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Hindbrain 5'-adenosine monophosphate-activated protein kinase Mediates Short-Term Food Deprivation Inhibition of the Gonadotropin-Releasing Hormone-Luteinizing Hormone Axis: Role of Nitric Oxide

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Abstract

Hindbrain-derived stimuli restrain the gonadotropin-releasing hormone (GnRH)-pituitary luteinizing hormone (LH) reproductive neuroendocrine axis during energy insufficiency. Interruption of food intake, planned or unplanned, is emblematic of modern life. This study investigated the premise that the hindbrain energy sensor adenosine 5'-monophosphate-activated protein kinase (AMPK) inhibits reproductive neuroendocrine function in short-term, e.g. 18 hr food-deprived (FD) estradiol (E)-implanted ovariectomized female rats. Intra-caudal fourth ventricular administration of the AMPK inhibitor Compound C (Cc) reversed FD-induced inhibition of rostral preoptic (rPO) GnRH protein expression and LH release in animals given E to replicate proestrus- (high-E dose), but not metestrus (low-E dose)-stage plasma steroid levels. FD caused Cc-reversible augmentation or diminution of preoptic norepinephrine (NE) activity in high- versus low-E rats, respectively, and AMPK-independent reductions in hypothalamic NE accumulation in the latter. Nitric oxide (NO) and kisspeptin are key stimulatory signals for the preovulatory LH surge. Here, FD inhibited rPO neuronal nitric oxide synthase protein expression in high-, but not low-E - dosed animals. Lateral ventricular delivery of the NO donor SIN-1 reversed inhibitory GnRH and LH responses to FD in high-E rats, and normalized rPO Vglut2, anteroventral periventricular KiSS1, and dorsomedial hypothalamic RFRP-3 mRNA and/or protein profiles. Data show that FD curtails reproductive neuroendocrine outflow by hindbrain AMPK-dependent mechanisms in the presence of peak estrous cycle E levels. Results indicate that neural networks linking this sensor to GnRH neurons likely involve NO signaling, which may function upstream of one or more neurotransmitters identified here by SIN-1 – reversible inhibitory responses to FD.

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Keywords

Food deprivation; estradiol; norepinephrine; GnRH-I; luteinizing hormone; neuronal nitric oxide synthase; KiSS1

Introduction

Clinical and experimental studies document strict control of reproduction in female mammals by metabolic status. Negative energy balance impairs fecundity in women, food animals, and laboratory species. Female reproduction (encompassing ovulation, conception, pregnancy, and lactation) is a high energy-cost activity; its impedance or suspension under such circumstances is logical as successful conclusion of this complex process is likely to be jeopardized and energy investment thereby wasted [Wade and Jones, 2004]. Specialized neurons in the caudal hindbrain continuously monitor cellular energy state [Oomura and Yoshimatsu, 1984]. Indeed, the hindbrain, not the hypothalamus, is the primary source of metabolic deficit cues that curb pituitary luteinizing hormone (LH) secretion [Ohkura et al., 2000]. Provision of glucose anti-metabolite drugs to the hindbrain suppresses GnRH neuron transcriptional activation and LH release, evidence that affirms a functional link between hindbrain metabolic sensors and the gonadotropin-releasing hormone (GnRH)-LH neuroendocrine axis [Singh and Briski, 2004a; Ibrahim and Briski, 2014].

Maintenance of energy homeostasis despite variable environmental nutrient provision and dynamic adjustments in internal energy needs poses a constant challenge. Eating behavior is a complex interplay of physiological, psychological, social, and genetic factors that influence meal timing, quantity of food intake, and food preference. The ideal circumstance of unfettered ability to eat-at-will in response to the above drives can be impractical in reality. Indeed, short-term suspension of food intake, planned or unplanned, is an unavoidable and unpredictable metabolic stressor inherent to modern life. Interrupted feeding over several hours elicits estradiol (E)-dependent patterns of hyperphagia and adjustments in circulating substrate fuel and energy deficit-sensitive hormone levels in ovariectomized (OVX) female rats [Ibrahim and Briski, 2015; Alenazi et al., 2016; Briski et al., 2016]. The ultra-sensitive energy sensor adenosine 5'-monophosphate-activated protein kinase (AMPK) acts within the caudal dorsomedial hindbrain to control feeding and forebrain AMPK and metabolic neuropeptide responses to short-term food deprivation (FD) [Alenazi et al., 2016]. A2 noradrenergic neurons are a plausible source of E-sensitive AMPK cues originating within that site as this neurochemical phenotype is the sole local cell population that is identified so far as AMPK-positive and, moreover, is capable of integrating steroidal and metabolic stimuli by virtue of estrogen receptor-alpha and -beta protein expression [Ibrahim et al., 2013]. E modulates effects of caudal hindbrain administration of the AMP mimic 5-aminoimidazole-4-carboxamide-riboside (AICAR) on AMPK activity in hindbrain metabolo-sensory A2 noradrenergic neurons [Ibrahim et al., 2013] and on sensor activation and metabolic neuropeptide transmitter expression in downstream hypothalamic loci [Alenazi et al., 2014]. Estrogen establishes preoptic and hypothalamic targets of hindbrain AMPK-impelled NE signaling in ovariectomized (OVX) female rats [Alenazi et al., 2016]. E likely modulates reproductive neuroendocrine sensitivity

to energy deficit as hormone-replaced OVX rats exhibit amplified anti-glucose metabolite-induced suppression of LH output [Nagatani et al., 1996]. Pituitary-gonadal hormones exhibit rapid reactivity to nutritional signals [Cameron and Norbisch, 1991; Schreihöfer et al., 1993]. The current study investigated the hypothesis that female reproductive neuroendocrine function is vulnerable to FD inhibition involving caudal hindbrain AMPK-dependent mechanisms.

E acts on the female brain to impose opposing negative/positive feedback actions on the GnRH-LH axis, and to regulate metabolic status via control of energy intake, storage, and expenditure. E secretion fluctuates dramatically over the female rat estrous cycle, increasing 4–5 fold from nadir to peak circulating levels. Basal plasma E levels inhibit reproductive neuroendocrine function, whereas the mid-cyclic increase in circulating E amplifies GnRH output to the anterior pituitary, which in turn initiates the proestrus pre-ovulatory LH surge. This dual mode of estrogenic control is accomplished, in part, by distinctive patterns of norepinephrine (NE) neurotransmission to the preoptic area/hypothalamus [Wise et al., 1981; Adler et al., 1983; Demling et al., 1985]. Cycle stage-specific patterns of E release impact metabolic stasis as food intake declines and energy state becomes (paradoxically) more negative as levels of this hormone levels rise [Giles et al., 2010]. The present project utilized a characterized E replacement paradigm that re-establishes plasma hormone levels at estrous cycle peak versus nadir concentrations [Goodman, 1978; Briski et al., 2001] to determine if physiologically distinct patterns of E secretion exert distinguishing control of caudal dorsomedial hindbrain AMPK-driven NE input to reproduction-relevant forebrain structures alongside GnRH-LH axis reactivity to short-interval FD in female rats. Micropunch-dissected loci evaluated for FD-mediated changes in NE activity in low- versus high-E – replaced OVX female rats included structures [e.g. rostral preoptic area (rPO) and anteroventral periventricular (AVPV), medial preoptic (MPN), and hypothalamic arcuate (ARH) nuclei] previously reported to exhibit adjusted neurotransmitter content during hypoglycemic metabolic stress [Briski and Shrestha, 2016]. A related aim of the current project was to identify neurochemicals within discernible forebrain targets of FD-triggered NE signaling that may potentially link hindbrain AMPK with the GnRH-LH axis during this metabolic stress. Effects of FD on the E-sensitive neurotransmitters nitric oxide (NO) [Watanobe et al 2001; Moreno and Franci, 2004; Lima et al., 2014], γ -amino-butyric acid (GABA) [Leonhardt et al., 1999], kisspeptin [Luque et al., 2007], glutamate [Brann and Mahesh, 1997], RFamide-related peptide-3 (RFRP-3) [Gibson et al., 2008; Klingerman et al., 2011; Leon et al., 2014], neuropeptide Y (NPY) [Pelletier et al., 1992; Jovanovic et al., 2010; Sainsbury and Zhang, 2010; Señarís et al., 2011], and proopiomelanocortin (POMC) [Bohler et al., 1990; Wise et al., 1990; Jovanovic et al., 2010; Sainsbury and Zhang, 2010; Señarís et al., 2011], were evaluated using high-sensitivity quantitative real-time RT-PCR and Western blotting for analysis of micropunch tissue samples containing neurons that synthesize these neurochemicals.

Methods and Materials

Animals

Adult female Sprague Dawley rats (2–3 months of age) were maintained under a 14 hr-light/10 hr-dark lighting schedule (lights on at 05:00h), and permitted free access to standard laboratory rat chow (Harlan Teklad LM-485; Harlan Industries, Madison, WI, USA) and tap water. Rats were handled daily for 7 days prior to experimentation. All protocols were conducted in accordance with NIH guidelines for care and use of laboratory animals, with approval by the ULM Institutional Animal Care and Use Committee.

Experimental Design

Experiment 1—On day 1, rats were bilaterally OVX and implanted with a PE-20 polyethylene cannula aimed at the caudal fourth ventricle (CV4) under ketamine/xylazine (0.1 mL/100 g *bw* *i.p.*; 90 mg ketamine/10 mg xylazine/mL; Henry Schein, Inc., Melville, NY, USA), as described [Singh and Briski, 2004a]. After surgery, animals were treated with Enrofloxacin (Baytril 2.27%, 10 mg/kg *bw*; intramuscular) and Ketoprofen [3 mg/kg *bw*; subcutaneous (*sc*)], and transferred to individual housing after surgery. On day 7, animals were anesthetized with isoflurane (5% for induction; 2.5% for maintenance) prior to *sc* implantation of a silastic capsule [10 mm/100 g *bw*; 0.062 in. i.d./0.125 in. o.d.] containing 17 β -estradiol-3-benzoate at a concentration of either 30 (E-30) or 300 (E-300) μ g/mL safflower oil. We reported that these disparate E doses yield circulating hormone levels that mimic plasma E levels measured on metestrus or proestrus, respectively, [Butcher et al., 1974] in ovary-intact female rats [Briski et al., 2001]. Groups of E-30 and E-300 rats were treated as follows: 1) full-fed (FF) plus intra-CV4 vehicle (V) dimethyl sulfoxide (DMSO) administration at 15.00 hr on day 11 [n=4 E-30; n=4 E-300]; 2) FD (initiated at 21.00 hr on day 10) plus CV4 V treatment at 15.00 hr on day 11 [n=4 E-30; n=4 E-300]; 3) FD plus CV4 administration of the AMPK inhibitor Compound C (Cc; 5.0 μ g/2.0 μ L DMSO) at 15.00 hr on day 11 [n=4 E-30; n=4 E-300]. Animals were sacrificed at 16.00 hr on day 11. Brains were cut into 100 micron-thick fresh frozen sections for micropunch dissection of individual preoptic/hypothalamic structures of interest. Trunk blood was collected for LH radioimmunoassay.

Experiment 2—On day 1, OVX rats were implanted with a PE-20 cannula into the left lateral cerebral ventricle (LV) [Singh and Briski, 2004a]. Animals received a *sc* silastic capsule [10 mm/100 g *bw*; 0.062 in. i.d./0.125 in. o.d.] filled with 300 (E-300) μ g estradiol benzoate/ μ g/mL safflower oil. Groups of E-300 rats (n=5/group) were treated as follows: 1) full-fed (FF) plus LV administration of the vehicle saline (V) at 15.00 hr on day 11; 2) FD (initiated at 21.00 hr on day 10) plus intra-LV delivery of V at 15.00 hr on day 11; 3) FD plus LV 3-morpholinosydnonimine (SIN-1; 100 μ g/2.0 μ L) [Yokotani et al., 1997; Briski, 1999] at 15.00 hr on day 11; 4) FD plus intra-LV administration of 200 μ g/SIN-1 at 15.00 hr on day 11. Animals were sacrificed at 16.00 hr on day 11. Brains were cut into 100 micron-thick fresh frozen sections for micropunch dissection of preoptic/hypothalamic structures of interest. Trunk blood was obtained for LH radioimmunoassay.

Brain tissue micro-punch dissection

Calibrated hollow needles (0.5 mm diameter; prod. no. 57401, Stoelting, Inc., Kiel, WI), were used to bilaterally dissect the following neural structures (relative to bregma) from serial frozen brain sections, using described methods [Singh and Briski, 2004b]: *Experiment 1*: rPO [+0.3 to 0.00 mm]; AVPV [−0.00 to −0.26 mm]; MPN [−0.26 to −0.60 mm]; paraventricular hypothalamic nucleus [PVN; −1.78 to −2.00 mm]; ARH [−1.78 to −3.25 mm]; ventromedial hypothalamic nucleus [VMN; −2.00 to −3.25 mm]; dorsomedial hypothalamic nucleus [DMN; −2.45 to −3.25 mm]; lateral hypothalamic area [LHA; −1.78 to −3.28 mm]. For each neural structure of interest, tissues harvested from separate sides of the forebrain were collected into appropriate buffers for NE ELISA (0.01N HCL containing 1 mM EDTA and 4 mM sodium metabisulfite) or Western blot (2% SDS, 0.05 M DTT, 10% glycerol, 1 mM EDTA, 60 mM Tris-HCl, pH 7.2) analyses. *Experiment 2*: rPO [+0.30 to 0.00 mm]; AVPV [0.00 to −0.26 mm]; ARH [−1.78 to −3.25 mm]; DMN [−2.45 to −3.25 mm]. For each structure described above, tissues harvested from right and left hemi-forebrains were respectively collected into buffer for quantitative real-time RT-PCR (guanidinium isothiocyanate and β-mercaptoethanol) or Western blot analyses.

Brain Tissue NE ELISA Analysis

For each brain structure of interest, aliquots from individual subjects were pooled to create triplicate samples, for each treatment group, for NE content analysis using Noradrenaline Research ELISA™ kit reagents (Labor Diagnostika Nord GmbH & Co KG, Nordhorn, Germany) [Shrestha et al., 2014]. Briefly, standards (10 µL), controls (10 µL) and sample aliquots (25 µL) were pipetted in into duplicate extraction plate wells and diluted to 100 µL with distilled water. Plates were sequentially incubated by shaking at 600 rpm with TE buffer (25 µL), acylation buffer (150 µL) plus acylation reagent (25 µL), and hydrochloric acid (100 µL). 90 µL of extracted samples, standards, and controls were transferred to individual microtiter plate wells, mixed with enzyme solution (25 µL), and incubated for 37 °C for 2 hr. Sample, standard, and control wells aliquots (100 µL) were transferred to pre-coated Noradrenaline Microtiter strip wells, mixed with 50 µL of primary antiserum, then incubated for 18 hr at 2–8 °C. After plate contents were discarded, wells were washed 4X, then incubated with enzyme conjugate (100 µL) for 30 min. Wells were emptied, washed 4X, then incubated for 30 min with 100 µL substrate. After termination of reactions with 100 µL stop solution, absorbance of well contents was read at 450 nm in an Emax Precision Microplate Reader (Molecular Devices, LLC, Sunnyvale, CA, USA). Assay sensitivity was 0.1 ng/ml × C* [C* (Correction factor) = volume of standards extracted / sample volume extracted].

Brain Tissue Western Blot Analysis

Heat-denatured micropunch tissues obtained in Experiment 1 were evaluated as previously described [Gujar et al., 2013]. In each treatment group, aliquots from individual subjects were pooled to create triplicate samples for immunoblotting of each protein of interest. Proteins were separated on 10–15% gradient Tris-glycine gels (90 V, 105 min; Tris-glycine SDS running buffer) and transblotted (30 V, overnight at 4°C; Towbin buffer) to 0.45-µm PVDF membranes. After pretreatment with Pierce Western blotting signal enhancer,

membranes were blocked with Tris-buffer saline (TBS), pH 7.4, 0.1% Tween-20 containing either 2% bovine serum albumin or 5% normal donkey serum. Proteins from various structures were probed with one of the following primary antibodies: rPO: rabbit anti-pAMPK $\alpha_{1/2}$ (Thr 172) [1:1000, sc-33524; Santa Cruz Biotechnol., Inc., Santa Cruz, CA], mouse anti-GnRH1 (HU11B) [1:1000, sc-32292; Santa Cruz Biotechnol.], rabbit anti-NOS1/neuronal nitric oxide synthase (nNOS) [1:1000, sc-648; Santa Cruz Biotechnol.], goat anti-GAD $_{65/67}$ (C-20) [1:1000, sc-7513; Santa Cruz Biotechnol.]; AVPV: goat anti-kisspeptin (C-20) [1:1000, sc-18134; 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 (1:5000; PerkinElmer, Boston, MA), goat anti-rabbit (1:5000; PerkinElmer), or donkey anti-goat (1:5000; sc-2020; Santa Cruz Biotechnol.) secondary antisera, followed by Supersignal West Femto maximum sensitivity chemiluminescent substrate (ThermoFisherScientific, Rockford, IL). Signal visualization was recorded using a Syngene G:Box Chemi (Syngene, Frederick, MD). Protein band optical densities (O.D.) were quantified with Syngene Genetool 4.01 software and normalized to α -tubulin. Immunoblots were performed in triplicate for each target protein. Protein molecular weight markers were included in each Western blot analysis. Proteins in micropunch samples obtained in Experiment 2 were separated as described above, except that BioRad TGX 10–12% stain-free gels were used [Colella et al., 2012; Gurtler et al., 2013; Gilda and Gomes, 2013, 2015]. After electrophoresis, gels were activated for 1 min by UV light in a BioRad ChemiDoc TM Touch Imaging System prior to protein transfer to PVDF membranes. After blocking, membranes were incubated with one of the following primary antisera: rPO: mouse anti-GnRH1 (see above), mouse anti-vesicular glutamate transporter-2 (Vglut2) [1:1000, MAB5504; EMD Millipore, Temecula, CA], rabbit anti-nNOS (see above); AVPV: goat anti-kisspeptin (see above); ARH: goat anti-kisspeptin (see above), goat anti-neuropeptide Y (NPY) [1:2000, NBP1-46535; Novus Biologicals], goat anti-proopiomelanocortin (POMC) [1:2000, NB100-1533; Novus Biologicals, Littleton, CO]; DMN: goat anti-RFamide-related peptide-3 (RFRP-3) (M-18) [1:500, sc-32380; Santa Cruz Biotechnol.]. Membranes were incubated sequentially with horseradish peroxidase-labeled secondary antisera, as described above, and SuperSignal West Femto Maximum Sensitivity substrate. Total in-lane protein and chemiluminescence band O.D. values were measured densitometrically with BioRad Image Lab 3.0.1 software. Protein bands were normalized to total protein content of their respective lane. Immunoblots were performed in triplicate for each target protein. Protein molecular weight markers were included in each Western blot analysis.

Quantitative Real-Time RT-PCR Analysis of Micropunched Forebrain Loci

Total RNA was isolated from micropunch tissue samples of individual neural structures using Qiagen RNeasy MiniElute Cleanup kit reagents. For each structure, RNA integrity, purity, and quantity was determined using an Agilent Technologies 2100 Bioanalyzer and Agilent RNA 6000 nano kits. Total RNA was transcribed into first-strand cDNA with Invitrogen SuperScriptTM III Reverse Transcriptase. Forward and reverse primers for target genes were designed with Beacon Designer software (Premier Biosoft International, Palo Alto, CA): GnRH-I [*forward*: CACTATGCTCACCAGCGGGG; *reverse*: AGAGCTCCTCGCAGATCCCT-AAGA]; Vglut-2 [*forward*: TCCCTCGGACAGATCTAC;

reverse: CATAGCGGAGCCTTCTTC], metastasis-suppressor KiSS-1 (KiSS1) [*forward*: TGGCACCTGTGGTGAACCTGACC; *reverse*: ATCAGGCGACTGCGGGTGGCA-CA], NPY [*forward*: TGTTTGGGCATTCTGGCTGAGG; *reverse*: CTGGGGGCATTTTCTGTGCTTT-C], POMC [*forward*: CTCCTGCTTCAGACCTCCATAGAC; *reverse*: AAGGGCTGTTTCATCTCCGT-TG], RFRP-3 [*forward*: GAGTCCTGGTCAAGAGCAAC; *reverse*: ACTGGCTGGAGGTTTCCTAT], GAPDH [*forward*: AAGTTCAACGGCACAGTCAAGG; *reverse*: CATACTCAGCACCAGCATCAC-C], CypA [*forward*: ATAATGGCACTGGTGGCAAGTC; *reverse*: ATTCCTGGACCCAAAACGCTC-C], and obtained from Genemed Synthesis (San Antonio, TX). PCR reactions with SYBR Green-based detection were performed, as described [Vavaiya et al., 2007; Vavaiya and Briski, 2007; 2008], in a BioRad iQTM 5 iCycler system as follows: initial 3 min denaturation at 95°C, followed by 40 cycles of denaturation at 1) 95°C for 15 sec, 2) temperature-specific annealing for 30 sec [GnRH-1, 58°; Vglut2, 55°; kisspeptin, 68°; NPY, 60°; POMC 60°; RFRP-3, 50°; GAPDH, 30°; CypA, 58°], and 3) extension at 72°C for 30 sec. No-template and -RT controls and melt curve analyses were performed for each RT-PCR analysis. Amplified PCR products were evaluated by agarose gel electrophoresis. mRNA data were analyzed by the 2^{-Ct} method.

LH Radioimmunoassay

Plasma LH concentrations were measured by radioimmunoassay, as described [Singh and Briski, 2004].

Statistical analyses

Mean tissue NE, mRNA, and normalized protein O.D. values and plasma LH levels were evaluated by one- or two-way ANOVA, according to experimental design, and Duncan's multiple range test. Differences of $p < 0.05$ were considered significant.

Results

Experiment 1 sought to determine if E concentration regulates forebrain targets of caudal hindbrain AMPK-driven NE signaling in response to FD. Figure 1 Panels A–C depict FD effects on NE accumulation in distinctive preoptic loci involved in reproduction, e.g. rPO (A), AVPV (B), and MPN (C), in OVX rats replaced with E to mimic baseline (E-30) or peak (E-300) plasma E concentrations. FD-exposed E-30 animals exhibited significant suppression of NE content in the rPO and MPN compared to FF controls [E-30/FD/V_{CV4} versus E-30/FF/V_{CV4}]; intra-CV4 Cc administration reversed this response in the rPO only [E-30/FD/Cc_{CV4} versus E-30/FD/V_{CV4}]. Yet, in E-300 rats, FD caused Cc-reversible augmentation of NE levels in each preoptic site [E-300/FD/V_{CV4} versus E-300/FF/V_{CV4} / E-300/FD/Cc_{CV4}]. FD-associated adjustments in NE accretion in hypothalamic metabolic structures are illustrated in Panels D–H. In these locations, E-30/FD/V_{CV4} animals were characterized by diminished NE content, which was not normalized by Cc. At the same time, the E-300/FD/V_{CV4} group showed an increase in DMN NE (2E), which was reversed by Cc. Results show that caudal hindbrain AMPK regulates preoptic NE input during FD,

where recipient sites and directionality of NE response vary in line with estrous cycle peak-versus nadir-like E levels.

Firsthand evidence here for AMPK-dependent NE signaling to the rPO, which encompasses a metabolic-sensitive subset of GnRH neurons [Briski and Sylvester, 1998], bolstered a related aim to examine whether GnRH-I and key rPO (NOS, GABA) and AVPV (kisspeptin) upstream neurochemicals exhibit similar or divergent reactivity to FD in E-30 versus E-300 animals. Figure 2 portrays effects of FD on rPO profiles of pAMPK (Panel A), GnRH-I (Panel B), nNOS (Panel C) and GAD_{65/67} (Panel D) protein profiles and AVPV kisspeptin protein expression (Panel E) in E- (E-30 versus E-300) implanted OVX rats. Panel F depicts representative Western blots of target proteins. E-300/FF/V_{CV4} animals showed elevated rPO pAMPK, GnRH-1, and nNOS expression relative to E-30/FF/V_{CV4}. FD-exposed E-30 animals exhibited increased pAMPK protein, a response that was abolished by Cc, but no change in other proteins evaluated. In E-300 rats, rPO pAMPK levels did not differ between FF/V_{CV4} and /FD/V_{CV4} groups, whereas FD caused Cc-revocable reductions in GnRH-I and nNOS profiles [E-300/FD/V_{CV4} versus E-300/FF/V_{CV4} / E-300/FD/Cc_{CV4}]. AVPV kisspeptin protein content was significantly greater in FF E-300 versus FF E-30 animals. FD caused a trend toward decreased kisspeptin expression that did not reach statistical significance in either E-30 or E-300 animals. AVPV kisspeptin content was significantly decreased in the E-300/FD/Cc_{CV4} group compared to E-300/FF/V_{CV4}. Data point to caudal hindbrain AMPK-dependent FD inhibition of rPO GnRH-I and NOS protein expression in the presence of estrous cycle peak, but not nadir E levels.

Effects of CV4 Cc administration on FD-associated adjustments in pituitary LH secretion in E-30 versus E-300 animals are shown in Figure 3. Outcomes reveal that FD suppressed circulating LH levels in E-300, but not E-30 rats, and that inhibitory patterns of hormone output in the former group were normalized by intra-CV4 administration of Cc.

Experiment 2 addressed the premise that NO down-regulation is crucial for FD suppression of GnRH-1 in E-300 rats. The excitatory neurotransmitter glutamate is a critical stimulus for the mid-cycle LH surge via actions that include NO up-regulation [Brann et al., 1997]. In order to assess FD effects on rPO glutaminergic function, expression of Vglut2 (the predominant vesicular glutamate transporter isoform expressed by glutamate neurons in the rPO [Lin et al., 2003]) was measured as an indicator of signaling activity. Data in Figure 4 depict impact of LV administration of the NO donor SIN-1 to FD-exposed E-300 animals concerning rPO GnRH-1 and Vglut2 mRNA and protein content. rPO GnRH-1 and Vglut2 mRNAs (Panels A and C, respectively, were insensitive to FD, but were each significantly elevated alongside GnRH-1 (Panel B) and Vglut2 (Panel D) proteins in response to the lower (e.g. 100 µg) [FD/S(100)_{LV} versus FD/V_{LV} / FF/V_{LV}], but not higher (200 µg) SIN-1 dose. SIN-1 treatment caused dose-dependent augmentation of rPO GnRH-1 and Vglut2 protein content in FD-exposed E-300 rats, thereby normalizing the latter profile. Results show that FD imposes a NO-reversible negative tonus on rPO GnRH-I and Vglut2 protein, but not mRNA expression.

AVPV KiSS1 and DMN RFRP-3 neurons respectively stimulate or inhibit the LH surge via rPO projections [Wu et al., 2009; Lehman et al., 2013; Yip et al., 2015]. Experiment 2

investigated the prospect of FD-driven nitroergic regulation of gene and/or gene product expression in these cell groups. Figure 5 illustrates effects of SIN-1 on FD-associated patterns of AVPV KiSS1 mRNA (Panel A) and kisspeptin protein (Panel B) and DMN RFRP-3 mRNA (Panel C) and protein (Panel D) expression in E-300 rats. AVPV KiSS1 gene expression was markedly suppressed due to FD, whereas corresponding kisspeptin protein levels were resistant to this metabolic stressor. Both SIN-1 dosages significantly increased KiSS1 gene transcription relative to both FF/V_{LV} and FD/V_{LV} groups. However, AVPV kisspeptin protein content was respectively elevated or not different in FD/S(100)_{LV} and FD/S(200)_{LV} groups compared to FD/V_{LV}. FD stimulation of DMN RFRP-3 mRNA and protein expression was attenuated at each SIN-1 dosage. Outcomes implicate NO down-regulation in FD augmentation of DMN RFRP-3 gene and gene product expression.

ARH tissue obtained from E-300 animals in Experiment 2 was similarly evaluated for NO involvement in reproduction-relevant neurotransmitter mRNA and protein responses to FD. FD regulation of KiSS1, NPY, and POMC gene and gene product expression in E-300 animals, with or without coincident SIN-1 treatment, is illustrated in Figure 6. Kisspeptin protein but not KiSS1 mRNA was reduced by FD [FD/V_{LV} versus FF/V_{LV}]; neither profile was affected by SIN-1 administration (Panels A and B). NPY (Panels C and D), but not POMC (Panels E and F) protein was elevated in FD versus FF groups; neither transcript was altered by FD. SIN-1 treatment normalized NPY protein (at the higher dose level), but not mRNA expression, and stimulated POMC gene (100 and 200 µg doses) and protein (200 µg dosage) levels. Data show that FD regulates ARH NPY protein, but not gene expression, via NO-dependent mechanisms.

Data in Figure 7 depict effects of SIN-1 administration on pituitary LH secretion in FD-exposed E-300 animals. Results show that FD caused a significant decline in hormone output, and that this inhibitory response was reversed by treatment with the lower, but not higher dose of SIN-1.

Discussion

Metabolic constraint of female reproduction ensures that this lengthy, energy-costly function proceeds only when sufficiency energy is available to ensure successful completion. This study examined the premise that distinctive estrous cycle-associated patterns of E release, embodying negative- versus positive-feedback signals to the reproductive neuroendocrine axis, differentially regulate GnRH-LH sensitivity to short-interval FD. Current data show that in the presence of estrous cycle peak (but not nadir) E levels, FD caused hindbrain AMPK-mediated diminution of rPO nNOS and GnRH-1 proteins, alongside decreased pituitary LH secretion. FD caused divergent Cc-reversible adjustments in preoptic NE accumulation in high- versus low-E – dosed rats, as well as diminished hindbrain AMPK-independent activity in select hypothalamic structures in the latter group. Results suggest that E positive-feedback activation of the GnRH-LH axis is associated with acquired sensitivity to hindbrain metabolic sensory input during FD. E stimulus strength likely establishes forebrain substrates of FD-initiated NE input as well as directionality of NE signaling in those target sites. Observations that the NO donor SIN-1 reverses FD repression of GnRH and several upstream regulatory neuron populations imply that NO is a critical

coordinating element in neural pathways that impose metabolic restriction of reproductive neuroendocrine function.

The present study addressed the theory that female rat forebrain NE activity may vary in a site-specific manner in response to FD according to estrous cycle stage-specific patterns of E secretion. Data show that FD decreased NE accumulation throughout the preoptic area and hypothalamus in low-E – dosed rats, but amplified NE activity in preoptic loci in the high-E group. Hindbrain AMPK-driven FD NE signaling was focused to reproduction-significant preoptic loci in both E dosage groups, but numbers of targeted structures and direction of NE accumulation in those sites depended upon E concentration. Intra-CV4 Cc administration normalized rPO NE activity in E-30 (reversal of FD inhibition) and –300 animals (reversal of FD stimulation), as well as NE accumulation in the AVPV and MPN in the latter group. E is thus a critical determinant of neural location and mode (positive versus negative) of FD-propelled NE activity. To date, the preoptic area has been subject to little scrutiny regarding potential involvement in metabolic homeostasis. Current results emphasize the need to clarify preoptic participation in coordinated corrective responses to metabolic stress, including characterization of local targets and functional sequelae (including counteractive autonomic, neuroendocrine, and/or behavioral outflow) of FD-driven NE signals to the rPO, AVPV, and MPN. Additional research is also necessary to discern if E dose-dependent diminution (low-E dose) versus augmentation (high-E dose) of NE activity in those sites involves different noradrenergic cell sources or if divergent signals reflect E dose-dependent control of local NE production and release from a single set of afferent neurons. It is unclear if disparities involving FD-driven NE signaling to hypothalamic metabolic structures involve high E dose ‘silencing’ of noradrenergic projections to those loci by inhibition of local NE axon firing or equivalent augmentation of rates of NE synthesis, degradation, and release, resulting in no net change in tissue NE content. No Cc effect on FD NE input to the hypothalamus of low-E – dosed animals may reflect activation of non-metabolic-sensory caudal hindbrain noradrenergic neurons or, instead, AMPK-expressing noradrenergic neurons situated outside diffusion range of the CV4.

Current results demonstrate that peak estrous cycle E levels render the GnRH-LH axis susceptible to inhibitory hindbrain metabolic cues. FF E-300 animals exhibited elevated baseline rPO AMPK activity relative to FF E-30. Although FD recruited hindbrain AMPK control of this rPO sensor in both E-300 and E-30, rPO pAMPK content did not differ between FF versus FD E-300 animals. These outcomes may reflect maximal activation of rPO AMPK by E positive-feedback despite uninterrupted feeding, as whole-body energy state becomes more negative as E secretion rises during proestrus [Giles et al., 2010], with the upshot that FD may have minimal capability to augment sensor activity further. An alternative (albeit less likely) explanation is that hindbrain augmentation of rPO pAMPK expression in FD-exposed E-300 rats may be offset by simultaneous cues for sensor deactivation. rPO GnRH-1 protein content was greater in FF E-300 versus E-30 rats, and was subject to Cc-reversible inhibition by FD, alongside reductions in LH secretion, in the former group. Because GnRH-1 profiles were refractory to FD in lowE animals despite elevated rPO pAMPK activity, this sensor may likely regulate non-reproductive responses to this metabolic stress when E secretion is at nadir. Previous studies showed that rPO GnRH

neurons express AMPK [Ibrahim and Briski, 2014; Shrestha et al., 2014]. Ongoing work aims to determine if, during peak E release, the GnRH-based sensor is selectively activated by FD relative to the rPO at-large, and whether such augmentation is causally related to down-regulated GnRH-I protein expression owing to FD. Additional effort will also examine whether hindbrain AMPK regulation of rPO pAMPK and GnRH-I expression is mediated by down- versus up-regulated NE signals in corresponding E-30 and E-300 animals.

The neurotransmitters γ -amino-butyric acid (GABA) and nitric oxide (NO) act directly to correspondingly inhibit [Wuttke et al., 1996; Han et al., 2002, 2004] or stimulate [Bhat et al., 1998; Dhandapani and Brann, 2000] GnRH neurons [Bhat et al., 1994; Christian and Moenter, 2010]. Glucoprivation augments rPO GABA neurotransmission [Singh and Briski, 2004] and enhances transcriptional activation of GABA- and nitrergic neurons in that location [Briski and Sylvester, 1999; Briski and Singh, 2008]. Experiment 1 therefore investigated the hypothesis that FD causes Cc-reversible changes in rPO expression of the marker biosynthetic enzymes GAD_{65/67} and nNOS in the presence of nadir and/or peak plasma E. Data reveal that while rPO GAD_{65/67} protein levels were refractory to FD irrespective of E dose, rPO nNOS content declined in FD-exposed E-300 animals but normalized despite FD by Cc treatment. These outcomes infer that rPO nitrergic neurons are downstream recipients of hindbrain AMPK signaling during FD stress. NE control of PVH magnocellular nerve cell nNOS has been reported [Grange-Messent V et al., 2004]; our premise that rPO nNOS may subject to similar control merits investigation. Observations here of augmented baseline nNOS content in FF E-300 versus FF E-30 groups align with proof of NO involvement in steroid positive-feedback induction of the LH surge [Bonavera et al., 1993; Rettori et al., 1994; Kohsaka et al., 1999]. A principle aim of Experiment 2 was to verify whether the parallel fall in rPO nNOS and GnRH-I protein expression in FD-exposed E-300 rats signifies a causal relationship. Data showing SIN-1 augmentation of GnRH-I expression and LH profiles bolster this view, as GnRH-1 mRNA and protein profiles and plasma LH concentrations were greater in SIN-1- versus V-injected FD-exposed E-300 rats. Glutamate is a requisite stimulatory signal for preovulatory LH release [Brann and Mahesh, 1997; Dhandapani and Brann, 2000], acting directly [Christian and Moenter, 2010] and indirectly, via activation of NO release [Brann et al., 1997; Ganapathy et al., 1998], to stimulate GnRH output. GnRH neurons reside in close proximity to large numbers of Vglut2-expressing glutaminergic neurons [Ziegler et al., 2002; Lin et al., 2003]. Current data show that FD provokes SIN-1 – reversible diminution of rPO Vglut2 protein expression, which implies that NO deficiency may down-regulate this key excitatory input to GnRH neurons [Figure 8]. The present studies do not clarify whether glutaminergic neurons are directly controlled by NO during FD, or if NO may indirectly suppress rPO glutamate transmission via diminished kisspeptin.

AVPV and ARH kisspeptinergic neurons are critical mediators of corresponding E positive- and negative-feedback cues to the GnRH-LH axis [Popa et al., 2008]. Kisspeptin stimulates preoptic GnRH, nitrergic, and glutaminergic neurons via GPR54 receptor signaling [Irwig et al., 2004; Messenger et al., 2005; Pielecka-Fortuna and Moenter, 2010; Hanchate et al., 2012]. As Experiment 1 identified the AVPV, but not ARH as a target of FD-driven NE cues in E-300 animals, we examined the premise that kisspeptin yield in the former site may be regulated by FD. As immunoblots disclosed a trend (albeit insignificant) toward decreased

protein in E-300/FD versus E-300/FF groups, we next addressed the premise in Experiment 2 that KiSS1 neurons in the former, but not latter site may exhibit transcriptional reactivity to that metabolic challenge. Outcomes of Experiment 2 document FD-associated decrements in AVPV KiSS1 gene (statistically significant) and protein (statistically insignificant) expression in E-300 animals, as well as notable suppression of ARH kisspeptin protein content. These results align with earlier reports that 48 hour fasting suppresses AVPV (but not ARH) KiSS-1 gene expression in male rats [Kalamatianos et al., 2008]. Moreover, data here show that SIN-1 reversed FD repression of AVPV KiSS1 mRNA and augmented kisspeptin protein levels, but did not modify ARH KiSS1 gene or protein expression. These outcomes implicate NO in AVPV kisspeptinergic cell reactivity to FD; however, it is not known if this nitrgic control involves direct or indirect signaling. It remains unclear if AVPV and/or ARH KiSS1 neurons mediate FD effects on positive-feedback patterns of LH release. ARH kisspeptin/neurokinin B/dynorphin (KNDy) neurons reportedly regulate the LH surge via dynorphin input to the AVPV [Helena et al., 2015]. Present outcomes thus raise the prospect that ARH KNDy cells may function in hindbrain AMPK/NE-independent pathways that curb GnRH during FD.

AVPV KiSS1- and DMN RFRP-3-expressing neurons act in response to circadian cues to respectively stimulate or inhibit the E positive-feedback – induced LH surge [Khan and Kauffman, 2002]. RFRP-3 neurons reside exclusively in the DMN [Kriegsfeld et al., 2006] and provide direct innervation to preoptic GnRH neurons [Wu et al., 2009; Rizwan et al., 2012]. Elevated E inhibits RFRP-3 transmission, thereby dampening inhibition of GnRH [Gibson et al., 2008]. Data forthcoming from Experiment 1 showed that the DMN is the sole hypothalamic target of hindbrain AMPK-controlled NE input in FD-exposed E-300 rats. An objective of Experiment 2 was thus to evaluate sensitivity of DMN RFRP-3 to FD. Results indicate that RFRP-3 mRNA and protein levels are both significantly augmented by FD, and that these responses were prevented by SIN-1. Conjecture that hindbrain AMPK-impelled DMN NE activity regulates RFRP-3 via NO-dependent mechanisms will require experimental validation. As nitrgic nerve cell bodies are absent from the DMN [Bhat et al., 1995], NO regulation of RFRP-3 transmission may involve local release of this gaseous transmitter from axons derived elsewhere in the forebrain or, alternatively, NO activity at extra-DMN substrates that in turn innervate the RFRPergic population.

ARH NPY is obligatory for E positive-feedback initiation of the LH surge [Kalra and Crowley, 1992], while POMC peptide products β -endorphin (β -END) and α -melanocyte-stimulating hormone (MSH) respectively inhibit or stimulate LH release [Kalra, 1985; Murray et al., 2000]. NPY and POMC gene transcripts or products are sensitive to FD of 24 hours or greater duration [Señaris et al., 2011; Sainsbury and Zhang, 2010; Jovanovic et al., 2010]. Previous work showed that in E-30 animals, hindbrain AMPK exerts an inhibitory versus stimulatory influence on ARH NPY and POMC protein expression, respectively, during FD [Alenazi et al., 2016]. Data here from E-300 rats indicate that FD-associated augmentation of NPY protein (but not mRNA) is reversible by SIN-1. Further effort is required to determine the neuroanatomical location of nitrgic neurons that mediate this impact of FD. There is also a need to examine whether such amplification of NPY expression is mediated by hindbrain AMPK through NE-independent mechanisms, and if intensified NPYergic input offsets stimuli that inhibit LH release. Repression of inhibitory

endorphinergic input to the GnRH-LH axis enables the LH surge. Here, FD imposed for 18 hours did not alter ARH POMC mRNA or protein content, suggesting that the duration of FD may be a critical determinant of POMC reactivity to this metabolic stress. Evidence here for high dose enhancement of POMC expression by SIN-1 raises the possibility that β -END suppressive action may be increased, thereby overshadowing stimulatory mechanisms that prevail at lower dosages of SIN-1

In summary, the present project yields novel proof of differential reproductive neuroendocrine sensitivity to short-term FD related to female rat estrous cycle patterns of E secretion. Data show that in the presence of peak (proestrus-like) circulating E levels, FD elicits hindbrain AMPK-dependent suppression of rPO nNOS and GnRH-1 protein expression and pituitary LH release. Results thus imply that E positive-feedback activation of the GnRH-LH axis heightens its sensitivity to hindbrain metabolic sensory input during FD. E stimulus strength likely establishes forebrain substrates of FD-initiated NE input as well as directionality of NE signaling in those target sites. Observations that the NO donor SIN-1 reverses FD repression of GnRH and several upstream regulatory neuron populations imply that NO may act simultaneously via preoptic and DMN NE-dependent mechanisms to curb pre-ovulatory LH secretion during metabolic stress [Figure 8].

Abbreviations

AMPK	adenosine 5'-monophosphate-activated protein kinase
ARH	arcuate hypothalamic nucleus
AVPV	anteroventral periventricular nucleus
Cc	Compound c
CV4	caudal fourth ventricle
DMN	dorsomedial hypothalamic nucleus
DMSO	dimethyl sulfoxide
E	estradiol
FD	food-deprivation
FF	full-fed
KISS1	metastasis-suppressor KiSS-1
GABA	γ -amino-butyric acid
GnRH	gonadotropin-releasing hormone
HPG	hypothalamic-pituitary-gonadal
LH	luteinizing hormone
LHA	lateral hypothalamic area

LV	left lateral ventricle
MPN	medial preoptic nucleus
NE	norepinephrine
NO	nitric oxide
NNOS	neuronal nitric oxide synthase
NPY	neuropeptide Y
OVX	ovariectomy
O.D.	optical density
pAMPK	phospho-AMPK
POMC	proopiomelanocortin
PVN	paraventricular hypothalamic nucleus
SIN-1	3-morpholinosydnonimine
RFRP-3	RFamide-related peptide-3
RPO	rostral preoptic area
Vglut2	vesicular glutamate transporter-2
VMN	ventromedial hypothalamic nucleus

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Highlights

- Metabolic cues restrain gonadotropin-releasing hormone (GnRH)-luteinizing hormone (LH) output
- Short-term food deprivation (FD) inhibits GnRH/LH when estradiol levels are at estrous cycle peak.
- Hindbrain Compound C (Cc) delivery reverses FD repression of reproductive neuroendocrine axis
- Cc reverses FD augmentation or suppression of preoptic norepinephrine and nitric oxide synthase
- SIN-1 attenuates FD inhibition of GnRH/LH

Figure 1. Effects of CV4 Administration of the AMPK Inhibitor Compound C (Cc) on Short-Term FD-Associated Adjustments in Preoptic/Hypothalamic Norepinephrine (NE) Activity in E-Implanted OVX Female Rats

E [30 (E-30) versus 300 (E-300) $\mu\text{g/mL}$] - implanted OVX rats were exposed to FD or served as full-fed (FF) controls. FF animals were injected into the CV4 with the vehicle dimethyl sulfoxide (DMSO; V) [solid bars], while FD rats received V [diagonal-striped bars] or Cc [5.0 $\mu\text{g}/2.0 \mu\text{L}$ DMSO; cross-hatched bars] 1 hour prior to sacrifice. Graphs depict mean rostral preoptic area (rPO; Panel A), anteroventral periventricular nucleus (AVPV; Panel B), medial preoptic nucleus (MPN; Panel C), arcuate hypothalamic nucleus (ARH; Panel D), dorsomedial hypothalamic nucleus (DMN; Panel E), ventromedial hypothalamic nucleus (VMN, Panel), paraventricular hypothalamic nucleus (PVN; Panel G), and lateral hypothalamic area (LHA, Panel H) tissue NE content $\pm$ S.E.M. in E-30/FF/V (solid white bars), E-30/FD/V (diagonal-striped white bars), E-30/FD/Cc (cross-hatched white bars), E-300/FF/V (solid grey bars), E-300/FD/V (diagonal-striped grey bars), and E-300/FD/Cc (cross-hatched grey bars) animals ($n=4/\text{group}$). * $p<0.05$ compared to FF/V; ** $p<0.05$ versus FD/V; # $p<0.05$ versus E-30/FF/V.

Figure 2. Hindbrain AMPK Regulation of rPO AMPK Activity and rPO/AVPV Reproduction-Regulatory Proteins in FD-Exposed E-Implanted OVX Rats

Panels A–E correspondingly depict mean normalized rPO phospho-AMPK (pAMPK), rPO GnRH-I, rPO neuronal nitric oxide synthase (nNOS), rPO glutamate decarboxylase_{65/67} (GAD_{65/67}) and AVPV kisspeptin protein optical density measures $\pm$ S.E.M. for FF/V, FD/V, and FD/Cc groups of E-30 or E-300 animals. Panel F shows typical Western blots of rPO pAMPK (Row A), GnRH-I (Row B), nNOS (Row C), GAD_{65/67} (Row D), kisspeptin (Row E) and α -tubulin (Row F). * $p < 0.05$ versus FF/V; ** $p < 0.05$ versus FD/V; # $p < 0.05$ versus E-30/FF/V.

Figure 3. Effects of FD, with or without CV4 Cc Administration, on Pituitary Luteinizing Hormone (LH) Secretion in FD-Exposed E-30 versus E-300 OVX Rats

Bars show mean plasma LH concentrations $\pm$ S.E.M. for FF/V, FD/V, and FD/Cc groups of E-30 and E-300 animals. * $p < 0.05$ compared to FF/V; # $p < 0.05$ versus E-30/FF/V.

Figure 4. Impact of Lateral Ventricular (LV) Delivery of the Nitric Oxide Donor 3-Morpholinodisodium (SIN-1) on FD-Associated Patterns of rPO GnRH-I and Vesicular Glutamate Transporter-2 (Vglut2) Protein Expression in E-Implanted OVX Rats
Mean rPO GnRH-I mRNA (Panel A) and protein (Panel B) and rPO Vglut2 transcript (Panel C) and protein (Panel D) measures $\pm$ S.E.M. are depicted for FF/V, FD/V, FD/S(100), and FD/S(200) groups of E-300 rats ($n=5$ /group). Graphs presented in Panels B and D are accompanied by representative immunoblots for GnRH-I and Vglut2, respectively. * $p<0.05$ compared to FF/V; ** $p<0.05$ versus FD/V.

Figure 5. Effects of LV SIN-1 Administration on AVPV Kisspeptinergic and DMN RFRPergic Responses to FD in E-Implanted OVX Rats

Panels A and B correspondingly depict mean AVPV KiSS1 mRNA and kisspeptin protein values $\pm$ S.E.M. for FF/V, FD/V, FD/S(100), and FD/S(200) groups of E-300 animals. Panels C and D illustrate mean normalized values for DMN RFRP mRNA and RFRP-3 protein content for those groups. Representative kisspeptin and RFRP-3 Western blots are presented in Panels B and D, respectively. * $p < 0.05$ compared to FF/V; ** $p < 0.05$ versus FD/V; # $p < 0.05$ versus E-30/FF/V.

Figure 6. Role of Nitric Oxide in FD-Associated Patterns of ARH Kisspeptin, Neuropeptide Y (NPY), and Pro-opiomelanocortin (POMC) Gene and Protein Expression in E-Implanted OVX Rats

ARH KiSS1 mRNA (Panel A) and kisspeptin protein (Panel B), NPY mRNA (Panel C) and protein (Panel D), and POMC mRNA (Panel E) and protein (Panel F) values $\pm$ S.E.M. are shown for FF/V, FD/V, FD/S(100), and FD/S(200) groups of E-300 rats (n=5 animals/group). Representative target protein Western blots are provided. *p<0.05 compared to FF/V; **p<0.05 versus FD/V.

Figure 7. Effects of FD, with or without LV SIN-1 Administration, on Pituitary Luteinizing Hormone (LH) Secretion in FD-Exposed E-300 OVX Rats

Bars depict mean plasma LH concentrations $\pm$ S.E.M. for FF/V, FD/V, FD/S(100), and FD/S(200) groups of E-300 rats. * $p < 0.05$ compared to FF/V; ** $p < 0.05$ versus FD/V.

Figure 8. Model for Caudal Hindbrain Metabolic Sensor Regulation of the Pituitary LH Surge in FD Female Rats

Current data show that FD curtails steroid positive-feedback activation of the HPG axis through caudal hindbrain AMPK-dependent mechanisms. ER-expressing A2 noradrenergic neurons are a probable source of E concentration-sensitive AMPK cues during FD [Insert A]. E stimulus strength also likely establishes forebrain targets of FD-initiated NE [Insert B. 1] and non-NE [Insert B.2] signals. Neural networks that impose caudal hindbrain metabolic inhibition of rPO GnRH-I neurons in the presence of peak E likely involve NO signaling [Insert C], which may function upstream of one or more neurotransmitters (e.g. rPO glutamate, DMN RFRP-3, ARH NPY, ARH kisspeptin) identified here by SIN-1 – reversible inhibitory responses to FD.