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Ventromedial hypothalamic glucose sensing and glucose homeostasis vary throughout the estrous cycle

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Abstract

Objective—17 β -Estradiol (17 β E) regulates glucose homeostasis in part by centrally mediated mechanisms. In female rodents, the influence of the ovarian cycle on hypoglycemia counterregulation and glucose tolerance is unclear. We found previously that in prepubertal females, 17 β E modulates glucose sensing in nonadapting glucose-inhibited (GI) and adapting GI (AdGI) neurons within the ventrolateral portion of the ventromedial nucleus (VL-VMN). Nonadapting GI neurons persistently decrease their activity as glucose increases while AdGI neurons transiently respond to a glucose increase. To begin to understand if endogenous fluctuations in estrogen levels across the estrous cycle impact hypothalamic glucose sensing and glucose homeostasis, we assessed whether hypoglycemia counterregulation and glucose tolerance differed across the phases of the estrous cycle. We hypothesized that the response to insulin-induced hypoglycemia (IIH) and/or glucose tolerance would vary throughout the estrous cycle according to changes in 17 β E availability. Moreover, that these changes would correlate with estrous-dependent changes in the glucose sensitivity of VL-VMN glucose-sensing neurons (GSNs).

Methods—These hypotheses were tested in female mice by measuring the response to IIH, glucose tolerance and the glucose sensitivity of VL-VMN GSNs during each phase of the estrous cycle. Furthermore, a physiological brain concentration of 17 β E seen during proestrus was acutely applied to brain slices isolated on the day of diestrus and the response to low glucose in VL-VMN GSNs was assayed.

Results—The response to IIH was strongest during diestrus. The response of nonadapting GI and AdGI neurons to a glucose decrease from 2.5 to 0.5mM also peaked during diestrus; an effect which was blunted by the addition of 17 β E. In contrast, the glucose sensitivity of the subpopulation of GSNs which are excited by glucose (GE) was not affected by estrous phase or exogenous 17 β E application.

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Conclusion—These data suggest that physiological fluctuations in circulating 17 β E levels across the estrous cycle lead to changes in hypothalamic glucose sensing and the response to IHH.

Keywords

estrogen; glucose-excited neurons; glucose-inhibited neurons; hypoglycemia counterregulation; glucose tolerance

1. INTRODUCTION

The counterregulatory response to hypoglycemia is a sympathoadrenal and hormonal mechanism that restores euglycemia by decreasing glucose utilization and stimulating hepatic glycogenolysis and gluconeogenesis [1]. Sex differences in hypoglycemia counterregulation, which are well documented in humans and rodents (recently reviewed by Lopez *et al.* [2]), suggest a role for gonadal hormones in glucose homeostasis. In both sexes, 17 β -estradiol (17 β E) acts centrally to regulate energy and glucose homeostasis [3]. However, the impact of fluctuating endogenous ovarian hormones on glucose homeostasis is unclear. This issue is rarely considered in studies involving female subjects, and when it is, conflicting results of the impact of the follicular phase (i.e., high circulating 17 β E) on insulin sensitivity are reported [4,5]. Conflicting data in both humans and rodents has also been generated concerning glucose homeostasis across the female cycle [6,7,8,9,10,11,12,13]. Additionally, we have found only one study conducted in rodents where the authors report impaired glucose tolerance in diestrus and improved glucose tolerance in ovariectomized females following 17 β E replacement [12]. Some variability in the literature may be due to different experimental protocols (i.e., hypoglycemic clamps versus bolus glucose/insulin injections), endpoints (i.e., homeostatic model assessment for insulin resistance [HOMA-IR] versus glucose tolerance tests [GTT]), sample sizes, cycle phase determination or animal strains utilized in these studies. In particular, the gold standard method used to assess hypoglycemia counterregulation, hyperinsulinemic-hypoglycemic clamp, may confound the effects of circulating 17 β E due to the prolonged hypoglycemic and hyperinsulinemic conditions required for this technique [9,14].

In the brain, ventromedial hypothalamic (VMH) estrogen receptors (ER) are particularly important for 17 β E regulation of food intake [15,16], energy expenditure [16,17] and reproduction [18,19]. The VMH, which is comprised of the arcuate (ARC) and ventromedial nuclei (VMN), is also critical for glucose homeostasis. The VMH contains glucose sensing neurons that either increase (glucose-excited or GE), or decrease (glucose-inhibited or GI) their activity as glucose increases [20]. VMH GI neurons can be further subdivided depending on whether their response to low glucose is sustained (nonadapting) or reverses (adapting or AdGI) prior to return to baseline glucose levels [21]. In studies using male rats, local VMH glucopenia [22] or glucose infusion during systemic hypoglycemia [23] initiates or attenuates the peripheral release of counterregulatory hormones, respectively. We have previously shown that male VMN GI neurons play a role in hypoglycemia detection and counterregulation [24,25]. While the basic hormonal counterregulatory response is similar in males and females, the magnitude of the response is blunted in females [26]. Interestingly, we have recently shown that in prepubertal females 17 β E blunts the ability of nonadapting

GI and AdGI neurons within the ventrolateral (VL)-VMN to respond to decreased glucose [21]. This suggests that the blunted response of female nonadapting GI and AdGI neurons to low glucose may contribute to their attenuated counterregulatory response compared to males.

In this study, we hypothesized that hypoglycemia counterregulation and glucose tolerance vary across the estrous cycle and are correlated with changes in the glucose sensitivity of VL-VMN nonadapting GI and AdGI neurons. Thus, we evaluated the response to insulin-induced hypoglycemia (IIH), glucose tolerance, and the response of VL-VMN glucose sensing neurons to low glucose during each phase of the mouse estrous cycle. We found that the recovery from IIH was strongest during diestrus when $17\beta\text{E}$ levels are lowest. Similarly, the response of VL-VMN nonadapting and AdGI neurons to low glucose also peaked during diestrus. Consistent with these findings, exogenous $17\beta\text{E}$ blunted the response of VL-VMN nonadaptive and AdGI neurons to low glucose during diestrus. These data suggest that $17\beta\text{E}$ regulation of hypothalamic glucose sensing may contribute to cyclic changes in the response to IIH.

2. MATERIALS AND METHODS

2.1. Animals and Tissue Preparation

Female C57Bl/6 mice (3–4 weeks and 6–7 weeks old) were maintained on a 12:12 light:dark cycle (7am-lights on; 7pm-lights off) with standard rodent chow and water ad libitum. Animal procedures were approved by the Institutional Animal Care and Use Committee at Rutgers, The State University of New Jersey, RBHS-New Jersey Medical School. Estrous phase was determined in 6–7 week old females by examining vaginal cytology via light microscopy. Vaginal smears were collected within 2 hours following the onset of the lights (7–9am). Brain slices were collected by 11am on each day of the estrous cycle. This allowed for initiation of the $17\beta\text{E}$ surge in the slices collected on proestrus [27]. The following vaginal cytology descriptions were used: Diestrus-circular leukocytes, Proestrus-nucleated epithelial cells, Estrus-cornified squamous epithelial cells, and Metestrus-clustered cornified squamous epithelial cells and polynucleated leukocytes [28]. We established that all mice cycled regularly (3–5 day cycles), and on the day of the experiments estrus phase was re-assessed. All 3–4 week old female mice were only used prior to vaginal opening, a visual marker of pubertal transition [28].

2.2. Electrophysiology

Compared to isolated primary cell or cell-line cultures, patch-clamp analysis in brain slice preparations allows for the study of neuronal behavior in an environment that more closely mimics that seen *in-vivo* [29]. Therefore, following transcardial perfusion with 4°C oxygenated (95% O_2 /5% CO_2) perfusion buffer (composition in mM: 2.5 KCl, 7 MgCl_2 , 1.25 NaH_2PO_4 , 28 NaHCO_3 , 0.5 CaCl_2 , 7 glucose, 1 ascorbate, 3 pyruvate; ~300mOsm, pH 7.4), brains were quickly removed and placed in 4°C oxygenated perfusion buffer. The hypothalamus was dissected and 250–300µm coronal slices were made on a vibratome (Vibroslice, Camden Instruments). VMH containing slices (3 per animal) were maintained in oxygenated artificial cerebrospinal fluid (aCSF, in mM: 126 NaCl, 1.9 KCl, 1.2 KH_2PO_4 , 26

NaHCO₃, 2.5 glucose, 1.3 MgCl₂, 2.4 CaCl₂; ~300mOsm, pH 7.4) for at least one hour prior to recording. Standard whole-cell current clamp recordings were made as previously described [30]. Briefly, borosilicate pipettes (4–7MΩ; Sutter Instruments) were filled with an intracellular solution containing (in mM): 128 K-gluconate, 10 KCl, 4 KOH, 10 HEPES, 4 MgCl₂, 0.5 CaCl₂, 5 EGTA, 2 Na₂ATP, 0.4 Na₂GTP; ~300 mOsm, pH 7.2. Pipette-solution junction potential was nulled prior to formation a GΩ seal, membranes were ruptured with mild suction followed by whole cell capacitance compensation. Cells were deemed viable for recording if action potentials crossed 0 mV and access resistance was less than 30 MΩ following membrane rupturing. Input resistance was calculated from hyperpolarizing current pulses given every three seconds (500ms; –10, –15 or –20pA).

2.3. Slice treatments

Slices were exposed to the various treatment solutions for ten minutes each based on previous studies showing this exposure time was sufficient to determine glucose and hormone responses in VMH neurons [20,31,32,33,34,35]. Moreover, based on years of study and published data, we have concluded that the duration of exposure to the media is not enough to alter the basal physiology and thus in vivo changes in physiological state do indeed translate to measureable changes in glucose sensing within hypothalamic slices which persist during slicing and several hours of subsequent recording [14, 25, 33, 34]. Additionally, by using a media which is free of exogenous hormones we specifically 'leveled the playing field so that regardless of the day of cycle, all cells were exposed to the same media. All recordings were performed between 12pm and 7pm following cycle determination. Glucose sensing was evaluated in response to a glucose decrease from 2.5mM to 0.1mM or 0.5mM. These glucose concentrations represent those typically seen in the brain during peripheral euglycemia (2.5mM) and moderate (0.5mM) or severe (0.1mM) hypoglycemia [36,37]. The tissue concentration of 17βE in the VMH during each phase of the estrous cycle has yet to be determined, therefore, the 17βE concentration utilized in this study was selected based on hippocampal studies [38]. Thus, to determine the effect of 17βE on hypothalamic glucose sensing, glucose was lowered in the presence of 4nM 17βE, the hippocampal 17βE concentration during proestrus [38], in brain slices collected during diestrus.

On average 4 neurons were recorded per slice (for approximately 1.5 hours each), but no slice was exposed to 17βE for >30 minutes. In some cases, DMSO was used as the vehicle for drug treatments, but the final DMSO concentration for all treatments was 0.1%. At this concentration no significant changes in basal neuronal activity are observed [21].

2.4. Glucose Tolerance Test (GTT)

On the morning of of GTT, mice were phased, weighed and food was subsequently removed approximately 2 hours after lights on. Five hours later mice were given an intraperitoneal (IP) injection of sterile D-glucose (1g/kg; Sigma) and blood glucose was measured at t=0, 20, 40, 60, 90, and 120 minutes post injection using a FreeStyle Lite glucose meter [39]. All blood sampling was collected via the tail snip method and less than 10μL of blood was collected at each time point. All testing was completed prior to 3pm; these mice were only

used for GTT. In total, glucose tolerance was evaluated in 8, 6, 9, and 9 mice in diestrous, proestrous, estrous and metestrous, respectively.

2.5. Insulin-Induced Hypoglycemia (IIH)

On the morning of IIH, mice were phased, weighed and food was subsequently removed approximately 2 hours after lights on. Five hours later mice were given a SC injection of sterile Humulin®-R (2U/kg, Lilly Pharmaceuticals) and blood glucose was measured at t= -30, 0, 15, 30, 45, 60, 75, 90, 105 and 120 minutes post injection using a FreeStyle Lite glucose meter. All blood sampling was collected via the tail snip method and less than 10µL of blood was collected at each time point. All testing was completed prior to 3pm; these mice were only used for ITT. Two mice exhibited signs of severe hypoglycemia during the course of this study and were euthanized and excluded from analysis. In total, IIH was evaluated in 8, 7, 8, and 8 animals in diestrous, proestrous, estrous and metestrous, respectively.

2.6. Data Analysis & Statistics

All data are expressed as average $\pm$ standard error. For GTT and IIH, area under the curve from time 0 to 120 min was calculated using baseline values to 90mg/dL and 40mg/dL, respectively. These baseline values were selected because they encompassed the standard deviation of the curves while minimizing background contribution. For electrophysiological recordings, treatment effects were quantified using the percent changes in input resistance (IR) and membrane potential (Vm). IR was calculated from the membrane voltage responses to hyperpolarizing pulses according to Ohm's law (Resistance = Voltage/Current) within the last minute of treatment application. For AdGI neurons, IR in low glucose was calculated for one minute once maximal activation of these neurons occurred (~5–6min after solution change). Percent change was calculated relative to 2.5mM glucose. Data outliers, if any, were identified using studentized residuals and excluded from any supplemental statistical analyses. Data was compared using paired student t-tests, standard one-way ANOVAs with Tukey posthoc tests or repeated two-way ANOVAs with Bonferonni posthoc tests as specified in the figure legends. Bartlett's test for equality of variances was used to ensure validity of ANOVA tests. For t-tests, *p<0.05, +p<0.01. For ANOVAs, columns with different symbols indicate significant differences.

3. RESULTS

3.1. Glucose Tolerance Tends to be Impaired During Proestrus

Fasting blood glucose levels were similar in all phases (diestrous: 105 $\pm$ 4 (n=16), proestrous: 101 $\pm$ 5 (n=13), estrous: 102 $\pm$ 4 (n=17) and metestrous: 108 $\pm$ 3 (n=17) mg/dL). Blood glucose levels peaked at 20 minutes in all groups following D-glucose injection (Figure 1A). Peak blood glucose levels in proestrous were significantly higher than peak levels in all other phases (proestrous: 250 $\pm$ 5 mg/dL; diestrous: 168 $\pm$ 7; estrous: 157 $\pm$ 4; metestrous: 205 $\pm$ 6; P<0.05). However, blood glucose levels returned to baseline by 40 min in all phases. A similar trend (p=0.07) was observed in area under the curve (AUC) for GTT, but no statistical inter-phase differences were observed.

3.2. Recovery from IIH is Fastest During Diestrous

Blood glucose levels fell below 70mg/dL in all groups following insulin injection (Figure 1B). However, in diestrous blood glucose fell to its lowest point at 30 minutes post-insulin injection and returned to baseline within 90 minutes. In all other phases, blood glucose reached its nadir at 60 minutes post-insulin injection and did not return to baseline within the 120 minute monitoring period. AUC was also significantly higher during diestrus compared to proestrous (Figure 1D). This suggests that the counterregulatory response to hypoglycemia varies during the estrous cycle, with the strongest recovery occurring during diestrous.

3.3. Glucose Sensitivity of VL-VMN Nonadapting GI and AdGI, but not GE, Neurons Varies Throughout the Estrous Cycle

Membrane capacitance (Cm), a measure of cell size, resting membrane potential (Vm) and input resistance (IR) were statistically similar in all phases for nonadapting GI, AdGI and GE neurons (Supplemental Figure 1). However, Bartlett's test for unequal variance revealed significant intragroup differences for resting IR in nonadapting GI neurons ($p=0.01$).

Representative current-clamp recordings for nonadapting GI and AdGI neurons are shown in Figure 2A. Nonadapting GI neurons depolarized and increased their action potential frequency and IR in low glucose (Figure 2A, left). AdGI neurons behaved similarly, but their response to low glucose reversed prior to return to 2.5mM glucose (Figure 2A, right). As seen in prepubertal females [21], a subpopulation of AdGI neurons showed a smaller amplitude transient depolarization in response to a subsequent glucose challenge from 0.1mM to 2.5mM. In total, 65% (15 of 23), 33% (4 of 12), 10% (1 of 10) and 10% (1 of 10) of AdGI neurons displayed this secondary glucose response in diestrous, proestrous, estrous and metestrous respectively (Figure 2A, arrow). The magnitude, but not the duration or time to peak of this response was significantly larger in diestrous than in proestrous ($p<0.05$; data not shown). IR changes in nonadapting GI neurons in response to a glucose decrease from 2.5mM to 0.5mM, but not to 0.1mM, fluctuated across the estrous cycle peaking in diestrus and reached a nadir at proestrous (Figure 2B). The IR response of AdGI neurons to both 0.5 and 0.1mM also peaked in diestrus but reached its nadir during estrous (Figure 2C). Nonadapting GI neurons from diestrous females increased their IR to a lesser degree in response to a glucose decrease from 2.5 to 0.1mM compared to nonadapting GI neurons from prepubertal females (Figure 2D). The converse was true for AdGI neurons (Figure 2E). In all cases the degree of depolarization in response to low glucose was similar. Finally, Bartlett's test for unequal variance revealed significant intergroup differences in AdGI neurons for the percent change in IR in response to 0.1mM glucose ($p=0.03$).

VL-VMN GE neurons hyperpolarized and decreased their IR and action potential frequency in response to low glucose to the same degree in all phases of the estrous cycle (Figure 3A–C). Furthermore, there was no significant difference in the response of GE neurons from diestrous and prepubertal females to decreased glucose (Figure 3D). This suggests that this population of glucose sensing neurons is not sensitive to fluctuations of hormones across the estrous cycle.

3.4. 17β E attenuates the response of VL-VMN Nonadapting GI and AdGI, but not GE, neurons to decreased glucose

We next tested whether a physiological brain concentration of 17β E, typically seen during proestrous [38], would modulate the glucose sensitivity of VL-VMN glucose sensing neurons from diestrous animals when applied exogenously to isolated brain slices. The response of nonadapting GI and AdGI neurons to reductions in glucose was significantly blunted in the presence of 17β E (Figure 4A, B). However, 17β E had no effect on the glucose sensitivity of GE neurons in diestrous (Figure 4A, C).

4. DISCUSSION

In this study, we demonstrate that glucose homeostasis and the glucose sensitivity of VL-VMN nonadapting GI and AdGI neurons varies across the estrous cycle. In general, the recovery from IIH *in vivo* and the response of both types of GI neurons to low glucose *in vitro* fluctuated according to cycle day. Furthermore, exogenous 17β E blunted the response of nonadaptive GI and AdGI neurons to low glucose during diestrous. Glucose tolerance also varied across the cycle although the effects of the estrous cycle on glucose tolerance were much milder than on IIH. Neither estrous phase nor 17β E affected the glucose sensitivity of VL-VMN GE neurons suggesting that these neurons are not sensitive to the effects of fluctuating levels of 17β E. Our data suggest that the preovulatory hormone surge, particularly increased plasma 17β E, affects glucose sensing in VL-VMN nonadapting GI and AdGI neurons and the response to IIH in a similar fashion. Paradoxically, glucose tolerance appears to be greatest when the responses of GI neurons to low glucose are highest. Thus, changes in hypothalamic glucose sensing may contribute, in part, to the changes we observed in the response to IIH across the estrous cycle. This suggests that clinically, insulin therapies may need to be titrated to account for fluctuations in insulin sensitivity over the menstrual cycle.

4.1. Reproductive Cycle and Glucose Tolerance

There is overwhelming evidence that 17β E is a key metabolic hormone. However, the impact of cyclic fluctuations in 17β E levels on glucose homeostasis is still unclear. In euglycemic or hypoglycemic clamps studies involving small cohorts of women (n = 8) insulin sensitivity and hypoglycemia counterregulation are similar throughout the menstrual cycle [7,8,9]. But studies using the minimal glucose model show reduced insulin sensitivity in humans following an intravenous GTT during the follicular phase (i.e., when circulating 17β E peak) [5]. Euglycemic or hypoglycemic clamps are the gold-standard for evaluating glucose homeostasis and they are designed to test small affects in glucose homeostasis. However, the prolonged exposure to insulin (or hypoglycemia) during these clamps is nonphysiologic [40].

Similarly, in two different strains of rats, both improved and impaired glucose tolerance following an oral GTT has been observed during the 17β E surge [12,41]. In our study we used an IP GTT, which increases blood glucose more rapidly than oral administration. We show that peak glucose levels are greater during proestrous, when plasma 17β E peaks in mice compared to the rest of the cycle. Moreover, glucose tolerance, as indicated by a

reduced AUC, tends to be greatest during diestrus when $17\beta\text{E}$ levels are low. The slower rise in glucose following an oral GTT may explain why earlier rodent studies did not observe consistent changes during the estrous cycle. Age may also be a factor in the disparate results as adult rats were used by Bailey *et al*, and we used adolescent mice in our study [12]. Our choice to use adolescent mice resulted from difficulty in obtaining viable electrophysiological recordings in older animals due to increased connective tissue which reduces visibility and cell viability [42]. Our findings suggest a trend toward improved glucose tolerance in diestrus.

4.2. Reproductive Cycle and Insulin Induced Hypoglycemia

The response to IIH and the response of VL-VMN nonadapting GI and AdGI neurons to low glucose were all enhanced during diestrus when $17\beta\text{E}$ levels are lowest. Moreover, we found here that a physiological concentration of $17\beta\text{E}$ typically seen by the brain in proestrus reduced the response of VL-VMN nonadapting GI and AdGI neurons to low glucose when isolated during diestrus. Together, these data suggest that when glucose levels fall during periods of low circulating $17\beta\text{E}$ levels, maximal hypothalamic glucose sensing occurs allowing for full initiation of a counterregulatory response. However, we did not measure $17\beta\text{E}$ levels in this study. Thus, we cannot rule out the contribution of other ovarian hormones (e.g., progesterone, androgens). It is also possible that our observed effects result from secondary influences of ovarian hormones on other neuroendocrine or physiological systems.

Our data are consistent with previous reports of attenuated feeding and hyperglycemia responses in female versus male rats following peripheral administration of 2-deoxyglucose, a non-metabolizable glucose analog that initiates counterregulatory processes [43]. That is, in this study, which did not account for estrous status, elevated $17\beta\text{E}$ levels and reduced hypothalamic glucose sensitivity may have contributed to the observed sex differences reported. One caveat to our interpretation is that we did not measure counterregulatory hormones. Thus, changes in insulin sensitivity may have contributed to our findings. While, the higher insulin dose employed here helps to overcome the potential influence of insulin sensitivity, it may still prove to be a factor if influenced by estrogens or the estrous cycle.

Interestingly, the response to low glucose in both nonadapting GI and AdGI neurons fluctuated with the estrous phase and peaked at diestrus. Furthermore, when these two populations of VL-VMN glucose sensing neurons are considered together the additive response to low glucose is blunted at every phase except diestrus. While direct evidence of a role for VMN glucose sensing neurons in hypoglycemia counterregulation is currently unavailable, we have previously shown a parallel relationship between the glucose sensitivity of nonadapting VMN GI neurons and hypoglycemia counterregulation under a number of conditions [14,24,25]. These data imply that even a slight reduction in the response to low glucose in either nonadapting GI or AdGI neurons is sufficient to blunt the response to IIH. At the present time we can only speculate as to the physiological relevance of adaptation in AdGI neurons. However, it is possible that they enhance sensitivity to slow declines in extracellular glucose. On the other hand, the lack of effect of estrous cycle on GE

neurons implies that these glucose sensing neurons, at least those in the vVMN, may not be linked to hypoglycemia detection.

Our finding that the response to IIH varies with the estrous cycle conflicts with data from earlier hyperinsulinemic-hypoglycemic clamp studies performed in healthy non obese women [7,8]. These clamp studies found no differences in hypoglycemia counterregulation in a small cohort of women during the late follicular phase and the mid-luteal phase. Furthermore, a longitudinal study (n>250 healthy non obese subjects) shows that HOMA-IR, a measure of insulin resistance, also did not differ in these phases [44]. Our data suggest that the response to IIH is similar during these two periods (i.e., during and just after the preovulatory surge). Thus, previous studies may have not detected a change in hypoglycemia counterregulation due to the time points chosen for evaluation and/or age differences in the subjects.

Changes in hypoglycemia counterregulation warrant special attention given the current diabetes epidemic. More women than men are projected to be diagnosed with Type 2 Diabetes and may require more insulin-intensive therapies [45]. In order to avoid hypoglycemia associated autonomic failure, which increases the risk of undetected life-threatening hypoglycemic episodes, insulin therapies may need to be titrated to account for ovarian hormone induced changes in hypothalamic glucose sensing.

In summary, this study describes changes in the response to IIH and hypothalamic glucose sensing that coincide with the preovulatory surge in plasma $17\beta\text{E}$. Furthermore, the directional changes in the response to IIH and the glucose sensitivity of VL-VMN nonadapting GI and AdGI neuron were similar. This suggests that physiological elevations in circulating $17\beta\text{E}$ impairs the ability of VL-VMN glucose sensing neurons to respond to glucose deficit and may blunt the response to IIH. These data have implications for cycling women utilizing intensive insulin therapy in Type 1 and 2 Diabetes Mellitus.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

$17\beta\text{E}$	17β -estradiol
AMPK	AMP-activated protein kinase
ARC	arcuate nucleus
Cm	membrane capacitance
ER	estrogen receptor

GE	glucose-excited
GGT	glucose tolerance test
GI	glucose-inhibited
GSNs	glucose-sensing neurons
HOMA-IR	homeostatic model assessment for insulin resistance
IIH	insulin-induce hypoglycemia
IP	intraperitoneal
IR	input resistance
NGS	non-glucose sensing
SC	subcutaneous
VMH	ventromedial hypothalamus
VL-VMN	ventrolateral VMN
V_m	membrane potential
VMN	ventromedial hypothalamic nucleus

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Highlights

- The response to IIH varied throughout the estrous cycle
- Changes in hypothalamic glucose sensing coincided with those seen in the response to IIH
- Natural fluctuations in $17\beta\text{E}$ levels may alter the response to IIH

Figure 1. Glucose tolerance and the response to insulin-induced hypoglycemia vary with estrous phase

(A, B) Blood glucose levels in response to injection of D-glucose (A, 1 g/kg IP) or insulin (B, 2 U/kg, SC) in cycling females. Data sets marked with an asterisk (*) are significantly different according to repeated measures two-way ANOVA followed by Bonferoni post-hoc tests. (C, D) Quantification of AUC for glycemia. Baseline was set at 90mg/dL and 40mg/dL for GTT and IIH curves, respectively. Numbers within columns indicate the n number for each group. Columns which share the same symbols are not significantly different from each other via standard one-way ANOVA followed by Tukey post-hoc tests. AUC: area under curve, IP: intraperitoneal, SC: subcutaneous, n.s.: not significantly different.

Figure 2. Glucose sensing in VL-VMN nonadapting GI and adapting GI neurons varies throughout the estrous cycle

Glucose sensing was evaluated in hypothalamic slices taken at each phase of the estrous cycle. (A) Representative whole cell current-clamp recordings of a nonadapting GI (left) and adapting GI (AdGI, right) neuron. Glucose changes are schematically displayed above each recording; dashed grey line represents resting Vm. (B, C) Quantification of % Vm and % IR in response to decreasing glucose from 2.5 mM to 0.1mM (2.5G→0.1G) or 0.5mM (2.5G→0.5G) in nonadapting GI (B) and AdGI neurons (C). For nonadapting GI, n=7, 6, 5, 8 at 0.1mM glucose and n=5, 5, 5, 7 at 0.5mM glucose. For AdGI, n=23, 12, 10, 10 at 0.1mM and n=6, 7, 9, 7 at 0.5mM respectively. n# indicated in this figure legend is for diestrous, proestrous, estrous and metestrous, respectively. (D, E) Quantification of % Vm and % IR in response to decreasing glucose from 2.5 mM to 0.1mM (2.5G→0.1G) or 0.5 mM (2.5G→0.5G) in nonadapting GI (D; prepubertal n=18 and diestrous n=8) and AdGI neurons (E; prepubertal n=37 and diestrous n=23) from prepubertal and diestrous females. *p<0.05, **p<0.01 via unpaired student t-tests. Columns which share the same symbols are not significantly different from each other via standard one-way ANOVA and Tukey posthoc tests. G: mM glucose, IR: input resistance, n.s.: not statistically different, Vm: membrane

potential. ¹For AdGI neurons only, Bartlett's test for unequal variances determined the variances between phases was significantly different ($p=0.03$) for % IR in response to 0.1mM glucose.

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Figure 3. Glucose sensing in VL-VMN GE neurons does not vary throughout the estrous cycle

Glucose sensing was evaluated in hypothalamic slices taken at each phase of the estrous cycle. **(A)** Representative whole cell current-clamp recording of a GE neuron. Glucose changes are schematically displayed above each recording; dashed grey line represents resting Vm. **(B)** Representative voltage responses to a hyperpolarizing pulse in response to 0.1mM glucose at each phase of the estrous cycle. Vm was normalized to 2.5mM glucose to emphasize changes in IR **(C)** Quantification of % Vm and % IR in response to decreasing glucose from 2.5 mM to 0.1mM (2.5G $\rightarrow$ 0.1G) (left; n=21, 9, 10, 7) or 0.5mM (2.5G $\rightarrow$ 0.5G) (right; n=6, 4, 5, 5). n# indicated in this figure legend is for diestrous, proestrous, estrous and metestrous, respectively. **(D)** Quantification of % Vm and % IR in response to decreasing glucose from 2.5 mM to 0.1mM (2.5G $\rightarrow$ 0.1G) in GE neurons from prepubertal (n=17) and diestrous (n=7) females. n.s: not statistically different via standard one-way ANOVA and Tukey posthoc test. G: mM glucose, IR: input resistance, Vm: membrane potential.

Figure 4. 17βE blunts the response of VL-VMN nonadapting GI and AdGI neurons to low glucose

In this experiment, 17βE was added to hypothalamic slices taken during diestrus only. (A) Representative voltage responses to a hyperpolarizing pulse in response to 0.1mM glucose in the presence and absence of 17βE (4nM, diestrus only). Vm was normalized to 2.5mM glucose to emphasize changes in IR. (B, C) Quantification of % Vm and % IR in response to decreasing glucose from 2.5 mM to 0.1mM (2.5G→0.1G) in nonadaptive GI (B left; n=4), AdGI (B right; n=6) and GE (C; n=6) neurons during diestrus in the presence and absence of 17βE (4nM). *p<0.05, +p<0.01 via paired students t-test. G: mM glucose, IR: input resistance, Vm: membrane potential.