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Effects of Estradiol on Lactoprivic Signaling of the Hindbrain upon the Contraregulatory Hormonal Response and Metabolic Neuropeptide Synthesis in Hypoglycemic Female Rats

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Abstract

Background—Caudal dorsomedial hindbrain detection of hypoglycemia-associated lactoprivation regulates glucose counter-regulation in male rats. In females, estradiol (E) determines hypothalamic neuroanatomical and molecular foci of hindbrain energy sensor activation. This study investigated the hypothesis that E signal strength governs metabolic neuropeptide and counter-regulatory hormone responses to hindbrain lactoprivic stimuli in hypoglycemic female rats.

Methods—Ovariectomized animals were implanted with E-filled silastic capsules [30 (E-30) or 300 ug (E-300)/mL] to replicate plasma concentrations at estrous cycle nadir versus peak levels. E-30 and E-300 rats were injected with insulin or vehicle following initiation of continuous caudal fourth ventricular L-lactate infusion.

Results—Hypoglycemic hypercorticonemia was greater in E-30 versus E-300 animals. Glucagon and corticosterone outflow was correspondingly fully or partially reversed by hindbrain lactate infusion. Insulin-injected rats exhibited lactate-reversible augmentation of norepinephrine (NE) accumulation in all preoptic/hypothalamic structures examined, excluding the dorsomedial hypothalamic nucleus (DMH) where hindbrain lactate infusion either suppressed (E-30) or enhanced (E-300) NE content. Expression profiles of hypoglycemia-reactive metabolic neuropeptides were normalized (with greater efficacy in E-300 animals) by lactate infusion. DMH RFamide-related peptide-1 and -3, arcuate neuropeptide Y and kisspeptin, and ventromedial nucleus nitric oxide synthase protein responses to hypoglycemia were E dosage-dependent.

Conclusions—Distinct physiological patterns of E secretion characteristic of the female rat estrous cycle elicit differential corticosterone outflow during hypoglycemia, and establish both common and different hypothalamic metabolic neurotransmitter targets of hindbrain lactate deficit

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signaling. Outcomes emphasize a need for insight on systems-level organization, interaction, and involvement of E signal strength-sensitive neuropeptides in counter-regulatory functions.

Keywords

estradiol; L-lactate; norepinephrine; RFamide-related peptide-1/-3; neuropeptide Y; pre-pro-kisspeptin; nitric oxide synthase

Introduction

Insulin-induced hypoglycemia (IIH) is a recurring complication of rigorous pharmacotherapeutic management of type I diabetes mellitus that deprives the brain of sufficient energy fuel provision. The nervous system responds to hypoglycemia by hypothalamic coordination of counter-balancing autonomic, neuroendocrine, and behavioral outflow that alleviates neuro-glucopenia. The hypothalamus is informed of neuro-metabolic instability by specialized intra- and extra-hypothalamic neuron populations that adjust synaptic firing in reaction to diminished energy substrate availability. The caudal dorsomedial hindbrain is a vital source of metabolic sensory input as local deficits of the oxidizable glycolytic end-product L-lactate trigger neural mechanisms that elevate blood glucose [Patil and Briski, 2005]. Lactate delivery to this area during hypoglycemia intensifies glucose decrements while normalizing hypothalamic hypoglycemia-sensitive metabolic neuropeptide expression, which denotes influence of local energy status on downstream hypothalamic elements of the brain gluco-regulatory network [Gujar et al., 2014]. Caudal hindbrain A2 noradrenergic neurons likely detect energetic sequelae of hypoglycemia as 5' adenosine monophosphate-activated protein kinase (AMPK) is activated in a lactate-reversible manner in this (but not other) hindbrain catecholamine cell group, alongside augmented hypothalamic norepinephrine (NE) levels [Shrestha et al., 2014].

Estradiol (E) controls metabolic status in female mammals through regulation of energy procurement, ingestion, metabolism, partitioning, storage, and expenditure [Wade and Schneider, 1992]. A2 cells express estrogen receptor-alpha and -beta proteins [Ibrahim et al., 2013] and metabolo-sensory biomarkers, e.g. glucokinase, K_{ATP} , and AMPK [Briski et al., 2009; Cherian and Briski, 2011; Ibrahim et al., 2013], which supports a likely function to input E influence on the gluco-regulatory network. E governs A2 neuron and hypothalamic nucleus AMPK, hypothalamic metabolic neurotransmitter, and counter-regulatory hormone responses to caudal hindbrain delivery of the AMP mimic 5-aminoimidazole-4-carboxamide-riboside (AICAR) in ovariectomized (OVX) female rats [Ibrahim et al., 2013; Alenazi et al., 2014; Ibrahim and Briski, 2014]. Endogenous E secretion fluctuates over the female rat estrous cycle; a 4- to 5-fold mid-cycle rise in plasma hormone levels from baseline (metestrus-/diestrus I-stage) to peak concentrations (proestrus-stage) [Butcher et al., 1974] transforms E feedback to the hypothalamic-pituitary-gonadal (HPG) axis from positive to negative. This project addressed the premise that opposite extremes of estrous cycle E secretion establish unique forebrain neuroanatomical and molecular foci of IIH-associated hindbrain lactoprivic signaling in female rats, and that disparities variation may coincide with differential counter-regulatory hormone outflow. OVX animals were implanted with subcutaneous (*sc*) E-filled silastic capsules designed to replicate circulating

E levels at estrous cycle baseline versus maximal concentrations [Goodman, 1978; Briski et al., 2001] in advance of *sc* insulin injection and concurrent caudal fourth ventricular infusion of artificial cerebrospinal fluid with or without L-lactate. Hypothalamic gluco-regulatory structures characterized by hindbrain lactoprivic-driven augmentation of NE accretion in hypoglycemic male rats [Shrestha et al., 2014] were micropunch-dissected for norepinephrine (NE) ELISA and Western blot analyses of relevant gluco-regulatory neurotransmitter protein expression, including glutamate decarboxylate_{65/67} (GAD_{65/67}) [ventromedial nucleus (VMH)], corticotropin-releasing hormone (CRH) [paraventricular nucleus (PVH)], neuropeptide Y (NPY) and pro-opiomelanocortin (POMC) [arcuate nucleus (ARH)] and orexin-A (ORX-A) [lateral hypothalamic area (LHA)]. Reproduction is tightly coupled to metabolic status, as gonadotropin-releasing hormone (GnRH) release is diminished by energy shortage [Clarke et al., 1990; Chen et al., 1992; Chen et al., 1992; Heisler et al., 1993; Singh and Briski, 2005]. IHH inhibits gonadotropin secretion in female mammals [Clarke et al., 1990; Goubillon and Thalabard, 1996; Cagampang et al., 1997; He et al., 1999; [Lado-Abeal et al., 2002]. A corollary aim of this study was to investigate the impact of E signal strength on lactoprivic regulation of NE activity and reproduction-relevant neurotransmitter protein expression in preoptic loci involved in female reproduction, e.g. rostral preoptic area (rPO) [gonadotropin-releasing hormone (GnRH)], anteroventral periventricular nucleus (AVPV) [prepro-kisspeptin], and medial preoptic nucleus (MPN), [GAD_{65/67}].

Methods and Materials

Animals

Adult female Sprague-Dawley rats (3-4 months of age) were maintained in groups (2-3 animals per cage) under a 14-h light/10-h dark lighting schedule (light on at 05:00h), while allowed free access to standard laboratory rat chow (Harlan Teklad LM-485; Harlan Industries, Madison, WI, USA) and tap water. Animals were accustomed to daily handling for a minimum of one week before surgery. All protocols were conducted in accordance with NIH guidelines for care and use of laboratory animals, under ULM Institutional Animal Care and Use Committee approval.

Experimental Design

On day 1, rats were bilaterally OVX and implanted with a 26-gauge stainless-steel cannula guide (Prod no. C315G/SPC; Plastic One, Inc., Roanoke, VA) aimed at the caudal fourth ventricle (CV4) [Coordinates: 0 mm lateral to midline; 13.3 mm posterior to *bregma*; 6.1 mm ventral to skull surface] under ketamine/xylazine anesthesia (0.1mL/100 g *bw ip*, 90 mg ketamine: 10 mg xylazine/mL; Henry Schein, Melville, NY). After surgeries, animals were treated by intramuscular injection of enrofloxacin (baytril 2.27%; 10 mg/kg) and subcutaneous (*sc*) injection of ketoprofen (3 mg/kg), then transferred to individual cages. On day 7, rats received a *sc* silastic capsule (10 mm/100 g *bw*, 0.062 in. i.d., 0.125 in. o.d.) filled with 30 [E-30] or 300 [E-300] µg estradiol benzoate/mL safflower oil under isoflurane anesthesia. Our studies [Briski et al., 2001] show that these disparate E doses replicate circulating physiological hormone levels measured on metestrus versus proestrus, respectively, in 4-day cycling rats [Butcher et al., 1974]. On day 11, E-30 and E-300 animals

were each divided into 3 treatment groups (n=5/group). Continuous intra-CV4 infusion of artificial cerebrospinal fluid (aCSF; groups 1 and 2) or aCSF containing L-lactate (L; 25μM/2.0μL/hr [Patil and Briski, 2005]; group 3) was performed between 08.50 and 11.00 hr, using 33-gauge 0.5 mm-projecting internal injection cannulas (prod. no. C315I/SPC; Plastics One). At 09.00 hr, rats in group 1 were injection *sc* with sterile vehicle (V; Eli Lilly & Co., Indianapolis, IN), while animals in groups 2 and 3 were treated by injection of neutral protamine Hagedorn insulin (INS; 12.5 U/kg *bw* [Paranjape and Briski, 2005]; Henry Schein). Animals were sacrificed by decapitation at 11.00 hr for brain and blood collection. Dissected brains were immediately snap-frozen in liquid nitrogen-cooled isopentane and stored at -80°C. Plasma was obtained by immediate centrifugation and stored at -20°C.

Western Blot Analysis of AMPK, phosphoAMPK (pAMPK), and Metabolic Neurotransmitter Protein Expression in Hypothalamic Gluco-Regulatory Loci

Forebrains were cut into serial 100 μm-thick frozen sections. The rPO (+0.48 to 0.00 mm), AVPV (0.00 to -0.30 mm), MPN (-0.20 to -0.06 mm), ARH (-2.00 to -3.20 mm), VMH (-2.00 to -3.20 mm), DMH (-2.40 to -3.20 mm), and LHA (-2.40 to -3.60 mm) were separately micro-punch dissected from the right hemi-forebrain, over pre-determined distances relative to *bregma*, and collected into separate 20 μL volumes of lysis buffer [2.0% sodium dodecyl sulfate (SDS), 0.05 M dithiothreitol, 10.0% glycerol, 1.0 mM EDTA, 60 mM Tris-HCl, pH 7.2]. Tissue samples were obtained using Stoelting (Kiel, WI) calibrated hollow punch tools of 0.50 mm (rPO, AVPV, MPN, ARH, VMH, DMH) or 0.76 mm (LHA) diameter. For each treatment group, heat-denatured tissue aliquots from individual subjects were combined and separated on 10–15% gradient Tris-glycine gels (90 V, 105 min; Tris-glycine SDS running buffer) [Cherian and Briski, 2011, 2012]. Proteins were transblotted (30 V, overnight at 4°C; Towbin buffer) to 0.45-μm PVDF membranes (Osmonics, Gloucester, MA). Membranes were pretreated with Western blotting signal enhancer (Pierce, Rockford, IL), blocked for 2 hr with Tris-buffer saline (TBS), pH 7.4, containing either 0.1 % Tween-20 (Sigma Aldrich, St. Louis, MO) and 2% bovine serum albumin (MP Biomedicals, Solon, OH) or 5% normal donkey serum, then incubated overnight at 4°C with primary antisera. Proteins of interest were probed with primary polyclonal antisera raised in rabbit against NPY (1:1500, NBP1-46535; Novus Biologicals, LLC, Littleton, CO), POMC (1:1000, H0000543-D01; Abnova Corp. Walnut, CA), GnRH-I (1:1000, sc-20941; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), RFamide related peptide-1 (RFRP-1; 1:1000, sc-67010; Santa Cruz Biotechnol.), or neuronal nitric oxide synthase (nNOS; 1:1000, sc-648; Santa Cruz Biotechnol.) or raised in goat against prepro-kisspeptin (1:1000, sc-18134; Santa Cruz Biotechnol.), GAD_{65/67} (1:1000, sc-7513; Santa Cruz Biotechnol.), corticotropin-releasing hormone (CRH; 1:1000, sc-1759; Santa Cruz Biotechnol.), RFamide related peptide-3 (RFRP-3; 1:1000, sc-32380; Santa Cruz Biotechnol.), ORX-A (1:1000, sc-8072; Santa Cruz Biotechnol.), or melanin-concentrating hormone (MCH; 1:1000, sc-14509; Santa Cruz Biotechnol.). The housekeeping protein α-tubulin was detected with mouse monoclonal antibodies (1:1,000; EMD Millipore, Billerica, MA). Membranes were incubated for 1 hr with peroxidase-conjugated goat anti-mouse (1:5,000; PerkinElmer, Boston, MA), goat anti-rabbit (1:5,000; PerkinElmer), or donkey anti-goat (1:5,000; sc-2020; Santa Cruz Biotechnol.) secondary antisera. After incubation with Supersignal West Femto maximum sensitivity chemiluminescent substrate (ThermoFisherScientific,

Rockford, IL), signals were visualized in a Syngene G:Box Chemi (Syngene, Frederick, MD). Protein band optical densities (O.D.) were quantified with Genetool 4.01 software (Syngene) and expressed relative to α -tubulin. Immunoblots were performed in triplicate at minimum for each target protein. Protein molecular weight markers were included in each Western blot analysis.

ELISA Analysis of NE Content of Hypothalamic Gluco-Regulatory Loci

For each animal, micropunch samples of individual neural loci described above were removed from the left hemi-forebrain, homogenized in 100 μ L volumes of 0.01 N HCl containing 1 mM EDTA and 4 mM sodium metabisulfite, sonicated, and stored at -80°C . Sample aliquots were analyzed using Noradrenaline Research ELISATM kit reagents (Labor Diagnostika Nord GmbH & Co KG, Nordhorn, Germany) [Shrestha et al., 2014]. Briefly, standards (10 μ L), controls (10 μ L) and sample aliquots (25 μ L) were pipetted in duplicate into individual extraction plate wells, then diluted to 100 μ L with distilled water. Plates were sequentially incubated by shaking at 600 rpm with TE buffer (25 μ L), acylation buffer (150 μ L) plus acylation reagent (25 μ L), and hydrochloric acid (100 μ L). 90 μ L of extracted samples, standards, and controls were transferred to individual microtiter plate wells, mixed with enzyme solution (25 μ L), and incubated for 37°C for 2 hr. Sample, standard, and control wells aliquots (100 μ L) were transferred to pre-coated Noradrenaline Microtiter strip wells, mixed with 50 μ L of primary antiserum, then incubated for 18 hr at $2-8^{\circ}\text{C}$. After plate contents were discarded, wells were washed 4X, then incubated with enzyme conjugate (100 μ L) for 30 min. Wells were emptied, washed 4X, then incubated for 30 min with 100 μ L substrate. After termination of reactions with 100 μ L stop solution, absorbance of well contents was read at 450 nm in an Emax Precision Microplate Reader (Molecular Devices, LLC, Sunnyvale, CA, USA).

Blood Analyte Measurements

Blood glucose levels were measured using an Accu-Check Aviva Plus glucometer (Roche Diagnostics, Indianapolis, IN; Kale et al., 2006). Plasma glucagon (EMD Millipore, Billerica, MA) and corticosterone (MP Biomedicals, Santa Ana, CA) concentrations were analyzed by radioimmunoassay, as described elsewhere (Briski and Nedungadi, 2009).

Statistics

Mean glucose, hormone, NE, and normalized protein O.D. values were evaluated by two-way analysis of variance and Student Newman Keul's test, using Graphpad Prism software. Differences of $p < 0.05$ were deemed significant.

Results

Data depicted in Figure 1 illustrate effects of IIH with or without coincident hindbrain L-lactate (L) infusion on circulating glucose (Panel 1A), glucagon (Panel 1B), corticosterone (Panel 1C), and leptin (Panel 1D) levels in OVX rats implanted with *sc* silastic capsules containing 30 μ g (E-30) versus 300 μ g (E-300) E/mL. INS injection caused a similar decline in blood glucose in E-30 and E-300 animals. In both E dosage groups, circulating glucose levels did not differ between CV4 V/*sc* INS (V/INS)- versus CV4 L/*sc* INS (L/INS)-treated

rats [$F_{5,24} = 36.40$, $p < 0.0001$; INS effect: $F = 33.60$, $p < 0.0001$]. IIH elevated plasma glucagon concentrations to a similar degree in the presence of nadir versus peak estrous cycle E levels (Panel 1B) [$F_{5,24} = 6.97$, $p < 0.001$; INS effect: $F = 33.60$, $p < 0.0001$]. This hormone profile was normalized by CV4 lactate infusion to hypoglycemic E-30 and E-300 rats. As shown in Panel 1C, baseline plasma corticosterone levels were lower in E-300 versus E-30 CV4 V/sc V (V/V) treatment groups [$F_{5,24} = 25.53$, $p < 0.0001$; INS effect: $F = 87.51$, $p < 0.0001$; E effect: $F = 22.05$, $p < 0.0001$]. Hypercorticonemia was elicited by INS injection, albeit to a higher magnitude in E-30 rats. Hindbrain lactate repletion partially regularized patterns of corticosterone release in hypoglycemic rats treated with either E dosage. Results presented in Panel 1D indicate that IIH elevated plasma leptin levels in E-300, but not E-30 animals; hormone release was equivalent in V- versus L-infused INS-injected rats given either dose of E [$F_{5,24} = 5.36$, $p < 0.005$; INS effect: $F = 23.41$, $p < 0.0001$].

Figure 2 illustrates effects of hindbrain L-lactate infusion to INS-injected low E- versus high E-dosed OVX female rats on tissue NE levels in micro-punch dissected rPO (Panel 2A; $F_{5,24} = 11.66$, $p < 0.0001$; INS effect: $F = 34.47$, $p < 0.0001$; E effect: $F = 18.50$, $p < 0.0001$), AVPV (Panel 2B; $F_{5,24} = 9.79$, $p < 0.0001$; INS effect: $F = 20.01$, $p < 0.0001$; E effect: $F = 11.54$, $p < 0.005$), MPN (Panel 2C; $F_{5,24} = 33.62$, $p < 0.0001$; INS effect: $F = 27.42$, $p < 0.0001$; E effect: $F = 83.31$, $p < 0.0001$), ARH (Panel 2D; $F_{5,24} = 76.42$, $p < 0.0001$; INS effect: $F = 156.01$, $p < 0.0001$; E effect: $F = 172.53$, $p < 0.0001$), DMH (Panel 2E; $F_{5,24} = 22.77$, $p < 0.0001$; INS effect: $F = 51.42$, $p < 0.0001$; E effect: $F = 40.82$, $p < 0.0001$), LHA (Panel 2F; $F_{5,24} = 19.53$, $p < 0.0001$; INS effect: $F = 22.02$, $p < 0.0001$; E effect: $F = 61.58$, $p < 0.0001$), PVH (Panel 2G; $F_{5,24} = 12.89$, $p < 0.0001$; INS effect: $F = 25.31$, $p < 0.0001$; E effect: $F = 15.02$, $p < 0.001$), and VMH (Panel 2H; $F_{5,24} = 78.72$, $p < 0.0001$; INS effect: $F = 18.53$, $p < 0.0001$; E effect: $F = 320.09$, $p < 0.0001$). In each structure, NE accumulation was significantly lower in V/V-treated E-300 versus E-30 rats. IIH stimulated NE accretion in each location (V/INS versus V/V) in E-30 and E-300 animals, excepting the DMH where NE content was elevated only in hypoglycemic E-30 rats. In all other sites, hypoglycemic augmentation of NE was greater in low E- versus high E-treated rats. In E-30 rats, hindbrain lactate infusion to hypoglycemic animals normalized NE content to the euglycemic range in all locations, apart from the MPN (where this treatment decreased NE below controls levels) and ARH (where regularization was only partial). In E-300 animals, L/INS treatment standardized NE accumulation in each location excluding the DMH, where levels were elevated above controls.

Figure 3 illustrates effects of IIH with or without hindbrain L-lactate infusion on expression profiles of preoptic proteins relevant to reproduction in E-30 versus E-300 rats. As shown in Panels 3A and 3B, respectively, rPO GnRH-I [$F_{5,12} = 39.59$, $p < 0.0001$; INS effect: $F = 128.52$, $p < 0.0001$; E effect: $F = 9.56$, $p < 0.01$; INS plus E interaction: $F = 8.34$, $p < 0.01$] and AVPV prepro-kisspeptin [$F_{5,18} = 46.62$, $p < 0.0001$; INS effect: $F = 72.24$, $p < 0.0001$; E effect: $F = 87.30$, $p < 0.001$; INS plus E interaction: $F = 30.39$, $p < 0.0001$] content was significantly decreased in V/INS versus V/V groups of E-30 and E-300 rats. Both proteins were lower in E-30 V/I versus E-300 VI groups. Hindbrain lactate repletion fully prevented hypoglycemia-associated repression of both proteins irrespective of E dosage groups. Data in Panel 3C indicate that MPN GAD_{65/67} protein profiles were suppressed to equal levels in E-30 and E-300 rats by IIH, and that expression levels were normalized or elevated above control

levels by hindbrain lactate infusion in E-30 versus E300 animals, respectively [$F_{5,18} = 13.62$, $p < 0.0001$; INS effect: $F = 17.38$, $p < 0.0001$].

Figures 4–6 depict effects of lactate on ARH (Figure 4), DMH/VMH (Figure 5), and LHA/PVH (Figure 6) gluco-regulatory protein expression in hypoglycemic E-30 versus E-300 animals. IIH caused lactate-reversible augmentation of ARH NPY protein expression in E-300, but not E-30 rats (Panel 4A) [$F_{5,18} = 11.36$, $p < 0.0001$; INS effect: $F = 10.50$, $p < 0.005$; E effect: $F = 33.39$, $p < 0.001$], but at the same time elevated tissue prepro-kisspeptin levels in low E-, but not high E-dose groups (Panel 4B) [$F_{5,18} = 27.82$, $p < 0.0001$; INS effect: $F = 40.06$, $p < 0.0001$; E effect: $F = 43.78$, $p < 0.0001$]. Data in Panel 4C disclose that POMC protein profiles were refractory to IIH at either E dosage level [$F_{5,24} = 9.62$, $p < 0.0001$; E effect: $F = 38.74$, $p < 0.001$].

Panels A and B of Figure 5 indicate that in E-30 and E-300 animals, RFRP-1 [$F_{5,12} = 14.37$, $p < 0.0001$; INS effect: $F = 10.63$, $p < 0.0001$; E effect: $F = 47.28$, $p < 0.0001$] and -3 [$F_{5,18} = 11.64$, $p < 0.0001$; E effect: $F = 8.58$, $p < 0.01$] proteins were correspondingly increased by IIH, and that these responses were reversed by L/INS. E-300 treated with L plus INS exhibited diminished RFRP-3 profiles compared to V/V controls. Data in Panel C show that lactate infusion partially or completely reversed hypoglycemic inhibition of VMH GAD_{65/67} content in E-30 versus E-300 animals, respectively. GAD_{65/67} protein was significantly lower in V/INS E-30 versus E-300 rats [$F_{5,18} = 27.21$, $p < 0.0001$; INS effect: $F = 72.23$, $p < 0.0001$; E effect: $F = 8.83$, $p < 0.01$; INS plus E interaction: $F = 11.01$, $p < 0.005$]. As shown in Panel D, IIH elevated VMH nNOS protein expression by lactoprivic-contingent mechanisms in E-300, but not E-30 animals [$F_{5,18} = 15.67$, $p < 0.0001$; INS effect: $F = 10.50$, $p < 0.005$; E effect: $F = 40.05$, $p < 0.0001$]. In E-30 rats, L/INS treatment suppressed nNOS profile relative to V/INS.

In both E-30 and E-300 rats, LHA ORX-A (Figure 6, Panel A) and MCH (Figure 6, Panel B) protein levels were inversely altered [$\uparrow$ ORX-A; $\downarrow$ MCH] after I injection. In E-30, L/INS treatment fully normalized ORX-A [$F_{5,18} = 20.74$, $p < 0.0001$; INS effect: $F = 34.57$, $p < 0.0001$; E effect: $F = 23.33$, $p < 0.0001$], and MCH [$F_{5,12} = 55.20$, $p < 0.0001$; INS effect: $F = 206.53$, $p < 0.0001$; E effect: $F = 33.48$, $p < 0.0001$] expression, whereas this treatment suppressed ORX-A levels below V/V controls levels in E-300. As shown in Figure 6C, IIH elevated PVH CRH protein expression in both E-30 and E-300 animals [$F_{5,18} = 20.28$, $p < 0.0001$; INS effect: $F = 19.36$, $p < 0.0001$; E effect: $F = 15.23$, $p < 0.001$]. Lactate infusion to hypoglycemic rats normalized this profile in E-30, but diminished CRH protein levels below V/V baseline levels in E-300.

Discussion

Caudal dorsomedial hindbrain signals of lactate availability impact glycemic control in rats of both sexes [Patil and Briski, 2005; Vavaiya and Briski, 2008]. Current research investigated whether negative- versus positive-feedback patterns of E release associated with the female rat estrous cycle establish unique forebrain neuroanatomical and molecular targets of hindbrain lactoprivic signaling in hypoglycemic female rats, and that such disparities may correlate with differential counter-regulatory responses to IIH. Results show

that IIH elicits lactate-reversible increases in NE accumulation in each preoptic/hypothalamic structure examined. The DMH was the sole target of bi-directional lactoprivic regulation of NE as lactate repletion suppressed or enhanced NE content in the presence of baseline versus peak plasma E levels, respectively. Expression profiles of forebrain hypoglycemia-reactive metabolic neuropeptides, which varied according to E dosage for specific neurochemicals, were also normalized (with greater efficacy in E-300 animals) by lactate infusion to insulin-injected rats, alongside impedance of glucagon and corticosterone secretion. Outcomes show that hindbrain lactate status regulates hypoglycemic patterns of NE activity in the female rat preoptic area and hypothalamus, unlike males in which this signaling is confined to the hypothalamus [Shrestha et al., 2014]. Dissimilar patterns of E release typical of the adult female rat estrous cycle evidently determine both common and unique forebrain metabolic neurotransmitter targets of hindbrain lactate deficit signaling. It remains unclear if E concentration-reliant neurochemical responses identified here uniquely regulate gluco-regulatory and/or reproductive responses, as well as non-metabolic functions regulated by those neurotransmitters.

Data show that hindbrain lactate repletion normalizes IIH-associated NE accretion and hypoglycemia-sensitive transmitter/biosynthetic enzyme protein expression in forebrain loci implicated in glucostasis and/or reproduction in female rats. Current results disclose similarities and differences in IIH-related hindbrain lactoprivic regulation of hypothalamic and preoptic NE activity, respectively, between female and male rats [Shrestha et al., 2014]. The 'critical period' of CNS differentiation during early neonatal life establishes a structurally- and functionally-dimorphic network that controls reproductive endocrine and behavioral functions in a sex-specific manner [Arnold and Gorski, 1984; Gorski, 1986]. Multiple preoptic structures, including the MPN and AVPV, are major brain substrate/biomarkers of endogenous E imprinting and integral anchor components of the neural circuitry that regulates sex-specific patterns of reproductive behavior and hormone secretion [Simerly, 2002]. It is reasonable to presume that female-specific hindbrain lactoprivic signaling to these and other preoptic loci is a consequence of sex-dimorphic organization of reproductive neural networks. In each forebrain structure examined here, baseline NE levels were significantly higher in E-30 versus E-300 animals; the magnitude of IIH augmentation of NE accumulation was likewise greater in the former group. Likely differences in intensity of lactoprivic-drive NE activity in individual sites may play a role in one or more instances of E dosage-reliant neuropeptide/enzyme protein responses to hypoglycemia observed here. As individual hypothalamic nuclei and areas exhibit neurochemical heterogeneity, it is should be noted that IIH may discriminatively impact noradrenergic input to distinctive neurotransmitter populations in each site. Micro-punch dissection affords substantial improvement in resolution compared to measures of whole-hypothalamus, but we cannot discount the possibility that averaged measurements reported here may obscure small local opposite-direction changes in NE tone that in turn regulate neurotransmitters that impact glucostasis or other functions. Current research emphasizes the ever-present need for high-resolution analytical techniques for quantification of NE input to specific cell targets. The DMH was unique among analyzed structures in that local NE levels were modified by IIH only in low E dose rats; moreover, hindbrain lactate infusion exerted opposite effects, e.g. inhibitory (E-30) versus stimulatory (E-300), on DMH NE activity. A plausible explanation

is that in the presence of peak circulating E, DMH versus other loci may be innervated by two distinctive noradrenergic cell populations, respectively characterized by insensitivity versus sensitivity to IIH; alternatively, maximum strength E signal may act on DMH substrates to stabilize NE tissue content despite hypoglycemia-driven increases in hindbrain noradrenergic signaling. Prior studies showed that DMH NE content was refractory to IIH in male rats, which raises the question of whether local metabolic-sensitive neurotransmitters, e.g. RFRP-1 and -3, respond to IIH only in females, or alternatively, if these neuropeptides are regulated by non-noradrenergic mechanisms in males.

Expression profiles of hypoglycemia-reactive forebrain proteins involved in gluco-regulation and/or reproduction were normalized by lactate infusion, albeit with greater efficacy in E-300 animals. Regardless of E dose, IIH decreased rPO GnRH, AVPV prepro-kisspeptin, MPN and VMH GAD_{65/67}, and LHA MCH protein profiles, while amplifying PVH CRH and LHA ORX-A expression; each of these adjustments was overturned by hindbrain lactate repletion, albeit with greater efficacy in E-300 animals. Notably, prepro-kisspeptin, MCH, and CRH profiles were significantly greater in insulin-injected E-300 versus E-30 animals. Conversely, DMH RFRP-1 and -3, ARH NPY and prepro-kisspeptin, and VMH nNOS protein responses to hypoglycemia were contingent on E dosage. Nadir versus peak estrous cycle E output may thus establish joint alongside distinguishing hypothalamic metabolic neurotransmitter targets of hindbrain lactate deficit signaling during hypoglycemia. We previously reported that IIH modifies ARH, but not AVPV prepro-kisspeptin profiles during the afternoon LH surge induced by E positive-feedback [Briski and Shrestha, 2016], but describe here a reduction in AVPV prepro-kisspeptin content in E-30 and E-300 rats injected with INS in the morning. Thus, when E is released at peak levels, circadian rhythm may determine which KiSS1 neuron population (AVPV vs ARH) is inhibited by IIH.

E-30 animals exhibited lactate-reversible augmentation of DMH RFRP-1, but not RFRP-3 profiles during IIH, while insulin injection of E-300 rats caused lactate-dependent intensification of RFRP-3 protein, but not RFRP-1. Since RFRP stimulates feeding [Ubuka et al., 2016], findings suggest that different RFRP cell populations may regulate feeding responses to energy shortage according to magnitude of E. Evidence for IIH-induced up-regulation of DMH RFRP-3 in the absence of increased NE activity implies that this protein may be subject to A2-driven non-catecholaminergic signals. Multiple neuropeptide transmitters, e.g. NPY, neurotensin, and dynorphin, are co-expressed with NE in distinct A2 subpopulations [Rinaman, 2011]; it would thus be beneficial to determine if one or more of these neurochemical(s) impose hindbrain control of this DMH protein target during peak E secretion, and to ascertain if that/those neurotransmitter(s) derive from A2 neurons or alternative hindbrain noradrenergic cell groups.

Results show that VMH nNOS protein expression is elevated in E-300, but not E-30 animals during IIH. NOS-directed yield of the gaseous neurotransmitter nitric oxide is vital for increased firing of medial-basal hypothalamic 'glucose-inhibitory' (GI) metabolic-sensory neurons during neuro-glucopenia and for optimal counter-regulatory hormone release *in vivo* [Routh et al., 2014]. Present data raise the intriguing prospect that VMH GI signaling of energy shortage may be comparatively enhanced when estrous cycle E secretion is at maximal versus baseline levels. Ventromedial hypothalamic γ -aminobutyric acid (GABA)

neurons function as ‘glucose-excited’ (GE) metabolic sensors [Zhu et al., 2010]. As IIH inhibition of VMH GAD_{65/57} protein occurred in both E30 versus E300 groups, GE detection of metabolic imbalance is presumably refractory to circulating E.

Current data show that IIH simultaneously augmented or decreased expression of the orexigenic LHA neurotransmitters ORX-A [Tsuino and Sakurai, 2013] and MCH [Georgescu et al., 2005; Pissios et al., 2006], results that align with evidence that neurons that synthesize the former, but not the latter neuropeptide undergo transcriptional activation in response to decreased brain glucokinase activity [Zhou et al., 2011] or IIH [Nishimura et al., 2014]. Collectively, these data support a role for ORX-A, but not MCH in metabolic hyperphagia.

MPN GAD_{65/67} protein expression in hypoglycemic E-30 versus E-300 rats was investigated because GABAergic neurons in this site and the neighboring AVPV are uniquely reactive to dual modes of E feedback within the preoptic area [Curran-Rauhut and Petersen, 2002]. Previous reports that hindbrain glucose anti-metabolite administration stimulates GABA release [Singh and Briski, 2004] and that preoptic GABA_A receptors mediate neuro-glucoprivic inhibition of reproductive neuroendocrine function [Singh and Briski, 2005] led us to hypothesize augmentation of MPN GAD_{65/67} protein during IIH, possibly in a E concentration-dependent manner. Instead, current outcomes reveal a decline protein content due to IIH in both E dosage groups. Current results suggest that metabolic-drive augmentation of intra-preoptic GABA release derives from non-MPN GABA neurons. Further effort is required to determine if MPN GABA neurons function similarly to those in the VMH, namely communicating energy sufficiency, to sustain reproduction during energy-amenable conditions.

Hypoglycemic stimulation of glucagon and corticosterone secretion in female rats was fully or partially reversed, in that order, by hindbrain lactate infusion, data that underscore the critical role of hindbrain input in optimal counter-regulatory hormone release in this sex. Further effort is needed to verify the distinctive involvement of hindbrain lactoprivic-sensitive transmitter and biosynthetic enzyme proteins identified here in hyperglucagonemic and -corticosteronemic responses to IIH. Interestingly, a number of these neurochemicals, e.g. NPY [Pralong et al., 2002; Crown et al., 2007; Hill et al., 2008; Garcia-Garcia, 2012], RFRP-1 [Garcia-Garcia, 2012; Ubuka et al., 2016], RFRP-3 [Johnson et al., 2007; Qi et al., 2009; Clarke et al., 2012; Xiang et al., 2012], CRH [Wade and Jones, 2004], ORX-A [Garcia-Garcia, 2012; Celik et al., 2015], and MCH [Wu et al., 2009; Naufahu et al., 2013] regulate both energy homeostasis and reproduction. As linked command of metabolism and reproduction occurs in the brain, one or more transmitters described above may consolidate control of diverse functions that remedy energy imbalance, including food intake, energy expenditure, and impedance of female procreation. Future goals of our research include systems-level characterization of the anatomical organization and functional interaction of metabolic-regulatory forebrain neurons in the female brain, as well as gain of insight on how E signal strength may regulate hindbrain metabolic input to these cells and/or cellular receptivity to this stimulus. It would also be informative to identify neurotransmitters that function as a nexus for neural control of coordinated physiological and behavioral responses to energy deficiency. A current critical barrier to such aims is a lack of high-resolution tools

for polysynaptic mapping and neurochemical characterization of metabolic deficit-activated hindbrain-forebrain neural pathways.

In summary, current results show that hindbrain lactoprovic-driven NE activity is elevated/enhanced in a majority of multiple preoptic and hypothalamic loci in the hypoglycemic female rat brain irrespective of circulating E levels representing estrous cycle nadir versus peak release. Yet, these divergent physiological patterns of E secretion likely establish both common and unique hypothalamic metabolic neurotransmitter targets of hindbrain lactate deficit signaling, while enabling divergent corticosterone responses to IIH. Results provide unique evidence that distinct forebrain neurochemicals exhibit E concentration-dependent sensitivity to hypoglycemia. Further studies are needed to investigate how these differential responses may impact gluco-regulatory as well as non-metabolic functions, and time frame for recovery from hypoglycemia and habituation to recurring episodes of this metabolic stress.

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Abbreviations

aCSF	artificial cerebrospinal fluid
AICAR	5-aminoimidazole-4-carboxamide-riboside
AMPK	5' adenosine monophosphate-activated protein kinase
ARH	arcuate hypothalamic nucleus
AVPV	anteroventral periventricular nucleus
CRH	corticotropin-releasing hormone
CV4	caudal fourth ventricle
DMH	dorsomedial hypothalamic nucleus
E	estradiol
GAD_{65/67}	glutamate decarboxylase _{65/67}
GnRH	gonadotropin releasing hormone
IIH	insulin-induced hypoglycemia
INS	insulin
LH	luteinizing hormone
LHA	lateral hypothalamic area
MCH	melanin-concentrating hormone

MPN	medial preoptic nucleus
NE	norepinephrine
nNOS	neuronal nitric oxide synthase
NPH	neutral protamine Hagedorn insulin
NPY	neuropeptide Y
ORX-A	orexin A
OVX	ovariectomy
pAMPK	phosphoAMPK
PVH	paraventricular hypothalamic nucleus
POMC	pro-opiomelanocortin
RFRP-1/-3	RFamide-related peptide-1/-3
RIIH	recurrent insulin-induced hypoglycemia
rPO	rostral preoptic area
sc	subcutaneous
SDS	sodium dodecyl sulfate
TBS	tris-buffered saline
VMH	ventromedial hypothalamic nucleus

References

- Arnold AP, Gorski RA. Gonadal steroid induction of structural sex differences in the central nervous system. *Annu Rev Neurosci.* 1984; 7:413–442. [PubMed: 6370082]
- 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]
- Briski KP, Nedungadi TP. Adaptation of feeding and counter-regulatory hormone responses to intermediate insulin-induced hypoglycemia in the ovariectomized female rat: effects of oestradiol. *J Neuroendocrinol.* 2009; 21:578–585. [PubMed: 19500228]
- Briski KP, Shrestha PK. Hindbrain estrogen receptor-beta antagonism normalizes reproductive and counter-regulatory hormone secretion in hypoglycemic steroid-primed ovariectomized female rats. *Neuroscience.* 2016; 331:62–71. [PubMed: 27316550]
- Butcher RL, Collins WE, Fugo NW. Plasma concentrations of LH, FSH, progesterone, and estradiol-17beta throughout the 4-day estrous cycle of the rat. *Endocrinology.* 1974; 94:1704–1708. [PubMed: 4857496]
- Cagampang FR, Cates PS, Sandhu S, Strutton PH, McGarvey C, Coen CW, O'Byrne KT. Hypoglycaemia-induced inhibition of pulsatile luteinizing hormone secretion in female rats: role of oestradiol, endogenous opioids and the adrenal medulla. *J Neuroendocrinol.* 1997; 9:867–872. [PubMed: 9419838]
- Castellano JM, Bentsen AH, Mikkelsen JD, Tena-Sempere M. Kisspeptins: bridging energy homeostasis and reproduction. *Brain Res.* 2010; 1364:129–138. [PubMed: 20800054]

- Celik O, Aydin S, Celik N, Yilmaz M. Peptides: Basic determinants of reproductive functions. *Peptides*. 2015; 72:34–43. [PubMed: 26074346]
- Chen MD, O'Byrne KT, Chiappini SE, Hotchkiss J, Knobil E. Hypoglycemia 'stress' and gonadotropin-releasing hormone pulse generator activity in the rhesus monkey: role of the ovary. *Neuroendocrinology*. 1992; 56:666–673. [PubMed: 1488100]
- Clarke IJ, Horton RJE, Doughton BW. Investigation of the mechanism by which insulin-induced hypoglycemia decreases luteinizing hormone secretion in ovariectomized ewes. *Endocrinology*. 1990; 127:1470–1476. [PubMed: 2201538]
- Clarke IJ, Smith JT, Henry BA, Oldfield BJ, Stefanidis A, Millar RP. Gonadotropin-inhibitory hormone is a hypothalamic peptide that provides a molecular switch between reproduction and feeding. *Neuroendocrinology*. 2012; 95:305–16. [PubMed: 22286004]
- Chan O, Cheng H, Herzog R, Czyzyk D, Zhu W, Wang A, McCrimmon RJ, Seashore MR, Sherwin RS. Increased GABAergic tone in the ventromedial hypothalamus contributes to suppression of counterregulatory responses after antecedent hypoglycemia. *Diabetes*. 2008; 57:1363–1370. [PubMed: 18375441]
- 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 MD, O'Byrne KT, Chiappini SE, Hotchkiss J, Knobil E. Hypoglycemia 'stress' and gonadotropin-releasing hormone pulse generator activity in the rhesus monkey: role of the ovary. *Neuroendocrinology*. 1992; 56:666–673. [PubMed: 1488100]
- 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. A2 noradrenergic nerve cell metabolic transducer and nutrient transporter adaptation to hypoglycemia: Impact of estrogen. *J Neurosci Res*. 2012; 90:1347–1358. [PubMed: 22431334]
- Crown A, Clifton DK, Steiner RA. Neuropeptide signaling in the integration of metabolism and reproduction. *Neuroendocrinology*. 2007; 86:175–182. [PubMed: 17898535]
- Fergani C, Routly JE, Jones DN, Pickavance LC, Smith RF, Dobson H. Kisspeptin, c-Fos and CRFR type 2 co-expression in the hypothalamus after insulin-induced hypoglycaemia. *Reprod Domest Anim*. 2014; 49:433–440. [PubMed: 24716653]
- Fernandez-Fernandez R, Martini AC, Navarro VM, Castellano JM, Dieguez C, Aguilar E, Pinilla L, Tena-Sempere M. Novel signals for the integration of energy balance and reproduction. *Mol Cell Endocrinol*. 2006; 254–255:127–132.
- Garcia-Garcia RM. Integrative control of energy balance and reproduction in females. *Vet Sci* 2012
- Georgescu D, Sears RM, Hommel JD, Barrot M, Bolaños CA, Marsh DJ, Bednarek MA, Bibb JA, Maratos-Flier E, Nestler EJ, DiLeone RJ. The hypothalamic neuropeptide melanin-concentrating hormone acts in the nucleus accumbens to modulate feeding behavior and forced-swim performance. *J Neurosci*. 2005; 25:2933–2940. [PubMed: 15772353]
- Goubillon ML, Thalabard JC. Insulin-induced hypoglycemia decreases luteinizing hormone secretion in the castrated male rat: involvement of opiate peptides. *Neuroendocrinology*. 1996; 64:49–56. [PubMed: 8811666]
- Gorski RA. Sexual differentiation of the brain: a model for drug-induced alterations of the reproductive system. *Environ Health Perspect*. 1986; 70:163–175. [PubMed: 3830102]
- Gujar AD, Ibrahim BA, Tamrakar P, Koshy Cherian A, Briski KP. Hindbrain lactostasis regulates hypothalamic AMPK activity and hypothalamic metabolic neurotransmitter mRNA and protein responses to hypoglycemia. *Amer J Physiol Regul Integr Comp Physiol*. 2014; 306:R457–R69. [PubMed: 24381179]
- He D, Funabashi T, Sano A, Uemura T, Minaguchi H, Kimura F. Effects of glucose and related substrates on the recovery of the electrical activity of gonadotropin-releasing hormone pulse

- generator which is decreased by insulin-induced hypoglycemia in the estrogen-primed ovariectomized rat. *Brain Res.* 1999; 820:71–76. [PubMed: 10023032]
- Heisler LE, Pallotta CM, Reid RL, Van Vugt DA. Hypoglycemia-induced inhibition of luteinizing hormone secretion in the rhesus monkey is not mediated by endogenous opioid peptides. *J Clin Endocrinol Metab.* 1993; 76:1280–1285. [PubMed: 8388404]
- Hill JW, Elmquist JK, Elias CF. Hypothalamic pathways linking energy balance and reproduction. *Amer J Physiol Endocrinol Metab.* 2008; 294:E827–E832. [PubMed: 18285524]
- Johnson MA, Tsutsui K, Fraley GS. Rat RFamide related peptide-3 stimulates GH secretion, inhibits LH secretion, and has variable effects on sex behavior in the adult male rat. *Horm Behav.* 2007; 51:171–180. [PubMed: 17113584]
- 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]
- Lado-Abeal J, Clapper JA, Chen Zhu B, Hough CM, Syapin PJ, Norman RL. Hypoglycemia-induced suppression of luteinizing hormone (LH) secretion in intact female rhesus macaques: role of vasopressin and endogenous opioids. *Stress.* 2002; 5:113–119. [PubMed: 12186689]
- Naufahu J, Cunliffe AD, Murray JF. The roles of melanin-concentrating hormone in energy balance and reproductive function: are they connected? *Reproduction.* 2013; 146:R141–150. [PubMed: 23884861]
- Nestor CC, Kelly MJ, Rønnekleiv OK. Cross-talk between reproduction and energy homeostasis: central impact of estrogens, leptin and kisspeptin signaling. *Horm Mol Biol Clin Invest.* 2014; 17:109–128.
- Nishimura Y, Mabuchi K, Taguchi S, Ikeda S, Aida E, Negishi H, Takamata A. Involvement of orexin-A neurons but not melanin-concentrating hormone neurons in the short-term regulation of food intake in rats. *J Physiol Sci.* 2014; 64:203–211. [PubMed: 24676683]
- Paranjape SA, Briski KP. Recurrent insulin-induced hypoglycemia causes site-specific patterns of habituation or amplification of CNS neuronal genomic activation. *Neuroscience.* 2005; 130:957–970. [PubMed: 15652993]
- Patil GD, Briski KP. Lactate is a critical ‘sensed’ variable in caudal hindbrain monitoring of CNS metabolic stasis. *Amer J Physiol Regul Integr Comp Physiol.* 2005; 289:R1777–R1786. [PubMed: 16037122]
- Pissios P, Bradley RL, Maratos-Flier E. Expanding the scales: the multiple role of MCH in regulating energy balance and other biological functions. *Endoc Rev.* 2006; 27:606–620.
- Pralong FP, Gonzales C, Voirol MJ, Palmiter RD, Brunner HR, Gaillard RC, Seydoux J, Pedrazzini T. The neuropeptide Y Y1 receptor regulates leptin-mediated control of energy homeostasis and reproductive functions. *FASEB J.* 2002; 16:712–714. [PubMed: 11978737]
- Qi Y, Oldfield BJ, Clarke IJ. Projections of RFamide-related peptide-3 neurones in the ovine hypothalamus, with special reference to regions regulating energy balance and reproduction. *J Neuroendocrinol.* 2009; 21:690–697. [PubMed: 19500220]
- Shrestha PK, Tamarkar 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]
- 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]
- Singh SR, Briski KP. Septopreoptic mu opioid receptor mediation of hindbrain glucoprivic inhibition of reproductive neuroendocrine function in the female rat. *Endocrinology.* 2004; 145:5322–5331. [PubMed: 15308614]
- Singh SR, Briski KP. Caudal hindbrain glucoprivation enhances GABA release in discrete septopreoptic structures in the steroid-primed ovariectomized rat brain: Role of mu opioid receptors. *Neuroendocrinology.* 2004; 80:201–209. [PubMed: 15591795]
- Singh SR, Briski KP. Central GABA_A, but not GABA_B receptors mediate suppressive effects of caudal hindbrain glucoprivation on the LH surge in the steroid-primed, ovariectomized female rat. *J Neuroendocrinol.* 2005; 17:407–12. [PubMed: 15946158]

- Simerly RB. Organization and regulation of sexually dimorphic neuroendocrine pathways. *Behav Brain Res.* 1998; 92:195–203. [PubMed: 9638961]
- Simerly RB. Wired for reproduction: organization and development of sexually dimorphic circuits in the mammalian forebrain. *Annu Rev Neurosci.* 2002; 25:507–536. [PubMed: 12052919]
- Tsuino N, Sakurai T. Role of orexin in modulating arousal, feeding, and motivation. *Front Behav Neurosci.* 2013; 7:28–78. [PubMed: 23616752]
- Ubuka T, Son YL, Tsutsui K. Molecular, cellular, morphological, physiological and behavioral aspects of gonadotropin-inhibitory hormone. *Gen Comp Endocrinol.* 2016; 227:27–50. [PubMed: 26409890]
- Vavaiya KV, Briski KP. Effects of caudal hindbrain lactate infusion on insulin-induced hypoglycemia and neuronal substrate transporter, glucokinase, and sulfonylurea receptor-1 gene expression in the ovariectomized female rat dorsal vagal complex: Impact of estradiol. *J Neurosci Res.* 2008; 86:694–701. [PubMed: 17941059]
- Wade GN, Jones JE. Neuroendocrinology of nutritional infertility. *Amer J Physiol Regul Integr Comp Physiol.* 2004; 287:R1277–R1296. [PubMed: 15528398]
- Wu M, Dumalska I, Morozova E, vanden PA, Alreja M. Melanin-concentrating hormone directly inhibits GnRH neurons and blocks kisspeptin activation, linking energy balance to reproduction. *Proc Natl Acad Sci.* 2009; 106(40):17217–17222. [PubMed: 19805188]
- Xiang W, Lai P, Zhang BY, Wang PQ, Chu MX, Fan Q, Liu CX, Tan Y. Effects of RFRP-3 on reproductive function and energy balance in mammals. *Yi Chuan.* 2012; 34:969–976. [PubMed: 22917901]
- Zhou L, Yueh CY, Lam DD, Shaw J, Osundiji M, Garfield AS, Evans M, Heisler LK. Glucokinase inhibitor glucosamine stimulates feeding and activates hypothalamic neuropeptide Y and orexin neurons. *Behav Brain Res.* 2011; 222:274–278. [PubMed: 21440571]
- Zhu W, Czyzyk D, Paranjape SA, Zhou L, Horblitt A, Szabó G, Seashore MR, Sherwin RS, Chan O. Glucose prevents the fall in ventromedial hypothalamic GABA that is required for full activation of glucose counterregulatory responses during hypoglycemia. *Amer J Physiol Endocrinol Metab.* 2010; 298:E971–E977. [PubMed: 20304763]

Highlights

- Ovariectomized female rats were replaced with estradiol (E) at estrous cycle nadir or peak levels.
- Rats were injected with insulin or vehicle after start of caudal fourth ventricular L-lactate infusion.
- Lactate fully or partially reversed hypoglycemic hyperglucagonemia and -corticosteronemia.
- Lactate caused dissimilar changes in dorsomedial hypothalamic norepinephrine between E groups.
- E signal strength defines unique metabolic transmitter targets of lactoprivic signals.

Figure 1. Effects of Hindbrain Lactate Repletion on Blood Glucose and Counter-Regulatory Hormone Concentrations in Insulin (INS)-Injected Estradiol (E)-Implanted Ovariectomized (OVX) Adult Female Rats

OVX rats were implanted with *sc* capsules filled with E at a dose of 30 (E-30) [left-hand side; bars 1-3] or 300 (E-300) [right-hand side; bars 4-6] $\mu\text{g/mL}$ to replicate estrous cycle baseline versus peak plasma hormone levels. Animals were subsequently injected *sc* with INS or vehicle (V) after initiation of caudal fourth ventricular (CV4) infusion of L-lactate (L) or the vehicle artificial cerebrospinal fluid (V) alone. Data depict mean circulating glucose (Panel 1A), glucagon (Panel 1B), corticosterone (Panel 1C), and leptin (Panel 1D) levels $\pm$ S.E.M. in groups ($n=5$ rats/group) of E-30 and E-300 rats treated as follows: CV4 V/*sc* V (V/V; solid white bars), CV4 V/*sc* INS (V/INS; solid grey bars), and CV4 L/*sc* INS (L/INS; cross-hatched grey bars). * $p<0.05$; versus V/V; ** $p<0.05$; versus V/I; # $p<0.05$; versus E-30 V/V.

Figure 2. Effects of Hindbrain Lactate Infusion on Hypoglycemic Patterns of Norepinephrine (NE) Accumulation in Forebrain Gluco-Regulatory- and Reproduction-Relevant Structures in E-Implanted OVX Female Rat Brain

At the conclusion of 2 hour infusion of V or L to the CV4, groups of V/V-, V/INS-, and L/INS-treated E-30 and E-300 rats were sacrificed for micro-punch dissection of rostral preoptic area (rPO; Panel 2A), anteroventral periventricular nucleus (AVPV; Panel 2B), medial preoptic nucleus (MPN; Panel 2C), arcuate hypothalamic nucleus (ARH; Panel 2D), dorsomedial hypothalamic nucleus (DMH; Panel 2E), lateral hypothalamic area (LHA; Panel 2F), paraventricular hypothalamic nucleus (PVH; Panel 2G), and ventromedial hypothalamic nucleus (Panel 2H) tissues from each left hemi-forebrain for ELISA analysis of NE content. Data in each Panel illustrate mean tissue NE content $\pm$ S.E.M. for groups (n=5/group) of V/V- (solid white bars), V/INS- (solid grey bars), and L/INS- (crosshatched grey bars) treated E-30 [bars 1-3] and E-300 [bars 4-6] rats. *p<0.05; versus V/V; **p<0.05; versus V/INS; #p<0.05; versus E-30 V/V; +p<0.05; versus INS-injected E-30 rats.

Figure 3. Effects of Hindbrain Lactate Infusion on Rostral Preoptic Area (rPO) Gonadotropin-Releasing Hormone-I (GnRH-I), Anteroventral Periventricular Nucleus (AVPV) PrePro-Kisspeptin, and Medial Preoptic Nucleus (MPN) Glutamate Decarboxylase _{65/67} (GAD_{65/67}) Protein Responses to Insulin-Induced (IIH) in E-Implanted OVX Female Rat Brain

Micropunched rPO (Panel 3A), AVPV (Panel 3B), and MPN (Panel 3C) tissues from right hemi-forebrains of E-30 and E-300 rats treated by V/V, V/INS, or L/INS were analyzed by Western blot for GnRH-I, prepro-kisspeptin, and GAD_{65/67} proteins, respectively. Protein band optical densities (O.D.) were quantified with Syngene Genetool 4.01 software and expressed relative to α -tubulin. Bars depict illustrate mean normalized protein O.D. measures $\pm$ S.E.M. for groups (n=5/group) of V/V- (solid white bars), V/INS- (solid grey bars), and L/INS- (cross-hatched grey bars) treated E-30 [bars 1-3] and E-300 [bars 4-6] rats. *p<0.05; versus V/V; **p<0.05; versus V/INS; #p<0.05; versus E-30 V/V.

Figure 4. Effects of Hindbrain Lactate Infusion on Arcuate Hypothalamic Nucleus (ARH) Neuropeptide Y (NPY), PrePro-Kisspeptin, and Pro-Opiomelanocortin (POMC) Protein Responses to Insulin-Induced (IIH) in E-Implanted OVX Female Rat Brain

Right hemi-forebrain – derived ARH tissue from E-30 and E-300 rats treated by V/V, V/INS, or L/INS was analyzed by Western blot for NPY (Panel 4A), prepro-kisspeptin (Panel 4B), and POMC (Panel 4C). Bars show mean normalized protein O.D. measures $\pm$ S.E.M. for groups (n=5/group) of V/V- (solid white bars), V/INS- (solid grey bars), and L/INS- (crosshatched grey bars) treated E-30 [bars 1-3] and E-300 [bars 4-6] rats. *p<0.05; versus V/V; **p<0.05; versus V/INS; #p<0.05; versus E-30 V/V.

Figure 5. Effects of Hindbrain Lactate Infusion on IIH-Associated Patterns of Dorsomedial Hypothalamic Nucleus (DMH) RFamide-Related Peptide-1 (RFRP-1) and -3 (RFRP-3) and Ventromedial Hypothalamic Nucleus (VMH) GAD_{65/67} and Neuronal Nitric Oxide Synthase (nNOS) Protein Expression in E-Implanted OVX Female Rat Brain

Right forebrain DMH and VMH tissues were analyzed by Western blot for RFRP-1 (Panel 5A), RFRP-3 (Panel 5B), GAD_{65/67} (Panel 5C), and nNOS (Panel 5D) proteins. Bars show mean normalized protein O.D. measures + S.E.M. for groups (n=5/group) of V/V- (solid white bars), V/INS- (solid grey bars), and L/INS- (cross-hatched grey bars) treated E-30 [bars 1-3] and E-300 [bars 4-6] rats. *p<0.05; versus V/V; **p<0.05; versus V/INS; #p<0.05; versus E-30 V/V.

Figure 6. Effects of Hindbrain Lactate Infusion on IIH-Associated Patterns of Lateral Hypothalamic Area (LHA) Orexin-A (ORX-A) and Melanin-Concentrating Hormone (MCH) and Paraventricular Hypothalamic Nucleus (PVH) Corticotropin-Releasing Hormone (CRH) Protein Expression in E-Implanted OVX Female Rat Brain

Right forebrain LHA and PVH tissues were analyzed by Western blot for ORX-A (Panel 6A), MCH-3 (Panel 6B), and CRH (Panel 6C) proteins. Bars show mean normalized protein O.D. measures + S.E.M. for groups (n=5/group) of V/V- (solid white bars), V/INS- (solid grey bars), and L/INS- (cross-hatched grey bars) treated E-30 [bars 1-3] and E-300 [bars 4-6] rats. *p<0.05; versus V/V; **p<0.05; versus V/INS; #p<0.05; versus E-30 V/V.