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Hindbrain Estrogen Receptor Regulation of Ventromedial Hypothalamic Glycogen Metabolism and Glucoregulatory Transmitter Expression in the Hypoglycemic Female Rat

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Abstract

Neural substrates for estrogen regulation of glucose homeostasis remain unclear. Female rat dorsal vagal complex (DVC) A2 noradrenergic neurons are estrogen- and metabolic-sensitive. The ventromedial hypothalamic nucleus (VMN) is a key component of the brain network that governs counter-regulatory responses to insulin-induced hypoglycemia (IIH). Here, the selective estrogen receptor- α (ER α) or - β (ER β) antagonists MPP and PHTPP were administered separately to the caudal fourth ventricle to address the premise that these hindbrain ER variants exert distinctive control of VMN reactivity to IIH in the female sex. Data show that ER α governs hypoglycemic patterns of VMN astrocyte glycogen metabolic enzyme, e.g. glycogen synthase and phosphorylase protein expression, whereas ER β mediates local glycogen breakdown. DVC ER also regulate VMN neurotransmitter signaling of energy sufficiency [γ -aminobutyric acid] or deficiency [nitric oxide, steroidogenic factor-1] during IIH. Neither hindbrain ER mediates IIH-associated diminution of VMN norepinephrine (NE) content. Both ER oppose hypoglycemic hyperglucagonemia, while ER β contributes to reduced corticosterone output. Outcomes reveal that input from the female hindbrain to the VMN is critical for energy reserve mobilization, metabolic transmitter signaling, and counter-regulatory hormone secretion during hypoglycemia, and that ER control those cues. Evidence that VMN NE content is not controlled by hindbrain ER α or - β implies that these receptors may regulate VMN function via NE-independent mechanisms, or alternatively, that other neurotransmitter signals to the VMN may control local substrate receptivity to NE.

Keywords

estrogen receptor-beta; ventromedial hypothalamic nucleus; glycogen; glycogen phosphorylase; nitric oxide synthase; glutamate decarboxylase

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Introduction:

The brain consumes a disproportionate amount of bodily energy in order to sustain fundamental nerve cell activities. Iatrogenic insulin-induced hypoglycemia (IIH) is an persistent complication of strict therapeutic control of insulin-dependent diabetes mellitus [Cryer et al., 2003; Cryer, 2010]. IIH deprives the brain of a sufficient energy fuel supply, and thus creates considerable risk for neurological dysfunction and injury. The CNS network that regulates glucose homeostasis receives constant appraisal of cellular energy paucity from dedicated metabolic sensors located in the brain and periphery, and acts on such cues to control contra-regulatory autonomic, neuroendocrine, and behavioral functions. The hindbrain dorsal vagal complex (DVC) supplies vital metabolic sensory input as blood glucose is elevated as a consequence of local shortage of the oxidizable glycolytic end-product L-lactate [Patil and Briski, 2005]. Lactate repletion of that area during hypoglycemia exacerbates circulating glucose reductions and normalizes hypothalamic metabolic neurotransmitter expression, signifying the impact of DVC energy status on downstream elements of the brain gluco-regulatory circuitry [Gujar et al., 2014]. A2 noradrenergic neurons likely monitor hypoglycemia-associated cellular energy disequilibrium in the DVC as lactate-reversible activation of the ultra-sensitive energy sensor adenosine 5'-monophosphate-activated protein kinase (AMPK) occurs in this but no other hindbrain catecholamine cell groups, in parallel with lactate-dependent increases in hypothalamic norepinephrine (NE) activity [Shrestha et al., 2014].

Estradiol is an important endocrine modulator of glucose homeostasis. Estrogen receptors (ER) occur in several structural elements of the central glucoregulatory circuitry, but neither the neuroanatomical location(s) of substrates for estrogenic input nor their impact on downstream network operation is well understood in either sex. While estradiol may likely govern sensory, integrative, and motor activities within this regulatory system, distinctive brain locations where visceral sensory information is processed, including the DVC nucleus of the solitary tract and area postrema, are a credible focus of interest as ER are highly expressed in those sites [Simerly et al., 1990]. Estradiol administration to caudal fourth ventricle modifies glycemic profiles in insulin-injected ovariectomized rats [Nedungadi and Briski, 2012]. A2 cells express estrogen receptor-alpha (ER α) and -beta (ER β) proteins [Ibrahim et al., 2013], and integrate steroid and metabolic stimuli as steroid positive-feedback activation of these cells is repressed by local substrate fuel deficiency [Ibrahim et al., 2014; Shrestha et al., 2014]. Estradiol regulates DVC metabolic sensory control of hypothalamic function. Pharmacological activation of DVC AMPK elicits estradiol-dependent hypothalamic structure-specific patterns of transcriptional activation [Ibrahim et al., 2013] and phospho-AMPK and metabolic transmitter protein expression [Alenazi et al., 2014].

The hypothalamus imposes ultimate control over counter-regulatory responses to IIH. The ventromedial hypothalamic nucleus (VMN) processes nutrient, endocrine, and neurochemical signals of metabolic state to control contra-regulatory responses to IIH [Watts and Donovan, 2010, Donovan and Watts, 2014]. In male rats, VMN neurotransmitter effectors of local and extrinsic metabolic-sensory readout include γ -aminobutyric acid (GABA), which suppresses pancreatic glucagon and adrenomedullary catecholamine release

during IHH [Chan et al., 2006], and signals that increases counterregulatory hormone secretion, e.g. nitric oxide (NO) and steroidogenic factor-1 (SF-1) [Fioramonti et al., 2011; Garfield et al., 2014; Routh et al., 2014; Meek et al., 2016]. SF-1 is also involved in neural regulation of energy expenditure in both sexes [Choi et al., 2013; Kinyua et al., 2016]. The role of these neurochemicals in counter-regulatory outflow in the female is not clear. In male rats, DVC NE regulates VMN metabolic sensory function and gluco-regulatory transmitter signaling as hypoglycemic VMN AMPK activation and SF-1 neuropeptide expression are altered by DVC catecholaminergic nerve cell ablation [Alhamami et al., 2018]. The current study utilized a characterized ovariectomized (OVX) female rat model involving re-establishment of circulating estradiol levels at estrous cycle-like concentrations [Briski et al., 2001] to address the premise that in the female, DVC ER α and/or -ER β regulate IHH-associated patterns of VMN NE activity together with gluco-inhibitory and -stimulatory neurotransmitter signaling and counter-regulatory hormone secretion. As NE is reported to stimulate glycogenolysis *in vitro* [Fillenz et al., 1999; Dong et al., 2012], a corollary aim of this research was to determine if these receptors control VMN glycogen metabolic enzyme protein expression and tissue glycogen content. The VMN and other characterized hypothalamic metabolic structures were micropunch-dissected from forebrains of insulin-injected animals pretreated by intra-caudal fourth ventricular (CV4) administration of the selective ER α antagonist 1,3-*Bis*(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1*H*-pyrazole dihydro-chloride (MPP), the ER β antagonist 4-[2-phenyl- 5,7-*bis*(trifluoromethyl)pyrazolo[1,5-*a*]pyrimidin-3-yl]phenol (PHTPP), or vehicle alone for Western blot, NE ELISA, and glycogen HPLC/mass spectrometry analyses.

Materials and Methods:

Experimental Design:

Adult female Sprague-Dawley rats (2–3 months of age) were housed 2–3 per cage, under a 14 hr light/10 hr dark lighting schedule (lights on at 05:00 hr). Animals had unrestricted access to tap water and laboratory rat chow (Harlan Teklad LM-485; Harlan Industries, Madison, WI). Rats were accustomed to daily handling before commencement of experimentation. Animal use protocol implementation was compliant with NIH guidelines, under approval by ULM Institutional Animal Care and Use Committee. On study day 1, rats were anesthetized by intraperitoneal injection of ketamine/xylazine anesthesia (0.1 mL/100 g *bw*, 90 mg ketamine:10 mg xylazine/mL; Henry Schein Inc., Melville, NY), then bilaterally OVX and stereotactically implanted with a PE-20 cannula directed to the CV4 at the following coordinates: 0.0 mm from midline; –13.3 mm posterior to bregma, 6.6 mm ventral to skull surface. After surgery, animals were injected with enrofloxacin (10mg/kg *im*; Norbrook Laboratories Limited, Newry, Northern Ireland) and ketoprofen (3mg/kg *sc*; Zoetis Inc., Kalamazoo MI), and transferred to individual cages for recovery. On day 7, rats were implanted *sc* with a silastic capsule (i.d. 0.062 in.; o.d. 0.125 in.; 10 mm/100 g *bw*) containing 30 ug 17 β -estradiol-3-benzoate/mL safflower oil, under isoflurane anesthesia. This steroid replacement regimen produces plasma estradiol concentrations of approximately 22 pg/ml [Briski et al., 2001], which correspond to circulating hormone levels characteristic of metestrus in 4-day cycling female rats [Butcher et al., 1974]. At 08.45 hr on Day 10, vehicle [dimethyl sulfoxide (DMSO, 200 nL; VWR, Solon, OH);

groups 1 (n=5) and 2 (n=5), the ER α antagonist MPP [10 μ M/200 nL; Tocris/Bio-Techne Corp., Minneapolis, MN; group 3 (n=5)], or ER β antagonist PHTPP [10 μ M/200 nL; Tocris; group 4 (n=5)] was delivered to the CV4. At 9:00 hr on Day 10, animals in group 1 were injected *sc* with the vehicle sterile diluent (V; Eli Lilly & Co., Indianapolis, IN), while remaining groups were injected with neutral protamine Hagedorn insulin (INS; 10 U/kg *bw*; Eli Lilly). At one hour post-injection (10:00 hr), animals were sacrificed by microwave fixation (1.45 sec exposure; In Vivo Microwave Fixation System, 5kW; Stoelting Co., Wood Dale, IL) for brain tissue and blood collection.

Micropunch Dissection of the VMN, Arcuate Hypothalamic Nucleus (ARH), Dorsomedial Hypothalamic Nucleus (DMN), and Lateral Hypothalamic Area (LHA):

Each hypothalamus was cut into serial 100 – 150 micron-thick frozen sections through the VMN, ARH, DMN, and LHA, using calibrated Brain Punch set punch tools (prod. no. 57401; Stoelting), for micropunch dissection. Distinct sets of sections were collected over specific rostro-caudal lengths posterior to *bregma* for different analytical objectives: 1) between –2.00 and –2.20 mm: two 100 μ m-thick sections; Western blot analysis; 2) between –2.20 and –2.40 mm: two 100 μ m sections, NE ELISA analysis; 3) between –2.40 and –2.70 mm: two 150 μ m sections, HPLC/mass spectrometric analysis; 4) beginning at –2.70 mm: six (VMN, ARH, LHA) or four (DMN) 100 μ m-thick sections, Western blotting. Micropunches used for immunoblotting were collected into lysis buffer consisting of 2.0% sodium dodecyl sulfate, 0.05 M dithiothreitol, 10.0% glycerol, 1.0 mM EDTA, 60.0 mM Tris-HCl, pH 7.2; tissues utilized for ELISA were saved in lysis buffer made of 0.01 N HCl supplemented with 1.0 mM EDTA, 4.0 mM sodium metabisulfite; punches for HPLC/mass spectrometric measurements were collected into 0.02 M Tris buffer, pH 7.2.

Western Blot Analysis:

VMN tissue punches from each animal were heat-denatured at 95°C. For each protein of interest, three separate tissue sample pools were created within each treatment group before separation on Bio-Rad Stain Free 10–12% acrylamide gels (prod. no. 161–0183; Bio-Rad, Hercules, CA). Gels were activated by UV light (1 min) in a BioRad ChemiDoc TM Touch Imaging System prior to overnight transblotting (4–5°C) to 0.45- μ m PVDF-Plus membranes (prod. no. PV4HY00010; ThermoFisherScientific, Waltham, MA). After blocking for 2 hr with Tris-buffered saline, pH 7.4, containing 0.1 % Tween-20 and 2% bovine serum albumin, membranes were incubated for 24–48 hrs (45°C) with rabbit primary polyclonal antisera against glycogen synthase (GS; 1:2,000; prod. no. 3893S; Cell Signaling Technology, Danvers, MA), glycogen phosphorylase (GP; 1:2,000; prod. no. NBP1–32799; Novus Biologicals, Littleton, CO), glutamate decarboxylase65/67 (GAD65/67; 1:10,000; prod. no. ABN904; MilliporeSigma (Burlington, MA), neuronal nitric oxide synthase (nNOS; 1:2,000; prod. no. NBP1–39681; Novus Biol.), brain-derived neurotrophic factor (BDNF; 1:2,000; prod. no. NBP1–46750; Novus Biol.), or steroidogenic factor-1 (SF1; 1:2,000; prod. no. PA5–41967; ThermoFisherSci.). Membranes were next incubated for 1 hr with goat anti-rabbit secondary antibodies (1:5,000; prod. no. NEF812001EA; PerkinElmer, Waltham, MA), then exposed to Supersignal West Femto maximum sensitivity chemiluminescent substrate (prod. no. 34096; ThermoFisherSci.). Membrane buffer washes and antibody incubations were carried out by Freedom Rocker™ BlotItbot® automation

(Next Advance, Inc., Troy NY). Chemiluminescence band optical density (O.D.) values obtained in the ChemiDoc MP system were normalized to total in-lane protein with Imagelab software (Image Lab™ 6.0.0; Bio-Rad). Precision plus protein molecular weight dual color standards (prod. no. 161–0374; Bio-Rad) were included in each Western blot analysis.

ELISA Measurement of VMN, ARH, DMN, and LHA NE Content:

For each animal, micropunched tissue from each structure was analyzed using Noradrenaline Research ELISA™ kit reagents (Labor Diagnostika Nord GmbH & Co KG, Nordhorn, Germany), as described [Shrestha et al., 2014].

Glycogen HPLC Analyses:

VMN tissue was heat-denatured and homogenized by ultra-sonification. Supernatant aliquots (20 µL) were hydrolyzed by incubation with 0.5 mg/ml amyloglucosidase (10 µL) and 0.1M sodium acetate (10 µL) for 2 hr prior to heating to 100°C (5 min), followed by cooling to room temperature. Supernatant glycogen concentrations were determined by reverse-phase HPLC in a Hitachi LaChrom Elite® System (Hitachi America, Ltd., Tarrytown, NY), by modification of published methods [Honda et al., 1989; Fuller et al., 2012; Bai et al., 2015]. Hydrolyzed and non-hydrolyzed sample aliquots were derivatized with 100 µL 0.5 M 1-phenyl-3-methyl-5-pyrazolone (PMP) reagent supplemented with 0.3 M NaOH. After acidification with 400 µL 0.75% formic acid, derivatized samples were extracted with chloroform, vacuum concentrated to remove chloroform and formic acid, transferred to fresh tubes, frozen at –80°C, and lyophilized. Lyophilized samples were diluted to 1.0 mL with acetonitrile/0.01 M triethylamine (65:35) v/v; 20 µL aliquots were then separated on a Zorbax ODS (4.6 cm × 250 mm, 5 µm), with an acetonitrile/0.005 M triethylamine (65:35) v/v mobile phase, at a flow rate of 1 ml/min. Wavelength of detection was 245 nm. Tissue glycogen concentrations, as calculated by subtracting from hydrolysis-derived total glucose concentrations, were determined using a calibration curve. Glycogen and D-glucose concentrations were expressed as ng/mL and ug/mL respectively. Prepared D-glucose-PMP and HPLC eluent containing D-glucose-PMP were analyzed in a Q®TRAP LC-MS/MS 3200 System (AB Sciex LLC, Framingham, MA). 1 µg/mL D-glucose-PMP was diluted with HPLC-grade methanol prior to injection, and eluted with acetonitrile/0.005 M triethylamine (65:35) v/v over consecutive 1–3 min intervals. HPLC eluent was evaporated at 45°C for 60 min, frozen to –80°C, lyophilized at –55°C, and reconstituted with methanol prior to injection.

Glucose and Counter-Regulatory Hormone Measurements:

Plasma glucose concentrations were measured using an ACCU-CHECK Aviva plus glucometer (Roche Diagnostic Corporation, Indianapolis, IN) [Kale et al., 2006]. Circulating plasma free fatty acid levels were analyzed with Free Fatty Acid Quantitation Kit reagents (MAK044; Sigma Aldrich, St. Louis, Mo) [Briski et al., 2017]. Plasma corticosterone (ADI-900–097; Enzo Life Sciences, Inc., Farmingdale, NY) and glucagon (EZGLU-30K, EMD Millipore, Billerica, MA) concentrations were determined using commercial ELISA kit reagents [Alhamami et al., 2018].

Statistical analyses:

Mean normalized Western blot protein O.D., micropunch tissue NE, VMN glycogen, glucose, glucagon, corticosterone, and free fatty acid values were evaluated between treatment groups by one-way analysis of variance and Student Newman Keuls post-hoc test. Differences of $p < 0.05$ were considered significant.

Results:

Figure 1 depicts effects of intra-CV4 administration of the selective ER α antagonist MPP or ER β antagonist PHTPP on VMN GAD_{65/57}, nNOS, SF-1, and BDNF protein responses to IIH in the female rat. Data presented in Panel A show that IIH significantly decreased VMN GAD_{65/67} profiles [V/INS versus V/V], and that DVC ER β [PHTPP/INS versus V/INS], but not ER α blockade prevented this decline in protein expression ($F_{(3,8)} = 14.37$; $p < 0.0001$). At the same time, IIH augmented VMN nNOS content (Panel B) by mechanisms involving DVC ER α and ER β [MPP/INS and PHTPP/INS versus V/INS] ($F_{(3,8)} = 74.20$; $p < 0.0001$). VMN SF-1 protein expression (Panel C) was unaffected by IIH, but was significantly elevated in MPP- or PHTPP-pretreated, INS-injected animals compared to vehicle-pretreated hypoglycemic animals [MPP/INS and PHTPP/INS versus V/INS] ($F_{(3,8)} = 39.51$; $p < 0.0001$). IIH significantly inhibited BDNF protein expression (Panel D), a response that was exacerbated by ER β antagonism [PHTPP/INS versus V/INS] ($F_{(3,8)} = 14.59$; $p = 0.0003$).

Data presented in Figure 2 portray the impact of DVC ER α or ER β antagonism on hypoglycemic patterns of VMN GS and GP protein expression and VMN tissue glycogen and glucose amassment. As illustrated in Panel A, female rats exhibited no change in VMN GS protein expression in response to hypoglycemia ($F_{(3,8)} = 7.67$; $p = 0.002$). Pretreatment with MPP caused a significant increase in GS profiles compared to V-pretreated groups [MPP/INS versus V/V and V/INS]. Data in Panel B show that INS-injected animals exhibited decreased VMN GP profiles compared to V-injected controls ($F_{(3,8)} = 16.16$; $p < 0.0001$). This decline in GP protein expression was amplified in MPP/INS rats relative to the V/INS group. As shown in Panel C, VMN tissue glycogen content was significantly decreased in response to IIH [V/INS versus V/V] ($F_{(3,16)} = 14.07$; $p = 0.0006$), which was reversed beyond levels measured in V/V by PHTT pretreatment. VMN free glucose concentrations were also suppressed in V/INS versus V/V groups (Panel D) ($F_{(3,16)} = 4.21$; $p = 0.022$). Tissue glucose levels did not differ between MPP- or PHTPP-pretreated versus V-pretreated INS-injected animals.

Figure 3 illustrates effects of IIH in the presence or absence of DVC ER antagonism on NE accumulation in micropunch-dissected hypothalamic metabolic loci. Data show that hypoglycemic animals exhibited significant diminution of VMN (Panel A; $F_{(3,16)} = 6.13$; $p = 0.018$), ARH (Panel B; $F_{(3,16)} = 13.73$; $p = 0.0026$), DMN (Panel C; $F_{(3,16)} = 6.30$; $p = 0.017$), and LHA (Panel D; $F_{(3,16)} = 15.29$; $p = 0.001$) NE content. MPP pretreatment normalized NE content in the ARH, but not other structures, whereas PHTPP did not alter these site-specific reductions in NE.

Data in Figure 4 depict effects of MPP or PHTPP pretreatment on glycemic, glucagon, corticosterone, and FFA responses to INS injection to female rats. As presented in Panel A, circulating glucose levels were suppressed after INS therapy [V/INS versus V/V] ($F_{(3,16)} = 124.90$; $p < 0.0001$). The magnitude of this decrement was further exacerbated in MPP-pretreated animals, but attenuated in the PHTPP/INS group. Plasma glucagon concentrations were elevated in hypoglycemic animals, a response that was amplified in MPP- or PHTPP-pretreated groups [MPP/INS and PHTPP/INS versus v/INS] (Panel B; $F_{(3,16)} = 36.78$; $p < 0.0001$). Data in Panel C indicate that IIH-associated inhibition of plasma corticosterone levels was not modified by ER antagonism ($F_{(3,16)} = 114.60$; $p < 0.0001$). Circulating FFA levels were refractory to IIH regardless of V versus MPP or PHTPP pretreatment (Panel D; $F_{(3,16)} = 3.76$; $p = 0.053$).

Discussion:

The role of estradiol in hindbrain metabolic sensor regulate of hypothalamic genomic activation, AMPK activity state, and metabolic transmitter signaling in female rats is established [Ibrahim et al., 2013; Alenazi et al., 2014], but it is unclear if that action occurs, in part, within the hindbrain. Current studies investigated whether DVC ER control counter-regulatory hormone and VMN glycogen metabolism and gluco-regulatory transmitter responses to hypoglycemia in that sex. Results reveal that ER α and ER β regulate VMN glycogen enzyme protein profiles and tissue glycogen accumulation, respectively. Both receptors up-regulate VMN nNOS protein, while ER β also inhibits GAD_{65/67} expression in insulin-injected animals. Neither ER directed hypoglycemic diminution of VMN NE levels. Both DVC ER oppose hypoglycemic hyperglucagonemia, while ER β suppresses corticosterone release. Present outcomes demonstrate DVC ER control of VMN glycogen metabolism and GABA- and nitrergic signals of cellular energy deficiency in hypoglycemic female rats, but do not implicate the latter in ER influence on counter-regulatory hormone release. The inability of hindbrain ER α or -beta antagonism to modify VMN NE responses to hypoglycemia suggests that these receptors may regulate VMN function through NE-independent mechanisms or rather, that ER-sensitive non-catecholaminergic signals from the DVC may govern VMN substrate receptivity to NE.

VMN GS protein levels did not differ between V- versus INS-injected rats, while GP profiles were down-regulated due to hypoglycemia. DVC ER α signaling was inhibitory to GS expression, but stimulatory to GP; this input to each target protein is evidently offset by as-yet-unidentified stimuli. There remains a need to establish the neurochemical signal(s) and signal transduction pathways that mediate bi-directional effects of ER α on VMN GS and GP protein profiles. Surprisingly, at the same time, VMN glycogen content was significantly diminished via ER β -dependent mechanisms. This apparent discordance between tissue GP and glycogen levels implies that total GP protein quantification may not reflect enzyme activation, e.g. phosphorylation state, in that GP activity might have been enhanced despite an overall reduction in protein expression, or that reduced GP profiles observed at the current time point were preceded by augmented protein production. It would be informative to address the issue of if and how IIH may affect VMN GS and GP phosphorylation, including questions pertaining to whether one or both enzyme activities are controlled by DVC ER β and how such control is achieved, but such effort is currently impeded by lack of

availability of antisera raised against phosphoGP. At present, the mechanism(s) by which ER α and ER β regulate different VMN astrocyte target proteins are not understood. One possibility is that stimuli from each receptor variant are conveyed by a different neurochemical(s) or, a more likely scenario that distinctive subsets of neurons within a single neurotransmitter population, characterized by distinctive VMN targets, may express a single ER variant or diverse ER α / ER β ratios where one or the other predominates.

Current data show that VMN tissue glucose levels were decreased in female rats by DVC ER α - and ER β -dependent mechanisms. A plausible explanation is that these receptors may up-regulate glucose transporters, including the constitutive and insulin-dependent nerve cell transporters GLUT3 and GLUT4 as well as the astrocyte transporter GLUT1. Augmented glucose uptake in the VMN could conceivably align with enhanced glycolytic yield of pyruvate for entry into the tricarboxylic acid (TCA) cycle, as estradiol is reported to stimulate key glycolytic, TCA, and respiratory chain enzyme activity [Kostanyan and Nazaryan, 1992; Nilsen et al., 2007; Chen et al., 2009] and oxidative respiration [Irwin et al., 2008] within brain regions or whole brain.

DVC ER α and ER β signaling is critical for hypoglycemia-associated up-regulated expression of VMN nNOS, a marker protein for the gluco-stimulatory transmitter NO. Input from ER β also mediates concurrent inhibition of VMN GAD_{65/67} profiles, which is apt to reflect diminished release of GABA, a counter-regulation – repressing transmitter. Recent male rat studies reveal that VMN nitrenergic, but not GABAergic neurons are sensitive to glycogen-derived substrate fuel provision as nNOS protein content is augmented by pharmacological inhibition of GP [Alhamami et al., 2018]. The prospect that in the female VMN, nNOS and/or GAD_{65/67} protein responses to hypoglycemia may reflect, in part, down-regulated GP expression and/or diminished glycogen mass cannot be discounted here. Further research is merited to examine whether these hindbrain ER variants control VMN NO and GABA neurons by astrocyte- dependent and/or -independent mechanisms. Data here also show that DVC ER α and ER β are each inhibitory to VMN SF-1 expression, which was unchanged during IIH owing to counter-balancing positive cues, while ER β opposes IIH-associated suppression of BDNF. The respective roles of these neurotransmitters in communicating cellular energy status in the female remain to be characterized.

Present data show that the NE content of the VMN and other hypothalamic metabolic structures, e.g. ARH, DMN, and LHA, was significantly reduced at one hour after onset of hypoglycemia in female rats. These findings deviate from observations of elevated VMN NE accumulation in males treated with a similar neutral protamine Hagedorn (NPH) INS dose, e.g. 10.0 U/kg *bw* [Alhamami et al., 2018]. Importantly, those studies showed that VMN NE exhibits bi-directional responses over a graded NPH INS dose range in male rats, as NE activity was decreased at lower dosages (5.0 U NPH/kg *bw*), yet significantly increased when injected with 10.0 U/kg. It should be noted that quantification of steadystate tissue NE mass does not provide definitive insight on how local NE synthesis/breakdown/exocytosis ratios may be affected by IIH. Thus, it is unclear if diminished VMN NE content in males and females injected with 5.0 or 10.0 U/kg *bw*, respectively, may mirror reductions in the local NE synthesis/release ratio relative to euglycemic controls. An additional issue requiring resolution is whether apparent sex differences in IIH-associated VMN NE

amassment at a given NPH dosage, namely 10.0 U/kg, involves discrepant signal strength conveyed by noradrenergic axons from the hindbrain to the hypothalamus or instead to sex-specific control of NE production, metabolism, and release. Yet, prior work in our laboratory disclosed divergent effects of equivalent NPH dosing of male versus female rats on A2 neuron nerve cell dopamine-beta-hydroxylase (D β H) mRNA expression [Cherian and Briski, 2011; 2012], results that were extended by observations of decreased D β H protein in females [Tamrakar et al., 2017]. Those data support likely differences in hindbrain noradrenergic input to the VMN and other hypothalamic structures.

Outcomes further reveal that DVC ER α signaling diminished ARH NE content, but did not control NE accumulation in other hypothalamic loci examined here, including the VMN; meanwhile, ER β had no apparent role in hypoglycemic patterns of NE activity. These unanticipated findings imply, on one hand, that one or both DVC ER alter net NE synthesis and exocytosis, albeit to a comparable extent so that local ratios of these activities may not change, with the exception of the ARH in the event of ER α antagonism. Then again, hindbrain DVC ER α and ER β may regulate VMN astrocyte glycogen mobilization and gluco-regulatory neurotransmitter stimuli by mechanisms unrelated to NE signal strength; for example, these ER may regulate hindbrain non-catecholaminergic stimuli that control VMN substrate receptivity to NE input. It is also plausible that DVC ER may differentially regulate noradrenergic input to distinctive neurotransmitter groups within the VMN, depending upon physiological or pathophysiological circumstances. Thus, NE quantification within the whole VMN may obscure small cellular-level changes in NE tone that modify, for example, transmitter cues that impact glucose homeostasis. This work underscores the ongoing need for high-resolution analytical techniques for quantification of NE input to specific neurochemical targets.

Present data disclose that DVC ER α and ER β oppose hypoglycemic hyperglucagonemia in the female within the first hour after INS injection. The latter receptor is also implicated in concurrent suppression of corticosterone release. This ER-driven blunting of counter-regulatory hormone outflow is inconsistent with receptor augmentation of VMN gluco-regulatory signaling of cellular energy deficiency, e.g. up-regulated NO alongside down-regulated GABA. It is thus reasonable to presume that VMN NO and GABA neurons may not function, over the timeframe investigated here, within pathways that impose DVC ER control of counter-regulatory hormone secretion. Additional research is justified to characterize the mechanisms whereby hindbrain ER control glucagon and corticosterone outflow in the female.

In the current experimental protocol, selective ER α or - β antagonists were delivered into the caudal fourth ventricle (CV4) in order to avoid destruction of DVC tissue by introduction of drug delivery devices into that site. Thus, the possibility that observed effects of MPP and/or PHTPP following administration into that site may reflect, to some undetermined extent, actions on hindbrain substrates located outside the DVC cannot be dismissed. As our research plan did not evaluate effects of either antagonist on parameters of interest in the absence of hypoglycemia, further work will be needed to determine if the extent of hindbrain ER influence on those functions is equivalent or different during the distinctive energy states of eu- versus hypoglycemia. At present, the possibility that data described here

may reflect, to some extent, additive effects of IHH and ER antagonist cannot be overlooked. A possibility that MPP may be cleaved *in vivo*, in the present experimental circumstances, to the ER α agonist methyl-pyrazole-triol after administration to the hindbrain cannot be discounted [Zhou et al., 2009]. Further studies are required to address if and to what extent post-delivery conversion of MPP occurs in our model, and to evaluate resulting antagonist versus agonist effects of MPP on hypothalamic parameters of interest.

Estradiol regulates gene transcription through genomic and non-genotropic mechanisms [reviewed by Marino et al., 2006]. The former involves both direct and indirect control of gene expression through nuclear ER contact with DNA estrogen response elements (ERE) or modulation of activity of other transcription factors such as activator protein-1, NF κ B, and stimulating protein-1, respectively. Alternatively, non-genotypic, e.g. rapid E actions on cytoplasmic and nuclear functions are mediated by control of signal transduction pathway [e.g. phospholipase C/protein kinase C; Ras/Raf/MAPK; phosphatidylinositol 3 kinase/AKT; cAMP/protein kinase A] activity by plasma membrane-associated nuclear and novel ER. In light of recent evidence for detectable changes in both gene and protein expression more rapidly than conventionally assumed, e.g. in less than one hour versus two or more hours in other experimental models, we presume that observed changes in hindbrain and hypothalamic tissue protein levels may reflect, in part, altered rate of production. We also consider that ER-sensitive and independent hypoglycemia-associated changes in detectable protein levels may, additionally, be a sign (of as yet an undeterminable extent) of adjustments in post-translational processing, including protein degradation, modification, and/or protein-protein complex formation [You and Yin, 2000].

Evidence for insulin receptor expression in the hypothalamus and hindbrain DVC raises the prospect that exogenous insulin administration, as performed here, could influence activity of these receptors and thereby modulate ER-dependent hindbrain signaling and/or reactivity of hypothalamic substrates to such cues. Current studies did not determine if and to what extent peripheral insulin injection may alter brain tissue insulin concentrations in vicinity of these receptor populations. The likelihood that the experimental paradigm carried out here did not substantially augment brain insulin content is supported by reports that saturable transport of insulin from plasma into brain cerebrospinal fluid is very slow under hyperinsulinemic conditions [Wallum et al., 1987; Schwartz et al., 1990], and that movement from CSF into brain parenchyma occurs at a much diminished rate relative to liver, muscle, and adipose tissue. [Gray et al., 2014]. However, further effort is justified to determine if hypoglycemia-sensitive DVC noradrenergic neurons that innervate the hypothalamus and/or their forebrain cellular targets are directly or indirectly controlled by insulin.

In summary, current research utilized selective ER α and ER β antagonists as pharmacological tools to investigate the respective roles of these DVC receptor variants in VMN glycogen metabolism and metabolic transmitter signaling as well as hypoglycemic patterns of counter-regulatory hormone secretion in the female rat. Data indicate that input from the female hindbrain to the VMN is critical for energy reserve mobilization, metabolic transmitter signaling, and counter-regulatory hormone secretion during hypoglycemia, and that ER control those cues. Results show that ER α and - β respectively control VMN astrocyte glycogen metabolic enzyme expression and glycogen mass during IHH. These

hindbrain ER also govern expression of VMN neurotransmitter signals of cellular energy state. Proof that IIH-associated diminution of VMN NE is unrelated to hindbrain ER signaling infers that control of VMN targets may not be conveyed by NE signal strength, but rather by ER-sensitive non-catecholaminergic stimuli that govern substrate receptivity to NE. DVC ER α and/or ER β inhibit hypoglycemic patterns of glucagon and corticosterone secretion by mechanisms that do not involve VMN neurotransmitters characterized as glucoregulatory in males.

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Abbreviations:

DVC	dorsal vagal complex
E	estradiol
GABA	γ -aminobutyric acid
GAD_{65/67}	glutamate decarboxylase _{65/67}
GP	glycogen phosphorylase
GS	glycogen synthase
IIH	insulin-induced hypoglycemia
LHA	lateral hypothalamic area
MPP	1,3- <i>Bis</i> (4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1 <i>H</i> -pyrazole dihydrochloride
NE	norepinephrine
NO	nitric oxide
nNOS	neuronal nitric oxide synthase
OVX	ovariectomy
PHTPP	4-[2-phenyl-5,7- <i>bis</i> (trifluoromethyl)pyrazolo[1,5- <i>a</i>]pyrimidin-3-yl]phenol
SF-1	steroidogenic factor-1
VMN	ventromedial hypothalamic nucleus

References:

Alhamami HN, Uddin MM, Mahmood ASMH, Briski KP. Lateral but not medial hypothalamic AMPK activation occurs at the hypoglycemic nadir in insulin-injected male rats: Impact of caudal dorsomedial hindbrain catecholamine signaling. *Neuroscience* 2018; 379:103–114. [PubMed: 29534973]

- Alhamami HN, Alshamrani A, Briski. Effects of the glycogen phosphorylase inhibitor 1,4-dideoxy-1,4-imino-D-arabinitol on ventromedial hypothalamic nucleus 5'-adenosine monophosphate-activated protein kinase activity and metabolic neurotransmitter biosynthetic enzyme protein expression in eu- versus hypoglycemic male rats. *Physiological Reports* 2018; 103: 236–249
- Alenazi FSH, Ibrahim BA, Briski KP. Estradiol regulates effects of hindbrain activator 5-aminoimidazole- 4-carboxamide-riboside administration on hypothalamic adenosine 5'-monophosphate-activated protein kinase activity and metabolic neurotransmitter mRNA and protein expression. *J. Neurosci. Res* 2014; 93: 651–659. [PubMed: 25476093]
- Bai W, Fang X, Zhao W, Huang S, Zhang H, Qian M. Determination of oligosaccharides and monosaccharides in Hakka rice wine by precolumn high-performance liquid chromatography. *J. Food Drug Anal.* 2015; 23: 645–651. [PubMed: 28911480]
- Briski KP, Koshy Cherian A, Genabai NK, Vavaiya KV. In situ coexpression of glucose and monocarboxylate transporter mRNAs in metabolic-sensitive dorsal vagal complex catecholaminergic neurons: transcriptional reactivity to insulin-induced hypoglycemia (IIH) and caudal hindbrain glucose or lactate repletion during IIH. *Neuroscience* 2009; 164: 1152–1160. [PubMed: 19744543]
- Briski KP, Marshall ES, Sylvester PW. Effects of estradiol on glucoprivic transactivation of catecholaminergic neurons in the female rat caudal brainstem. *Neuroendocrinology* 2001; 73: 369–377. [PubMed: 11408778]
- Chan O, Paranjape SA, Horblitt A, Zhu W, Sherwin RS. Lactate-induced release of GABA in the ventromedial hypothalamus contributes to counterregulatory failure in recurrent hypoglycemia and diabetes. *Diabetes* 2013; 62: 4239–4246. [PubMed: 23939392]
- Chan O, Zhu W, Ding Y, McCrimmon RJ, Sherwin RS. Blockade of GABA(A) receptors in the ventromedial hypothalamus further stimulates glucagon and sympathoadrenal but not the hypothalamo-pituitary-adrenal response to hypoglycemia. *Diabetes* 2006; 55: 1080–1087. [PubMed: 16567532]
- Chen JQ, Brown TR, Russo J. Regulation of energy metabolism pathways by estrogens and estrogenic chemicals and potential implications in obesity associated with increased exposure to endocrine disruptors. *Biochim. Biophys. Acta* 2009; 1793: 1128–1143. [PubMed: 19348861]
- Cherian A, Briski KP. Quantitative RT PCR and immunoblot analyses reveal acclimated A2 noradrenergic neuron substrate fuel transporter, glucokinase, phospho-AMPK, and dopamine-beta-hydroxylase responses to hypoglycemia. *J. Neurosci. Res* 2011; 89: 1114–1124. [PubMed: 21488089]
- Cherian A, Briski KP. Estradiol regulates phosphoAMPK, glucokinase, sub-strate transporter, and dopamine-beta-hydroxylase adaptation to repeated insulin-induced hypoglycemia in female rat A2 noradrenergic neurons. *J. Neurosci. Res* 2012; 90: 1347–1358. [PubMed: 22431334]
- Dong JH, Chen X, Cui M, Yu X, Pang Q, Sun JP. B2-adrenergic receptor and astrocyte glucose metabolism. *J. Mol. Neurosci* 2012; 48: 456–463. [PubMed: 22399228]
- Fillenz M, Lowry JP, Boutelle MG, Fray AE. The role of astrocytes and noradrenaline in neuronal glucose metabolism. *Acta Physiol. Scand* 1999; 167: 275–284. [PubMed: 10632627]
- Fioramonti X, Marsollier N, Song Z, Fakira KA, Patel RM, Brown S, Duparc T, Pica-Mendez A, Sanders NM, Knauf C, Valet P, McCrimmon RJ, Beuve A, Magnan C, Routh VH. Ventromedial hypothalamic nitric oxide production is necessary for hypoglycemia detection and counterregulation. *Diabetes* 2010; 59: 519–528. [PubMed: 19934009]
- Fuller M, Duplock S, Turner C, Davey P, Brooks DA, Hopwood JJ, Meikle PJ. Mass spectrometric quantification of glycogen to assess primary substrate accumulation in the Pompe mouse. *Anal. Biochem* 2012; 421: 759–763. [PubMed: 22239964]
- Garfield AS, Shah BP, Madara JC, Burke LK, Patterson CM, Flak J, Neve RL, Evans ML, Lowell BB, Myers MG, and Heisler LK. A parabrachial-hypothalamic cholecystokinin neurocircuit controls counterregulatory responses to hypoglycemia. *Cell Metab.* 2014; 20: 1030–1037. [PubMed: 25470549]
- Gray SM, Meijer RI, Barrett EJ. Insulin regulates brain function, but how does it get there? *Diabetes* 2014; 63: 3992–3997. [PubMed: 25414013]

- Honda S, Akao E, Suzuki S, Okuda M, Kakehi K, Nakamura J. High-performance liquid chromatography of reducing carbohydrates as strongly ultraviolet-absorbing and electrochemically sensitive 1-phenyl-3-methyl-5-pyrazolone derivatives. *Anal. Biochem* 1989; 180: 351–357. [PubMed: 2817366]
- Ibrahim BA, Briski KP. Role of dorsal vagal complex A2 noradrenergic neurons in hindbrain glucoprivic inhibition of the luteinizing hormone surge in the steroid-primed ovariectomized female rat: Effects of 5-thioglucose on A2 functional biomarker and AMPK activity. *Neuroscience* 2014; 269: 199–214. [PubMed: 24631866]
- Ibrahim BA, Tamrakar P, Gujar AD, Koshy Cherian A, Briski KP. Caudal fourth ventricular administration of the AMPK activator 5-aminoimidazole-4-carboxamide-riboside regulates glucose and counterregulatory hormone profiles, dorsal vagal complex metabolosensory neuron function, and hypothalamic Fos expression. *J. Neurosci. Res* 2013; 91: 1226–1238. [PubMed: 23825033]
- Irwin RW, Yao J, Hamilton RT, Cadenas E, Brinton RD, Nilsen J. Progesterone and estrogen regulate oxidative metabolism in brain mitochondria. *Endocrinology* 2008; 149: 3167–3175. [PubMed: 18292191]
- Kale AY, Paranjape SA, Briski KP. I.c.v. administration of the nonsteroidal glucocorticoid receptor antagonist, CP4–72555, prevents exacerbated hypoglycemia during repeated insulin administration. *Neuroscience* 2006; 140: 555–565. [PubMed: 16626867]
- Kostanyan A, Nazaryan K. Rat brain glycolysis regulation by estradiol-17 beta. *Biochim. Biophys. Acta* 1992; 1133: 301–3–6.
- Marino M, Galluzzo P, Ascenzi P. Estrogen signaling multiple pathways to impact gene transcription. *Curr. Genomics* 2006; 7: 497–508. [PubMed: 18369406]
- Meek TM, Nelson JT, Matsen ME, Dorfman MD, Guyenet SJ, Damian V, Allison MB, Scarlett JM, Nguyen HT, Thaler JP, Olson DP, Myers MG, Schwartz MW, Morton GJ. Functional identification of a neurocircuit regulating blood glucose. *Proc. Natl. Acad. Sci* 2016; 113: E2073–E2082. [PubMed: 27001850]
- Nedungadi TP, Briski KP. Site-specific effects of intracranial estradiol administration on recurrent insulin-induced hypoglycemia in ovariectomized female rats. *Neuroendocrinology* 2012; 96: 311–323. [PubMed: 22572755]
- Nilsen J, Irwin RW, Gallaher TK, Brinton RD. Estradiol *in vivo* regulation of brain mitochondrial proteome. *J. Neurosci* 2007; 27: 14069–14077. [PubMed: 18094246]
- Routh VH, Hao L, Santiago AM, Sheng Z, Zhou C. Hypothalamic glucose sensing: making ends meet. *Front. Syst. Neurosci* 2014; 8:236. doi: 10.3389/fnsys.2014.00236. [PubMed: 25540613]
- Schwartz MW, Sipols A, Kahn SE, Lattemann DF, Taborsky GJ Jr, Bergman RN, Woods SC, Porte D Jr. Kinetics and specificity of insulin uptake from plasma into cerebrospinal fluid. *Amer. J. Physiol* 1990; 259: E378–E383. [PubMed: 2205107]
- Simerly RB, Chang C, Muramatsu M, Swanson LW. Distribution of androgen and estrogen receptor mRNA-containing cells in the rat brain: an *in situ* hybridization study. *J Comp Neurol.* 1990; 294: 76–95. [PubMed: 2324335]
- Shrestha PK, Briski KP. Hindbrain lactate regulates preoptic gonadotropin-releasing hormone (GnRH) neuron GnRH-I protein but not AMPK responses to hypoglycemia in the steroid-primed ovariectomized female rat. *Neuroscience* 2015; 298:467–474. [PubMed: 25934033]
- Shrestha PK, Tamrakar P, Ibrahim BA, Briski KP. Hindbrain medulla catecholamine cell group involvement in lactate-sensitive hypoglycemia-associated patterns of hypothalamic norepinephrine and epinephrine activity. *Neuroscience* 2014; 278: 20–30. [PubMed: 25084049]
- Tamrakar P, Briski KP. Impact of recurrent hypoglycemic stress on metabolic signaling in dorsal vagal complex neurons: modulation by estradiol. *Acta Neurobiol. Exper* 2017; 77: 31–44.
- Tamrakar P, Shrestha PK, Briski KP. Dorsomedial hindbrain catecholamine regulation of hypothalamic astrocyte glycogen metabolic enzyme protein expression: Impact of estradiol. *Neuroscience* 2015; 292: 34–45. [PubMed: 25701713]
- You L, Yin J. Patterns of regulation from mRNA and protein time-series. *Metab. Eng* 2000; 2: 210–217. [PubMed: 11056063]

- Wallum BJ, Taborsky GJ Jr, Porte D Jr, Figlewicz DP, Jacobson L, Beard JC, Ward WK, Dorsa D. Cerebrospinal fluid insulin levels increase during intravenous insulin infusions in man. *J Clin Endocrinol Metab.* 1987; 64: 190–194. [PubMed: 3536982]
- Watts AG, Donovan CM. Sweet talk in the brain: glucosensing, neural networks, and hypoglycemic counterregulation. *Front. Neuroendocrinol* 2010; 31: 32–43. [PubMed: 19836412]
- Zhou HB, Carlson KE, Stossi F, Katzenellenbogen BS, Katzenellenbogen JA. Analogs of methylpiperidinopyrazole (MPP): antiestrogens with estrogen receptor alpha selective activity. *Bioorg. Med. Chem. Lett* 2009; 19: 108–110. [PubMed: 19014882]

Highlights:

- Estrogen receptor-alpha (ER α) or -beta (ER β) antagonist was injected *icv* to hypoglycemic female rat hindbrain (HB).
- HB ER α governs ventromedial hypothalamic nucleus (VMN) glycogen enzyme protein levels; ER β controls VMN glycogen mass.
- HB ERs regulate VMN transmitters that signal metabolic stability, e.g. γ -aminobutyric acid and nitric oxide.
- Hypoglycemic diminution of VMN norepinephrine content is HB ER-independent.
- HB ER α and - β oppose hypoglycemic hyperglucagonemia, while ER β suppresses corticosterone output.

Figure 1. Effects of Caudal Fourth Ventricular (CV4) Administration of the ER α Antagonist 1,3-Bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole dihydrochloride (MPP) or ER β Antagonist 4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl]phenol (PHTPP) on Ventromedial Hypothalamic Nucleus (VMN) Metabolic Neurotransmitter Marker Protein Responses to Insulin-Induced Hypoglycemia (IIH) in the Female Rat.

Micropunch-dissected VMN tissue obtained from estradiol-implanted ovariectomized female rats pretreated by CV4 delivery of MPP, PHTPP, or vehicle prior to *sc* insulin (INS) injection was pooled within treatment groups for Western blot analysis of glutamate decarboxylase_{65/67} (GAD_{65/67}; Panel A), neuronal nitric oxide synthase (nNOS; Panel B), steroidogenic factor-1 (SF-1; Panel C), or brain-derived neurotrophic factor (BDNF; Panel D). Data depict mean normalized protein optical density (O.D.) values $\pm$ S.E.M. for vehicle-pretreated animals injected *sc* with vehicle- (V/V; solid white bars; n=5) or INS (V/INS;

solid gray bars; n=5) or INS- injected rats pretreated with MPP (MPP/INS; diagonal-striped gray bars; n=5) or PHTPP (PHTPP/INS; cross-hatched gray bars; n=5. *p<0.05; **p<0.01; ***p<0.001.

Figure 2. Impact of DVC ER α or ER β Antagonism on Hypoglycemic Patterns of VMN Glycogen Synthase (GS) and Glycogen Phosphorylase (GP) Protein Expression and Tissue Glycogen and Glucose Concentrations.

Panels A and B depict mean normalized GS and GP O.D. values $\pm$ S.E.M., respectively, from triplicate Western blot analyses of pooled VMN tissue from V/V (solid white bars), V/INS (solid gray bars), MPP/INS (diagonal-striped gray bars), and PHTPP/INS (cross-hatched gray bars) treatment groups. Panels C and D illustrate effects of INS in the presence or absence of pharmacological blockade of DVC ER on mean HPLC measures of VMN tissue glycogen and glucose content $\pm$ S.E.M., respectively, for $n=5$ animals per treatment group. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Figure 3. Effects of DVC ER α or ER β Antagonism on Hypoglycemic Site-Specific Norepinephrine (NE) Accumulation in Hypoglycemic Female Rats.

Micropunches of the VMN (Panel A), arcuate hypothalamic nucleus (Panel B), dorsomedial hypothalamic nucleus (DMN), and lateral hypothalamic area (LHA) were analyzed by ELISA methods for assessment of tissue NE content. Data depict mean NE levels + S.E.M. for V/V (solid white bars; n=5), V/INS (solid gray bars; n=5), MPP/INS (diagonal-striped gray bars; n=5), and PHTPP/INS (cross-hatched gray bars; n=5) treatment groups. *p<0.05; **p<0.01; ***p<0.001.

Figure 4. CV4 MPP versus PHTPP Pretreatment on Glucose, Glucagon, Corticosterone, and Free Fatty Acid (FFA) Responses to Insulin Injection of Female Rats.

Data show circulating glucose (Panel A), glucagon (Panel B), corticosterone (Panel C), and FFA (Panel D) levels + S.E.M. levels in V/V (solid white bars; n=5), V/INS (solid gray bars; n=5), MPP/INS (diagonal-striped gray bars; n=5), and PHTPP/INS (cross-hatched gray bars; n=5) treatment groups. *p<0.05; **p<0.01; ***p<0.001.