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Sex-Dimorphic Aromatase Regulation of Ventromedial Hypothalamic Nucleus Glycogen Content in Euglycemic and Insulin-Induced Hypoglycemic Rats

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Abstract

Estrogen receptors control hypothalamic astrocyte glycogen accumulation *in vitro*. Glycogen metabolism impacts metabolic transmitter signaling in the ventromedial hypothalamic nucleus (VMN), a key glucoregulatory structure. Aromatase, the enzyme that converts testosterone to estradiol, is expressed at high levels in the VMN. Here, the aromatase inhibitor letrozole (LZ) was used alongside high-resolution microdissection/UPHLC-electrospray ionization-mass spectrometric methods to determine if neuroestradiol imposes sex-specific control of VMN glycogen content during glucostasis and/or glucoprivation. Testes-intact male and estradiol-replaced ovariectomized female rats were pretreated by lateral ventricular letrozole (Lz) infusion prior to subcutaneous insulin (INS) injection. Vehicle-treated female controls exhibited higher VMN glycogen content compared to males. Lz increased VMN glycogen levels in males, not females. INS-induced hypoglycemia (IIH) elevated (males) or diminished (females) rostral VMN glycogen accumulation. Induction of IIH in Lz-pretreated animals reduced male VMN glycogen mass, but augmented content in females. Data provide novel evidence for regional variation, in both sexes, in glycogen reactivity to IIH. Results highlight sex-dimorphic neuroestradiol regulation of VMN glycogen mass during glucostasis, e.g. inhibitory in males versus insignificant in females. Locally-generated estradiol is evidently involved in hypoglycemic enhancement of male VMN glycogen, but conversely limits glycogen content in hypoglycemic females. Further research is needed to characterize mechanisms that underlie the directional shift in aromatase regulation of VMN glycogen in eu- versus hypoglycemic male rats and gain in negative impact in hypoglycemic females.

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Keywords

letrozole; aromatase; insulin-induced hypoglycemia; ventromedial hypothalamic nucleus; glycogen; sex differences

Introduction:

The ventromedial hypothalamic nucleus (VMN) maintains glucostasis through integration of multiple regulatory cues, including nutrient profiles and indicators of local (glycogen) and peripheral (adipose tissue) energy stores. Astrocytes store the complex carbohydrate glycogen as a vital metabolic fuel reserve [Stobart and Anderson, 2013]. Augmentation of brain glycogenolysis during energy imbalance, e.g. seizure, sleep deprivation, and hypoglycemia [Brown, 2004] liberates glucose for conversion to the oxidizable metabolic fuel L-lactate, which supports neuronal oxidative respiration [Falkowska et al., 2015]. Inhibition of VMN glycogenolysis alters glucoregulatory transmitter marker protein expression [Alhamami et al., 2018; Alshamrani and Briski, personal communication], inferring that decreased VMN glycogen mass or turnover may signal energy shortage to those neurons.

Estradiol exerts sex-dimorphic control over VMN metabolic transmission via mechanisms that include differential regulation of astrocyte glycogen accumulation [Mahmood et al., 2018; Ibrahim et al., 2020]. The enzyme aromatase catalyzes conversion of the androgenic hormone testosterone to estradiol. High VMN aromatase expression [Roselli and Klosterman, 1998] raises the prospect that locally-produced estradiol may regulate the glycogen reserve. Current research investigated the premise that aromatase-generated neuroestradiol controls VMN glycogen amassment during glucostasis and/or glucoprivic disassembly in a sex-specific manner. Here, rats of each sex were pretreated by continuous intracerebroventricular (*icv*) Alzet pump delivery of the aromatase inhibitor letrozole (Lz) prior to subcutaneous (*sc*) vehicle or neutral protamine Hagedorn insulin (INS) injection. Rostral and caudal VMN tissue was obtained separately by micropunch dissection for glycogen content analysis by UHPLC-electrospray ionization-mass spectrometric (LC-ESI-MS) methodology [Bheemanapally et al., 2019].

Materials and Methods:

Animals:

Adult male and female Sprague Dawley rats (3–4 months of age) were housed in groups of 2–3 according to sex, under a 14 h light/10 h dark light cycle (lights on at 05.00 h). Animals had *ad-libitum* access to standard laboratory chow and water, and were acclimated to daily handling. All surgical and experimental protocols were performed in compliance with NIH guidelines for care and use of laboratory animals, under approval by the ULM Institutional Animal Care and Use Committee.

Experimental Design:

Animals of each sex were divided into four treatment groups (n=12 males and n=12 females per group). On day 1, female rats were bilaterally ovariectomized (OVX) and implanted *sc* with a silastic capsule (i.d. 0.062 inch, o.d. 0.125 inch; 10 mm/100 g *bw*) filled with 30 µg 17β estradiol-3-benzoate/mL safflower oil to reproduce circulating steroid levels measured in ovary-intact metestrus female rats [Briski et al., 2001]. On day 5, male and female animals were implanted with a 28-gauge stainless-steel cannula (Alzet Brain Infusion Kit 1, DURECT™ Corporation, Cupertino, CA) into the left lateral ventricle (LV) [coordinates: 0.0 mm posterior to *bregma*; 1.5 mm lateral to *bregma*; 3.5 mm ventral to brain surface] [Ibrahim et al., 2020], connected to a primed Model 1007D Alzet osmotic minipump (0.5 uL/hour) containing vehicle [V; 30% artificial cerebrospinal fluid (aCSF)/70% dimethyl sulfoxide (DMSO); groups V/V-M; V/INS-M; V/V-F/ V/INS-F; n=6/group] or Lz (1.67 µg/µL [Nelson et al., 2016]; prod. no. L0248; Tokyo Chemical Industries, Tokyo, Japan; groups Lz/V-M; Lz/INS-M; Lz/V-F; Lz/INS-F; n=6/group). Table 1 summarizes experimental treatment group identifier abbreviations. Post-surgery, animals were injected with ketoprofen (1 mg/kg *bw sc*) and enrofloxacin (10 mg/0.1 mL IM), and transferred to individual cages. At 9.00 hr on day 11, animals were injected *sc* with sterile diluent (V; Eli Lilly & Co., Indianapolis, IN) or INS (10.0 U/kg *bw*; Henry Schein [Napit et al., 2019]). Later, at 10.00 hr, animals were anesthetized with isoflurane for blood collection by cardiac puncture, then sacrificed by microwave fixation (1.45 sec; In Vivo Microwave Fixation System, 5kW; Stoelting Co., Wood Dale, IL). Each brain was snap-frozen in a liquid nitrogen-cooled isopentane and stored at -80 °C. Plasma was stored at -20 °C.

LC-ESI-MS VMN Tissue Glycogen Analysis:

From each brain, consecutive 100 µm-thick frozen sections were cut through the VMN at two distinct rostro-caudal levels: 1) between -2.30 and -2.50 mm and 2) between -2.80 and -3.00 mm posterior to *bregma*. The VMN was identified using coronal rat brain section topographic features and neuroanatomical landmarks illustrated in rat brain atlases. VMN tissue was bilaterally removed from each tissue section using a calibrated 0.5 mm hollow punch tool (prod. no. 57401; Stoelting Co., Kiel, WI). Figure 1 depicts approximate positioning of the micropunch tool (prod. no. 57401; Stoelting Co., Wood Dale, IL) over the VMN. Accuracy of use of micropunch methodology for collection of distinctive hypothalamic loci of interest, including the VMN, as indicated by marker protein expression, has been verified [Mandal et al., 2017, 2018]. VMN punch samples were combined within each level to create two separate tissue pools per animal. Pooled tissue samples were heat-denatured, ultra-sonicated, stored at -80 °C, and analyzed for glycogen concentrations, as reported [Bheemanapally, et al., 2020]. Supernatant aliquots were hydrolyzed by incubation with amyloglucosidase and 0.1 M sodium acetate, pH 6.0. Hydrolyzed and non-hydrolyzed samples were derivatized with 1-phenyl-3-methyl-5-pyrazolone (PMP) reagent and NaOH, acidified, extracted with chloroform, vacuum-concentrated, and lyophilized. Lyophilized samples were diluted with 10 mM ammonium acetate, vortexed (30s), and centrifuged. Supernatant aliquots were transferred to 350 µL inserts, which were placed into 2 mL Surestop vials in an autosampler tray. D-(+)-Glucose-PMP derivative was resolved through a chromatographic column (Shodex™ Asahipak™ NH2P-40 3E) with mobile phase (75:25 v/v) acetonitrile:10 mM ammonium acetate (0.2

mL/min) in a ThermoFisherScientific Vanquish UHPLC+ System equipped with Thermo Scientific™ Dionex™ Chromeleon™ 7 Chromatography Data System software and coupled to an ISO ISQ EC mass spectrometer (ThermoFisherScientific). Analysis of D-(+)-Glucose-PMP was performed in negative ionization mode. D-(+)-Glucose-PMP ion chromatograms were extracted from Total Ion Current (TIC) at m/z 510.2 to generate area-under-the-curve data.

Plasma Glucose Analyses:

Circulating glucose levels was measured with an ACCU-CHECK Aviva Plus glucometer (Roche Diagnostics USA, Indianapolis, IN) [Kale et al., 2006].

Statistical Analyses:

Mean VMN tissue glycogen and plasma glucose measures were evaluated by three-way analysis of variance and Student-Newman-Keuls *post-hoc* test. Differences of $p < 0.05$ were considered significant. In each figure, statistical differences between specific pairs of treatment groups are denoted as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Results:

Figure 2A depicts effects of intra-LV infusion of the aromatase enzyme inhibitor Lz on LC-ESI-MS measures of glycogen content in male versus female rat rostral VMN [$F_{(7,40)} = 22.45$; $p < 0.0001$]. Data show that baseline glycogen levels were higher in females compared to males [V/V-F (solid gray bar) versus V/V-M (solid white bar)] (Table 2). IINS injection significantly increased tissue glycogen in male [V/INS-M (diagonal-striped white bar) versus V/V-M], but diminished content in females [V/INS-F (diagonal-striped gray bar) versus V/V-F].

Data in Figure 2A also show that *icv* aromatase infusion increased rostral VMN glycogen levels in euglycemic male [Lz/V-M (cross-hatched white bar) versus V/V-M], but not female rats. INS dosing of Lz-pretreated animals significantly reduced tissue glycogen in males [Lz/INS-V (stippled white bar) versus Lz/V-M], but, in contrast, conspicuously increased rostral VMN glycogen levels in females [Lz/INS-F (stippled gray bar) versus Lz/V-F (cross-hatched gray bar)].

Effects of INS dosing of *icv* Lz- versus V-pretreated male and female rats on caudal VMN glycogen amassment are presented in Figure 2B [$F_{(7,40)} = 19.67$; $p < 0.0001$]. In this segment of the VMN, females exhibited greater basal glycogen content versus male rats [V/V-F versus V/V-M]. INS injection did not alter caudal VMN tissue glycogen levels in either sex. Aromatase stimulated caudal VMN glycogen buildup in male [Lz/V-M versus V/V-M], but not female rats. INS dosing of Lz-infused rats did not alter local glycogen accumulation in males, but significantly elevated caudal VMN glycogen in females [Lz/INS-F versus Lz/V-F].

Data in Figure 3 depict significant reductions in plasma glucose concentrations in each sex after INS administration [V/INS-M versus V/V-M; V/INS-F versus V/V-F] [$F_{(7/40)} = 68.61$;

$p < 0.0001$]. Lz pretreatment did not prevent INS-induced reductions in glucose in male or female rats.

Discussion:

Estrogen receptors regulate astrocyte glycogen accumulation in each sex [Ibrahim et al., 2020]. While aromatase is highly expressed in the VMN, involvement of locally-generated estradiol in glycogen buildup and stimulus-induced mobilization remains unclear. Here, pharmacological, high-neuroanatomical resolution microdissection, and LC-ESI-MS analytical techniques were used to determine if neuroestradiol exerts sex-contingent control of VMN glycogen content during glucostasis and/or glucoprivation. Results document sex differences in VMN glycogen accumulation during euglycemia and hypoglycemic regulation of glycogen mass. Notably, in both sexes, glycogen reactivity to IHH was region-specific. Lz infusion increased baseline glycogen levels in sampled regions of the male, but not female VMN, inferring that neuroestradiol limits buildup of this energy depot in the former sex. Lz pretreatment prevented IHH augmentation of glycogen mass in males, but stimulated accumulation in hypoglycemic females. Current outcomes infer that brain-derived estradiol exerts energy state-dependent control of glycogen accumulation in each sex. Further research seeks to identify mechanisms that underlie the directional, e.g. negative-to-positive shift in aromatase regulation of VMN glycogen in eu- versus hypoglycemic male rats and gain of inhibitory aromatase influence on glycogen buildup in hypoglycemic females.

Results confirm earlier reports of sex discrepancy in baseline VMN glycogen concentrations, e.g. levels associated with females exceed those in the other sex [Bheemanapally et al., 2019]. Surprisingly, sex-contingent effects of hypoglycemia on glycogen mass, e.g. augmentation in males versus depletion in females were observed in the rostral VMN, but not more caudally. A reasonable interpretation of these outcomes is that mobilization of glucosyl units from local glycogen is correspondingly reduced or intensified, respectively. Functional ramifications of segment-specific glycogen disassembly in hypoglycemic females remain unclear, but may reflect, to some extent, disparate regional cell needs for alternative metabolic substrate fuels aside from blood-derived glucose that emerge over the current interval between insulin injection and VMN tissue sampling. Outcomes here do not clarify if or to what degree reductions in glycogen mass may occur in the caudal VMN in hypoglycemic male and female rats at time points before or after the +1 hour time point examined here. It is noted that a lack of discernible treatment effect on net tissue glycogen content may obscure parallel, i.e. matched alterations in rates of glucose incorporation into versus liberation from glycogen that accelerate or diminish quantity of glucose derived from glycogen relative to euglycemic state. Observations of hypoglycemic up-regulation of rostral (statistically significant) and caudal (non-significant trend) glycogen in male rats is consistent with emerging evidence that glucogenic amino acids may be metabolized to generate energy during acute hypoglycemia in at sex [Briski et al., 2020]. Ongoing efforts aim to extend current results by mapping sex-specific patterns of glycogen reactivity to hypoglycemia over contiguous segments over the entire VMN length.

Data here provide unique evidence for sex-dimorphic neuroestradiol regulation of glucostatic patterns of VMN glycogen amassment. Here, *icv* Lz administration heightened

content in both sampled segments of male, but not female VMN, inferring that neuroestradiol inhibits expansion of this energy reserve in the former sex, but does not impose such control in the latter. As Lz was delivered to the LV, the prospect that demonstrable drug effects on VMN glycogen might reflect, in part, inhibition of aromatase activity both within and external to that structure cannot be overlooked. Yet, high aromatase expression profiles attributed to the VMN support the likelihood that suppression of local enzyme action accounts substantially for observed treatment outcomes. It is unclear if neuroestradiol is the sole regulatory stimulus that may act to limit glycogen increase in the male VMN. While these data prompt speculation on whether sex disparities in tissue neuroestradiol content may account for differential control of baseline glycogen, evidence for comparable basal VMN aromatase protein expression between sexes [Uddin and Briski, personal communication] argues against this notion. The present experimental design did not include assessment of Lz treatment effects on regional VMN tissue aromatase activity or estradiol content as quantitative methods of requisite sensitivity for analysis of these parameters in small-volume tissue samples obtained from region-based VMN microdissection are not yet available. In the brain, aromatase is reportedly expressed mainly or exclusively in neurons [Wagner and Morrell, 1996; Horvath and Wikler, 1999]. As aforementioned studies did not involve analysis of the VMN, there is justification for additional effort to characterize the VMN cell type(s) that generate neuroestradiol, and to determine how aromatase expression in that(those) cell(s) may be regulated.

Notable outcomes include sex-specific effects of Lz pretreatment on hypoglycemic regulation of VMN, as Lz/INS males exhibited a reduction in rostral VMN glycogen (whereas this mass was increased in V/INS animals), while Lz/INS females showed significant augmentation of glycogen content (while levels were unchanged in V/INS). Data in males support the possibility that neuroestradiol signaling may be required for hypoglycemic patterns of glycogen metabolic enzyme activity that mediate expansion of glycogen mass, namely diminished glycogen phosphorylase and/or increased glycogen synthase activity. In that sex, neuroestradiol facilitation of hypoglycemic amplification of VMN glycogen contrasts with inhibitory effects of this signal on glycogen accumulation during euglycemia, which likely reflects opposite effects of locally-produced estradiol on those enzymes. Mechanisms underlying bi-directional metabolic state-specific neuroestradiol action on the male VMN glycogen reserve will require elucidation. On one hand, it is possible to speculate whether volume of this signal is sensitive to departure from glucose homeostasis and thus imposes variable regulation over glycogen accumulation. Alternatively, astrocyte receptivity to this brain-derived signal may change in response to hypoglycemia-associated regulatory inputs. In females, on the other hand, neuroestradiol control of VMN glycogen changes from none to inhibitory during eu- versus hypoglycemia. There is a parallel need to determine how this regulatory shift occurs in this sex.

In summary, current research provides novel evidence for sex-dimorphic regulation of VMN glycogen content by brain-derived estradiol in the rat (Figure 4). Results support the view that neuroestradiol imposes energy state-dependent control of glycogen accumulation in each sex. Additional studies are needed to identify mechanisms that underlie the directional, e.g. negative-to-positive shift in aromatase regulation of VMN glycogen in eu-

versus hypoglycemic male rats and gain of inhibitory aromatase influence on glycogen buildup in hypoglycemic females.

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Highlights:

- Ventromedial hypothalamic nucleus (VMN) glycogen metabolism affects glucoregulatory function
- Rats of each sex were infused *icv* with letrozole (LZ) or vehicle before insulin-induced hypoglycemia (IIH).
- LZ elevated VMN glycogen levels in euglycemic male, but not female rats.
- Brain-derived estradiol exerts energy state-dependent control of glycogen accumulation in each sex.

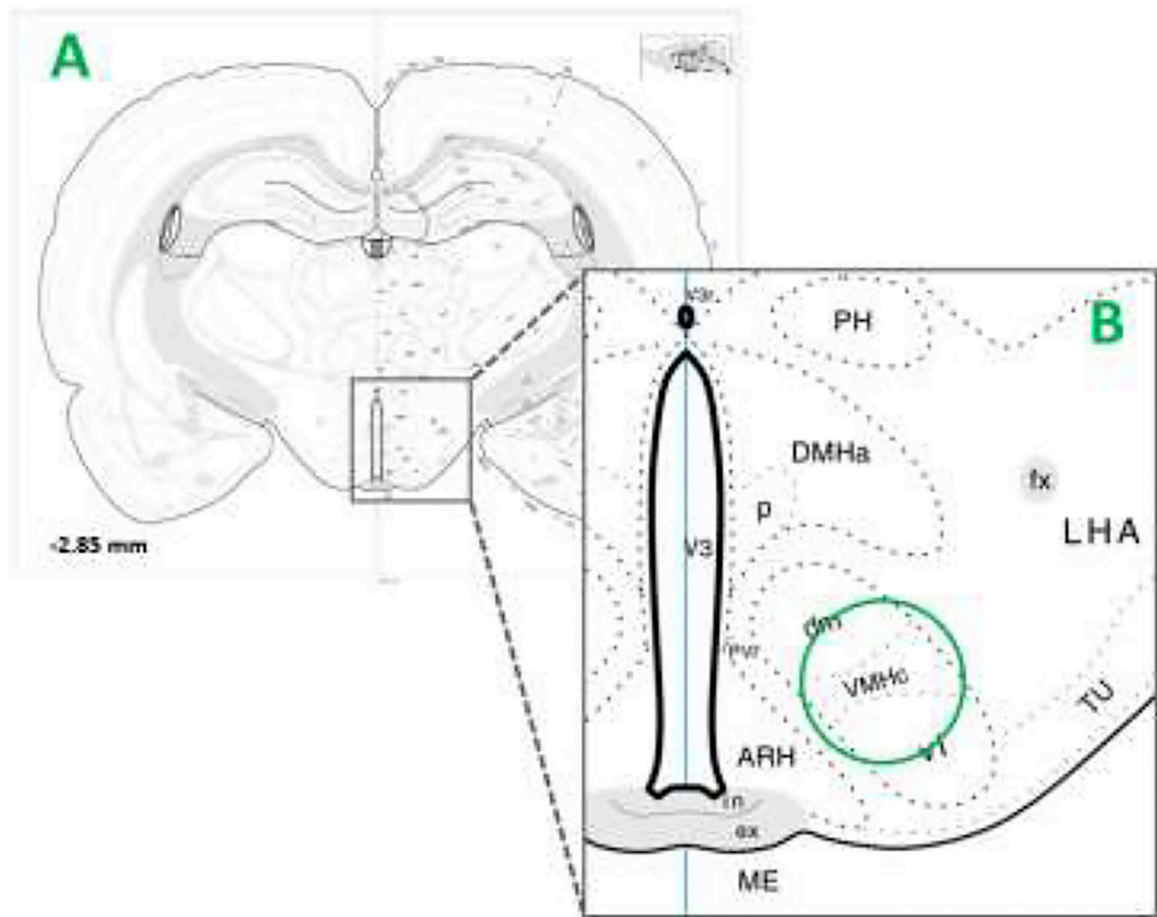


Figure 1.

Ventromedial Hypothalamic Nucleus (VMN) Micropunch Dissection. The rectangle in the Panel A brain map (-2.85 mm posterior to *bregma*) depicts the VMN within the mediobasal hypothalamus, and is enlarged (Panel B) to illustrate the location of VMN in that region. The blue circle denotes positioning of a 0.50 mm diameter circular micropunch tool over the center of the elliptical-shaped VMN, enabling sampling of tissue from dorsomedial, central, and ventrolateral divisions of the VMN. *Abbreviations in Panel B:* ARH: arcuate hypothalamic n.; DMHa,p: anterior, posterior dorsomedial hypothalamic n.; fx: fornix; LHA: lateral hypothalamic area; ME: median eminence; PVi: intermediate periventricular hypothalamic n.; VMHc, dm, vl: central, dorsomedial, ventromlateral ventromedial hypothalamic n.; TU: tuberal n.; V3: third ventricle.

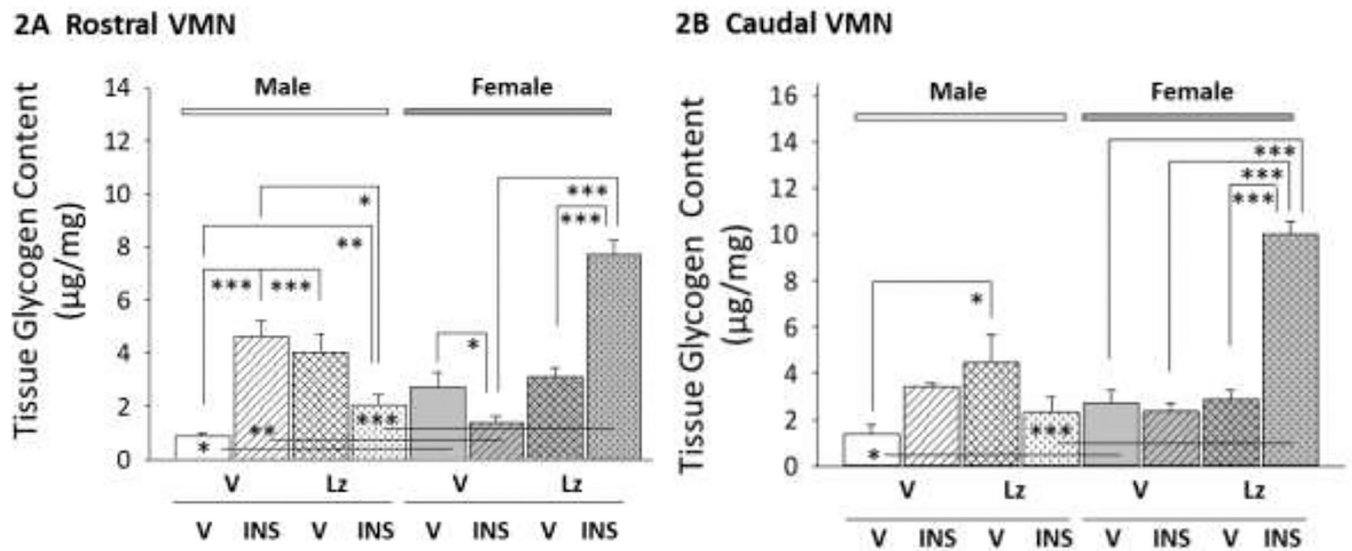


Figure 2. Impact of Intracerebroventricular (*icv*) Administration of the Aromatase Enzyme Inhibitor Letrozole (Lz) on Rostral versus Caudal Ventromedial Hypothalamic Nucleus (VMN) Glycogen Content in Eu- and Hypoglycemic Male and Female Rats.

Groups of Lz- or vehicle (V)- pretreated testes-intact male and estradiol-implanted, ovariectomized (OVX) female rats were injected subcutaneously (*sc*) with insulin (INS) or V one hour before sacrifice. VMN tissue collected between -2.30 and -2.50 mm posterior to *bregma* was analyzed by uHPLC-electrospray ionization-mass spectrometry for glycogen content. Data show mean rostral (Figure 2A) and caudal (Figure 2B) VMN glycogen measures \pm S.E.M. for groups of male (M; *left-hand* side; solid or patterned white bars) and female (F; *right-hand* side; solid or patterned gray bars) rats treated as follows: V infusion/V injection (V/V-M, solid white bars, $n=6$; V/V-F, solid gray bars; $n=6$), V infusion/INS injection (V/INS-M; diagonal-striped white bars; $n=6$; V/INS-F; diagonal-striped gray bars; $n=6$), Lz infusion/V injection (Lz/V-M; cross-hatched white bars; $n=6$; Lz/V-F; cross-hatched gray bars, $n=6$), Lz infusion//INS injection (Lz/INS-M; stippled white bars; $n=6$; Lz/INS-F; stippled gray bars; $n=6$). * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.

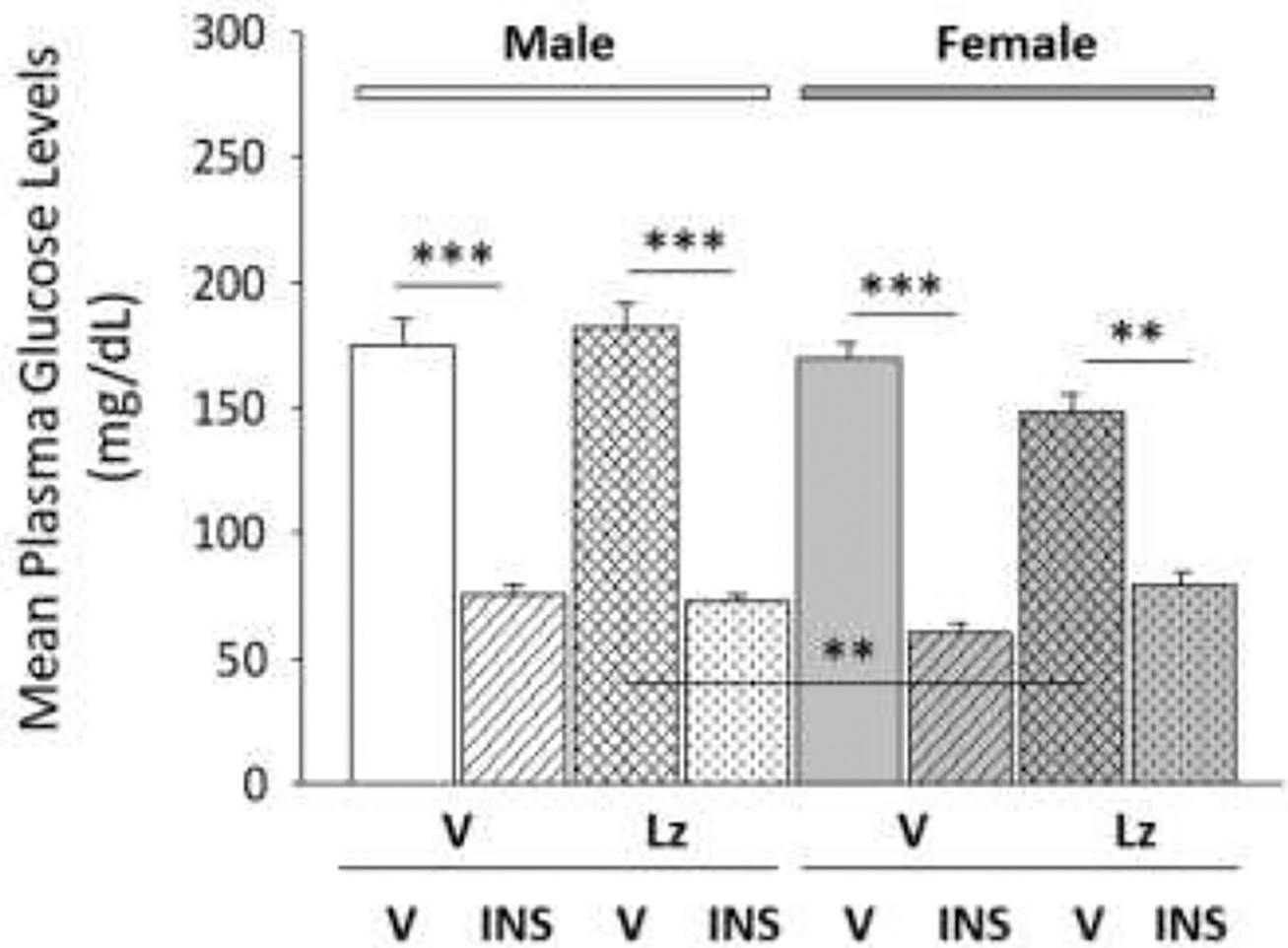


Figure 3. Effects of Lz Pretreatment on Insulin-Induced Hypoglycemia in Male versus Female Rats.

Data depict mean plasma glucose levels \pm S.E.M. for V/V, V/INS, Lz/V, and Lz/INS groups of male (M; *left-hand* side) and female (F; *right-hand* side) rats. * p <0.05; ** p <0.01; *** p <0.001; **** p <0.0001.

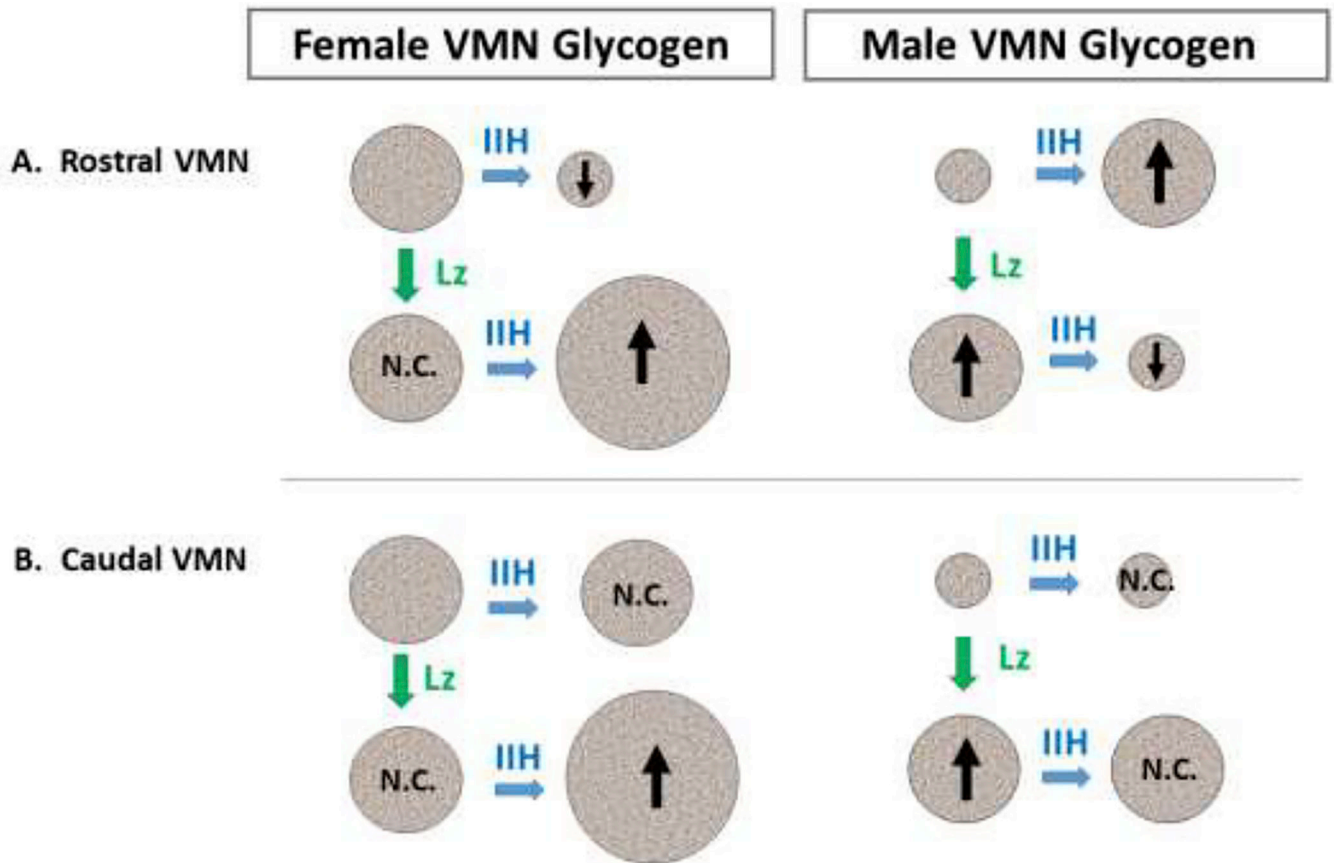


Figure 4. Summary of Singular or Combinatory Effects of Letrozole and Insulin-Induced Hypoglycemia on Male versus Female Rat VMN Glycogen Content.

Abbreviations: IIH, insulin-induced hypoglycemia; Lz, letrozole; N.C., no change. *Symbols:* ↑, increased glycogen; ↓, decreased glycogen. Top row (rostral VMN): IIH caused divergent changes in glycogen levels in female (reduction) versus male (augmentation) rats. Lz did not affect (female) or increased (male) basal glycogen content, indicating no role versus inhibitory neuroestradiol regulation of this energy reserve. Lz pretreatment promoted hypoglycemic amplification (female) or diminution (male) of rostral VMN glycogen, denoting inhibitory versus stimulatory neuroestradiol regulation during IIH. Bottom row (caudal VMN): IIH did not modify glycogen content in either sex. Lz did not alter (female) or elevated (male) baseline glycogen levels, inferring that neuroestradiol respectively does not regulate or inhibits glycogen mass. Lz-pretreated hypoglycemic rats exhibited increased (female) or unaltered (male) caudal VMN glycogen, inferring neuroestradiol inhibition versus no control of caudal VMN glycogen in those sexes.

Table 1.

Experimental Design

	<i>Icv</i> ^a	Pretreatment		
		Day 5-Day 11	Day 11	subcutaneous Injection
Treatment Groups:				
Male:	n=6	V ^b	V ^c	V/V-M
		V	INS ^d	V/INS-M
		Lz ^e	V	Lz/V-M
Female:	n=6	Lz	INS	Lz/INS-M
		V	V	V/V-F
		V	INS	V/INS-F
		Lz	V	Lz/V-F
		Lz	INS	Lz/INS-F

^a left lateral ventricle

^b artificial cerebrospinal fluid (30%)/dimethyl sulfoxide (70%)

^c sterile diluent; 100 µL/100 g *bw*

^d 10.0 U neutral protamine Hagedorn insulin/*kg bw*

^e 1.67 µg/µL dosage, 0.5 µL/hr infusion rate, 7 day duration of pretreatment

Table 2.

DATA SUMMARY Effects of Insulin (INS) Dosing of Vehicle (V)- or Letrozole (Lz)- Pretreated Male and Female Rats on Rostral versus Caudal Ventromedial Hypothalamic Nucleus (VMN) Glycogen Content

<i>VMN Level</i>	<i>Treatment Groups</i>			
	<i>V/V^a</i>	<i>V/INS^b</i>	<i>Lz/V^c</i>	
<i>Lz/INS^d</i>				
Rostral VMN				
Male (M)	↑ vs. V/V-M	↑ vs. V/V-M	↓ vs. Lz/V-M	
Female (F)	↑ vs. V/V-M	↓ vs. V/V-F	N.C. ^e vs. V/V-F	↑ vs. Lz/V-F
Caudal VMN				
Male		N.C. vs. V/V-M	↑ vs. V/V-M	N.C. vs. Lz/V-M
Female	↑ vs. V/V-M	N.C. vs. V/V-F	N.C. vs. V/V-F	↑ vs. Lz/V-F

^a intracerebroventricular (*icv*) V infusion days 5–11; subcutaneous (*sc*) V injection day 11

^b *icv* V infusion days 5–11; *sc* neutral protamine Hagedorn insulin (INS; 10 U/kg bw) day 11

^c *icv* letrozole (Lz; 1.67 µg/µL dosage, 0.5 µL/hr infusion rate) infusion days 5–11; *sc* V injection day 11

^d *icv* Lz infusion days 5–11; *sc* INS injection day 11

^e No Change compared to referenced treatment group