SUCCESSFUL REPRODUCTION is highly dependent on availability of an ample supply of oxidizable metabolic fuels, and food availability is a primary environmental factor affecting fertility, particularly in female mammals (3). Animals in negative energy balance, as a consequence of decreased food intake or increased energy expenditure (e.g., during cold exposure or excessive exercise) often exhibit nutritional infertility (3, 11, 27). However, even animals in positive energy balance may display a form of nutritional infertility resulting from altered partitioning of metabolic fuels. For example, infertility is associated with both naturally occurring and experimentally induced obesities (1, 9, 23, 26).

Treatment with pharmacological doses of insulin diverts circulating metabolic fuels away from tissues where they are oxidized and into adipose tissue storage depots. When these high doses of insulin are given either acutely or chronically, reproductive function is impaired. Insulin-induced changes include a suppression of pulsatile luteinizing hormone (LH) release and an inhibition of ovulatory cycles (4, 10, 16, 17, 26). These effects of insulin on reproduction are almost certainly due to a diversion of metabolic fuels away from oxidative processes and into storage, rather than to elevated insulin levels per se, because these inhibitory effects of insulin on reproduction can be overcome by concurrent infusion of glucose (4) or simply by allowing animals to increase their food intake (26).

Although a significant body of work has examined the suppression of LH pulses and ovulatory cycles by treatment with pharmacological doses of insulin, there is almost no information available on the effects of insulin-induced repartitioning of metabolic fuels on reproductive behaviors. The sole exception (26) showed that treatment of gonadally intact Syrian hamsters with insulin for 48 h immediately after estrus significantly decreased the likelihood that the animals would display lordosis at the next expected estrus. However, this work made no attempt to quantify the changes in estrous behavior. Nor did it determine whether the inhibition of estrous behavior was due to suppression of estradiol and progesterone (P) secretion, to a reduced neural responsiveness to ovarian steroids, or to both. Other energetic challenges, including food deprivation, treatment with pharmacological inhibitors of glucose and fatty acid oxidation, and cold exposure, inhibit estrous behavior in steroid-treated ovariectomized hamsters, indicating a decreased behavioral responsiveness to ovarian hormones (6, 13; A. K. Panicker, R. A. Mangels, J. B. Powers, G. N. Wade, and J. E. Schneider, unpublished observations). One aim of this work was to determine whether insulin-induced repartitioning of metabolic fuels would decrease behavioral responsiveness to estradiol and P.

Nutritional infertility may be due in part to changes in neural receptors for estradiol and P. Nutritional manipulations that suppress estrous behavior also decrease the number of detectable estrogen receptor immunoreactive (ERIR) cells in the ventromedial hypothalamus (VMH), the principal site where estradiol is thought to act to facilitate estrous behavior in rodents (2, 19). In contrast, these nutritional manipulations increase the number of detectable ERIR cells in the medial preoptic area (mPOA) (13). A second aim of this work was to determine whether insulin treatments would affect neural ERIR in ovariectomized hamsters.

The physiological cues signaling metabolic fuel availability appear to reach the forebrain circuits controlling estrous behavior via the area postrema (AP). The AP is a circumventricular organ situated in the floor of the fourth ventricle, ideally situated to sense changes in the plasma or cerebrospinal fluid (14). Lesions of the AP prevent the suppression of estrous behavior that is induced by food deprivation (Panicker et al., unpublished observations), treatment with metabolic inhibitors (13), or cold exposure (A. H. Early, G. N. Wade, and...
R. L. Lempicki, unpublished observations) in female hamsters. The last experiment determined whether the AP played a role in insulin-induced suppression of estrous behavior in Syrian hamsters.

MATERIALS AND METHODS

Animals and Housing

Female Lak:LVG Syrian hamsters weighing 90–100 g were purchased from Charles River Breeding Laboratories (Wilmington, MA) and housed in stainless steel, wire-bottom cages. The animals were housed in a 14:10-h light-dark cycle (lights on at 0600) at an ambient temperature of 22 ± 2°C. Purina laboratory rodent chow (no. 5001, Ralston Purina, St. Louis, MO) was given ad libitum unless otherwise indicated. Water was available at all times. Animals were given 1 wk to acclimate to the laboratory, and then they were bilaterally ovariectomized under pentobarbital sodium anesthesia (80 mg/kg ip). Two weeks of recovery were allowed before the start of each experiment. All procedures were conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and were approved by the University of Massachusetts Institutional Animal Care and Use Committee.

AP Lesions

AP lesions were performed as described previously (13). Hamsters were anesthetized with pentobarbital sodium (80 mg/kg ip) and positioned in a stereotaxic instrument. An incision was made in the dorsal neck region to expose the muscles underneath. With use of blunt dissection, the skull was exposed, and the occipitoatlantal ligament, the dura mater, and arachnoid membranes were incised to reveal the occipital foramen magnum. The overlying meninges were removed to expose the fourth ventricle, and the AP was aspirated using a 5-µl Wiretrol (Drummond Scientific Company, Broomall, PA). Surgical wounds were closed with silk. Sham lesions were performed by exposing the dorsal hindbrain.

Histology

AP-lesioned animals were given a lethal dose of pentobarbital sodium (120 mg/kg ip) and were perfused with saline for 30 s followed by 2% acrolein for 10 min at a pressure of 100 mmHg and a flow rate of 25 ml/min. The medulla was removed and stored overnight in 0.1 M sodium phosphate buffer containing 20% sucrose. Forty-micrometer coronal sections were cut and stained with thionine. Sections were examined to confirm the presence of the lesion. Animals with incomplete lesions of the AP were omitted from the data analyses, as were animals with significant damage to adjacent neural structures. All the animals included in the study had either no or minimal damage to the underlying nucleus of the solitary tract.

Behavior Testing

Two weeks after ovariectomy (experiment 1) or AP lesions (experiment 3), hamsters were injected subcutaneously with 2 µg of estradiol benzoate (EB) followed 44 h later by 500 µg of P. They were tested for lordosis 4 h after the injection of P in the presence of an experienced male hamster in a Plexiglas arena. Lordosis was quantified by recording the number of seconds spent by the female in the lordosis posture during a 3-min test. To provide a consistent level of flank stimulation to elicit lordosis, a soft 1-cm artist’s paint brush was used to stroke the female’s flank region during the test period (25).

Immunocytochemistry

Hamsters used for ERIR mapping were killed with an overdose of pentobarbital sodium and were perfused with 2% paraformaldehyde and 0.4% glutaraldehyde. Brains were then processed for estrogen receptor immunocytochemistry as described previously (13). In brief, 40-µm coronal brain sections were incubated with the H-222 monoclonal primary antibody (Abbott Laboratories, North Chicago, IL) at a concentration of 1 µg/ml for 72 h. They were then incubated with the secondary antiserum, biotinylated rabbit anti-rat immunoglobulin, at a concentration of 6 µg/ml for 90 min. The sections were then incubated for another 90 min in avidin-DH-biotinylated horseradish peroxidase-H complex and reacted with diaminobenzidine in the presence of hydrogen peroxide to give a dark-brown-colored end product, indicating the presence of estrogen receptors. Two sections, separated by 120 µm, from each brain area (Fig. 1) were chosen from each animal after careful matching of their landmarks under dark-field illumination. The experimenter was always blind to the treatment groups of the sections being analyzed. ERIR cells were counted with a computer-aided image analysis system using the public domain NIH Image program developed at NIH and available at http://rsb.info.nih.gov/nih-image/. At the beginning of the analysis of the first sample, the camera gain and black level were adjusted to give a normal distribution of gray levels ranging from 0 to 254, falling within the parameters of the imaging system. This light level was maintained throughout the analysis in order to obtain consistent measurements. The mean pixel density and standard deviation for each section was determined, and the criterion for a labeled cell nucleus to be counted was set at two standard deviations above the mean pixel density of the region to be analyzed.
Analysis of Data

Data from each experiment were analyzed by t-tests or one-way analyses of variance. Significant treatment effects were further analyzed by Newman-Keuls post hoc tests. All tests were based on two-tailed tests of significance. Results were considered significant if \( P < 0.05 \).

Procedures

Experiment 1: Effect of insulin treatment on steroid-induced estrous behavior. Animals were divided into two groups matched for mean body weight and baseline food intake. One group (\( n = 6 \)) was given subcutaneous injections of either 5 U/100 g body wt of NPH recombinant human insulin (Eli Lilly, Indianapolis, IN) every 12 h for 72 h; the other group (\( n = 6 \)) was given an equal volume (0.05 ml/100 g body wt) of physiological saline. During the entire injection period, each animal was limited to \( \sim 110\% \) of its ad libitum food intake, as measured during the preinjection baseline period. Animals were fed one time every 12 h, at the time of the insulin injections. Hamsters were injected with EB immediately after the second injection of insulin or saline. After the completion of injections, all animals were tested for estrous behavior.

Experiment 2: Effect of insulin treatment on neural ERIR. As in experiment 1, ovariectomized hamsters matched for mean body weight and baseline food intake were given subcutaneous injections of 5 U/100 g body wt insulin (\( n = 7 \)) or physiological saline (\( n = 7 \)) every 12 h for 72 h. Food intake was limited to \( \sim 110\% \) of baseline levels during injections as in experiment 1. At the end of 72 h, animals were perfused for estrogen receptor immunocytochemistry.

Experiment 3: Effects of AP lesions on insulin-induced suppression of estrous behavior. Two weeks after ovariectomy, animals were given aspiration lesions of the AP (\( n = 16 \)) or were sham lesioned (\( n = 14 \)). Two weeks later, one-half of the animals in each group were treated with insulin or with physiological saline, and EB + P-induced estrous behavior was measured as in experiment 1. As in experiments 1 and 2, animals were prevented from overeating during insulin treatment by limiting them to \( \sim 110\% \) of their baseline intake.

RESULTS

Experiment 1: Effect of Insulin Treatment on Steroid-Induced Estrous Behavior

Insulin-treated animals showed a significant decrease in lordosis duration compared with saline-injected animals (\( P < 0.001 \)) (Fig. 2). The insulin-injected animals also showed a longer latency to the first lordosis compared with the saline-treated controls (data not shown). As we have observed previously (26), the insulin-treated hamsters exhibited no signs of ill health. At the time of behavioral testing, they were fully conscious and active. Unreceptive animals were clearly capable of rebuffing the males’ advances.

Experiment 2: Effect of Insulin Treatment on ERIR in the VMH and mPOA

Insulin treatment significantly decreased (\( P < 0.05 \)) the number of detectable ERIR cells in the VMH compared with the saline-injected animals (Fig. 3). In contrast, the number of detectable ERIR cells in the mPOA was significantly increased after insulin treatment (\( P < 0.05 \)) (Fig. 3).

Experiment 3: Effects of AP Lesions on Insulin-Induced Suppression of Estrous Behavior

Thirteen out of sixteen animals sustained complete destruction of the AP. The final group sizes were: sham lesioned, saline injected (\( n = 7 \)); AP lesioned, saline injected (\( n = 7 \)); sham lesioned, insulin injected (\( n = 7 \)); and AP lesioned, insulin injected (\( n = 6 \)). The sham-lesioned, insulin-injected animals showed the expected inhibition of estrous behavior (\( P < 0.05 \)), but AP lesions prevented this effect (Fig. 4). Insulin treatment significantly increased body weight gain in both AP-lesioned and sham-lesioned animals (\( P < 0.01 \)) (Fig. 4).
Insulin treatment resulted in site-specific changes in the number of detectable ERIR cells in the VMH and mPOA, just as does food deprivation and treatment with metabolic inhibitors (13), indicating that neural estrogen receptors respond similarly regardless of the method by which fuel availability is restricted. The fact that induction of these changes in neural estrogen receptors requires both glucoprivation and lipoprivation (13) again reinforces the position that insulin treatment reduces the availability of glucose and fatty acids for oxidation, in contrast to the common assumption that insulin effects on reproduction are primarily due to changes in glucose metabolism.

The insulin-induced decreases in VMH ERIR probably contribute to the decreases in sexual receptivity; the association between VMH ERIR and behavioral responsiveness to ovarian steroids has now been demonstrated consistently using a variety of metabolic and neurological manipulations (13; Panicker et al., unpublished observations). On the other hand, the increases in the number of detectable mPOA ERIR cells is probably unrelated to the deficits in estrous behavior, because lesions of the AP or total subdiaphragmatic vagotomy can readily dissociate these two responses (13). The role, if any, of the increases in mPOA ERIR in nutritional infertility is unclear at this time. It has been suggested that the increased mPOA ERIR may contribute to the paradoxical increase in positive feedback sensitivity of LH secretion to estradiol in food-deprived animals (15). Whether a similar phenomenon is seen in insulin-treated animals is not known.

The AP has been implicated in conveying information regarding metabolic fuel status to forebrain circuits controlling estrous behavior (13, 27; Panicker et al., unpublished observations), just as it does for circuits controlling eating behavior (21). The results of experiment 3 show that an intact AP is required for the inhibition of estrous behavior by insulin treatment, just as it is required in hamsters that have been food deprived (Panicker et al., unpublished observations), treated with metabolic inhibitors (13), or subjected to cold (5°C) temperatures (Early et al., unpublished observations). Again, this would seem to reveal a commonality of mechanisms mediating the effects of metabolic fuel availability on estrous behavior, regardless of the source of the energetic challenge.

Finally, the present findings give some credence to our assertion that at least some types of obesity-associated infertility could be due to deficits in metabolic fuel availability (26, 27). A corollary of this position is that in order to support reproduction, metabolic fuels must be readily available for oxidation. The mere presence of large energy stores in the body is insufficient to maintain fertility unless these calories can be mobilized and made available for oxidation by other tissues. An analogous situation may be seen in untreated or poorly controlled diabetes mellitus. Despite the existence of greatly elevated levels of circulating glucose, diabetics are often infertile, because glucose energy is not readily available for oxidation in most tissues (7, 8, 12, 24).
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