction of LHRH secretion control in the diabetic rat.

In summary, the present findings suggest that the endocrinopathy associated with short-term STZ-induced diabetes is not a result of LHRH neuron loss or their responsiveness to α-adrenergic stimulation. Moreover, stimulus secretion coupling in LHRH neurons, as evidenced by KCl depolarization, appears to be normal in short-term STZ-treated rat hypothalami. Additionally, LHRH content in several hypothalamic nuclei is not altered in STZ-treated rats. In contrast, significant alterations in hypothalamic catecholamines

are known, and thus, it is hypothesized that endocrinopathy associated with STZ-induced diabetes in rats may involve altered neurochemical control of LHRH release.

Materials and Methods

Animals

Adult male Sprague-Dawley rats (Harlan Industries, Indianapolis, IN) were housed in a temperature-controlled (22°C) vivarium with a 12:12 light:dark cycle. Food (TekLab Rat Diet, Madison, WI) and tap water were available ad libitum. All animal experiments of the present report were approved by the Institutional Animal Care and Use Committee and are in full accord with state and federal guidelines. The rats were made diabetic by injection of streptozotocin (STZ; 50 mg/kg, ip, in 0.01*M* citrate buffer, pH 4.5) or were treated with injection vehicle only (controls). Beginning the morning after STZ treatment, insulin replacement was given to a subgroup of diabetic animals. Replacement consisted of 5 IU/kg of insulin (protamine zinc and ileti I; Eli Lilly, Indianapolis, IN) injected just prior to lights off and 2 IU/kg of insulin injected within 1 h of lights on. Animals were weighed twice weekly, and the insulin dose was adjusted accordingly. Blood glucose was determined at autopsy for assessment of diabetes. Glucose was assayed by a glucose oxidase procedure using a kit purchased from Sigma (St. Louis, MO).

Experiment 1: LHRH Neuron Quantitation

Randomly selected and deeply anesthetized (chloral hydrate/pentobarbital) control and short-term (4-wk) STZ-treated rats were transcardially perfused with 4.0% ice-cold paraformaldehyde in preparation for immunohistochemistry (IHC). LHRH neurons in control ($n = 2$) and STZ-treated rats ($n = 2$/group) were counted in complete serial sections through the rostral hypothalamus following IHC localization using an avidin–biotin method (*elite* ABC kit, Vector Labs, Burlingame, CA). Sucrose-equilibrated brains were sectioned at 40-μm thickness. Free-floating sections were incubated for 30 min in blocking serum followed by 48 h of incubation in primary antiserum diluted in 0.1% Triton X-100-PBS (0.1*M*, pH 7.4) in the cold (4°C). The antisera used for IHC was the same as that used for LHRH radioimmunoassay (RIA; *see below*). However, the final working dilution for IHC was 1/10,000. After incubation in anti-LHRH, sections were rinsed 10X, and incubated for 2 h in solution B (biotinlyated antirabbit IgG) of the ABC kit. After additional rinsing, sections were incubated in the final solution (ABC solution) of the kit for two additional hours. Visualization of the reaction used 3,3'-diaminobenzidine tetrahydrochloride (Sigma) in the presence of hydrogen peroxide. After mounting on gel-dipped slides, slides were coded, randomized, and analyzed by an observer blind to the treatment groups. Each of 45 sections/animal were drawn in camera lucida, and each LHRH-positive neuron was mapped and counted.

Experiment 2: In vitro LHRH Release

POA-MBH-ME explants were removed from control, STZ-treated, or STZ-treated insulin-replaced rat brains as previously described *(18)*. Three perifusion runs were necessary, since the maximum number of explants/perifusion was six. For each perifusion run, six explants (2/treatment group) were collected into chilled Gibco F-12 culture media (Gibco Labs., Grand Island, NY) and successively rinsed with sterile F-12 medium containing penicillin/streptomycin to deter contamination and then were place into F-12 culture media supplemented with 5% fetal calf serum, CaCl2 (to achieve 1.2 m*M*) and penicillin/streptomycin. Bacitracin (Sigma, $2 \times 10^{-4}M$), was added to prevent breakdown of LHRH *(27)*. Explants were individually transferred to 200-μL microchambers and placed into a perifusion apparatus (Acusyst S, Endotronics, Coon Rapids, MN). Explants were continuously perifused with warmed (37°C) and oxygenated (95%) culture medium from a common source using a peristaltic pump set at a flow rate of 25 μL min. Effluents of the microchambers were collected in a multiple-tube fraction collector set in a refrigerator (4°C). Following a 2-h equilibration period, fractions were collected for 7.5 h, during which explants were exposed to 2×30 min pulses of phenylephrine ($2 \times 10^{-4}M$; Sigma), separated by 1.75 h, in medium containing 0.03% ascorbic acid. A final pulse of KCl (45 m*M*) was used to assess stimulus-secretion coupling in these explants as an assessment of their viability. Fifteen-minute fractions were collected (approx 400-μL vol) and were subsequently frozen for later RIA for LHRH. All fractions were assayed for LHRH in a single assay. At sacrifice, blood from each animal was collected on ice, allowed to clot, and serum was drawn off for assay of LH, FSH, and T.

RIA

A single-antibody nonequilibrium RIA was used to measure the concentration of LHRH in aliquots of culture medium effluents. The antibody used was provided by Chen and Ramirez (anti-LHRH R11B73, University of Illinois, Urbana, IL) and has been extensively characterized *(28)*. Briefly, duplicate 100-μL aliquots of medium were incubated for 48 h in the presence of a 1/250,000 dilution of the R11 serum in 0.1% gelatin–0.1*M* sodium phosphate-buffered saline (PBS, pH 7.4) containing 0.15 m*M* sodium EDTA and 0.2% normal rabbit serum (NRS). Nonspecific binding was assessed by incubation of the NRS-EDTA buffer with no anti-LHRH serum. Specific binding was measured by omitting the reference standard (synthetic LHRH, Bachem, Torrance, CA) in the presence of the anti-LHRH serum. Approximately 4500 cpm of iodinated LHRH tracer (SA. 1680 μCi/ug; New England Nuclear, Boston, MA) was added to each tube after a 48-h preincubation period. After an additional 72 h of incubation, the antibody-bound and free LHRH were separated using ice-cold 95% ethanol followed by centrifugation. The bound fraction was monitored for γ-radioactivity. Nonspecific

binding was approx 4.5% and specific binding was 31%. The sensitivity of assay (10% displacement from 0) was 1 pg/tube. The effective range of the assay was 1–50 pg LHRH. Our intra- and interassay variabilities, based on a homogeneous pool of MBH tissue, is approx 4 and 11% respectively. Blank medium, medium containing phenylephrine, and medium containing KCl were assayed to evaluate possible interference with the RIA. Serum LH, FSH, and T were assayed as previously described *(8)* using reagents provided by the National Pituitary Hormone Program of the NIAMDD.

Experiment 3: Feedback of T on LHRH in the ME

Control, STZ-treated, and STZ-treated insulin-replaced rats were subjected to experiments to determine the effect of T replacement therapy to castrated rats on LHRH content in the median eminence. Fifteen days following STZ treatment, rats of each group were castrated under ether anesthesia. Rats were given injections (sc) of T propionate (100 µg, Sigma) or oil vehicle on d 2, 4, and 6 after castration. One day after the last T injection (d 7 post-castration), rats were sacrificed and the median eminence was dissected out and prepared for subsequent RIA of LHRH. ME fragments were sonicated for 20 s in 1 mL 0.1 HCl. Ten-microliter aliquots were then assayed as described in experiment 3.

Experiment 4: LHRH Content in the Diabetic Brain

Control, STZ-treated, STZ-treated continuous-insulin-replaced, and STZ-treated delayed-insulin-replaced rats were sacrificed 45 d following STZ treatment to compare LHRH content in the ME, anterior hypothalamus-POA, MBH, and olfactory bulbs. In the continuous-insulin-replaced group, insulin was administered starting the day after STZ treatment and continued until sacrifice (45 d). In the delayed-insulin-replaced group, insulin replacement was initiated 33 d after STZ treatment and continued until sacrifice (45 d).

Data Analysis

For experiment 1, data were collected from 45 brain sections for each of two animals ($n = 2$). Therefore, only descriptive statistics are indicated. For experiments 2 and 4, differences between groups were analyzed by two-way ANOVA. All other comparisons used one-way ANOVA.

Acknowledgments

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