Published in final edited form as:

J Neurosci Res. 2017 July ; 95(7): 1427–1437. doi:10.1002/jnr.23892.

Hindbrain A2 Noradrenergic Neuron Adenosine 5' - Monophosphate-Activated Protein Kinase (AMPK) Activation, Upstream Kinase/Phosphorylase Protein Expression, and Receptivity to Hormone and Fuel Reporters of Short-Term Food Deprivation are Regulated by Estradiol

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Abstract

Estradiol (E) mitigates acute and post-acute adverse effects of 12 hr-food deprivation (FD) on energy balance. Hindbrain 5'-monophosphate-activated protein kinase (AMPK) regulates hyperphagic and hypothalamic metabolic neuropeptide and norepinephrine responses to FD in an E-dependent manner. Energy state information from AMPK-expressing hindbrain A2 noradrenergic neurons shapes neural responses to metabolic imbalance. Here, we investigated the hypothesis that FD causes divergent changes in A2 AMPK activity in E versus oil (O)-implanted ovariectomized female rats, alongside dissimilar adjustments in circulating metabolic fuel [glucose, free fatty acids (FFA)] and energy deficit-sensitive hormone [corticosterone, glucagon, leptin] levels. FD decreased blood glucose in oil (O)-, but not E-implanted ovariectomized female rats, and elevated or reduced glucagon levels in O and E, respectively. FD decreased circulating leptin in O and E, but increased corticosterone and FFA concentrations in E only. Western blot analysis of laser-microdissected A2 neurons showed that glucocorticoid receptor type II and very long-chain acyl-CoA synthetase 3 protein profiles were amplified in FD/E versus FD/O. A2 total AMPK protein was elevated without change in activity in FD/O, while FD/E exhibited increased AMPK activation along with decreased upstream phosphatase expression. The catecholamine biosynthetic enzyme, dopamine- β -hydroxylase (D β H), was increased in FD/O, but not FD/E A2 cells. Data show discordance between A2 AMPK activation and glycemic responses to FD as sensor activity was refractory to glucose decrements in FD/O, but augmented in FD/E despite stabilized glucose and elevated FFA levels. E-dependent amplification of AMPK activity may reflect adaptive conversion to fatty acid oxidation and/or glucocorticoid stimulation. FD augmentation of A2 D β H protein profiles in FD/O but not FD/E animals suggests that FD may

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Conflict of Interest Statement: The authors have no conflict of interest to declare.

Statement of Author Contributions: KPB and FSHA designed the work, FSHA and MS collected and analyzed the data; KPB, FSHA, and PWS interpreted the data, KPB drafted the manuscript, KPB and FSHA revised and approved the final version to be published.

correspondingly regulate NE synthesis versus metabolism/release in the absence versus presence of E. Mechanisms underlying translation of E-contingent A2 neuron responses to FD into regulatory signaling remain to be determined.

Graphical Abstract

Brackets indicating effects of short-term food deprivation on A2 nerve cell total and phosphorylated AMPK protein content show that this metabolic challenge elevated total AMPK without altering in activity in ovariectomized (OVX) animals implanted with oil, but augmented AMPK activation alongside decreased upstream phosphatase expression in OVX rats replaced with estradiol.

Keywords

A2 noradrenergic neurons; estradiol; adenosine 5'-monophosphate-activated protein kinase; free fatty acid levels; corticosterone; glucocorticoid receptor; very long-chain acyl-CoA synthetase 3

Introduction

The brain is integral for preservation of systemic energy homeostasis, owing to its ability to monitor and initiate compensatory responses to body-wide disturbances in nutrient and stored energy fuel availability. The enzyme 5'-monophosphate-activated protein kinase (AMPK) provides crucial input on brain cell ATP availability to neural pathways that safeguard systemic energy stability. This ultrasensitive, evolutionarily-conserved energy gauge is activated via phosphorylation in response to metabolic stressors (exercise, starvation, hypoxia, etc.) that increase the intracellular AMP/ATP ratio [Hardie, 2003; Kahn et al., 2005]. Phosphorylated AMPK (pAMPK) inhibits ATP-consuming biosynthetic pathways, while augmenting ATP-producing metabolic functions such as fatty acid oxidation. AMPK is expressed in hypothalamic and hindbrain elements of the sizable brain metabolic regulatory network. Hindbrain caudal dorsal vagal complex (cDVC) AMPK is implicated in neural regulation of feeding [Hayes et al., 2009; Alenazi et al., 2015] and glucostasis [Ibrahim et al., 2013]. DVC AMPK responds to substrate fuel availability as activation of this sensor by insulin-induced hypoglycemia is normalized by localized infusion of the oxidizable glucose metabolite L-lactate [Gujar et al., 2014].

Short-term abstinence from food intake, planned or unplanned, is unavoidable in modern life, but is negatively correlated with appetite control and obesity. The ovarian hormone estradiol (E) limits acute re-feeding after short-term feeding suspension (12-hour food deprivation; FD), but augments acute re-feeding when FD coincides with hindbrain energy depletion, mimicked by caudal fourth ventricular (CV4) administration of the AMPK activator 5-aminoimidazole-4-carboxamide-riboside (AICAR) [Ibrahim and Briski, 2015]. This compound metabolic stress also exerts E-dependent effects during later resumption of circadian-induced food intake, e.g. increased consumption versus weight gain in the presence versus absence of E. These results signify that E mitigates acute and post-acute adverse effects of disrupted fuel acquisition on systemic energy balance. cDVC AMPK impacts E influence as E- versus oil (O)-implanted ovariectomized (OVX) female rats

exposed to FD display divergent patterns of cDVC AMPK activation [Ibrahim and Briski, 2014], as well as disparate adjustments in feeding and hypothalamic AMPK phosphorylation, metabolic neurotransmitter expression, and norepinephrine activity after intra-CV4 delivery of the AMPK inhibitor compound C [Alenazi et al., 2015]. Cc treatment of FD/E, but not FD/O animals normalizes food intake to full-fed *ad-libitum* levels, implying that in the presence of E, primary if not exclusive impetus to re-feed emanates from this sensor. A2 noradrenergic neurons are a plausible source of E-dependent AMPK signaling during FD as this neurochemical phenotype is the sole cDVC cell population identified to-date as AMPK-positive and is, moreover, capable of integrating steroidal and metabolic stimuli by virtue of estrogen receptor-alpha (ER α) and -beta (ER β) protein expression [Ibrahim et al., 2013]. Application of microlaser technology to optical systems provides an unparalleled means for selective procurement of pure nerve cell populations from native tissue environment for gene and/or protein expression analysis. Here, pure A2 nerve cell samples obtained by single-cell laser-catapult microdissection were analyzed for protein content by high-sensitivity Western blotting to address the premise that E regulates FD-induced patterns of A2 nerve cell AMPK and/or pAMPK expression in OVX female rats. Our prior work showed that hypoglycemic effects on A2 AMPK activity correlate with altered expression of the AMPK upstream kinase Ca⁺⁺/calmodulin-dependent protein kinase-beta (CAMKK β) and protein phosphatase PP2A [Tamarkar et al., 2015]. In light of evidence that cDVC AMPK activity is enhanced to a greater degree in FD/E versus FD/O female rats following CV4 AICAR administration [Ibrahim and Briski, 2015], we speculate here that upstream kinase and/or phosphatase proteins may be reactive to FD in an E-conditional manner.

A further aim of this project was to investigate the impact of E on substrate fuel and endocrine signals of FD, as well as A2 nerve cell receptivity to these FD surrogates. Available studies focus on male rats, in which FD of 4- to 10-hr duration reportedly decreases blood glucose, while elevating free fatty acid (FFA) [Le Magnon et al., 1980]. Here, we examined whether FD alters circulating metabolic fuel [glucose, FFA] and energy deficit-sensitive hormone [corticosterone, glucagon, ghrelin] levels in females, and if E is involved in those responses. We also addressed the hypothesis that A2 profiles of fatty acid (FA) metabolic proteins, e.g. acyl-coenzyme A synthetase long-chain FA activator (ACSVL-3; also referred to as FA transporter protein FATP3); SLC5A8, a Na⁺-coupled L-lactate and short-chain FA transporter [Martin et al., 2006]; and the FA translocase FAT/CD36, which is implicated in FA sensing in the hypothalamus [Moulle et al., 2013, 2014], are modified differently in OVX + O versus OVX + E. The type II glucocorticoid receptor (GR) transduces regulatory effects of normal and elevated corticosterone secretion on cell targets throughout the central neuroaxis. GR immunoreactivity (-ir) is localized to the nucleus of the solitary tract, the sensory component of the DVC [Ahima and Harlan, 1990; Cintra et al., 1994], where GR-ir-positive neurons are transcriptionally activated in response to hypoglycemia [Kale et al., 2007]. The present study analyzed GR protein expression in A2 neurons by Western blot to verify that A2 cells express this receptor protein, and to determine if FD exerts differential effects on this protein in the presence versus absence of E.

Methods and Materials

Animals

Adult female Sprague Dawley rats (250–300 g *bw*) were maintained under a 14 hr light: 10 hr dark schedule (lights on at 0.500 hr), and allowed free access to standard laboratory rat chow (Harlan Teklad LM-485; Harlan Industries, Madison, WI) and water. Animals were accustomed to daily handling. All experimental protocols were conducted in accordance with NIH guidelines for the care and use of laboratory animals, with approval by the University of Louisiana at Monroe Institutional Animal Care and Use Committee.

Experimental Design

On day 1, rats were anesthetized with ketamine:xylazine (0.1 ml/100 g *bw i.p.*, 90 mg ketamine:10 mg xylazine/ml; Henry Schein, Melville, NY) and bilaterally ovariectomized (OVX) and implanted with a subcutaneous silastic capsule (0.062 in. *i.d.* x 0.125 in. *o.d.*; 10 mm/100 g *bw*) loaded with 30 µg estradiol E)/mL safflower oil or oil alone (O) (Briski et al., 2001). This steroid replacement regimen yields approximate plasma E concentrations of 22 pg/ml, replicating circulating hormone levels characteristic of metestrus in 4-day (Butcher et al., 1974) or diestrus day 2 in 5-day ovary-intact cycling animals (Goodman, 1978). Animals were injected with ketoprofen (1 mg/kg *bw sc*) before and after surgeries; closed incisions were treated topically with 0.25% bupivacaine. At 21.00 hr on day 7, E and O animals were randomly divided into full-fed (FF; n=3 FF/E, n=3 FF/O) and FD groups, at which time food was removed from n=3 FD/E and n=3 FD/O. On day 8, rats were sacrificed at 09.00 hr for trunk blood and brain tissue collection. Brains were immediately snap-frozen in liquid nitrogen-cooled isopentane prior to storage at –80°C. Trunk blood was collected for blood glucose and plasma hormone measurements. No animals were excluded from the study.

Western Blot Analysis of Laser-Catapult Microdissected Hindbrain Dorsal Vagal Complex (DVC) A2 Noradrenergic Neurons

Serial 10 µm-thick frozen sections were cut from DVC at the location of A2 neurons, e.g. –14.36 to –14.86 mm posterior to *bregma*, and mounted on polyethylene naphthalate membrane slides (prod. no. 911724; Carl Zeiss MicroImaging, Inc., Thornwood, NY). Tissue sections were fixed with cold acetone (prod. no. 179124, Sigma-Aldrich Co. LLC, St. Louis, MO), blocked with 5% normal horse serum (prod. no. PK-6120; Vectastain Elite ABC mouse IgG kit; Vector Laboratories, Inc., Burlingame, CA) diluted in 0.05 M Tris-buffered saline, pH 7.4, containing 0.05% Triton X-100 (prod. no. T9284; Sigma-Aldrich) (TBS-Tx), and incubated for 24 hr at 4°C with a mouse monoclonal primary antiserum against tyrosine hydroxylase (TH) (prod. no. 22941, 1:1,000; ImmunoStar, Inc., Hudson, WI; RRID: AB_2313787) diluted in TBX-Tx. Sections were sequentially incubated with Vectastain IgG Elite ABC mouse IgG kit biotinylated secondary antibody, ABC reagent, and Vector DAB kit reagents (prod. no. SK-4100; Vector Laboratories) to visualize TH-immunoreactive (-ir) neurons. Individual TH-ir cells exhibiting a visible nucleus and complete labeling of the cytoplasmic compartment were harvested with a Zeiss P.A.L.M. UV-A microlaser (Carl Zeiss MicroImaging). For each protein of interest, a minimum of three separate pools of 50 TH-ir neurons per treatment group (16–17 cells/animal) were collected into 30 µL tissue lysis buffer (2% sodium dodecyl sulfate, 0.05 M dithiothreitol, 10% glycerol, 1 mM EDTA,

60 mM Tris HCl, pH 7.2). Denatured A2 nerve cell proteins were electrophoresed in 4–20% gradient Tris–glycine gels and transblotted to 0.45- μ m polyvinylidene fluoride-Plus membranes (prod. no. PV4HY00010; Osmonics, Gloucester, MA). Membranes were pretreated with Quentix Western blot signal enhancer (ThermoFisherScientific, Rockford, IL), then were blocked with 0.1% Tween-20 (Sigma-Aldrich) and 2% bovine serum albumin (MP Biomedicals, Solon, OH) or 5% donkey serum prior to overnight incubation at 4°C with primary antisera [rabbit polyclonal AMPK $_{\alpha 1/2}$ (H-300), 1:500 (prod. no. sc-25792, Santa Cruz Biotechnology, Inc., Santa Cruz, CA; RRID: AB_2169546); rabbit anti-pAMPK $_{\alpha 1/2}$ phospho(Thr 172) (Thr172), 1:500 (prod. no. sc-33524, Santa Cruz Biotechnol.; RRID: AB_2169714); rabbit anti-dopamine- β -hydroxylase (H-213) (D β H), 1:500 (prod. no. sc-15318, Santa Cruz Biotechnol.; RRID: AB_2089347); mouse anti-PP2A-C $_{\alpha/\beta}$ (7A6), 1:500 (prod. no. sc-81601, Santa Cruz Biotechnol.; RRID: AB_81601); goat anti-CAMKK β (L-19), 1:500 (prod. no. sc-9629, Santa Cruz Biotechnol.; RRID: AB_2243844); rabbit anti-GR (M-20), 1:500 (prod. no. sc-1004, Santa Cruz Biotechnol.; RRID: AB_2155786); goat anti-ACSVL3 (Q-12), 1:500 (prod. no. sc-137275, Santa Cruz Biotechnol.; RRID: AB_10838921); goat anti-SLC5A8 (A-12), 1:500 (sc-34189; Santa Cruz Biotechnol.; AB_2189838); goat anti-FAT/CD36 (N-15), 1:500 (sc-5522, Santa Cruz Biotechnol.; RRID: AB_638143); mouse anti- α -tubulin, 1:1,000 (prod. no. CP06, Calbiochem., Gibbstown, NJ); RRID: AB_212802] diluted in appropriate blocking solution. Membranes were subsequently incubated with peroxidase-conjugated goat anti-mouse (1:5,000; prod. no. NEF 822001EA; Perkin Elmer, Boston, MA), goat anti-rabbit (1:5,000; NEF812001EA, Perkin Elmer) or donkey anti-goat (1:5,000; prod. no. sc-2020, Santa Cruz Biotechnol.; RRID: AB_631728) secondary antisera, followed by SuperSignal West Femto Maximum Sensitivity substrate (prod. no. 34096; ThermoFisherSci.). Protein band chemiluminescence imaging and densitometric analysis were performed using a Syngene G:Box Chemi and Genetool 4.01 software. Protein optical density (O.D.) measures were normalized to α -tubulin. Protein molecular weight markers were included in each analysis. Each protein was analyzed in triplicate at minimum. Each primary antiserum detected a single band protein as illustrated on representative blots.

Glucose, Hormone, and Free Fatty Acid Analyses

Blood glucose was analyzed with an ACCU-CHECK Aviva plus glucometer (Model: NC; Roche Diagnostics, Indianapolis, IN) (Kale et al., 2006). Plasma glucagon (GL-32K; EMD Millipore, Billerica, MA) and corticosterone (07120102; MP Biomedicals, LLC, Santa Ana, CA) concentrations were determined by radioimmunoassay, as described (Briski and Nedungadi, 2009). Serum ghrelin concentrations were measured using ELISA kit reagents (EZRGRA-09K; EMD Millipore), per kit instructions. Briefly, 20 μ L aliquots of serum obtained from Pefabloc-treated blood samples and assay standards were added to individual plate wells along with matrix solution, assay buffer, and antibody solution (50 μ L), and incubated for 2 hr at room temperature at 400–500 rpm. After plate contents were discarded, wells were washed 3x, then incubated with enzyme conjugate (100 μ L) for 30 min while shaken. Wells were emptied, washed 6x, then incubated for 5–20 min with 100 μ L of substrate. After termination of reactions with 100 μ L stop solution, contents absorbance for each well was read at 450 nm in an Emax Precision Microplate Reader (Molecular Devices, LLC, Sunnyvale, CA). Circulating free fatty acid levels were analyzed by colorimetric

coupled enzyme assay (MAK044; Sigma-Aldrich), per manufacturer's instructions. In brief, serum sample aliquots were added to individual plate wells and diluted to a 50 uL volume with assay buffer. Acyl-CoA synthase (2 uL) and master reaction mix (50 uL; consisting of 44 uL fatty acid assay buffer, 2 uL fatty acid probe, 2 uL enzyme mix, and 2 uL enhancer) were sequentially incubated in each well for 30 min at 37 °C in the dark. Absorbance readings for individual wells were read at 570 nm.

Statistical Analyses

Mean glucose, hormone, free fatty acid, and normalized A2 nerve cell protein O.D. measures were evaluated by two-way analysis of variance, with food deprivation and estradiol treatment as factors in the 2×2 factorial design, and Duncan's multiple range test. Differences of $p < 0.05$ were considered significant.

Results

Figure 1 illustrates effects of 12-hour food deprivation [FD; 21.00 hr to 09.00 hr] on plasma glucose (Figure 1.A), free fatty acid (Figure 1.B), glucagon (Figure 1.C), corticosterone (Figure 1.D), and ghrelin (Figure 1.E) concentrations in estradiol (E)- versus oil (O)-implanted ovariectomized female rats. Data demonstrate that each of these five parameters was statistically equivalent in full-fed E (FF/E; white diagonal-striped bar) versus O (FF/O; gray diagonal-striped bar) groups. As shown in Figure 1.A, FD reduced glucose levels in O, but not E rats [$F_{3,8} = 21.69$, $n=3$, $p=0.0003$, $1-\beta=1.0$; FD effect: $F=41.28$, $p=0.0002$; E and FD interaction: $F = 19.98$, $p=0.002$]. Circulating free fatty acid concentrations (Figure 1.B) were elevated in FD/E (solid gray bar), but not FD/O (solid white bar) groups relative to respective FF controls [$F_{3,8} = 7.01$, $n=3$, $p=0.012$, $1-\beta=0.87$; FD effect: $F = 16.04$, $p=0.003$]. FD augmented (statistically significant) or reduced (did not reach statistical significance) plasma glucagon concentrations (Figure 1.C) in O and E rats, respectively [$F_{3,8} = 5.004$, $n=3$, $p=0.03$, $1-\beta=0.68$; E and FD interaction: $F=12.79$, $p=0.009$]. Corticosterone values (Figure 1.D) were significantly elevated in FD/E, but not FD/O [$F_{3,8} = 13.03$, $p=0.008$]; FD effect: $F = 26.75$, $n=3$, $p=0.003$, $1-\beta=0.94$; E and FD interaction: $F = 9.79$, $p=0.02$]. Data in Figure 1.E show that serum ghrelin concentrations were increased to a similar extent by FD in O and E rats [$F_{3,8} = 7.22$, $n=3$, $p=0.01$, $1-\beta=0.86$; FD effect: $F = 19.99$, $p=0.002$]. FD did not modify circulating insulin in E or O (Figure 1.F), whereas serum leptin levels were reduced to an equal extent in FD/E versus FD/O (Figure 1.G) [$F_{3,8} = 19.59$, $n=3$, $p=0.0005$, $1-\beta=0.9$; FD effect: $F = 49.53$, $p=0.0001$; E and FD interaction: $F = 6.38$, $p=0.35$].

Figure 2 depicts effects of FD on A2 noradrenergic neuron AMPK (Figure 2.A) and pAMPK (Figure 2.B) protein expression. Data show that both protein profiles were significantly increased in FD/O, but not FD/E [AMPK: $F_{3,8} = 7.53$, $n=3$, $p=0.01$, $1-\beta=0.89$; E effect: $F = 9.43$, $p=0.01$; E and FD interaction: $F = 11.85$, $p=0.008$] [pAMPK: $F_{3,12} = 6.34$, $n=4$, $p=0.008$, $1-\beta=0.89$; E effect: $F = 5.93$, $p=0.03$; FD effect: $F = 10.75$, $p=0.006$]. Figure 2.C shows FD did not alter the mean ratio of A2 nerve cell pAMPK/AMPK protein content in O animals, but did significantly enhance this protein ratio in E rats. Figure 3 illustrates the impact of FD on upstream AMPK kinase CAMKK β (Figure 3.A) and phosphatase PP2A

(Figure 3.B) profiles in A2 neurons. FD did not alter A2 CAMKK β protein content in O or E rats relative to FF controls, but resulted in elevated levels of expression of this protein in FD/O versus FD/E [$F_{3,8} = 3.85$, $n=3$, $p=0.05$, $1-\beta=0.60$; E effect: $F = 5.36$, $p=0.04$; E and FD interaction: $F = 6.00$, $p=0.03$]. A2 PP2A protein levels were elevated in FF/E versus FF/O, and were significantly reduced by FD in E, but not O animals [$F_{3,12} = 7.80$, $n=4$, $p=0.003$, $1-\beta=0.94$; FD effect: $F = 14.34$, $p=0.002$; E and FD interaction: $F = 8.38$, $p=0.01$]. Data in Figure 4 depict FD effects on A2 nerve cell GR and ACSVL3 proteins in E versus O rats. FD did not alter GR levels in E or O animals relative to FF controls, but caused this protein to be relatively higher in FD/E versus FD/O (Figure 4A) [E effect: $F = 688$, $p=0.03$; $1-\beta=0.73$]. ACSVL3 protein was augmented in FF/E versus FF/O and in FD/E versus FD/O [$F_{3,8} = 6.78$; $n=3$, $p=0.01$, $1-\beta=0.99$; E effect: $F = 19.06$; $p=0.02$]. FD did not modify this profile in either E or O. SCL5A8 and FAT/CD36 proteins were not detected in A2 neurons.

Data in Figure 5 indicate that A2 nerve cell D β H protein expression was significantly increased in FD/O, but not FD/E relative to FF controls [$F_{3,8} = 4.78$, $p=0.03$; $1-\beta=0.70$; E effect: $F = 5.47$, $p=0.04$; FD effect: $F = 6.12$, $p=0.03$].

Discussion

Previous studies show that that caudal hindbrain AMPK crucially shapes hypothalamic AMPK and metabolic neurotransmitter responses to FD in female rats, and that this sensor is the primary if not exclusive source of impetus to re-feed in FD/E animals [Alenazi et al., 2015]. AMPK-expressing A2 noradrenergic neurons are an important E-sensitive [Ibrahim et al., 2013] sensory element of the brain metabolic regulatory network [Gujar et al., 2014]. Here, pure A2 cell samples obtained by laser-catapult microdissection were analyzed by Western blot to address the overarching hypothesis that A2 AMPK activation by FD is conditional on E, correlating with hormone effects on metabolic fuel and endocrine indicators of this metabolic imbalance. Results show that FD elevated A2 total AMPK protein but not net sensor activity in O, while augmenting A2 AMPK activity without adjustment of total protein in E. Intriguingly, A2 sensor activity was refractory to glucose decrements in FD/O, but augmented in FD/E rats despite unaltered glucose and elevated FFA levels. E-contingent amplification of AMPK activity may reflect adaptive reliance of A2 neurons on fatty acid oxidation and/or glucocorticoid regulation of cellular energy metabolism or sensor function. FD augmentation of A2 D β H protein profiles in FD/O but not FD/E suggests that FD may correspondingly regulate NE synthesis versus metabolism/release in the absence versus presence of E. Further research is needed to determine how E governs integration of diverse metabolic and endocrine (corticosterone, glucagon) cues to regulate A2 neurotransmission during FD.

E is a critical factor in systemic metabolic fuel supply in the event of FD, as transient interruption of feeding over a 12-hour period resulted in differential changes in blood glucose and FFA levels in E- versus O-implanted OVX female rats. Results show indicate that alike to gonad-intact male rats [Le Magnon et al., 1983], FD/O animals displayed reductions in circulating glucose; yet, contrary to that earlier work, FFA levels were not coincidentally elevated. In contrast, FD/E animals exhibited no glucose decrements alongside augmented FFA, implying that enhanced substrate fuel availability is a mechanisms of E

defense against cell energy deficiency. A2 nerve cell ACSVL3 protein content was greater in FF/E versus FF/O (despite equivalent plasma FFA levels) groups and in FD/E versus FD/O animals, data that suggest that E may increase FFA utilization by these cells during energy sufficiency as well as short-term suspension of fuel acquisition. FD elevated plasma glucagon (statistically significant) or reduced (did not reach statistical significance) concentrations in O and E rats, respectively, to the extent that hormone levels diverged significantly in FD/O versus FD/E. The FD-associated decline in FD/E glucagon may be causally related, in part, to FFA augmentation as FFA reportedly inhibit glucagon secretion [Gerich et al., 1974; Luyckx et al., 1978]. Plasma corticosterone levels were significantly elevated in response to FD in E, resulting in disparate output in FD/E versus FD/O. At the same time, A2 neuron GR protein levels were significantly higher in FD/E versus FD/O. These data imply that E enhances glucocorticoid regulation of A2 function during FD through mechanisms involving amplification of both strength of hormone signal and cell sensitivity to this stimulus. Since FFA increase corticosterone output via putative central actions [Widmaier et al., 1995], FFA may mediate E-dependent amplification of circulating corticosterone. FD elevated plasma ghrelin levels to an equivalent extent in E and O rats. Ghrelin receptor immunoreactivity (-ir) is localized to the DVC, but is absent from resident catecholamine neurons [Bron et al., 2013]; thus, we did not analyze ghrelin receptor protein in A2 neurons. However, we cannot discount the possibility that E may regulate ghrelin receptor expression in local or distant sources of afferent input to these cells under normo-energetic or energy deficit condition.

Current data point to E regulation of A2 nerve cell AMPK reactivity to FD as total AMPK protein content was increased without adjustment of activity in FD/O, whereas net AMPK levels were stabilized alongside augmented phosphorylation in FD/E. In previous work, we found that FD did not affect cDVC AMPK levels in O rats [Ibrahim et al., 2015]. This deviation of A2 cells from the averaged cDVC response suggests that FD-associated intensification of A2 total AMPK protein is evidently masked by dissimilar changes in this protein profile in non-A2 cells. Unlike total AMPK, pAMPK profiles were elevated in both cDVC tissue and A2 neurons in FD/O animals. In FD/E rats, A2 neurons are excluded from the trend of FD-associated reduction in cDVC pAMPK levels. These results support the unique concept that A2 neurons respond differently to FD versus non-A2 AMPK-expressing cells in the female rat cDVC, regardless of the presence or absence of E. Data also show that the pAMPK/AMPK ratio was increased in A2 neurons from FD/E, but not FD/O rats. On one hand, FD may result in net ATP deficiency or AMP excess in A2 cells in the presence of E, a premise that remains speculative as analytical methods of requisite sensitivity for measurement of AMP/ATP levels in A2 cells *in vivo* are not currently available. Alternatively, A2 AMPK activity may be augmented in FD/E independent of ATP status, through mechanisms involving receptor-mediated actions of endocrine/neurotransmitter signals such as FFA and corticosterone. Indeed, FFA is reported to exert allosteric effects that promote AMPK phosphorylation independent of energy state [Watt et al., 2006], while corticosterone is likewise observed to directly regulate hypothalamic AMPK activity [Shimizu et al., 2008]. The latter possibility of a non-AMP/ATP ratio-based mechanism for FD regulation of A2 AMPK activity in the presence of E is supported by current evidence for down-regulation of the AMPK upstream protein phosphatase PP2A in FD/E, an outcome

that would likely attenuate AMPK deactivation via de-phosphorylation. It is noted that present measures of upstream kinase and phosphatase protein expression do not address the possibility that food deprivation may influence enzyme activity via phosphorylation or methylation. Further research is required to determine if E-dependent adjustments in net AMPK activity in response to FD are achieved by direct hormone action on A2 neurons or through E-driven chemical inputs to these cells.

E regulates effects of FD on A2 nerve cell expression of the catecholamine biosynthetic enzyme D β H as this protein profile was increased in FD/O, but not FD/E. Current evidence for parallel up-regulation of D β H versus total/phosphorylated AMPK suggests that expression of these proteins may be governed in tandem. In FD/E, A2 D β H content was unchanged despite increased AMPK activation. Previous studies show that cDVC hindbrain AMPK is a critical determinant of hyperphagic and hypothalamic AMPK responses in FD/E rats as pharmacological inhibition of this sensor normalizes those functions. If the premise that increased D β H expression is an accurate indicator of elevated norepinephrine synthesis and release is correct, our data would imply that A2 cells do not convey cDVC AMPK-initiated signals to the brain metabolic regulatory circuitry. As norepinephrine is co-expressed with other neurotransmitters, including neuropeptide Y, neurotensin, and dynorphin, an alternative possibility is that A2 neurons communicate FD effects via non-noradrenergic stimuli. On the other hand, we cannot disregard the prospect that, notwithstanding a lack of change in D β H protein expression, FD may regulate A2 norepinephrine transmission through control of NE metabolism and release within projection sites. Indeed, FD elicits adjustments in tissue norepinephrine content in different forebrain structures in O versus E animals, responses that are attenuated by the AMPK inhibitor Cc [Alenazi et al., 2015]. We speculate that E may determine downstream targets of norepinephrine noradrenergic signals of hindbrain sensor status through actions at noradrenergic axon terminals to ‘silence’ or ‘un-silence’ AMPK-sensitive norepinephrine release within distinct forebrain loci.

In summary, current research shows that FD respectively reduces or does not impact circulating glucose in O and E animals, while elevating FFA in E rats only (Figure 6). A2 nerve cell AMPK activity was refractory to FD/O, but augmented in FD/E. E-contingent amplification of AMPK activity may reflect adaptive conversion of energy metabolism from carbohydrate to fatty acid oxidation and/or glucocorticoid stimulation. FD augmentation of A2 D β H protein profiles in FD/O but not FD/E implies that FD may correspondingly regulate NE synthesis versus metabolism/release in the absence versus presence of E. Further studies are needed to clarify how metabolic and hormonal surrogates of FD are translated by A2 neurons into regulatory inputs to the brain-wide circuitry that defends energy balance.

List of Abbreviations

ACSVL3	very long-chain acyl-CoA synthetase 3
AICAR	5-aminoimidazole-4-carboxamide-riboside
AMPK	5'-monophosphate-activated protein kinase

CAMKKβ	Ca ⁺⁺ /calmodulin-dependent protein kinase-beta
cDVC	caudal dorsal vagal complex
CV4	caudal fourth ventricle
DβH	dopamine-beta-hydroxylase
E	estradiol
EDTA	ethylenediaminetetraacetic acid
FD	12-hour food deprivation
FF	full-fed
GR	type II glucocorticoid receptor
O	oil
OVX	ovariectomy
pAMPK	phosphorylated AMPK
PP2A	protein phosphatase PP2A
TBS-Tx	0.05 M Tris-buffered saline, pH 7.4, containing 0.05% Triton X-100

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Significance Statement

Short-term abstinence from food intake is unavoidable in modern life, but is negatively correlated with appetite control and obesity. Estradiol (E) mitigates acute and post-acute adverse effects of short-term feeding suspension (12-hour food deprivation; FD) on energy balance. Hindbrain 5'-monophosphate-activated protein kinase (AMPK) regulates feeding and hypothalamic metabolic neuropeptide and norepinephrine responses to FD in an E-dependent manner. AMPK-expressing hindbrain A2 noradrenergic neurons convey energy state information to the hypothalamus. We investigated the hypothesis that E-reliant A2 AMPK activation during FD correlates with E regulation of circulating levels and A2 receptivity to metabolic fuels and energy deficit-sensitive hormones.

Figure 1. Effects of 12-Hour Food Deprivation (FD) on Circulating Glucose, Free Fatty Acid (FFA), Glucagon, Corticosterone, and Ghrelin Levels in Estradiol (E)- and Oil (O)-Implanted Ovariectomized (OVX) Female Rats

Bars depict mean glucose (Figure 1.A), FFA (Figure 1.B), glucagon (Figure 1.C), corticosterone (Figure 1.D), ghrelin (Figure 1.E), insulin (Figure 1.F), and leptin (Figure 1.G) measures $\pm$ S.E.M. for groups of FD/O (white bars), FD/E (gray bars), full-fed (FF)/O (diagonal-striped white bars), and FF/E (diagonal-striped gray bars) animals. * $p < 0.05$, FD versus FF; ** $p < 0.05$, E versus O.

Figure 2. Effects of FD on Hindbrain Dorsal Vagal Complex (DVC) A2 Noradrenergic Nerve Cell Adenosine 5'-Monophosphate-Activated Protein Kinase (AMPK) and PhosphoAMPK (pAMPK) Protein Profiles in E versus O Female Rats

Individual tyrosine hydroxylase (TH)-immunoreactive (-ir) A2 neurons were laser-microdissected from 10 μ m-thick sections of the caudal DVC of FD and FF groups of E and O rats. For each protein of interest, lysates of pooled A2 cells (n=50 per treatment group; n=10 per rat) were analyzed by Western blot in triplicate at minimum; protein band optical densities (O.D.) were quantified with Syngene Genetool 4.01 software and expressed relative to α -tubulin. Panels depict mean normalized O.D. values $\pm$ S.E.M. for A2 neuron AMPK (Figure 2.A) and pAMPK (Figure 2.B) proteins for the following treatment groups: FD/O (white bars), FD/E (gray bars), FF/O (diagonal-striped white bars), FF/E (diagonal-striped gray bars). Representative immunoblots of individual proteins of each interest and α -tubulin are shown below each graph. * $p < 0.05$, FD versus FF; ** $p < 0.05$, E versus O.

Figure 3. Effects of FD on Hindbrain Dorsal Vagal Complex (DVC) A2 Noradrenergic Nerve Cell Ca^{++} /Calmodulin-Dependent Protein Kinase-Beta (CaMKKβ) and Protein Phosphatase 2A (PP2A) Protein Profiles in E versus O Female Rats

CaMKKβ and PP2A proteins were analyzed by Western blot in at minimum triplicate pools of laser-microdissected A2 neurons (n=50 neurons/pool) collected from each treatment group. Bars illustrate mean normalized CaMKKβ (Figure 3.A) and PP2A (Figure 3.B) O.D. values $\pm$ S.E.M. for FD/O (white bars), FD/E (gray bars), FF/O (diagonal-striped white bars), FF/E (diagonal-striped gray bars) treatment groups. Representative immunoblots of proteins of interest are depicted below each graph. * $p < 0.05$, FD versus FF; ** $p < 0.05$, E versus O.

Figure 4. Effects of FD on Hindbrain Dorsal Vagal Complex (DVC) A2 Noradrenergic Nerve Cell Glucocorticoid Receptor (GR) and Fatty Acyl-CoA Synthetase Very-Long Chain 3 (ACSVL3) Protein Profiles in E versus O Female Rats

GR and ACSVL3 proteins were analyzed by Western blot in at minimum triplicate pools of laser-microdissected A2 neurons (n=50 neurons/pool) collected from each treatment group. Bars illustrate mean normalized GR (Figure 4.A) and ACSVL3 (Figure 4.B) O.D. values $\pm$ S.E.M. for FD/O (white bars), FD/E (gray bars), FF/O (diagonal-striped white bars), FF/E (diagonal-striped gray bars) treatment groups. Representative immunoblots of proteins of interest are depicted below each graph. * $p < 0.05$, FD versus FF; ** $p < 0.05$, E versus O.

Figure 5. Effects of FD on Hindbrain Dorsal Vagal Complex (DVC) A2 Noradrenergic Nerve Cell Dopamine-Beta-Hydroxylase (D β H) Protein Expression in E versus O Female Rats

D β H protein was analyzed by Western blot in triplicate pools of laser-microdissected A2 neurons (n=50 neurons/pool) harvested obtained from each treatment group. Bars illustrate mean normalized D β H protein O.D. values $\pm$ S.E.M. for FD/O (white bars), FD/E (gray bars), FF/O (diagonal-striped white bars), FF/E (diagonal-striped gray bars) treatment groups. Representative immunoblots of D β H and α -tubulin are presented below the graph.

* $p < 0.05$, FD versus FF; ** $p < 0.05$, E versus O.

Figure 6. Estradiol-Dependent 12-Hour FD Effects on A2 Neuron Upstream AMPK Kinase/Phosphatase Protein Expression Coincide with Steroid-Mediated Sustainment of Glucose Profiles and Augmentation of Free Fatty acid Availability

At the top of the Figure, side-by-side illustrative depictions of the systemic vasculature system list blood substrate fuel and hormone surrogates of metabolic imbalance potentially reactive to FD accompanied by brackets containing arrows denoting direction of change (↑: increase; ↓: decrease) or symbolic portrayal of absence of change (Δ covered by an X) in the presence (left-hand side) versus absence (right-hand side) of estradiol. In the lower half of the figure, representative A2 noradrenergic neurons illustrate FD effects on glucocorticoid receptor (GR) and FFA transporter (ACSVL3) protein content in A2 cells harvested from rats implanted with estradiol (left) or oil (right). The box associated with each A2 neuron shows FD effects on AMPK upstream kinase and phosphorylase, total and phosphorylated AMPK, and pAMPK/AMPK ratio. Below each box brackets indicate effect or lack of effect of FD on A2 DβH protein expression. *Abbreviations:* ACSVL3, very long-chain acyl-CoA synthetase 3 (also referred to as fatty acid transport protein 3 or FATP3); AMPK, 5'-monophosphate-activated protein kinase; CaMKKβ, Ca⁺⁺/calmodulin-dependent protein kinase-beta; DβH, dopamine-beta-hydroxylase; GR; type II glucocorticoid receptor; OVX, ovariectomy; pAMPK, phosphorylated AMPK; PP2A, protein phosphatase PP2A.

Table 1

Description of Primary Antibodies Used

Antigen	Description of Immunogen	Source, Host Species, Cat. #, Clone/Lot # RRID	Concentration Used
AMPK	Amino acids 251–550 of C terminus of human AMPK $\alpha_{1/2}$ catalytic subunit	Santa Cruz Biotechnol.; rabbit; sc-25792; Lot no. 1009; AB_216946	0.4 ug/mL
pAMPK	Phosphorylated Thr 172 of human AMPK $\alpha_{1/2}$	Santa Cruz Biotechnol.; rabbit; sc-33524; Lot no. J0915; AB_216714	0.4 ug/mL
DBH (H-213)	Amino acids 391–603 of C terminus of human DBH	Santa Cruz Biotechnol.; rabbit; sc-15318; Lot no. B1413; AB_2089347	0.4 ug/mL
PP2A-C alpha/beta (7A6)	Unmethylated C-terminus amino acids 302–309 of human PP2A-C	Santa Cruz Biotechnol.; mouse; sc-81601; Lot no. J1410; AB_1128773	0.4 ug/mL
CaMKK β (L-19)	C-terminus of rat CaMKK β	Santa Cruz Biotechnol.; rabbit; sc-9629; Lot no. H0411; AB_2243844	0.4 ug/mL
GR (M-20)	N-terminus of mouse GR-alpha	Santa Cruz Biotechnol.; rabbit; sc-1004; Lot no. A2609; AB_2155786	0.4 ug/mL
ASCVL3 (Q-17)	Internal region of human ASCVL3	Santa Cruz Biotechnol.; goat; sc-137275; Lot no. I1114; AB_10838921	0.4 ug/mL
SLC5A8 (A-15)	Internal region of human SLC5A8	Santa Cruz Biotechnol.; goat; sc-34189; Lot no. I1614; AB_2189838	0.4 ug/mL
CD36 (N-15)	N-terminus of human CD36	Santa Cruz Biotechnol.; goat; sc-5522; Lot no. J 2315; AB_638143	0.4 ug/mL
α -tubulin	Amino acids 426–450	EMD Millipore (Calbiochem.); mouse; CP06-100UG; Lot no. D00175772; AB_212802	0.1 ug/mL
TH	34 kDa catalytic core	ImmunoStar; mouse; 22941; Lot no. 1552001; AB_2313787	Not available
Mouse IgG	Not available	PerkinElmer; goat; NEF 822001EA Lot no. 120709	0.2 ug/mL
Rabbit IgG	Not available	PerkinElmer; goat; NEF 812001EA Lot no. 120454	0.2 ug/mL
Goat IgG	Not available	Santa Cruz Biotechnol.; donkey; sc-2020; Lot no. C1615; AB_631728	0.08 ug/mL