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Hypoglycemia Does Not Affect Gonadotroph Responsiveness to Gonadotropin-Releasing Hormone in Rhesus Monkeys

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Hypoglycemia inhibits gonadotropin secretion in primates by an undefined mechanism. Some evidence suggests that hypoglycemia inhibits gonadotropin secretion independent of gonadotropin-releasing hormone (GnRH) inhibition. To this end, the effect of insulin-induced hypoglycemia on the luteinizing hormone (LH) and follicle-stimulating hormone (FSH) response to graded doses of GnRH (25, 75, and 250 ng/kg) administered at 120-min intervals was determined in rhesus monkeys. A crossover design was employed such that each animal received GnRH under both hypoglycemic and euglycemic conditions. Experiments were performed in the follicular phase. Gonadotroph responsiveness to GnRH was quantified by determining the change in area under the LH (ΔAULHC) and FSH (ΔAUFSHC) curves that occurred in the first 60 min following each GnRH pulse. There was no statistical difference in ΔAULHC between euglycemic and hypoglycemic animals at any GnRH dose (25 ng/kg: $p = 0.19$; 75 ng/kg: $p = 0.41$; 250 ng/kg: $p = 0.46$). Similarly, changes in AUFSHC following GnRH administration were comparable in euglycemic and hypoglycemic animals (25 ng/kg: $p = 0.59$; 75 ng/kg: $p = 0.90$; 250 ng/kg: $p = 0.33$). We conclude that hypoglycemia had no effect on gonadotroph responsiveness to GnRH. These results are consistent with the conclusion that hypoglycemia inhibits gonadotropin secretion by acting primarily at the level of the hypothalamus to reduce GnRH secretion rather than affecting pituitary responsiveness to GnRH.

Key Words: Hypoglycemia; gonadotropin-releasing hormone; luteinizing hormone; follicle-stimulating hormone; rhesus monkeys.

Introduction

It is generally accepted that stimulation of gonadotropin secretion is the integrated response to hypothalamic stimulation in the form of gonadotropin-releasing hormone (GnRH) and gonadotroph responsiveness to GnRH *(1)*. Conversely, inhibition of gonadotropin secretion may result from inhibition of GnRH release, reduced responsiveness to GnRH, stimulation of a gonadotropin-release inhibiting factor (GnRIF), or some combination of these. Several hypothalamic peptides including endogenous opioids, corticotrophin-releasing hormone (CRH), vasopressin, γ-aminobutyric acid, norepinephrine, and neuropeptide Y have been implicated in the inhibition of gonadotropin release *(2)*. Stimuli that acutely activate the hypothalamic-pituitary-adrenal axis such as restraint *(3)*, hypoglycemia *(4)*, alcohol *(5)*, food restriction *(6)*, and endotoxins *(7)* inhibit gonadotropin secretion by hypothalamic mechanisms involving these mediators.

Although most evidence suggests that these neuromodulators inhibit gonadotropin secretion by inhibiting GnRH, there is evidence that gonadotropin secretion may be inhibited by a mechanism independent of GnRH inhibition. Lesions to the median eminence of rats increased gonadotropin secretion *(8)* and enhanced pituitary responsiveness to GnRH *(9)*. Pituitary stalk transection in rats *(10)* and monkeys *(11)* also increased gonadotroph responsiveness to GnRH. Furthermore, a glycoprotein extracted from the rat hypothalamus was shown to inhibit GnRH-induced luteinizing hormone (LH) release, whereas an antibody to the extract potentiated estrogen-induced LH release *(12)*. These data suggest that an inhibitory hypothalamic factor is released into the hypophyseal circulation, where it opposes the action of GnRH at the gonadotroph.

Insulin-induced hypoglycemia has been demonstrated to inhibit gonadotropin secretion in several species including rat *(13)*, sheep *(14)*, and primates *(15,16)*. The mechanism whereby hypoglycemia exerts its negative effect on gonadotropin secretion in the primate remains unresolved. The objective of the present study was to test the hypothesis that hypoglycemia inhibits gonadotropin secretion independent of inhibiting GnRH. If such a mechanism were in operation, we reasoned that gonadotroph responsiveness to GnRH would be reduced in hypoglycemic animals.

Results

Mean LH responses (±SEM) to 25, 75, and 250 ng of GnRH/kg under euglycemic and hypoglycemic conditions

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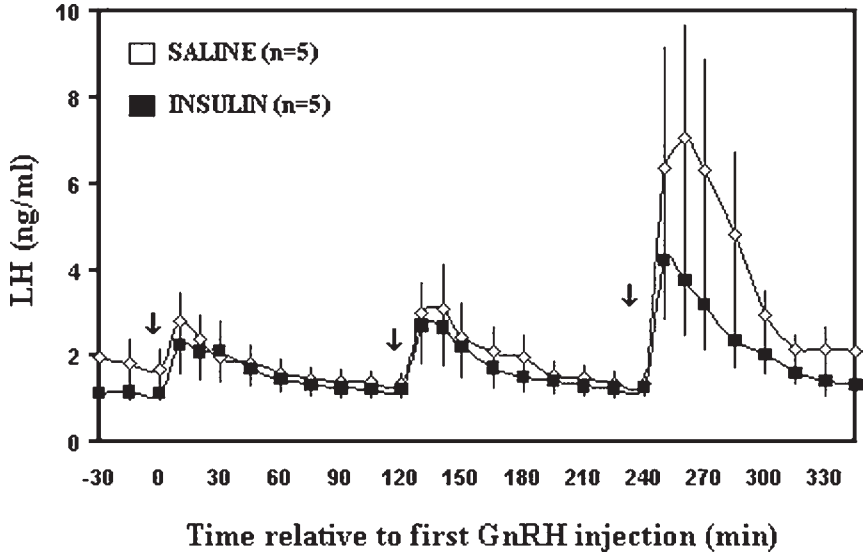

Fig. 1. Effect of hypoglycemia on GnRH-stimulated LH release in rhesus monkeys. Mean LH responses (±SEM) in five rhesus monkeys injected with three graded doses of GnRH (25, 75, and 250 ng/kg) under euglycemic and hypoglycemic conditions are shown. Arrows denote the time of GnRH injections.

are displayed in Fig. 1. A 25 ng/kg GnRH bolus (given at $t = 0$ min) increased LH concentrations from 1.70 ± 0.51 to 2.81 ± 0.68 ng/mL in euglycemic monkeys. A similar LH response to a 25 ng/kg GnRH bolus was seen during hypoglycemia, as circulating LH levels increased from 1.15 ± 0.20 to 2.27 ± 0.70 ng/mL. A higher dose of GnRH (75 ng/kg at $t = 120$ min) stimulated a greater release of LH under both experimental conditions. LH concentrations peaked at 3.11 ± 1.09 ng/mL in euglycemic animals and 2.70 ± 0.90 ng/mL in hypoglycemic monkeys. LH concentrations were greatest following injection of 250 ng/kg of GnRH ($t = 240$ min). Circulating LH concentrations increased to 7.05 ± 2.66 and 4.23 ± 1.43 ng/mL in euglycemic and hypoglycemic monkeys, respectively. Mean glucose levels decreased from 4.1 ± 0.15 to 1.29 ± 0.09 mmol/L following insulin administration. Mean glucose levels throughout the hypoglycemic period were 2.37 ± 0.10 mmol/L.

The corresponding follicle-stimulating hormone (FSH) responses to increasing GnRH doses are depicted in Fig. 2. In euglycemic animals, a 25 ng/kg GnRH pulse increased FSH concentrations (±SEM) from 0.75 ± 0.19 to 1.37 ± 0.33 ng/mL. Similar increases were seen at higher doses of GnRH, with levels reaching 1.54 ± 0.49 and 1.71 ± 0.39 ng/mL following injections of 75 and 250 ng/kg of GnRH, respectively. FSH responses to GnRH were similar under hypoglycemic conditions. Peak FSH levels were 1.34 ± 0.48, 1.61 ± 0.22, and 1.84 ± 0.77 ng/mL following administration of increasing GnRH dose (25, 75, and 250 ng/kg, respectively).

Figure 3 illustrates the mean change in area under the LH curve (ΔAULHC ± SEM) at each GnRH dose. In euglycemic monkeys, administration of 25 ng/kg of GnRH increased AULHC by 26.27 ± 13.15 ng·min/mL). A larger increase was detected after injection of 75 ng/kg of GnRH (56.67 ± 27.53 ng·min/mL. ΔAULHC was greatest in response to a 250 ng/kg bolus of GnRH (221.946 ± 116.95 ng·min/mL. Similar responses to 25, 75, and 250 ng/kg of GnRH were detected in hypoglycemic animals. No statistical difference was detected between euglycemic and hypoglycemic animals at any GnRH dose (25 ng/kg: $p = 0.19$; 75 ng/kg: $p = 0.41$; 250 ng/kg: $p = 0.46$).

Mean changes in area under the FSH curve (ΔAUFSHC ± SEM) in response to increasing GnRH doses are depicted in Fig. 4. In euglycemic animals, ΔAUFSHC increased from 23.09 ± 11.36 to 38.05 ± 16.76 and 44.73 ± 17.95 ng·min/mL following administration of 25, 75, and 250 ng/kg of GnRH, respectively. Comparable changes were seen in hypoglycemic animals (32.03 ± 14.60, 36.14 ± 9.28, and 56.68 ± 20.89 ng·min/mL, respectively. When compared to euglycemic controls, hypoglycemia had no effect on ΔAUFSHC at any dose (25 ng/kg: $p = 0.59$; 75 ng/kg: $p = 0.90$; 250 ng/kg: $p = 0.33$).

Discussion

The precise mechanism that mediates hypoglycemia-induced inhibition of gonadotropin secretion is unresolved and appears to vary with species. In rats *(13)* and ewes *(14)*, hypoglycemia-induced inhibition of gonadotropin secretion was reversed by naloxone, indicating involvement of an opioid mechanism. By contrast, endogenous opioid peptides do not mediate hypoglycemia-induced inhibition of LH secretion in monkeys since LH inhibition was not reversed by opiate antagonism *(15,16)*. CRH is considered a possible candidate for mediating hypoglycemia-induced inhibition of gonadotropin secretion, since CRH mRNA in the

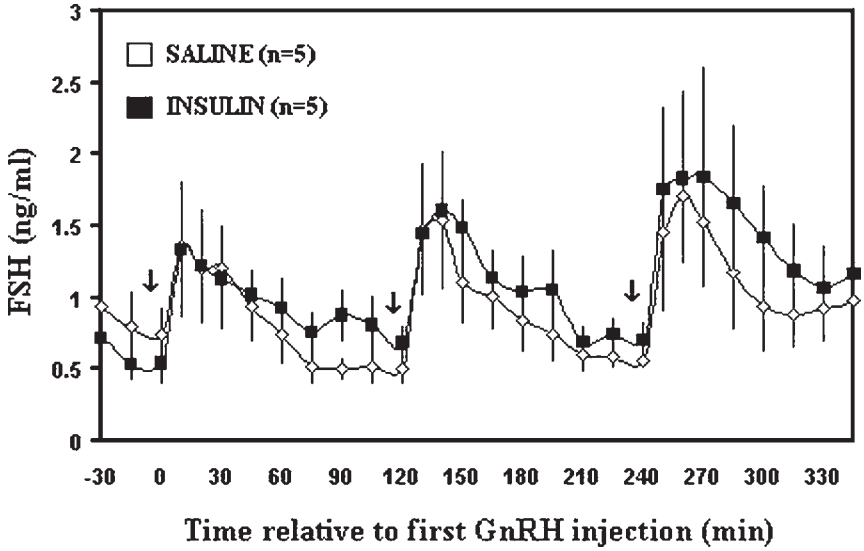

Fig. 2. Effect of hypoglycemia on GnRH-stimulated FSH release in rhesus monkeys. Mean FSH responses (±SEM) in five rhesus monkeys given three graded doses of GnRH (25, 75, and 250 ng/kg) under euglycemic and hypoglycemic conditions are shown. Arrows denote the time of GnRH injections.

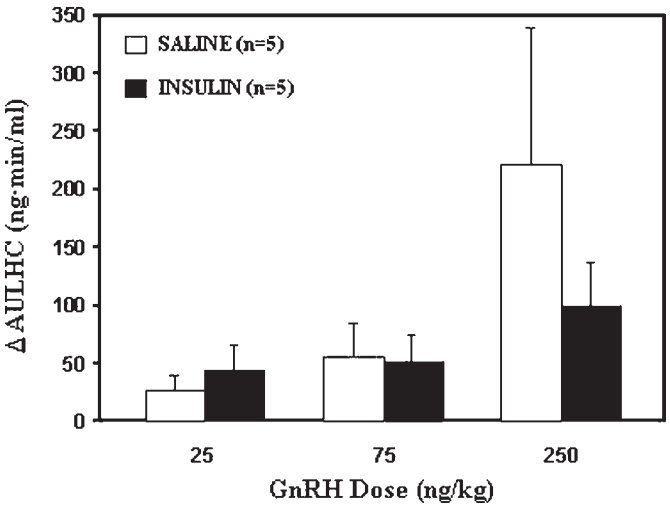

Fig. 3. Effect of hypoglycemia on GnRH-stimulated LH release as quantified by change in ΔAULHC (±SEM). Total LH released in response to graded doses of GnRH was not affected by hypoglycemia (25 ng/kg: $p = 0.19$; 75 ng/kg: $p = 0.41$; 250 ng/kg: $p = 0.46$).

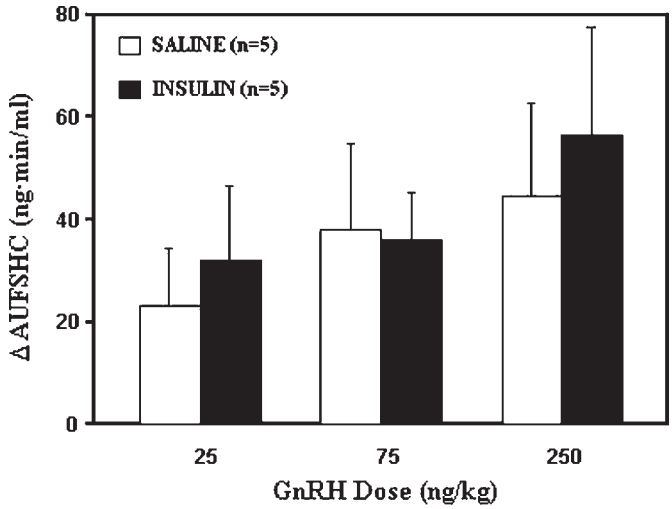

Fig. 4. Effect of hypoglycemia on GnRH-stimulated FSH release as quantified by change in ΔAUFSHC (±SEM). Total FSH released in response to graded doses of GnRH was not affected by hypoglycemia (25 ng/kg: $p = 0.59$; 75 ng/kg: $p = 0.90$; 250 ng/kg: $p = 0.33$).

hypothalamus *(19,20)* and CRH concentrations in portal blood are elevated in hypoglycemic animals *(21–23)*. To date there are two studies in the primate substantiating CRH involvement in hypoglycemia-induced suppression of LH *(17,24)*. A study conducted in our laboratory using alprazolam, a potent benzodiazepine that inhibits CRH release, concluded that impaired CRH secretion blocked hypoglycemia-induced inhibition of LH secretion in rhesus monkeys *(17)*. Furthermore, Chen et al. *(24)* reported that intraventricular infusion of a CRH antagonist delayed hypoglycemia-induced inhibition of hypothalamic multiunit activity in rhesus monkeys. Nevertheless, it is difficult to reconcile why CRH antagonism, but not opioid antagonism, reversed hypoglycemia-induced inhibition of gonadotropin secretion *(15)*, since several studies in primates have concluded that the inhibitory effects of CRH on gonadotropin secretion are mediated by endogenous opioid peptides *(25–27)*.

Vasopressin also has been implicated in hypoglycemia-induced inhibition of LH secretion. Vasopressin concentrations in portal blood are increased in hypoglycemic animals *(22,28)*, and vasopressin antagonism reversed hypoglycemia-induced suppression of LH secretion in rhesus monkeys

(4,29). Moreover, there is evidence indicating that hypoglycemia preferentially stimulates vasopressin secretion over CRH into the hypophyseal portal circulation *(22,23)*. Interestingly, vasopressin may inhibit gonadotropin secretion in primates independent of an effect on GnRH since intraventricular administration of vasopressin to rhesus monkeys suppressed pulsatile gonadotropin secretion without reducing pulse generator frequency *(24)*. A direct effect of vasopressin on the gonadotroph seems unlikely given that in vitro studies report either increased or no change in basal gonadotropin release from isolated pituitaries incubated with vasopressin *(30–33)*. However, it is possible that vasopressin induces the release of hypothalamic factors that inhibit the gonadotroph. In 1987, Hwan and Freeman *(12)* isolated GnRIF from the rat hypothalamus. There is reason to speculate the existence of a GnRIF in primates since pituitary stalk transection in monkeys caused an initial rise in gonadotropin secretion and increased pituitary responsiveness to GnRH *(11)*.

We tested the hypothesis that hypoglycemia inhibits gonadotropin secretion independent of inhibiting GnRH release by measuring gonadotrope responsiveness to GnRH in hypoglycemic animals. Our results do not support the conclusion that gonadotroph responsiveness to GnRH is reduced during hypoglycemia. Hence, if hypoglycemia activates neuromodulators that inhibit gonadotropin secretion by a direct effect on the gonadotroph, it does so without interfering with GnRH signaling.

Although the trend for increased LH secretion with increasing GnRH doses in euglycemic monkeys was slightly reduced in some hypoglycemic animals, mean responses at 25, 75, and 250 ng/kg of GnRH were not significantly different between groups. Failure to detect a statistically significant dose response over a 10-fold GnRH dose range reflects the inherent variation in gonadotroph responsiveness to GnRH. This individual variation may have precluded the detection of a significant effect of hypoglycemia on gonadotroph responsiveness. Based on the variability of the current study, a post-hoc power calculation determined that a sample size of 16–20 animals would be required to detect a significant dose response. There was no evidence of compromised FSH secretion in hypoglycemic monkeys at any GnRH dose.

While concomitant insulin administration has been reported to have no effect on GnRH-induced gonadotropin release in humans *(34,35)*, it should be noted that the objectives of these earlier studies differ significantly from those of the current study. Those earlier studies determined whether pituitary reserve could be determined from a challenge that combined administration of insulin and hypophysiotropic-releasing hormones. To that end, pituitary hormone responses to a single dose of thyrotropin-releasing hormone and GnRH were compared under euglycemic and hypoglycemic conditions. An important difference between our study and these previous studies is that we used multiple doses of GnRH. This is an absolute requirement for determining whether hypoglycemia altered gonadotroph responsiveness to GnRH. In addition, we used a regimen of insulin-induced hypoglycemia, which we and others have documented to decrease LH secretion *(15–17,24)*. Since the mechanism of hypoglycemia-induced suppression of LH secretion was not an objective of these earlier studies, the strength of the hypoglycemic challenges may not be comparable.

Although chronic alterations in insulin homeostasis have been correlated to reproductive axis dysfunction in humans *(36)*, a direct effect of insulin has yet to be shown. In fact, hyperinsulinemic-euglycemic clamp studies concluded that hypoglycemia, rather than insulin, is the factor responsible for acutely decreasing gonadotropin secretion in primates *(37)*. These results point to the involvement of glucose-sensitive neurons within the HPG axis. Electrophysiologic studies examining GnRH neurons have confirmed glucose availability as the cause of hypoglycemia-induced suppression of LH secretion in rats *(38)*. Studies analyzing LH pulsatility in both rats *(39)* and sheep *(14)* have shown that glucose administration reversed insulin-induced inhibition of LH secretion. Although insulin has been reported to have direct effects on isolated rat gonadotrophs, it should be noted that insulin exposure spanned 2 d and caused increased sensitivity to GnRH *(40)*.

In conclusion, hypoglycemia did not affect gonadotroph responsiveness to GnRH. Therefore, it is likely that hypoglycemia acts primarily at the level of the central nervous system to disrupt GnRH secretion. While these data do not preclude the involvement of vasopressin or a GnRIF in the mediation of hypoglycemia-induced inhibition of gonadotropin secretion, they do favor the participation of neuropeptides such as CRH, which effectively arrest the GnRH pulse generator and inhibit GnRH neurosecretion.

Materials and Methods

Animal Husbandry

Experiments were conducted in five adult female rhesus monkeys, *Macaca mulatta*. Ages ranged from 5 to 9 yr and weights ranged from 5.4 to 6.5 kg. Animals were grouped or individually housed in a light- and temperature-controlled environment (lights on from 7:00 AM to 7:00 PM; temperature at 22ºC). Their diet consisted of a twice-daily ration of Purina monkey chow (Ralston Purina, St. Louis, MO) supplemented with fruit and vegetables. Water was available ad libitum. Experiments were conducted in the follicular phase, ascertained from menstrual cycle data and progesterone measurements. A progesterone value of <3.5 ng/mL was considered indicative of the follicular phase. This cutoff was based on progesterone measurements over six consecutive cycles in five rhesus monkeys with regular ovulatory cycles (mean follicular-phase progesterone levels = 1.8 ± 0.1 ng/mL with a range of 1.0–3.7 ng/mL; mean luteal-phase progesterone levels = 8.5 ± 0.4 ng/mL with a range

of 4.0–21.9 ng/mL). All animal husbandry practices and experimental procedures conformed to guidelines of the Canadian Council on Animal Care and were approved by the Queen's University Animal Care Committee.

Experimental Protocol

Monkeys were sedated with ketamine HCl (5–10 mg/kg) (Rogarsetic, Montreal, QC, Canada) and placed in primate chairs. Angiocatheters were inserted into a femoral vein for blood collection and saphenous vein for drug administration. Approximately 2 h later, a baseline blood sample was collected followed by injection of insulin (1 U/kg) (Eli Lilly Canada, Scarborough, Ontario, Canada) or an equivalent volume of saline. Escalating doses of GnRH (Factrel®; 25, 75, and 250 ng/kg; Wyeth-Ayerst Canada, St. Laurent, QC, Canada) were administered intravenously 1, 3, and 5 h after saline or insulin treatment. Our laboratory has shown that hypoglycemia effectively inhibits pulsatile gonadotropin secretion in rhesus monkeys within 1 h of insulin injection *(16,17)*. Blood samples were collected at 10-min intervals for the first 30 min following each GnRH pulse and every 15 min thereafter until the next GnRH pulse. Blood glucose was monitored frequently, and levels were maintained below 50% of baseline by administration of supplemental doses of insulin (0.05 U/kg). A crossover design was employed such that each animal was randomly tested under both hypoglycemic and euglycemic conditions. Experiments were conducted 1 wk apart to minimize differences in estrogen priming owing to ovarian cycle–related secretory changes.

Radioimmunoassays

Blood was refrigerated and permitted to clot overnight. Following centrifugation, serum was separated and stored at −20ºC until assayed. LH and FSH were assayed using reagents provided by the National Hormone and Pituitary Program. Unknowns were assayed in triplicate. Standard curves used LH and FSH reference preparations AFP 6936A and AFP 6940A, respectively. Serum samples were incubated with LH (AFP 6936A) or FSH (AFP 782594) antibodies followed by the addition of ^{125}I-radiolabeled LH (AFP 6936A) and FSH (AFP 6940A). A sheep anti–rabbit γ-globulin (Prince, Toronto, Ontario, Canada) was used to precipitate the antigen-antibody complex. Precipitation was facilitated by adding 12.5% Carbowax® (Sigma, St. Louis, MO) prior to centrifugation. Assay sensitivity, defined as the amount of reference preparation required to reduce binding by 2 SDs below the zero standard divided by the sample volume, was 0.6 ng/mL for LH and 0.4 ng/mL for FSH. The intraassay coefficient of variation (CV) was 7.4 and 9.6% for LH and FSH, respectively. The interassay CV determined from high and low serum pools included in each assay was 11.7% for LH. All FSH measurements were determined in a single assay.

Progesterone was assayed in 100-µL replicates using solid-phase radioimmunoassay kits purchased from Diagnostic Products (Los Angeles, CA). Assay sensitivity as quoted by the manufacturer was 0.02 ng/mL. Intra- and interassay CVs were 3.6 and 3.9%, respectively. Glucose levels were measured using a glucometer (Accu-Chek Advantage; Boehringer Mannheim, Laval, QC, Canada) and expressed as millimoles per liter.

Data Analysis

LH and FSH measurements were used to calculate AUC using the trapezoid rule *(18)*. Gonadotropin responsiveness to GnRH was quantified by determining the change in the AUC (ΔAUC) that occurred over the first 60 min following each pulse. ΔAUC was determined for each monkey, at each dose, under both hypoglycemic and euglycemic conditions. Paired *t*-tests were used to determine whether differences in the gonadotropin response to each GnRH dose were significant between euglycemic and hypoglycemic animals.

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