Published in final edited form as:

Horm Metab Res. 2015 July ; 47(8): 611–621. doi:10.1055/s-0034-1387792.

Deferred Feeding and Body Weight Responses to Short-Term Interruption of Fuel Acquisition: Impact of Estradiol

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Abstract

Short-term abstinence from food intake, planned or unplanned, is unavoidable in modern life, but negatively correlated with appetite control and obesity. This study investigated the role of estradiol in feeding and body weight (*bw*) reactions to short-span cessation of feeding. During acute 1-6 hr re-feeding, FD (12 hr food-deprived)-estradiol benzoate (EB)-implanted ovariectomized rats ate less food and gained less weight than FD animals implanted with oil (O). Full fed (FF)- and FD-EB consumed equal amounts of food over 24 hr, but weight gain was greater in the latter; 24 hr food intake and *bw* gain in FD-O exceeded FD-EB. Caudal fourth ventricular administration of the AMPK activator AICAR increased dorsal vagal complex AMPK activity in FD-EB and -O, but elicited dissimilar adjustments in hypothalamic metabolic neuropeptide transmitter expression, while respectively enhancing or reducing acute re-feeding in these animals and reversing FD-O weight gain. Drug-treated FD-EB and FD-O exhibited respective feeding and weight gain increases between 6-24 hrs. AICAR enhanced 24 hr consumption in FD-EB versus FF-EB, but cumulative intake and *bw* gain were greater in AICAR-treated FD-O versus FD-EB. Results show that estradiol limits acute re-feeding after short-term feeding suspension, but augments acute re-feeding when energy depletion coincides with suspended feeding. This compound metabolic stress exerts steroid-dependent effects during later resumption of circadian-induced feeding, e.g. increased consumption versus weight gain in the presence versus absence of estradiol. These studies provide novel evidence that estrogen mitigates acute and post-acute adverse effects of disrupted fuel acquisition on energy balance.

Keywords

ovariectomy; deferred feeding; estradiol benzoate; dorsal vagal complex; AICAR; body weight

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Authorship:

B.A.I. designed the study, performed the research, analyzed the data, contributed to discussion, and reviewed the manuscript. K.P.B. contributed to discussion and wrote the manuscript.

Conflict of Interest: None

Introduction:

The CNS maintains energy equilibrium through control of nutrient acquisition, energy expenditure, and energy storage. Detection and appropriate reaction to variable environmental nutrient provision and dynamic adjustments in internal energy needs are critical for maintenance of energy balance. Eating behavior is a complex interplay of physiological, psychological, social, and genetic factors that influence meal timing, quantity of food intake, and food preference [1]. The ideal circumstance of unfettered ability to eat-at-will, in response to the above drives, can be impractical in reality. Short-term suspension of food intake, planned or unplanned, is often unavoidable and unpredictable in modern life. Epidemiological studies have identified a strong inverse relationship between eating frequency, body weight, and obesity [2]. Reduced eating frequency, e.g. meal omission, in human subjects negatively affects appetite control [3]. Identification of mechanisms that cause or exacerbate adverse effects of interrupted feeding on rebound consumption and diversion of fuel to storage can yield potential targets for therapeutic mitigation of outcomes that promote obesity.

The ovarian hormone, estradiol, acts on central and peripheral substrates to regulate energy balance [4-6]. Long-term effects of estrogen in female rats include anorexia and reduced body weight [7-9]. The influence of estrogen on short-span energy depletion/repletion remains controversial as available studies have typically evaluated its influence on rebound feeding after 48 hours or more of food withdrawal [10,11]. In order to more closely replicate meal omission(s) within a single day, the present studies utilized a more physiologically-relevant model for limited interruption of feeding, involving suspension of food intake for 12 hours commencing two hours after lights off, to investigate the hypothesis that estradiol attenuates immediate (e.g. 1-6 hour) feeding, metabolic rate, and body weight responses to brief feeding suspension in female rats. Since the above functions normalize within 1-3 hours after feeding recommencement, it was of interest to examine whether short-term feeding pause/re-feeding influences energy intake or storage during consequent routine feeding. We thus evaluated food intake and body weight between 6 and 24 hours after feeding resumption, as this period encompasses light-to-dark transition, the onset of the next circadian-entrained meal time [12].

Tonic suppressive effects of estrogen on food intake are mediated, in part, by action within the hindbrain dorsal vagal complex (DVC) [13]. DVC metabolic-sensory neurons, e.g. A2 noradrenergic neurons, are directly sensitive to estrogen by virtue of estrogen-alpha and -beta receptor protein expression [14]. The ultra-sensitive metabolic sensor 5'-monophosphate-activated protein kinase (AMPK) is activated via phosphorylation in response to stressors that deplete ATP, including exercise, starvation, and hypoxia [15,16]. Our studies show that estrogen regulates effects of caudal fourth ventricular administration of the AMPK activator 5-aminoimidazole-4-carboxamide-riboside (AICAR) on A2 nerve cell Fos expression, hyperglycemia, and genomic activity within hypothalamic metabolic structures [14]. This pharmacological approach was used here to investigate the premise that estrogen controls the direction and magnitude of feeding and body weight responses to hindbrain AMPK signals of energy depletion during disrupted versus uninterrupted fuel acquisition. In order to identify potential downstream hypothalamic targets of estradiol-

dependent versus -independent hindbrain AMPK activation during these distinctive energy states, Western blot methods for protein analysis were utilized in conjunction with micropunch-dissection to examine expression of hypothalamic catabolic [arcuate nucleus pro-opiomelanocortin (POMC)] and anabolic [arcuate nucleus neuropeptide Y (NPY); lateral hypothalamic area orexin-A (ORX-A)] neurotransmitters implicated in feeding.

Methods and Materials:

Animals:

Adult female Sprague Dawley rats (240–290 g *bw*) were obtained from the ULM College of Pharmacy Vivarium in-house breeding program, and maintained under a 14 hr light:10 hr dark lighting schedule (light on at 05.00 hr). Animals were allowed free access to standard laboratory rat chow and water, and were gentled by daily handling. 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:

Experiment 1: Effects of short-term (12 hr) food deprivation with or without hindbrain AICAR administration on acute- and post-acute refeeding and body weight in full-fed (FF) versus estradiol benzoate (EB)- versus oil-implanted OVX female rats.—On day 1, animals were implanted with a PE-20 polyethylene cannula into the caudal fourth ventricle (CV4) [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, USA), and 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 inches, o.d. 0.125 inches; 10 mm/100 g *bw*) filled with 30 µg EB/mL safflower oil or oil alone [17]. This replacement strategy results in plasma estradiol levels that approximate the physiological nadir in the 4-day estrous cycle [18]. Animals received *sc* injections of ketoprofen (1 mg/kg *bw*) before and after each surgery, and were treated by topical administration of 1-3 drops of 0.25% bupivacaine after incision closure. For three consecutive days before the experiment (days 8-10), animals were acclimated to the presence of standard laboratory rat chow in food jars inside their home cages and regular removal of those jars for weighing. Beginning at 21.00 hr on day 11, groups of OVX + EB (EB) and OVX + oil rats were food-deprived (FD) over a 12 hr period prior to time zero (t_0) at 0.900 hr on the day of the experiment (day 12). Other animals were allowed to feed *ad-libitum*, e.g. full-fed (FF), over the same interval. At 09.00 hr (time zero; t_0) on the day of the study (day 12), groups of FD rats ($n=5$ per group) were injected into the CV4 with AICAR (0.5 or 2.5 µg/µL) or the vehicle, saline (SAL) in a 2.0 µL total volume; administered drug doses were thus 1.0 or 5.0 µg/animal. FF animals received either SAL or 1.0 µg AICAR. Pre-weighed jars containing chow were placed in each cage at t_0 and weighed hourly between +1 and +6 hours and again at +24 hr, as described [19]. For each time point, measures of consumed food were adjusted by subtracting the weight of food crumbs on underlying paper. All animals used in the present studies exhibited cerebrospinal fluid reflux

from the cannula tip on day 12; post-mortem histological examination confirmed accuracy of cannula placements within the CV4.

Experiment 2: Effects of hindbrain AICAR administration on hypothalamic metabolic effector neurotransmitter protein expression in FF and FD E- versus oil-implanted OVX female rats.—Groups of EB and oil animals were injected into the

CV4 with 1.0 ug AICAR [n=3 FF-EB; n=3 FD EB; n=3 FF-oil; n=3 FD-oil] or SAL [n=3 FF-EB; n=3 FD EB; n=3 FF-oil; n=3 FD-oil], as described in Experimental 1 above, then sacrificed at +1 hr. Food was removed after CV4 treatments. Each fore- and hindbrain were snap-frozen in liquid nitrogen-cooled isopentane, stored at -80°C , and cut into serial 150 μm or 200 μm serial sections, respectively. Palkovits-style hollow micropunch needles of 0.76 mm diameter (prod. no. 57401, Stoelting, Inc., Kiel, WI) were used to dissect the hypothalamic arcuate nucleus (ARH) and lateral hypothalamic area (LHA) from sections cut between -2.00 mm to -2.60 mm from bregma, using neuroanatomical landmarks depicted in *Brain Maps: Structure of the Rat Brain* (L.W. Swanson, Elsevier, 1999) as a guide. The DVC was similarly removed from two consecutive 200 μm frozen sections cut through the hindbrain beginning at 14.40 mm posterior to bregma. ARH, LHA, and DVC tissues from each animal were collected in microcentrifuge tubes containing 20 μL tissue lysis buffer (2% SDS, 0.05 M DTT, 10% glycerol, 1 mM EDTA, 60 mM Tris-HCl, pH 7.2) and heat-denatured. For each treatment group, aliquots of heat-denatured tissue from each animal were combined and separated on 10–15% gradient Tris-glycine gels (90 V; 105 min; Tris-glycine SDS running buffer), as described [Cherian and Briski, 2011; 2012]. Proteins were transblotted (30 V; overnight; 4°C ; Towbin buffer) to 0.45 μm PVDF-Plus membranes (prod. no. PV4HY00010; Osmonics Inc., Gloucester, MA). Membranes were treated with Western blot signal enhancer (prod. no. 21050; Pierce, Rockford, IL); blocked (2 hr) with Tris-buffered saline, pH 7.4, containing 0.1 % Tween-20 (prod. no. P9416; Sigma Aldrich, St. Louis, MO) and 2% bovine serum albumin (prod. no. 81003; MP Biomedicals, Solon, OH) (TBS-T-BSA); and incubated (overnight; 4°C) with primary antisera. DVC tissue samples were probed with Santa Cruz Biotechnology, Inc. [Santa Cruz, CA] rabbit primary polyclonal antisera against AMPK α 1/2 (prod. no. sc-25792; 1:1,000) or phosphoAMPK α 1/2 (Thr 172) (pAMPK) (prod. no. sc-33524; 1:1,000). Hypothalamic tissue samples were probed with rabbit anti-POMC (prod. no. sc-20148; 1:500), anti-NPY (prod. no. sc-28943; 1:500), or anti-ORX-A (prod. no. sc-8070; 1:500) antisera from Santa Cruz Biotechnol. Sample levels of the housekeeping protein α -tubulin were detected using monoclonal antibodies (prod. no. CP06; 1:1,000; EMD Millipore, Billerica, MA). Membranes were incubated (1 hr) with peroxidase-conjugated goat anti-mouse (prod. no. NEF822001EA; 1:5,000; PerkinElmer, Boston, MA) or goat anti-rabbit (1:5,000; prod. no. NEF812001EA; PerkinElmer). After incubation with Supersignal West Femto Maximum Sensitivity chemiluminescent substrate (prod. no. 34096; Thermo Fisher Scientific, Inc., Rockford, IL), signals were visualized in a Syngene G: box Chemi. Band optical densities (O.D.) were quantified with Genetool 4.01 software (Syngene; Frederick, MD), and expressed relative to α -tubulin. Protein molecular weight markers were included in each Western blot analysis. Immunoblots were performed in triplicate.

Statistics:

Food intake and body weight measures between +1 and +6 hrs were analyzed by repeated measures analysis of variance and Bonferroni's test. Cumulative food intake and body weight measures at +6 and +24 hrs were evaluated by three-way analysis of variance and Student-Newman-Keuls (SNK) test. Mean normalized protein O.D. values were analyzed by two- or three-way ANOVA and SNK test. Differences of $p < 0.05$ were considered significant.

Results:

The data in Figure 1 depict effects of 12 hr food deprivation on acute feeding in EB versus oil rats after re-introduction of food at 09.00 hr. Temporal patterns of food intake by FF groups of EB and oil animals did not differ over the 6 hr period between 0.900 and 15.00 hours (Panel 1.A). Both FD-EB and FD-oil ate more chow compared to their FF controls at +1 hr and at +1, 3, and 6 hrs, respectively. Food intake by FD-oil exceeded that of FD-EB at +1, 2, and 3 hrs. The data in Panel 1.B show that net food consumption between 1 and 6 hrs was significantly greater in both FD groups relative to their FF controls, and that acute consumption by FD-oil was approximately twice that of FD-EB. Figure 1.C illustrates changes in body weight between 1 and 6 hrs after re-feeding in EB versus oil. Mean adjustments in body weight of FF-EB and FF-oil were equivalent at each time point over this interval (Figure 1.D). FD-EB and FD-oil exhibited augmented body weight, relative to FF controls, at +1 and +6 hrs and at +1, 2, and 3 hrs, respectively. Weight gain in FD-oil exceeded that in FD-EB at +1 and +2 hrs. Both FD-EB and FD-oil exhibited increases in body weight relative to FF controls over initial 6 hour of re-feeding, a gain that was significantly greater in FD-oil versus FD-EB.

Figures 2 and 3 depict cumulative food intake and body weight changes between 6-24 and 1-24 hours, respectively, after re-feeding of EB versus oil animals. Results show that FF and FD groups consumed equivalent quantities of food between 6 and 24 hrs after re-feeding, e.g. 15.00 hr and 09.00 hr (Panel 3.A), but that FD-EB, but not FD-oil gained significantly more weight than FF controls over this interval (Panel 3.B). Panels 3.C and 3.D show that net 24 hour food consumption and weight gain, respectively, were both higher in FD animals versus their FF controls, and that both parameters were significantly greater in FD-oil versus FD-EB.

The data in Table 1 depict effects of AICAR delivery to the CV4 on food intake and body weight changes over a 24 hr period in FF EB and O animals. AICAR did not alter mean chow consumption between 1-6 hrs, between 6-24 hrs, or net 24 hr food intake in FF EB or FF O, compared to corresponding SAL-injected FF EB and FF O rats. However, this treatment did result in a reduction in body weight in FF EB, but not FF O, between 1-6 hrs. Mean measures of body weight did not vary among treatments groups between 6-24 hrs after treatments.

Figure 4 illustrates effects of intra-CV4 AICAR administration on patterns of re-feeding by EB versus oil animals after 12 hours of food deprivation. The data in Panel 4.A show that FD-EB rats injected with the lower (1.0 ug), but not the higher dose (5.0 ug) of AICAR

coincident with re-introduction of food at 09.00 hr consumed more than SAL-treated FD controls at +1 hr. However, FD-EB animals treated with 1.0 or 5.0 ug AICAR exhibited a net increase in food intake between +1 and +6 hrs compared to FD-EB + SAL rats (Panel 4.B). In contrast to EB rats, AICAR did not augment food consumption in FD-O at +1 hr (Panel 4.C), and attenuated feeding relative to the FD-oil + SAL group at +3 and +6 hrs. This drug treatment resulted in a dose-dependent decline in net food intake by these animals, relative to SAL-injected FD controls, over the first 6 hrs of re-feeding (Panel 4.D). The data in Figure 5 depict effects of AICAR on changes in body weight during re-feeding of EB versus oil rats after 12 hrs of absence of food. FD-EB animals injected with 1.0 ug AICAR at 09.00 hr exhibited significantly greater increase in body weight at +1 hr, and attenuated weight gain at +6 hrs, compared to FD + SAL controls (Panel 5.A). Neither drug dose influenced net change in body weight between 1 and 6 hrs of re-feeding of FD-EB rats (Panel 5.B). FD-oil rats injected with SAL or AICAR showed equivalent increases in body weight at +1 hr of re-feeding (Panel 5.C). Administration of the higher drug dose caused a significant reduction in body weight of FD-oil animals between 1 and 6 hrs, relative to the FD + SAL group (Panel 5.D).

Data in Figure 6 show that intra-CV4 delivery of AICAR at 09.00 hr to FD-EB rats enhanced food intake between 6 and 24 hrs after treatment, compared to FD-EB + SAL controls (Panel 6.A), but did not alter body weights of these animals over that interval (Panel 6.B). AICAR did not modify food intake (Panel 6.C), but stimulated weight gain (5.0 ug dose; Panel 6.D) between +6 and +24 hrs in FD-oil rats. AICAR did not modify chow consumption or weight gain over this 18 hr interval in FF-EB (insets to Figures 6.A and 6.B) or FF-O controls (insets to Figures 6.C and 6.D). Cumulative effects of AICAR on feeding and body weight over the 24 hr period after delivery to FD rats are depicted in Figure 7. The data in Panels 7.A and 7.C show that this drug treatment augmented net food intake in EB, but not oil. Panels 7.B and 7.D indicate that AICAR did not modify net changes in body weight in FD-EB or FD-oil over this period of time.

Figure 8 depict effects of CV4 AICAR administration on AMPK and pAMPK protein expression in the EB- (Figure 8.A) and oil-implanted (Figure 8.B) OVX rat DVC. Results indicate that AMPK protein levels did not differ between SAL-injected FF-EB versus FD-EB (8.A) or between SAL-injected FF-oil versus FD-oil (8.B) groups; moreover, AICAR did not modify AMPK expression in FF or FD animals regardless of estrogen status. Short-term suspension of feeding (FD) elicited divergent adjustments in pAMPK protein expression in EB versus oil animals, as levels were reduced in FD-EB versus FF-EB (8.A) or increased in FD-oil versus FF-oil (8.B). AICAR treatment respectively suppressed or elevated pAMPK expression in FF-EB and FF-oil groups compared to SAL controls, but augmented DVC pAMPK in both FD-EB (vs FD-EB + SAL) and FD-oil (vs FD-oil + SAL).

Figures 9, 10, and 11 depict effects of hindbrain AICAR administration on ARH NPY, ARH POMC, and LHA ORX-A protein levels, respectively, in EB- and oil-implanted OVX rats. ARH NPY neuropeptide levels were significantly reduced in FF-EB versus FF-oil rats, and were diminished by FD in EB (vs. FF-EB) and oil (vs. FF-oil) (Figure 9). AICAR administration decreased ARH NPY levels in FF-EB (vs. FF-EB + SAL) and FF-oil (vs. FF-oil + SAL). On the other hand, FD animals treated with AICAR exhibited either elevated

NPY (FD-EB + AICAR > FD-EB + SAL) or no change in protein expression (FD-oil + AICAR = FD-oil + SAL). As shown in Figure 10, ARH POMC expression was decreased in FF-EB versus FF-oil rats, and was suppressed by FD [FD-EB vs. FF-EB; FD-oil vs. FF-oil]. AICAR suppressed ARH POMC levels in FF but not FD groups. LHA prepro-ORX-A protein levels were lower in FF-EB versus FF-oil animals (Figure 11). Following FD, ORX-A expression was elevated in EB [FD-EB vs. FF-EB], but inhibited in oil [FD-oil vs. FF-oil]. In FF groups treated with CV4 AICAR, LHA ORX-A levels were unchanged in EB (vs. FF-EB + SAL), but reduced in oil (vs FF-oil + SAL). In FD groups given AICAR, this protein profile was increased in EB (vs. FD-EB + SAL), but unchanged in oil (vs. FD-oil + SAL).

Discussion:

The present studies utilized a physiologically-relevant model for short-term feeding abstinence to examine effects of the ovarian hormone, estradiol, on deferred feeding and body weight gain after short-span suspension of fuel gain with or without concomitant energy depletion. Data show that estrogen limits the magnitude of acute (1-6 hr) re-feeding and controls temporal weight gain patterns in FD animals. The AMP mimic, AICAR, did not stimulate chow consumption in FF rats, but enhanced acute and post-repletion feeding (6-24 hr) in FD-EB, while inhibiting acute food intake and reversing deferred weight gain between 6-24 hr in FD-O animals. These results are consistent with evidence for respective reduction or augmentation of DVC AMPK activation 1 hr after AICAR administration to FF-EB vs. FD-EB animals. Moreover, AICAR-induced sensor activation in FD-EB and -oil rats resulted in disparate adjustments in hypothalamic metabolic transmitter protein expression at this time point. Current results demonstrate estrogenic control of metabolic responses to energy depletion-repletion, including food intake and diversion of fuel to storage, over both acute and post-acute time frames. Results suggest that estradiol exerts these effects, in part, by action within the hindbrain to regulate AMPK sensitivity to distinctive energy states of suspended vs. non-interrupted fuel acquisition. Evidence that hypothalamic metabolic neuropeptides exhibit differential responses to simultaneous short-term feeding cessation and hindbrain energy depletion in EB and oil animals suggest that this hormone may also control signaling from hindbrain AMPK to the hypothalamus and/or reactivity of hypothalamic substrates to that input.

Within the first six hr of re-feeding, FD-EB and FD-oil ate more food and gained more weight than their respective FF controls; the magnitude and duration of these augmented responses were greater in FD-O [food intake and weight gain were 8.5 and 15 g greater than FF-O] versus FD-EB [food intake and weight gain were 1.5 and 5.5 g greater than FF-EB]. Moreover, FD-EB but not FD-O rats continued to gain weight, relative to controls, between 6-24 hr after resumption of feeding. Over that period, FF-EB animals ate approximately 15 g, but did not gain additional weight, whereas FD-EB rats ate a similar amount, e.g. 16 g, and gained 6 g in body weight. These results suggest that estrogen may promote beneficial post-acute responses to metabolic deprivation, namely enhanced allocation of fuels for reserve versus utilization after resumption of normal feeding patterns. It would be informative to understand the mechanisms, including relevant neural circuitries and endocrine signals that mediate this putative deferred, estrogen-dependent shift from fuel expenditure to storage. Our results differ from earlier reports that after 48 hr of food

deprivation, initial re-feeding and net 24 hr food intake and weight gain are comparable between EB- versus oil-treated OVX rats [10]. These discrepant outcomes imply that the duration of food abstinence may be a critical determinant of direction and magnitude of estrogenic regulation of these responses. It is also noted that different experimental models were used, since in the prior work, female rats were long-term OVX [from 21 to 90 days of age] before institution of estrogen replacement therapy.

Intra-CV4 AICAR administration did not alter food consumption by FF animals, but initiated an acute reduction in body weight in FF-EB rats. This lack of drug effect on food intake in FF females post-delivery to the hindbrain is consistent with previous reported involving *ad-libitum*-fed male rats [20]. In contrast to FF animals, AICAR treatment enhanced re-feeding in FD-EB. Drug treatment elevated food consumption, relative to FF baseline, over the 24 hr period of study, whereas SAL-injected FD-EB rats increased their intake solely between +1 and +6 hrs. AICAR-induced augmentation of food intake in FD-EB rats did not further increase body weight, suggesting that additional consumed metabolic fuels were utilized, not stored, over initial fuel repletion. Thus, in the ovary-intact female, as-yet-undetermined parameters of body-wide energy status may be determinants of selective metabolic responses to hindbrain AMPK activation. Interestingly, CV4 AICAR elicited an acute decline, e.g. between 1 and 6 hrs, in both feeding and body weight in FD-oil animals, findings that demonstrate novel inhibitory effects of this compound on metabolic functions in the absence of estrogen. In contrast to FD-EB rats, AICAR did not further enhance food intake by FD-oil between +6 and +24 hrs, yet stimulated weight gain over this period, results that imply a shift toward storage versus metabolism of ingested fuels. Further experimentation is necessary to determine if weight gain observed between 6 and 24 hrs in FD-oil animals is a secondary response to or, alternatively, is independent of initial weight loss in this group.

Input to the central metabolic network on hindbrain energy state likely originates, in part, from caudal DVC A2 noradrenergic neurons, which express the metabolic sensory biomarkers glucokinase (GCK) [21], ATP-dependent potassium channel, K_{ATP} [21], and AMPK [22,23]. A2 neurons couple substrate fuel availability to AMPK activity as hindbrain lactate repletion normalizes hypoglycemia-associated activation of this sensor in these cells [24]. We thus presume that observed AICAR effects on feeding, body weight, and hypothalamic neurotransmitter synthesis are communicated, in part, by A2 nerve cells as this drug alters pAMPK protein expression in these cells [14]. However, it is possible that other hindbrain neuron populations may also report AICAR-induced changes in AMPK activity, as we have recently found A1 and C1 catecholamine cell AMPK to be hypoglycemia-sensitive [Shrestha and Briski; personal communication]. Documentation here that estrogen regulates feeding and body weight responses to pharmacologic hindbrain energy depletion highlights the need to determine where and how such action occurs. The DVC is a likely substrate as it contains high estrogen receptor expression [25] by catecholaminergic neurons [26,27], including A2 neurons [14]. Intra-DVC estradiol implants enhance fasting-induced inhibition of pituitary gonadotropin secretion and inhibit food intake, implying that DVC metabolic sensing functionality is estrogen-dependent [13,28]. Estradiol may also control reactivity of metabolic network elements downstream to A2 and other AICAR-sensitive hindbrain neurons to drug-induced signaling from the hindbrain. Estrogen receptor

expression by ARH POMC and NPY neurons [29-31] implies that one or both populations may be direct targets of such steroidal action. Current data document hindbrain AMPK activation effects on feeding versus substrate fuel stores during suspended fuel intake and uninterrupted feeding, respectively, in the presence of metestrus-like estradiol levels. It is noted that the continuum of growth and development over the female lifespan involves transition between distinct reproductive states, including juvenile quiescence, fecundity, and reproductive senescence, which exhibit unique patterns of ovarian estradiol secretion. Ongoing work in our laboratory seeks to determine if diverse physiological patterns of estrogen secretion, including low prepubertal estrogen secretion, dynamic fluctuation of estradiol over the estrous cycle, elevated ovarian hormone release during pregnancy, and significant decline in estrogen output during old age exert characteristic effects on feeding and body weight under conditions of metabolic balance versus imbalance. It would also be informative to learn if and how physiological patterns of the other ovarian steroid hormone, progesterone, may influence estrogen actions on those parameters.

FD of 12 hr duration increased or decreased DVC pAMPK protein expression in oil and EB rats, respectively, results that correlate with the lesser amount of food consumed by the latter during acute refeeding. These data suggest in the presence of estradiol, FD elicits counteractive mechanisms to maintain cellular energy balance in the DVC, despite suspended fuel acquisition. We do not know if hindbrain sensor activity remains suppressed over longer periods of deprivation or if this decline is unique to the DVC. FD-oil and FD-EB rats both showed diminished ARH POMC and NPY protein levels, but divergent LHA ORX-A expression profiles. These results do not support a role for these anabolic neurotransmitters in acute re-feeding by either group, but do not discount a role for diminished signaling by POMC peptide products, e.g. α MSH, that inhibit food intake. CV4 AICAR administration to FF-oil and FF-EB similarly elevated or reduced DVC pAMPK expression, implying drug induction of a more positive energy state in the presence of estradiol. A plausible explanation of AMPK deactivation at 1 hr post-AICAR treatment of FF-EB is that it was likely preceded by augmented pAMPK expression, an outcome that would initiate compensatory energy-yielding metabolic functions that inhibit sensor activity. Elevated DVC pAMPK levels in FF-oil + AICAR at the same post-treatment time point may reflect longer activation of this sensor in the absence of estradiol. These sensor reaction patterns correlate with a null drug effect on feeding in FF-EB but not FF-oil, indicating that hindbrain sensor activation in the latter is ineffectual in producing either a feeding or bw response. Importantly, current evidence that reductions in DVC pAMPK expression in FF-EB + AICAR and FD-EB correspond respectively to decreased body weight and increased feeding suggest that estradiol determines the nature of metabolic response, e.g. catabolic versus anabolic, to hindbrain energy imbalance based upon energy state. As ORX-A expression patterns deviated between FF-EB + AICAR and FD-EB, this neuropeptide may play a role in differential metabolic outflow observed in these groups.

Interestingly, CV4 AICAR administration during FD increased DVC pAMPK protein in both oil and EB rats, yet stimulated feeding only in the latter. Augmented NPY and ORX-A protein expression in FD-EB + AICAR but not FD-oil + AICAR infers a role for these neuropeptides in this divergent behavior response. The lack of impact of hindbrain energy

depletion in FD-oil animals on downstream hypothalamic metabolic neurotransmitters implies a surprising functional disconnect between these metabolic circuit elements.

In summary, this research demonstrates the ability of estradiol to mitigate acute and post-acute adverse effects of disrupted fuel acquisition on energy balance. The current physiologically-relevant experimental model for short-term suspension of fuel gain has value for investigation of neural substrates and molecular mechanisms for endocrine manipulation of rebound consumption and diversion of fuel to storage in response to feeding abstinence with or without energy depletion. Such insight has the potential benefit of identifying potential targets for therapeutic mitigation of outcomes that promote obesity. The current work also provides novel evidence for that estradiol regulates DVC AMPK activity and control of hypothalamic metabolic neuropeptide expression and metabolic outflow by this sensor during distinctive states of suspended versus noninterrupted fuel acquisition.

Acknowledgements:

This research was supported by National Institutes of Health grant DK085122.

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Figure 1. Effects of estradiol benzoate (EB) on acute re-feeding after 12 hr food deprivation of ovariectomized (OVX) female rats.

Groups of EB- and oil-implanted OVX rats were full-fed (FF) or food deprived (FD) from 21.00 to 0.900 hr, at which time food was re-introduced. Panel 1.A depicts hourly measures of non-cumulative food intake by these animals between 10.00 and 15.00 hr. Food intake did not differ between FF-EB [●] and FF-oil [▼] rats over this 6 hr period. Both FD-EB [○] (+1 hr) and FD-O [▲] (+1, +3, and +6 hrs) ate more food than their FF controls, with consumption by FD-oil exceeding that of EB at +1, +2, and +3 hrs ($F_{0.95}(6,7,32,42) = 8.17$; $p < 0.001$). Degrees of freedom are presented for raw effect, column effect, subject matching, and interaction between raw and column effect, respectively. The data in Panel 1.B show that FF-EB [white bar] and FF-oil [gray bar] ate equivalent quantities of food between +1 and +6 hrs, and that this intake was significantly less than that by matched FD groups ($F_{0.95}(9,39) = 39.85$; $p < 0.001$), where treatment and residual sums of squares are respective degrees of freedom. Net food intake by FD-oil [diagonal-stripe gray bar] exceeded that of FD-EB [diagonal-stripe white bar] over this time interval. *, $p < 0.05$ vs FF controls; **, $p < 0.05$ vs EB. Panel 1.C depicts changes in bodyweight in FF-EB[●], FF-oil [▼], FD-EB [○], and FD-oil [▲] animals over a six hr period after re-introduction of food at 0.900 hr. Data show that hourly measures of FF-EB and FF-O body weights were similar over this period. FD-EB and FD-oil exhibited augmented body weight, relative to FF controls, at +1 and +6 hrs and at +1, +2, and +3 hrs, respectively. Weight gain in FD-oil exceeded that in FD-EB at +1 and +2 hrs ($F_{0.95}(6,7,32,42) = 5.02$; $p < 0.001$). Panel 1.D shows that FF-EB [white bar] and FF-oil [gray bar] groups exhibited smaller adjustments in body weight in the interval between 0.900 and 15.00 hrs than did matched FD groups ($F_{0.95}(9,39) = 37.29$; $p < 0.001$). FD-oil [diagonal-stripe gray bar] showed greater net weight gain over this period than FD-EB [diagonal-stripe white bar]. *, $p < 0.05$ vs FF controls; **, $p < 0.05$ vs EB.

Figure 2. Effects of EB on food intake and body weight adjustments between 6- 24 hours after re-feeding of acute food-deprived OVX rats.

The data in Panel 2.A show that food intake was equivalent among the four treatment groups [FF-EB (white bar); FF-oil (gray bar); FD-EB (diagonal-stripe white bar); FD-oil (diagonal-stripe gray bar)] between 6 and 24 hrs after re-feeding, e.g. 15.00 to 09.00 hrs ($F_{0.95}(9,40) = 8.01$; $p < 0.001$). FD-EB, but not FD-oil gained significantly more weight than FF controls over this interval (Panel 2.B) ($F_{0.95}(9,39) = 11.39$; $p < 0.001$). *, $p < 0.05$ vs FF controls; **, $p < 0.05$ vs EB.

Figure 3. Effects of EB on cumulative 24 hr food intake and body weight adjustments during re-feeding of acute food-deprived OVX rats.

Panels 3.A and 3.B show that net 24 hr food consumption ($F_{0.95} (9,39) = 38.30$; $p < 0001$) and weight gain ($F_{0.95} (9,38) = 62.45$; $p < 0001$), respectively, were both higher in FD animals versus their FF controls, and that both measures were significantly greater in FD-oil versus FD-EB. *, $p < 0.05$ vs FF controls; **, $p < 0.05$ vs EB.

Figure 4. Effects of caudal fourth ventricular (CV4) administration of the AMPK activator 5-aminoimidazole-4-carboxamide-riboside (AICAR) on acute re-feeding by FD-EB versus FD-oil rats.

After 12 hours of food deprivation (21.00 to 09.00 hr), groups of FD-EB (Panels 4.A, 4.B) and FD-oil (Panels 4.C, 4.D) rats were re-introduced to chow after injection into the CV4 of AICAR [$\blacktriangledown$, 1.0 ug AICAR; $\blacktriangle$, 5.0 ug AICAR] or SAL [$\circ$]. FF-EB and FF-oil controls [$\bullet$] were treated with SAL at 0.900 hr. FD-EB rats injected with the lower, but not the higher AICAR dose ate more chow than SAL-injected FD controls at +1 hr (Panel 4.A) ($F_{0.95}(6,7,32,42) = 8.17$; $p < 0.001$). Drug treatment did not increase food consumption in FD-oil at +1 hr (Panel 4.C), and attenuated feeding relative to SAL-injected FD-oil rats at +3 and +6 hrs. Panels 4.B (EB) and 4.D (O) depict cumulative food intake between 09.00 and 15.00 hr in FF + SAL [white bars]; FD + SAL [gray bars]; FD + 1.0 ug AICAR [horizontal-stripe gray bars]; FD + 5.0 ug AICAR [cross-hatch gray bars] groups. Net chow consumption over this 6 hr period was greater in FD-EB rats given 1.0 or 5.0 ug AICAR versus FD-EB + SAL (Panel 4.B; $F_{0.95}(9,39) = 39.85$; $p < 0.001$), whereas intake by FD-oil was reduced in a dose-dependent manner by AICAR (Panel 4.D; $F_{0.95}(9,39) = 39.85$; $p < 0.001$).

Figure 5. Effects of CV4 AICAR administration on body weight changes during re-feeding of FD-EB versus FD-oil rats.

After 12 hr of food deprivation (21.00 to 09.00 hr), groups of FD-EB (Panels 5.A, 5.B) and FD-oil (Panels 5.C, 5.D) rats were re-introduced to food and injected with either AICAR [▼], 1.0 ug AICAR; ▲, 5.0 ug AICAR] or SAL [○] into the CV4. FF-EB and FF-oil controls [●] received SAL at 09.00 hr. The data in Panel 5.A show that the lower (1.0 ug), but not higher (5.0 ug) AICAR dose augmented weight gain at +1 hr, and suppressed weight gain at +6 hrs in FD-EB rats ($F_{0.95}(6,7,32,42) = 5.02$; $p < 0.001$). Panels 5.B depicts net adjustments in body weight between +1 and +6 hrs after re-feeding of EB animals treated as follows: FF + SAL [white bars]; FD + SAL [gray bars]; FD + 0.5 ug AICAR [horizontal-stripe gray bars]; FD + 5.0 ug AICAR [cross-hatch gray bars]; results show that drug treatment had no net impact on weight gain over this interval ($F_{0.95}(9,36) = 37.29$; $p < 0.001$). AICAR treatment of FD-oil rats did not enhance weight gain at +1 hr, but resulted in a dose-dependent attenuation of this response at +3 hrs (Panel 5.C). Panel 5.D shows that FD-oil + 5.0 AICAR had a net reduction in weight gain relative to FD-oil + SAL between +1 and +6 hrs.

Figure 6. Effects of CV4 AICAR administration on feeding and body weight changes between 6-24 hrs after re-feeding of FD-EB versus FD-oil rats.

Effects of AICAR on food intake and body weight respectively between 15.00 and 09.00 hr are depicted for EB (Panels 6.A and 6.B) and oil (Panels 6.C and 6.D) rats treated as follows: FF + SAL [white bars]; FD + SAL [gray bars]; FD + 1.0 ug AICAR [horizontal-stripe gray bars]; FD + 5.0 ug AICAR [cross-hatch gray bars]. AICAR increased mean chow consumption relative to FD-EB + SAL controls ($F_{0.95} (9,40) = 8.01$; $p < 0001$), but body weight changes were equivalent between SAL- and drug-treated FD-EB rats over this interval ($F_{0.95} (9,38) = 11.39$; $p < 0001$). Drug treatment did not alter food intake between +6 and +24 hrs in FD-oil rats, but augmented weight gain, at the higher dose, over this period.

Figure 7. Effects of CV4 AICAR administration on cumulative 24 hr food intake and body weight changes after re-feeding of FD-EB versus FD-oil rats.

Effects of AICAR on food intake ($F_{0.95}(7,32) = 38.14$; $p < 0.0001$) and body weight ($F_{0.95}(7,32) = 38.14$; $p < 0.0001$) respectively between +1 and +24 hrs are depicted for EB (Panels 7.A and 7.B) and oil (Panels 7.C and 7.D) rats treated as follows: FF + SAL [white bars]; FD + SAL [gray bars]; FD + 1.0 ug AICAR [horizontal-stripe gray bars]; FD + 5.0 ug AICAR [cross-hatch gray bars]. Data show that this drug treatment increased cumulative food intake in EB, but not oil, but did not modify net adjustments in body weight in FD-EB or FD-oil over this period of time.

Figure 8: Effects of CV4 AICAR on dorsal vagal complex (DVC) adenosine 5'-monophosphate-activated protein kinase (AMPK) and phosphoAMPK (pAMPK) protein expression in FF and FD groups of EB and oil rats.

Aliquots of heat-denatured micropunched DVC tissue from EB and oil animals treated by intra-CV4 administration of SAL or AICAR (A; 1.0 ug) were combined within treatment groups and analyzed by Western blot for AMPK, pAMPK, or α-tubulin. Immunoblots were performed in triplicate. Figure 8.A depicts mean normalized pAMPK protein optical density (O.D.) measures ± S.E.M. for FF-oil + SAL [solid white bar], FF-oil + A [diagonal-striped white bar], FD-oil + SAL [solid grey bar] and FD-oil + A [diagonal-striped grey bar] treatment groups. Figure 8.B shows normalized pAMPK protein O.D. values ± S.E.M. for FF-EB + SAL [solid white bar], FF-EB + A [diagonal-striped white bar], FD-EB + SAL [solid grey bar], and FD-EB + A [diagonal-striped grey bar] treatment groups.

Representative immunoblots of pAMPK, AMPK and α-tubulin proteins are presented below each graph. Data labeled by dissimilar letter symbols differ statistically. Results show that in oil animals, DVC pAMPK protein was increased in both FF-oil + A and FD-oil + SAL groups (vs. FF-oil + SAL), and further augmented in the latter by A treatment [FD-oil + A > FD-oil + SAL] ($F_{0.95}(3,11) = 328.2$; $p < 0.0001$). In EB rats, pAMPK profiles were decreased in both FF-EB + A and FD-EB + SAL (vs. FF-EB + SAL); this decline in the latter group was reversed by A [FD-EB + A > FD-EB + SAL] ($F_{0.95}(3,11) = 6.35$; $p < 0.016$).

Figure 9: Effects of CV4 AICAR on arcuate hypothalamic nucleus (ARH) neuropeptide Y (NPY) protein expression in FF and FD groups of EB and oil rats.

Aliquots of heat-denatured micropunched ARH tissue from EB and oil animals injected with SAL or AICAR (A; 1.0 ug) into the CV4 were combined within treatment groups and analyzed by Western blot for AMPK, pAMPK, or α -tubulin. Immunoblots were performed in triplicate. The graph depicts mean normalized NPY protein O.D. measures + S.E.M. for on left-hand side: FF-oil + SAL [solid white bar], FF-oil + A [diagonal-striped white bar], FD-oil + SAL [solid grey bar] and FD-oil + A [diagonal-striped grey bar] treatment groups, and on right-hand side: FF-EB + SAL [solid white bar], FF-EB + A [diagonal-striped white bar], FD-EB + SAL [solid grey bar], and FD-EB + A [diagonal-striped grey bar] groups. Representative immunoblots of ARH NPY and α -tubulin proteins are shown below each graph. Data labeled by dissimilar letter symbols differ statistically. Results show that ARH NPY neuropeptide content was decreased in SAL-injected FF-EB versus FF-oil rats, and diminished by FD in both EB (vs. FF-EB) and oil (vs. FF-oil) ($F_{0.95}(7,23) = 36.08$, $P < 0.0001$). AICAR inhibited NPY protein levels in FF-EB (vs. FF-EB + SAL) and FF-oil (vs. FF-oil + SAL), but elevated this profile in FD-EB (vs. FD-EB + SAL) in the absence of effect in FD-oil (vs. FD-oil + SAL).

Figure 10: Effects of CV4 AICAR on ARH pro-opiomelanocortin (POMC) protein expression in FF and FD groups of EB and oil rats.

The graph depicts mean normalized ARH POMC protein O.D. measures $\pm$ S.E.M. from triplicate analyses of pooled micropunch tissue aliquots from following treatment groups: left-hand side: FF-oil + SAL [solid white bar], FF-oil + A [diagonal-striped white bar], FD-oil + SAL [solid grey bar], FD-oil + A [diagonal-striped grey bar]; right-hand side: FF-EB + SAL [solid white bar], FF-EB + A [diagonal-striped white bar], FD-EB + SAL [solid grey bar], FD-EB + A [diagonal-striped grey bar] groups. Representative immunoblots of ARH POMC and α -tubulin proteins are depicted below the graph. Data labeled by dissimilar letter symbols differ statistically. Results show that ARH POMC protein levels were decreased in SAL-injected FF-EB versus FF-oil rats, and suppressed by FD [FD-EB vs. FF-EB; FD-oil vs. FF-oil] ($F_{0.95}(7.23) = 41.98$, $P < 0.0001$). Treatment with A reduced this protein profile in FF but not FD groups.

Figure 11: Effects of CV4 AICAR on lateral hypothalamic area (LHA) orexin-A (ORX-A) protein expression in FF and FD groups of EB and oil rats.

The graph depicts mean normalized LHA prepro-ORX-A protein O.D. measures + S.E.M. from triplicate analyses of pooled micropunch tissue aliquots from following treatment groups: left-hand side: FF-oil + SAL [solid white bar], FF-oil + A [diagonal-striped white bar], FD-oil + SAL [solid grey bar], FD-oil + A [diagonal-striped grey bar]; right-hand side: FF-EB + SAL [solid white bar], FF-EB + A [diagonal-striped white bar], FD-EB + SAL [solid grey bar], FD-EB + A [diagonal-striped grey bar] groups. Representative immunoblots of LHA ORX-A and α -tubulin proteins are depicted beneath the graph. Data labeled by dissimilar letter symbols differ statistically. LHA ORX-A protein was decreased in SAL-injected FF-EB versus FF-oil animals ($F_{0.95}(7.23) = 42.06$, $P < 0.0001$). FD augmented or inhibited this protein profile in EB [FD-EB > FF-EB] and oil [FD-oil < FF-oil], respectively. Treatment with A did not modify ORX-A expression in FF-EB (vs. FF-EB + SAL), but decreased this protein in FF-oil (vs FF-oil + SAL). LHA ORX-A levels were increased in FD-EB + A vs. FD-EB + SAL, but resistant to A in FD-oil (vs. FD-oil + SAL).

Table 1.

Effects of Caudal Fourth Ventricular Administration of the AMPK Activator, 5-aminoimidazole-4-carboxamide-riboside (AICAR), on Food Intake and Body Weight in Estradiol Benzoate (EB)- Versus Oil (O) -Implanted Ovariectomized (OVX) Female Rats

		1 – 6 Hours	6- 24 Hours	Cumulative 24 Hours
Treatment Groups				
OVX + EB + SAL	Food Intake ^a	2.34 ± 0.53	14.82 ± 0.91	17.16 ± 0.90
	Change in bw ^b	-2.40 ± 1.07	-0.80 ± 1.23	-3.20 ± 0.97
OVX + EB + AICAR ^c	Food Intake	1.52 ± 0.48	12.94 ± 1.00	14.46 ± 1.24
	Change in bw	-8.60 ± 1.97 ^d	0.50 ± 1.66	-8.10 ± 1.90
OVX + O + SAL	Food Intake	1.34 ± 0.60	15.70 ± 0.65	17.04 ± 1.18
	Change in bw	-3.20 ± 1.41	4.00 ± 1.29	0.80 ± 0.73
OVX + O + AICAR	Food Intake	1.60 ± 0.92	14.06 ± 1.67	15.50 ± 2.51
	Change in bw	-7.50 ± 2.88	1.35 ± 2.23	-6.20 ± 4.84

^a grams food consumed

^b grams change in body weight

^c administered at a dose of 0.5 ug/rat

^d p< 0.05, versus SAL controls