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Energy Status Determines Hindbrain Signal Transduction Pathway Transcriptional Reactivity to AMPK in the Estradiol-Treated Ovariectomized Female Rat

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Abstract

Dorsal vagal complex (DVC) AMPK regulation of food intake in the estradiol-treated ovariectomized (OVX) female rat is energy state-dependent. Here, RT-PCR array technology was used to identify estradiol-sensitive AMPK-regulated DVC signal transduction pathways that exhibit differential reactivity to sensor activation during energy balance versus imbalance. The AMP mimetic AICAR correspondingly reduced or stimulated cDVC phosphoAMPK (pAMPK) and estrogen receptor-beta (ER β) proteins in *full-fed* (*F*) versus *12 hr food-deprived* (*D*) estradiol-treated ovariectomized (OVX) rats, but elevated ER-alpha (ER α) in *F* only. Estradiol suppressed DVC ER β protein and hypoxia, NF κ B, STAT3, STAT6, and Hedgehog signaling pathway marker genes against oil-implanted OVX controls. *F* + (A)ICAR and *D* + (S)aline groups each exhibited further inhibition of NF κ B, STAT3, and Hedgehog pathway genes, and diminished PPAR, Notch, and STAT5 transcripts versus *F*+*S*. Conversely, genes in these six pathways were up-regulated by AICAR treatment of *D*. Results show that in this animal model, acute AMP augmentation or feeding cessation each inhibit both pAMPK and ER β expression, but in combination increase these protein profiles. pAMPK protein and DVC TNF (NF κ B), SOCS3 (JAK/STAT), WNT6 (Hedgehog), and FABP1 (PPAR) mRNAs were down- or upregulated in parallel by AICAR in *F* versus *D* states, respectively. Further research is needed to determine the impact of ER β on opposing directionality of these responses, and to characterize the role of the aforementioned signaling pathways in hyperphagic responses in the female to AICAR-induced DVC AMPK activation during acute interruption of feeding.

Keywords

AICAR; estradiol; dorsal vagal complex; estrogen receptor-alpha; estrogen receptor-beta; Hedgehog

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Introduction

The ovarian steroid hormone 17 β -estradiol exerts significant influence on the metabolic status of female mammals via central and peripheral mechanisms that control procurement, ingestion, metabolism, partitioning, storage, and expenditure of energy fuels [Wade and Schneider, 1992]. Estradiol functions as a regulatory transmitter stimulus to distinct nerve cell components of the CNS circuitry that regulates energy balance; specific central neuron populations mediate effects of this hormone on body weight and composition, food intake, and metabolic rate [Xu et al., 2011]. Estradiol also acts to enhance neuron energy stability brain-wide [Nilsen et al., 2007], thereby providing ‘neuro-protection’ against bio-energetic insults such as stroke [Nilsen and Brinton, 2004]. Our research seeks to clarify how estradiol shapes neural control of systemic energy homeostasis through management of neuro-metabolic state, with reasoning that understanding of where and how estradiol affects metabolo-sensory input to neural networks will yield potential new targets for therapeutic simulation of beneficial hormonal effects on metabolic state.

Adenosine 5'-monophosphate-activated protein kinase (AMPK) is an ultra-sensitive energy sensor [Hardie, 2003; Kahn et al., 2005] that is activated via phosphorylation in response to increases in intracellular AMP/ATP ratio. Phosphorylated AMPK (pAMPK) inhibits ATP-consuming biosynthetic pathways, while augmenting ATP-producing metabolic pathways such as fatty acid oxidation. In the CNS, AMPK regulates both cellular and systemic responses to energy dyshomeostasis [Ronnett et al., 2009]. AMPK is expressed in select brain regions, namely the hypothalamus and hindbrain. Initial research on central AMPK regulation of body-wide metabolic equilibrium focused on the hypothalamus, but recent studies implicate hindbrain dorsal vagal complex (DVC) AMPK in neural control of glucostasis [Ibrahim et al., 2013] and food intake [Ibrahim and Briski, 2014]. Our work was the first to localize this sensor to a specific neurochemical phenotype in the caudal DVC (cDVC), e.g. A2 noradrenergic neurons, where it is revealed to be hypoglycemia-sensitive [Cherian and Briski, 2011; 2012]. The ability of the AMP mimic 5-aminoimidazole-4-carboxamide-riboside (AICAR) to stimulate feeding upon delivery to the DVC varies between sexes, as this treatment is reported to lack effect in male rats [Hayes et al., 2009]. Estradiol is likely involved in this sex difference as it governs effects of locally-administered AICAR on A2 nerve cell AMPK activation and Fos protein expression in the caudal DVC (cDVC) [Ibrahim et al., 2013]. Indeed, estradiol is reported to act directly on the hindbrain to regulate feeding [Asarian and Geary, 2007; Thammacharoen et al., 2008]. However, our current work indicates that this permissive action of estradiol toward AICAR induction of hyperphagia in females may depend, in part, upon prevailing energy status [Ibrahim and Briski, 2014]. Our results show that AICAR delivery into the fourth ventricle (V4) stimulates food intake in estradiol-, but not oil-implanted ovariectomized (OVX) female rats that did not feed over a 12 hour period prior to drug treatment. However, this AICAR treatment paradigm did not elicit hyperphagia in OVX + estradiol animals engaged in uninterrupted food consumption. Moreover, that research reveals that the efficacy of AICAR to activate cDVC AMPK differs between OVX + estradiol and OVX + oil groups during either continuous or suspended feeding. At present, there is little understanding of the signal transduction pathways that may mediate AMPK regulation of hindbrain cellular responses to

energy deficiency and signaling of hindbrain energy imbalance to the metabolic regulatory circuitry of the brain [Watts and Donovan, 2010]. Here, PCR array technology was used to identify estradiol-sensitive signal transduction pathways that respond to energy sufficiency-versus deficit-dependent adjustments in cDVC AMPK.

Estradiol regulation of CNS substrates is mediated, in part, by the ligand-activated nuclear receptors/transcription factors, estrogen receptor- α (ER α) and - β (ER β) [McEwen, 1999]. While these receptor variants share moderate homology of their ligand-binding domains and bind 17 β -estradiol with similar affinity [Kuiper et al., 2007], N-terminal domain differences [Kuiper et al., 2006] support the possibility that ER α versus and ER β activation may exert differential effects on gene transcription [Paech et al., 1997], and that changes in absolute or proportionate activation of one or both isoforms may elicit dissimilar cellular responses. ER α is implicated in the estrogenic regulation of energy balance based upon research involving pharmacologic manipulation or genetic knockout of receptor function; however, ER β involvement in this hormone action is less clear [Foryst-Ludwig and Kintscher, 2010; Barros and Gustafsson, 2011]. Chronic reduction of food intake by 50% over a 12 day interval decreases hypothalamic ER α mRNA [Lindblom et al., 2005], but effects of our short-term, e.g. 12 hr feeding cessation paradigm on cDVC receptor variant protein expression remain unclear. The current studies thus evaluated effects of AICAR and short-term interruption of feeding, alone or in combination, on ER α and ER β protein levels in micropunch-dissected cDVC tissue samples.

Methods and Materials

Animals

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

Experimental Design

On day 1, animals were implanted with a PE-20 cannula into the V4 [coordinates: 6.6 mm ventral to skull surface; 0 mm lateral to midline; 13.3 mm posterior to bregma] under ketamine/xylazine anesthesia (0.1 mL/100 g *bw ip*; 90 mg ketamine/10 mg xylazine/mL; Henry Schein, Inc., Melville, NY), then transferred to individual cages. On day 7, after re-anesthetization, rats were bilaterally ovariectomized (OVX) and implanted *sc* with a pre-soaked silastic capsule (i.d.: 0.062 in., o.d.: 0.125 in; 10 mm/100 g *bw*) filled with 30 μ g estradiol benzoate (E)/mL safflower oil or oil (O) alone. This steroid replacement regimen yields plasma estradiol levels, e.g. 22 pg/mL [Briski et al., 2001], that approximate circulating hormone concentrations measured on metestrus in 4-day [Butcher et al., 1974] or on diestrus day-2 in ovary-intact 5-day cycling animals [Goodman, 1978]. Animals received ketoprofen injections (1 mg/kg *bw sc*) before and after each surgery, and were treated by topical administration of 0.25% bupivacaine (1–3) drops after incision closures. Beginning at

21.00 hr on day 11 until the conclusion of the study, food was withdrawn from n=8 E rats [food deprived; D]. An additional n=8 E animals, as well as n=4 O controls, were allowed to feed ad-libitum over the same period [full-fed; F]. At 09.00 hr (time zero; t_0) on day 12, groups of DE and FE rats were injected into the V4 with AICAR (A; 0.5 ug/uL; 2.0 uL total volume; n=4 DE-A and n=4 FE-A) or the vehicle, 0.9% NaCl (S) n=4 DES and n=4 FE-S). Full-fed O-implanted OVX animals injected with S (FO-S; n=4). Animals were sacrificed at 10.00 hr on day 12. Brains were snap-frozen by plunge immersion in liquid nitrogen-cooled methyl butane. Two consecutive 200 μ m frozen sections were cut through the hindbrain at the level of the cDVC, beginning at 14.40 mm posterior to bregma. Bilateral punches of the cDVC were removed from each section with the aid of a hollow micropunch needle of 0.76 mm internal diameter (prod. no. 57401; Stoelting Inc., Kiel, WI). For each animal, punches from one side of the cDVC were pooled and evaluated by PCR array analysis, while tissue from the other side was saved for Western blot evaluation. Additional FE (n=8) and DE (n=8) animals were acclimated to the presence of standard laboratory rat chow in food jars placed inside their home cages and regular removal of those jars for weighing on days 9–11. At t_0 on day 12, rats were injected with A (0.5 ug/uL; 2.0 uL volume; n=4 DE-A and n=4 FE-A) or S (n=4 DE-S and n=4 FE-S) into the V4; at the same time, pre-weighed chow-filled jars were placed in each cage. Jars were removed at both +1 and +2 hours after t_0 injections and weighed, as described [Briski and Nedungadi, 2009]. For each time point, measures of consumed food were adjusted by subtracting the weight of food crumbs on underlying paper. All animals used in this study exhibited cerebrospinal fluid reflux from the cannula tip on day 12; post-mortem histological examination confirmed accuracy of cannula placements within the V4.

Western Blot Protocol

For each treatment group, aliquots of heat-denatured cDVC tissue from each animal were combined and separated on 4–12% gradient Tris-glycine gels (prod. no. 412W1 5M10; Jule Biotechnologies, Milford, CT), as described [Ibrahim et al., 2013]. Proteins were transferred overnight in a BioRad Criterion blotter to 0.45 μ m PVDF-Plus membranes (prod. no. PV4 HY00010; Osmonics Inc., Gloucester, MA). Membranes were blocked for 2 hr with Tris-buffered saline, pH 7.4 (TBS), containing 0.1% Tween-20 (T) and 2% bovine serum albumin (BSA), then incubated overnight with at 4°C with primary antisera (rabbit polyclonal pAMPK α 1/2 (Thr 172) (1:1,000; prod. no. sc-33524; Santa Cruz Biotechnology, Inc, Santa Cruz, CA), rabbit polyclonal AMPK α 1/2 (1:1,000; prod. no. sc-25792; Santa Cruz Biotechnol.), rabbit polyclonal ER α (1:1,000; prod. no. sc-542; Santa Cruz Biotechnol.), rabbit polyclonal ER β (1:1,000; prod. no. sc-8974; Santa Cruz Biotechnol.), or mouse monoclonal anti- α -tubulin (1:1,000; prod. no. CP06; Calbiochem., Gibbstown, NJ) diluted in TBS-T-BSA. Membranes were then incubated for 1 hr with peroxidase-conjugated goat anti-mouse (1:5,000; prod. no. NEF822001EA; PerkinElmer, Boston, MA) or goat anti-rabbit (1:5,000; prod. no. NEF812001EA; PerkinElmer) secondary antibodies prior to application of SuperSignal West femto substrate (prod. no. 34080; ThermoScientific, Rockland, IL). Chemiluminescent signals were captured in a G:Box Chemi (Syngene, Cambridge, UK). Protein band optical densities (O.D.) were quantified using Syngene Gene Tools Gel Analysis software and normalized to α -tubulin. Protein

molecular weight markers were included in each Western blot analysis. For each protein of interest, immunoblot analyses were performed in triplicate.

PCR Array

For each treatment group, RNA was extracted from pooled micropunch tissue samples using RNeasy MiniElute Cleanup kit reagents (prod. no. 74204; Qiagen, Frederick MD). Isolated total RNA was evaluated for integrity, purity, and quantity in an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA), using Agilent RNA 6000 nano kit reagents (prod. no. 5067-1511) and RNA nano chips (prod. no. 5067-1521). All RNA samples had an RNA integrity number (RIN) equal to or greater than 9.5. cDNA was synthesized using RT² preAMP cDNA synthesis kits (prod. no. 330451; Qiagen), then amplified by Signal Transduction PathwayFinder RT² preAMP pathway primer mix technology (prod. no. PBR-014Z; Qiagen). cDNA from each treatment group was analyzed in a Signal Transduction PathwayFinder RT² Profiler PCR array (prod. no. PARN-014ZA-12; Qiagen). For each gene, values for change in cycle threshold (ΔC_t) were determined by subtracting the mean threshold cycle (C_t) of the housekeeping genes ACTB, B2M, HPRT1, LDHA, and RPLP1. Data analysis was carried out using RT² profiler PCR array data analysis version 3.5 (<http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php?target=upload>). Normalized transcription was calculated as $2^{(-\Delta C_t)}$. ΔC_t was computed as the difference between C_t of individual genes and C_t of full-fed S-injected OVX controls (FOS). Two-fold up- or down-regulation of each gene was identified relative to FOS and compared among treatment groups. Scatter plots were constructed using the above website-based software. The network analysis program, <http://geneweb.sabiosciences.com/gncpro/gncpro.php>, was used to generate maps of potential functional interactions between genes characterized here by treatment-induced two-fold or greater change in expression.

Statistical Analyses

Mean protein O.D. and food intake measures were analyzed by two-way ANOVA and Student-Newman-Keuls test. The criterion for significance was set to $p=0.05$ in all comparisons. PCR array data showing a two-fold up- or down-regulation were interpreted as indicative of change in expression.

Results

Figure 1 depicts effects of intra-V4 AICAR administration on cDVC pAMPK (Row A), AMPK (Row B), ER α (Row C), and ER β (Row D) protein expression in E-implanted OVX rats that engaged in unrestricted feeding or were food-deprived for 12 hr prior to drug treatment. For each protein, representative immunoblots are organized in the following order according to treatment group: 1) Full-fed OVX+E (FE) injected with (S)aline, e.g. FE-S; 2) Full-fed OVX+E (FE) injected with (A)ICAR, e.g. FE-A; 3) Food-Deprived OVX+E (DE) injected with (S)aline; e.g. DE-S; and 4) Food-deprived OVX+E injected with (A)ICAR, e.g. DE-A. Bars linking pairs of Western blot bands denote a statistical difference between mean normalized protein O.D. values; single or double asterisks above the second band indicate a relative reduction or augmentation, respectively, of mean O.D. compared to the first band. Quantitative O.D. data in Table 1 show that relative to FE-S, DE-S rats exhibited

decreased expression of pAMPK and ER β proteins. FE animals injected with A showed elevated cDVC ER α , but diminished pAMPK and ER β content versus FE-S. DE rats injected with A displayed augmented pAMPK and ER β protein levels against DE-S. Significant main effects on pAMPK expression ($F_{3,11} = 6.35$; $p = 0.016$) reflected factor interaction ($F_{1,11} = 18.7$; $p = 0.0025$). Significant changes in ER α protein profiles ($F_{3,15} = 7.14$; $p = 0.005$) reflected independent effects of feeding status ($F_{1,15} = 11.5$; $p = 0.005$) and drug treatment ($F_{1,15} = 6.7$; $p = 0.02$). Main effects on ER β protein ($F_{3,11} = 33.02$; $p = 0.0001$) were due to AICAR ($F_{1,11} = 323.9$; $p = 0.001$), with significant interaction with feeding status ($F_{1,11} = 70.3$ ($p < 0.0001$)). Collectively, these data show that OVX+E rats exhibit parallel reductions in cDVC AMPK activation and ER β protein expression at either + 1 hr after AICAR administration or at +12 hr after cessation of feeding; yet, AICAR treatment of 12 hr food deprived rats paradoxically elevates both pAMPK and ER β profiles. AICAR modified ER α expression in full-fed, but not food-deprived.

Table 2 depicts, in the following order, Hedgehog, hypoxia, JAK/STAT, NF κ B, Notch, oxidative stress, p53, PPAR, and TGF β signal transduction pathway marker genes probed by PCR microarray analysis in the cDVC of FE-S, FE-A, DE-S, and DE-A animals relative to full-fed Oil-treated (FO) animals injected with (S)aline. The dendrogram in Figure 2 illustrates outcomes of hierarchical cluster analysis of these gene profiles across treatment groups.

In Figure 3, circles depict cDVC signal transduction gene expression profiles that were up- (red) or downregulated (green) in FE-S versus FO-S by a factor of two or greater. The upper and lower halves of the figures contain lists of genes, identified by pathway, that were over- and under-expressed, respectively, in the FE-S cDVC relative to FO-S. While Hedgehog and hypoxia genes, e.g. WNT1, WNT3A, and SLC2A1, were up-regulated by estrogen, Hedgehog WNT6 and hypoxia SERPINE1 genes were down-regulated to a greater extent. Compared to FO-S, FE-S animals were also characterized by suppression of cDVC NF κ B (BIRC3, TNF), STAT3 (CEBPD), STAT6 (GATA3) and TGF β (EMP1) pathway mRNA profiles. Figure 4 compares marker gene expression patterns in DE-S versus FE-S. Disruption of feeding for 12 hr did not augment any gene profile relative to FE-S, but did suppress E-insensitive PPAR (FABP1), Notch (HEY1), STAT3/5 (SOCS3), and NF κ B (ICAM1) genes. The DE-S group also exhibited a reduction in TNF and WNT6 mRNAs against FE-S. Figure 5 illustrates effects of intra-V4 administration of A on cDVC pathway marker gene expression in FE animals. This treatment decreased E-insensitive p53 (BBC3), PPAR (FABP1, SORBS1), Notch (JAG1, NOTCH1), STAT3 (LRG1), STAT3/5 (SOCS3), and Hedgehog (PTCH1) gene profiles. FE-A rats also exhibited reductions in E-sensitive Hedgehog WNT1 and -6 and NF κ B TNF mRNA expression. Figure 6 depicts cDVC pathways marker gene expression in DE-A rats. The data show that compared to DE-S, the DE-A group exhibited elevated E-insensitive PPAR (FABP1), Notch (HES1), STAT3/5 (SOCS3) and E-sensitive STAT3 (CEBPD), NF κ B (TNF), and Hedgehog (WNT6) mRNAs. DE-A animals also showed comparative reductions in E-sensitive STAT6 (GATA3) and E-insensitive Notch (NOTCH1) and WNT (WISP1) gene profiles. Figure 7 depicts potential interactions between TNF, SOCS3, and FABP1 identified by network analysis. Taken together, these data document inhibition of DVC ER β protein and hypoxia, NF κ B, STAT3,

STAT6, and Hedgehog signaling pathway marker genes by estradiol. OVX+E rats either injected with AICAR or fasted for 12 hr showed further reductions in NF κ B, STAT3, and Hedgehog pathway genes, and diminished PPAR, Notch, and STAT5 transcripts, whereas combining these treatments, e.g. administration of AICAR to food deprived animals, up-regulated genes in these six pathways.

Figure 8 illustrates effects of V4 administration of AICAR on food intake by FE versus DE rats. This treatment did not increase consumption at either +1 (Figure 8.A) or +2 (Figure 8.B) hours post-treatment in the FE group, but significantly augmented feeding by DE between t_0 and +1 hr. Main effect on DE-A versus DE-S ($F_{3,19} = 33.97$; $p = 0.0001$) was due to feeding status ($F_{1,19} = 87.2$, $p < 0.0001$) and AICAR treatment ($F_{1,19} = 8.2$, $p = 0.01$), with significant interaction between these factors ($F_{1,19} = 6.5$, $p = 0.02$).

Discussion

The present studies demonstrate that energy status is a critical determinant of direction of change in cDVC AMPK activity in estradiol-implanted OVX female rats treated by intra-V4 administration of the AMP mimic AICAR. Results show that pAMPK levels in that structure were decreased 1 hr after AICAR treatment of full-fed OVX+E animals, but elevated at a similar time point in rats that did not feed over a 12 hr period prior to drug treatment. This stimulatory effect of AICAR on AMPK in short-term food-deprived animals represents augmentation of activity relative to a decline in baseline pAMPK levels caused by interruption of feeding. Expression of cDVC ER β protein was similarly suppressed after AICAR treatment of full-fed animals or cessation of food intake, but was increased by combination of these treatments. The current research shows that estradiol down-regulates transcription of genes associated with multiple, e.g. hypoxia, NF κ B, TGF β , STAT3, STAT6, and Hedgehog signaling transduction pathways in the female rat cDVC. Our data identify estradiol-sensitive and -insensitive signaling genes in the cDVC that are responsive to AICAR, and illustrate that the former and a subset of the latter exhibit opposite, e.g. inhibitory versus stimulatory transcriptional responses to this drug during energy sufficiency versus deficiency, respectively. Taken together, these findings suggest that in the presence of estrogen, activating effects of acute energy imbalance on cDVC AMPK may be thwarted or truncated during energy stasis, a construal that is consistent with our evidence that locally-administered AICAR does not induce feeding in full-fed OVX+E rats. Results also reveal that cDVC AMPK activity is paradoxically reduced after short-term cessation of feeding in estradiol-implanted animals, but that exacerbation of metabolic stress (signaled here by simulated elevation of AMP levels) may override input to AMPK conveying positive energy status.

Total cDVC AMPK and pAMPK content was correspondingly down- or up-regulated by estradiol versus non-steroid - treated OVX controls. We previously found that levels of both proteins are equivalent in A2 noradrenergic neurons from the cDVC of OVX+E versus OVX +O rats [Tamrakar et al., 2012]. While A2 neurons are the only population in the cDVC currently known to express this metabolic sensor, the present data support the possibility that other cells in that site may do so as well, and that baseline AMPK and pAMPK profiles in non-A2 neurons may be regulated by estradiol. cDVC pAMPK levels were significantly

decreased by either 1) AICAR treatment of full-fed OVX+E rats or 2) acute, e.g. 12 hr cessation of feeding, data that imply that both experimental manipulations elicit a more positive energy state in this neural structure. We predicted an elevation in AMPK activity in DE-S versus FE-S, but our data demonstrate instead a relative reduction in pAMPK protein expression in the former group. These results imply the operation of counteractive mechanisms over the prior 12 hour period that prevent cellular energy imbalance in this brain site, despite suspended substrate fuel ingestion. As we did not evaluate cDVC AMPK activity after longer periods of food restriction, the time course of this putative protective response is unclear. Neither is it known if the magnitude or duration of this reduction in sensor activity is estrogen-dependent or brain region-specific. AICAR administration to food-deprived rats caused an increase in pAMPK expression relative to this down-adjusted baseline. These results are consistent with our report that this treatment paradigm elevates pAMPK levels in A2 neurons from DE animals. A plausible explanation of AICAR-induced deactivation of AMPK at 1 hr post-treatment of full-fed animals is that it was likely preceded by drug-induced enhancement of pAMPK, an outcome that would initiate compensatory energy-yielding metabolic functions that inhibit sensor activity. Since cDVC pAMPK levels were elevated in food-deprived animals 1 hr after AICAR, activation of this sensor may persist longer in DE-S versus FE-S animals, possibly due to construal of drug-mediated AMP/ATP imbalance as a second instance of metabolic deficit (of perhaps greater intensity) within 12 hr after interruption of feeding. It would be informative to learn if acclimation to multiple metabolic disturbances over the course of a several hours involves development of heightened AMPK sensitivity, and if such adaptation is estrogen-dependent. An alternative, though less probable account is that activation of cDVC AMPK in FE-S rats may be paradoxically at- or approaching-maximal, despite energy influx, and acute signals of metabolic distress may transiently inhibit pAMPK by reducing energy-requiring cell functions to prevent exacerbation of existing energy shortage. Yet, the possibility that AICAR may exert non-specific drug effects on AMPK synthesis, phosphorylation, or de-phosphorylation under the current conditions cannot be overlooked.

Estradiol replacement of full-fed OVX animals did not modify ER α , but decreased ER β protein expression in the cDVC. AICAR administration increased ER α protein levels in this site irrespective of systemic energy status, whereas food deprivation surprisingly did not. ER α up-regulation may thus depend upon the magnitude and/or duration of change in the AMP/ATP ratio from normal steady-state, as elevations in cell levels of AMP analogs or endogenous AMP owing to pharmacological versus physiological stimuli, respectively, may likely differ and be more pronounced in response to the former. On the other hand, AICAR effects on ER β protein were correlated with energy status as this drug suppressed or elevated this profile in full-fed versus food-deprived rats, in that order. Evidence that pAMPK and ER β proteins exhibit analogous responses to either AICAR or food deprivation suggests that this estrogen receptor subtype may be expressed in the cDVC in direct proportion to AMPK activation during metabolic stress; if so, this receptor may play a vital role in local AMPK-initiated responses to energy deficits. If our speculation of a dissimilar time course for AICAR-induced pAMPK expression in FE versus DE is ultimately proved correct, then it is plausible that ER β protein may be sensitive to temporal patterns of AMPK activation. Our ongoing efforts aim to determine if a causal relationship between pAMPK and ER β

expression exists, and if so, to elucidate the basis for functional interaction between these proteins. In light of recent evidence that the ER β antiserum used here, along with several other commercially available antibodies, is capable of detecting rat ER as well as proteins in receptor knockdown or null tissue [Snyder et al., 2011], we cannot discount the possibility that present results may reflect, in part, treatment effects on non-ER β proteins.

This study identified several estradiol-sensitive genes in hypoxia [SCL2A1, SERPINE1], NF κ B [BIRC3, TNF], TGF β [EMP1], STAT3 [CEBPD], STAT6 [GATA3], and Hedgehog [WNT1, -3, -6] signaling pathways in the cDVC. With the exception of SCL2A1, WNT1, and WNT3, the above genes were down-regulated in estradiol- versus oil-implanted OVX rats. Our evidence for estrogenic suppression of cDVC TNF gene expression is consistent with reports that this hormone inhibits astrocyte TNF mRNA levels [Kipp et al., 2007; Lewis et al., 2008; Valles et al., 2012]. Estradiol is reported to increase GLUT1 mRNA and protein expression in whole brain microvessels [Shi and Simpkins, 1997]; current evidence for hormonal augmentation of cDVC SCL2A1 gene expression confirms those results within the context of that brain site. Interestingly, the estradiol-sensitive genes, TNF and WNT6, as well as the estradiol-insensitive genes, FABP1 and SOCS3, showed a transcriptional decline after either AICAR delivery to full-fed rats or 12 hr food deprivation, as these four genes were each decreased in the cDVC of FE-A and DE-S compared to FES. As FABP1-encoded protein governs long-chain fatty acid transport and oxidation [Anonenkov et al., 2006], down-regulation of this gene alongside decreased AMPK activity is consistent with the acknowledged role of this sensor in fatty acid partitioning [Lam et al., 2005; Steinberg and Kemp, 2009]. Conversely, TNF, WNT6, FABP1, and SOCS3 were each up-regulated in DE-A versus DE-S animals. The parallel direction of these transcriptional reactions with pAMPK and ER β protein responses supports the possibility that these genes may function independently or in concert up- or downstream of cDVC AMPK. Further research is needed to investigate the role of ER β in opposing directionality of E-sensitive gene responses to AICAR; to verify that AICAR-sensitive gene and protein product response profiles are aligned, and to determine if and how cross-talk between TNF, WNT6, SOCS3, and FABP1.

Our data show that V4 AICAR treatment did not stimulate feeding in full-fed OVX+E rats, but increases consumption in these animals after short-term cessation of feeding. As these dissimilar behavioral responses to AICAR coincide with down- versus up-regulation of pAMPK expression in FE and DE groups, in that order, they seemingly reflect, in part, energy state-dependent effects of this drug on AMPK sensor activity. The current data point to animal model energy balance as a possible experimental design factor in conflicting reports on behavioral or physiological responses to hindbrain AICAR administration. It should be noted that putative relationships between described feeding and gene transcription responses to AICAR here remain speculative at this point. Indeed, it is our goal to discover if products of one or more genes characterized here by opposite transcriptional adjustments to AICAR in full-fed versus food-deprived animals, TNF, WNT6, SOCS3, and FABP1, regulate the distinctive feeding responses characteristic of each energy state. AICAR also exerted energy state-specific effects on additional gene profiles in the female rat cDVC. For example, this treatment decreased HEY1 and ICAM1 mRNAs in full-fed, but diminished NOTCH1, WISP1, and GATA3 transcripts while increasing CEBPD and HES1 transcription

in food-deprived rats. The impact of these gene responses on AMPK-controlled cDVC cell function and input to other metabolic structures in the brain remains to be investigated.

In summary, results show that AICAR-induced simulation of increased cellular AMP or short-term cessation of feeding independently inhibit cDVC pAMPK and ER β protein profiles in estradiol-treated OVX female rats, but in combination elevate expression of both proteins. Moreover, up- or down-regulation of cDVC AMPK activity by AICAR during systemic energy balance versus imbalance respectively coincides with suppressed or enhanced expression of estradiol-sensitive (TNF, WNT6) and -insensitive (FABP1, SOCS3) signal transduction pathway marker genes, as well as no change or augmentation of food intake. Further effort is required to establish how estradiol controls cDVC AMPK reactivity to cellular ATP decline during uninterrupted versus suspended fuel acquisition, and to discover if ER β mediates energy state-dependent effects of AICAR on sensor activity or, alternatively, is regulated by sensor activity state. Identification here of signal transduction pathway genes that exhibit opposing transcriptional reactivity to DVC AMPK activation full-fed versus short term food-deprived estradiol-treated female animals provides novel insight on molecular mechanisms by which this hormone may elicit appropriate metabolic reactions to acute energetic challenge during systemic energy homeostasis versus dyshomeostasis.

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Highlights

- Estradiol (E) regulates energy state-dependent effects of DVC AMPK on feeding in the female rat
- AICAR increased caudal DVC ER α protein expression in both full-fed and acute food-deprived rats
- AICAR effects on both pAMPK and ER β differed according to feeding status
- AICAR up- or downregulated TNF, SOCS3, WNT6, and FAPB1 transcription in fed- versus non-feeding animals
- Research on interactive roles of ER β and these genes in DVC AMPK control of energy homeostasis is needed.

Figure 1. Effects of the AMP mimic 5-aminoimidazole-4-carboxamide-riboside (AICAR) on Caudal Dorsal Vagal Complex (cDVC) 5' Adenosine Monophosphate-Activated Protein Kinase (AMPK), PhosphoAMPK (pAMPK), Estrogen Receptor-Alpha (ERα), and Estrogen Receptor-Beta (ERβ) Protein Expression in Full-Fed versus Short-Term Food Deprived Estradiol-Treated Ovariectomized (OVX) Female Rats

At time zero, groups of Full-Fed + Estradiol (FE; n=8) and Food-Deprived + Estradiol (DE; n=8) – treated OVX rats were injected into the fourth ventricle (V4) with (A)ICAR (1.0 ug/2.0 uL; n=4 DE-A and n=4 FE-A) or (S)saline (n=4 DE-S and n=4 FE-S). Animals were sacrificed at +1 hr after treatments. Pools of micropunched DVC tissue for each treatment group were analyzed by Western blot for pAMPK (Row A), AMPK (Row B), ERα (Row C); ERβ (Row D), or the housekeeping protein, α-tubulin (Row E) proteins. Protein band optical density (O.D.) values were normalized to tubulin. For each protein of interest, immunoblot analyses were performed in triplicate. Vertical lines connecting pairs of blots denote a significant reduction [*p<0.05; black line] or elevation [**p<0.05; grey line] in O.D. values in the second versus the first blot in those pairs.

Figure 2. Self-Organizing Tree Algorithm Hierarchical Cluster Analysis of DVC Signal Transduction Pathway Gene Expression

Panel 2.A: Each horizontal row of colored boxes in the dendrogram represents a single gene, *identified at left*, while treatment groups, e.g. FO-S, FE-S, FE-A, DE-S, and DE-A, *shown at top*, are signified by individual vertical columns. Red and green box colors indicate increased and decreased expression, respectively, relative to the average expression level of each gene. The representative cluster outlined in blue is depicted at higher magnification in Panel 2.B; signal transduction pathways associated with genes in this cluster are identified to the right.

Figure 3. Scatter Plot Depiction of Changes in cDVC Gene Expression Profiles in FE-S versus FO-S Female Rats

Red and green circles depict cDVC signal transduction genes that were up- or down-regulated, respectively, by a factor or two or more in FE-S [Full-fed Estradiol (FE) + injection of (S)aline] versus FO-S [Full-fed Oil (FO) + Injection of (S)aline]. The upper left and lower right halves of the figure contain listings of genes by pathway that were over-and under-expressed, respectively, in the FE-S cDVC relative to FO-S.

Figure 4. Scatter Plot Depiction of Changes in cDVC Gene Expression Profiles in DE-S versus FE-S Female Rats

Red and green circles depict cDVC signal transduction genes that were up- or down-regulated, respectively, by a factor or two or more in DE-S [Deprived Estradiol (DE) + injection of (S)aline] versus FE-S [Full-fed Estradiol (FE) + injection of (S)aline]. The upper left and lower right halves of the figure contain listings of genes by pathway that were over- and under-expressed, respectively, in the DE-S cDVC relative to FE-S.

Figure 5. Scatter Plot Depiction of Changes in cDVC Gene Expression Profiles in FE-A versus FE-S Female Rats

Red and green circles depict cDVC signal transduction genes that were up- or down-regulated, respectively, by a factor of two or more in FE-A [Full-fed Estradiol (FE) + injection of (A)ICAR] versus FE-S [Full-fed Estradiol (FE) + injection of (S)aline]. The upper left and lower right halves of the figure contain listings of genes by pathway that were over-and under-expressed, respectively, in the FE-A cDVC relative to FE-S.

Figure 6. Scatter Plot Depiction of Changes in cDVC Gene Expression Profiles in DE-A versus DE-S Female Rats

Red and green circles depict cDVC signal transduction genes that were up- or down-regulated, respectively, by a factor of two or more in DE-A [Deprived Estradiol (DE) + injection of (A)ICAR] versus DE-S [Deprived Estradiol (DE) + injection of (S)aline]. The upper left and lower right halves of the figure contain listings of genes by pathway that were over-and under-expressed, respectively, in the DE-A cDVC relative to DE-S.

Figure 7. Potential Interactions Among cDVC Genes Exhibiting Energy State-Controlled Transcriptional Response Directionality to AICAR

Colored lines indicate possible chemical, physical, and regulatory interactions recognized by network analysis involving TNF, SOCS3, and FABP1; functional connections with the estrogen-sensitive gene, WNT6, were not identified. *Abbreviations in alphabetical order:* *ADIPOQ*: adiponectin; *AKT1*: RAC-alpha serine/threonine-protein kinase; *CCL5*: chemokine (C-C motif) ligand 5; *FABP1*: fatty acid binding protein 1, liver; *FAS*: fatty acid synthase; *ICAM1*: intercellular adhesion molecule 1; *LEPR*: leptin receptor; *PPARα*: peroxisome proliferator-activated receptor alpha; *SERPINE1*: Serpin peptidase inhibitor, clade E, member 1; *SOCS3*: suppressor of cytokine signaling 3; *STAT3*: signal transducer and activator of transcription 3 (acute-phase response factor); *TNF*: tumor necrosis factor-alpha.

Figure 8. Effects of AICAR on Food Intake in Full-Fed versus Short Term Food-Deprived Estradiol-Treated OVX Female Rats

Bars depict mean grams of food consumed + S.E.M. at +1 (Figure 8.A) and +2 (Figure 8.B) hours after administration of A (1.0 ug) or S into the V4 of FE and DE rats. The following treatment groups are shown at each time point: FE-S (white bar; n=4); FE-A (diagonal-striped white bar; n=4); DE-S (solid grey bar; n=4); DE-A (diagonal-striped grey bar; n=4). *p<0.05 versus FES; **p<0.05 versus DE-S.

Table 1

Effects of AICAR on Mean Western Blot Optical Density (O.D.) Measures of Caudal Dorsal Vagal Complex AMPK, pAMPK, and Estrogen Receptor-Alpha (ER α) and -Beta (ER β) Proteins in Full-Fed versus Short Term Food-Deprived Estradiol-Treated Ovariectomized Rats

	Mean Protein O.D. + S.E.M.			
	AMPK	pAMPK	ER α	ER β
Treatment Groups				
FE-S ^a	1.000 + 0.0	1.000 + 0.0	1.000 + 0.0	1.000 + 0.0
FE-A ^b	0.890 + 0.081	0.769 + 0.085 *	1.172 + 0.073 *	0.833 + 0.052 *
DE-S ^c	1.058 + 0.156	0.724 + 0.081 *	0.936 + 0.108	0.705 + 0.018 *
DE-A ^d	1.055 + 0.118	1.020 + 0.029	0.968 + 0.086	1.338 + 0.155 *

^a Full-fed OVX+E (FE) + injection of (S)aline

^b Full-fed OVX+E (FE) + injection of (A)ICAR

^c Short Term Food-Deprived OVX+E (DE) + injection of (S)aline

^d Short Term Food-Deprived OVX+E (DE) + injection of (A)ICAR

* p < 0.05 versus FE-S

Table 2

Pathway	Unigene	Gene Bank	Symbol	Gene Name
Hedgehog	Rn.9996	NM_016993	Bcl2	Bcl-2
	Rn.90931	NM_017178	Bmp2	-
	Rn.10318	NM_012827	Bmp4	BOMPR4A
	Rn.105585	NM_053566	Ptch1	Ptch, Ptch2
	Rn.138108	NM_001105714	Wnt1	Int1
	Rn.17219	NM_342308	Wnt2b	
	Rn.21821	NM_001107005	Wnt3a	
	Rn.48749	NM_022631	Wnt5a	
	Rn.22149	NM_001108226	Wnt6	
Hypoxia	Rn.10232	NM_012715	Adm	Ap, H39316, RATAP
	Rn.10520	NM_012780	Arnt	Arnt1
	Rn.162391	NM_001107956	Car9	-
	Rn.11365	NM_017001	Epo	-
	Rn.107896	NM_017025	Ldha	Ldh1
	Rn.29367	NM_012620	Serpine1	PAI1A, Pai1, Pai1aa, Planh, RATPAI1A
	Rn.3205	NM_138827	Slc2a1	GLUTB, GTG1, Glut1, Gtg3, RATGTG1
	Rn.1923	NM_031836	Vegfa	VEGF164, Vegf
JAK/STAT	Rn.202620	NM_013154	Cebpd	C, EBPd, CELF
	Rn.10326	NM_133550	Fcer2	CD23, Fcer2a, MGC93219
	Rn.92350	NM_133293	Gata3	-
	Rn.6396	NM_012591	Irf1	-
	Rn.17847	NM_001009717	Lrg1	MGC95065
	Rn.129914	NM_021846	Mcl1	-
	Rn.127801	NM_053565	Socs3	Cish3, Socs-3, Ssi-3
NFκB	Rn.19770	NM_133416	Bcl2a1d Bcl2a1	
	Rn.10323	NM_031535	Bcl2l1	Bcl-xl, Bcl2l, Bclx, bcl-X
	Rn.64578	NM_023987	Birc3	Birc2, IAP1, MGC93416
	Rn.8019	NM_031116	Ccl5	Rantes, Scya5
	Rn.83632	NM_023981	Csf1	-
	Rn.12	NM_012967	Icam1	CD54, ICAM
	Rn.10795	NM_138880	Ifng	IFNG2
	Rn.33229	NM_032612	Stat1	-
	Rn.2275	NM_012675	Tnf	MGC124630, RATTNF, TNF-alpha, Tnfa
Notch	Rn.19727	NM_024360	Hes1	-
	Rn.22422	NM_024383	Hes5	-
	Rn.98202	XM_342216	Hey1	Herp2
	Rn.58672	NM_130417	Hey2	Herp1
	Rn.22252	NM_001107977	Heyl	-

Pathway	Unigene	Gene Bank	Symbol	Gene Name
Oxidative	Rn.2113	NM_012797	Id1	ID125A, Idb1, MGC156482
	Rn.88804	NM_019147	Jag1	-
	Rn.127809	NM_133393	Lfng	-
	Rn.25046	NM_001105721	Notch1	NOTCH, TAN1
	Rn.54447	NM_012848	Fth1	Fth
	Rn.8365	NM_012815	Gclc	Gclc, MGC93096
	Rn.19721	NM_053906	Gsr	-
	Rn.3160	NM_012580	Hmox1	HEOXG, Heox, Hmox, Ho-1, Ho1, hsp32
	Rn.11234	NM_017000	Nqo1	Dia4, MGC93075
	Rn.107103	NM_181550	Sqstm1	Osi, ZIP, ZIP3
p53	Rn.29777	NM_053800	Txn1	Txn
	Rn.67581	NM_031614	Txnrd1	MGC93353, Tr
	Rn.2460	NM_017305	Gclm	Gclr
	Rn.10668	NM_017059	Bax	-
	Rn.25176	NM_173837	Bbc3	Puma
	Rn.27923	NM_017259	Btg2	Agl, An, Pc3, Tis21, an-1
	Rn.10089	NM_080782	Cdkn1a	Cip1, Waf1
	Rn.37227	NM_031507	Egfr	ERBB1, ErbB-1, Errp
	Rn.162521	NM_139194	Fas	Tnfrsf6
	Rn.10250	NM_024127	Gadd45a	Ddit1, Gadd45
PPAR	Rn.223	NM_022381	Pcna	PCNAR, Pcna, cyclin
	Rn.55115	NM_017045	Rb1	-
	Rn.54820	NM_057107	Acs13	Acs3, Fac13
	Rn.87821	NM_053623	Acs14	Acs4, Fac14
	Rn.105862	NM_053607	Acs15	Acs5, Fac15
	Rn.11389	NM_012930	Cpt2	CPTII
	Rn.87449	NM_133306	Olr1	LOX-1, Oldlr1, Oldr1
	Rn.145068	XM_231115	Slc27a4	-
	Rn.110441	XM_001066536	Sorbs1	SH3P12
	Rn.36412	NM_012556	Fabp1	Fabplg, MGC108756, RATFABPLG
TGFβ	Rn.2423	NM_024403	Atf4	-
	Rn.29897	NM_031762	Cdkn1b	CDKN4, Cdk1b, Kip1, P27KIP1, p27
	Rn.19723	NM_012843	Emp1	CL20, EMP1, ENP1MR, MGC93627, TMP
	Rn.35886	NM_001008321	Gadd45b	MGC105966
	Rn.4028	NM_053523	Herpud1	Sup
	Rn.3723	NM_019242	Ifrd1	MGC93482, Pc4
	Rn.12072	NM_012603	Myc	MGC105490, RNCMYC, c-myc, mMyc
	Rn.83627	NM_145681	Tnfsf10	Trail
	Rn.162212	NM_024355	Axin2	axil
WNT	Rn.22279	NM_171992	Ccnd1	-

Pathway	Unigene	Gene Bank	Symbol	Gene Name
	Rn.96083	NM_022267	Ccnd2	-
	Rn.161784	NM_024159	Dab2	Doc-2, Doc2
	Rn.11306	NM_012953	Fosl1	Fra-1, fra-1
	Rn.10282	NM_012864	Mmp7	MPMM
	Rn.96181	NM_013141	Ppard	Pparb
	Rn.63486	NM_031716	Wisp1	MGC93340
House-keeping	Rn.94978	NM_031144	Actb	Actx
	Rn.1868	NM_012512	B2m	-
	Rn.47	NM_012583	Hprt1	Hgprtase, Hprt, MGC112554
	Rn.107896	NM_017025	Ldha	Ldh1
	Rn.973	NM_001007604	Rplp1	MGC72935