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Norepinephrine Regulation of Ventromedial Hypothalamic Nucleus Metabolic Transmitter Biomarker and Astrocyte Enzyme and Receptor Expression: Impact of 5' AMP-Activated Protein Kinase

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Abstract

The ventromedial hypothalamic energy sensor AMP-activated protein kinase (AMPK) maintains glucostasis via neurotransmitter signals that diminish [γ -aminobutyric acid] or enhance [nitric oxide] counter-regulation. Ventromedial hypothalamic nucleus (VMN) 'fuel-inhibited' neurons are sensitive to astrocyte-generated metabolic substrate stream. Norepinephrine (NE) regulates astrocyte glycogen metabolism *in vitro*, and hypoglycemia intensifies VMN NE activity *in vivo*. Current research investigated the premise that NE elicits AMPK-dependent adjustments in VMN astrocyte glycogen metabolic enzyme [glycogen synthase (GS); glycogen phosphorylase (GP)] and gluco-regulatory neuron biomarker [glutamate decarboxylase_{65/67} (GAD); neuronal nitric oxide synthase (nNOS); SF-1] protein expression in male rats. We also examined whether VMN astrocytes are directly receptive to NE and if noradrenergic input regulates cellular sensitivity to the neuro-protective steroid estradiol. Intra-VMN NE correspondingly augmented or reduced VMN tissue GAD and nNOS protein despite no change in circulating glucose, data that imply that short-term exposure to NE promotes persistent improvement in VMN nerve cell energy stability. The AMPK inhibitor Compound C (Cc) normalized VMN nNOS, GS, and GP expression in NE-treated animals. NE caused AMPK-independent down-regulation of α_2 -, alongside Cc-reversible augmentation of β_1 -adrenergic receptor protein profiles in laser-microdissected astrocytes. NE elicited divergent adjustments in astrocyte estrogen receptor-beta (AMPK-unrelated reduction) and GPR-30 (Cc-revocable increase) proteins. Outcomes implicate AMPK in noradrenergic diminution of VMN nitrergic metabolic-deficit signaling and astrocyte glycogen shunt activity. Differentiating NE effects on VMN astrocyte adrenergic and estrogen receptor variant expression suggest that noradrenergic regulation of glycogen metabolism may be mediated, in part, by one or more receptors characterized here by sensitivity to this catecholamine.

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Keywords

Ventromedial hypothalamic nucleus; norepinephrine; compound C; neuronal nitric oxide synthase; glycogen synthase; GPR-30

Introduction:

Insulin-induced hypoglycemia (IIH) poses a significant threat to neurological function as neurons require a continuous glucose supply to maintain vital high energy-demand functions. Neuroglucopenia activates a cohesive array of counter-active autonomic, neuroendocrine, and behavioral responses that collectively augment systemic glucose availability. The ventromedial hypothalamic nucleus (VMN) is a key brain site for assimilation of nutrient, endocrine, and neurochemical indicators of metabolic state that are involved in shaping neural counter-regulatory outflow [Watts and Donovan, 2010, 2014]. Dedicated metabolic-sensory neurons in the VMN supply a dynamic cellular energy readout by augmenting ('fuel-inhibited') or decreasing ('fuel-excited') synaptic firing as ambient energy substrate levels decline [Oomura et al., 1969; Ashford et al., 1990; Silver and Erecinska, 1998]. Ventromedial hypothalamic detection of neuro-energetic sequelae of hypoglycemia is required for optimal counter-regulatory endocrine and gluconeogenic outflow [Borg et al., 1997; 2003]. Neurotransmitter effectors of ventromedial hypothalamic cues of energy imbalance include γ -aminobutyric acid (GABA), which inhibits glucagon and adrenomedullary catecholamine release during hypoglycemia [Chan et al., 2006], as well as stimulatory signals, e.g. nitric oxide (NO) and steroidogenic factor-1 (SF-1), that intensity counter-regulatory hormone secretion [Fioramonti et al., 2011; Garfield et al., 2014; Routh et al., 2014; Meek et al., 2016].

The catecholamine neurotransmitter norepinephrine (NE) is a vital gluco-regulatory signal to the ventromedial hypothalamus. NE increases GABA levels in this area during IIH [Beverly et al., 2000, 2001]; however, noradrenergic regulation of gluco-stimulatory neurochemical signals from this area is not clear. Cortical astrocytes respond directly to NE via α_1 (α_1), α_2 (α_2) and β_1 (β_1) adrenoreceptors (A-R) [Hertz et al., 2010], and undergo glycogenolysis upon beta A-R stimulation [Fillenz et al., 1999; Dong et al., 2012]. Thus, NE regulation of VMN neuro-metabolic stability and metabolic effector signaling may involve, in part, control of glycogen-derived energy fuel provision. The astrocyte-neuron lactate shuttle hypothesis posits that glucose is taken up from the circulation mainly into the astrocyte cell compartment, where it is either stored as the complex polymer glycogen or metabolized to L-lactate for export to neurons [Magistretti et al., 1993; Pellerin et al., 1998]. Lactate trafficking between these cell compartments is achieved by glial- and nerve cell-specific monocarboxylate transporters (MCT), e.g. MCT1 and MCT2, respectively [Broer et al., 1997]. Ventromedial hypothalamic lactoprivation is a stimulus for counter-regulation, as local lactate repletion dampens glucagon and catecholamine secretory responses to IIH [Borg et al., 2003] by increasing GABAergic transmission [Chan et al., 2013]. The brain glycogen depot exhibits dynamic turnover during normal brain activity and metabolic homeostasis, and is a critical source of lactate equivalents during states of heightened activity or glucose deficiency [Stobart and Anderson, 2013]. The astrocyte glycogen shunt,

involving sequential glucose incorporation into and liberation from glycogen prior to entry into the glycolytic pathway, is a dynamic process accounting for a significant fraction of glucose catabolism in these cells [Walls et al., 2009; Schousboe et al., 1020]. Recent studies show that inhibition of VMN astrocyte glycogen phosphorylase activity enhances VMN 5'-AMP-activated protein kinase (AMPK) activity alongside nitrergic neuron signaling, suggesting that astrocyte glycogen-derived fuel stream may affect neuro-metabolic stability in that site [Alhamami et al., 2018]. The current project utilized pharmacological and high-spatial resolution microdissection/molecular analytical approaches to examine the hypothesis that NE regulates astrocyte glycogen metabolic enzyme and gluco-regulatory neuron biomarker protein profiles in the male rat VMN.

Astrocytes, which express nuclear and membrane estrogen receptors (ER) [Azcoitia et al., 1999; Garcia-Segura et al., 1999; Garcia-Ovejero et al., 2005; Pawlak et al., 2005], mediate neuroprotective effects of estradiol against degenerative insults including bio-energetic stress [Dhandapani and Brann, 2007; Arevalo et al., 2010]. Estradiol acts on the female rat VMN to regulate glucostasis [Nedungadi and Briski, 2012]. Recent studies in females show that estradiol-dependent VMN astrocyte glycogen synthase (GS) and glycogen phosphorylase (GP) protein responses to IIH require noradrenergic input [Tamrakar et al., 2015]. Since testosterone is converted, by aromatase enzyme action, to estradiol in the male brain, it is conceivable that VMN astrocytes may be a convergent focus for estrogenic and noradrenergic regulation of glucostasis in males. Combinatory immunocytochemistry/laser-cataapult microdissection enables selective procurement of astrocytes for quantitative assessment of protein expression *in vivo* [Tamrakar et al., 2015]. Here, this technique was used to characterize VMN astrocyte adrenoreceptor and ER subtype protein profiles in male rats, and to determine if and how noradrenergic stimuli may regulate astrocyte receptivity to estradiol and NE in this sex.

Results:

Figure 1 depicts effects of intra-VMN administration of NE, with or without Cc pretreatment, on VMN GAD_{65/67} (Panel A), nNOS (Panel B), and SF-1 (Panel C) protein content. Results show that GAD profiles were significantly increased by NE (NE/V versus V/V), and that this stimulatory effect was augmented by Cc (NE/Cc versus NE/V) [$F_{(2,6)} = 58.08$; $p < 0.0001$]. As shown in Panel B, NE caused Cc-reversible inhibition of nNOS expression [$F_{(2,6)} = 23.64$; $p = 0.0003$]. VMN SF-1 protein levels were refractory to NE, but were elevated compared to V/V controls by combinatory NE/Cc treatment [$F_{(2,6)} = 18.39$; $p = 0.0007$].

Data in Figure 2 illustrate adjustments in VMN GS (Panel A) and GP (Panel B) protein expression in response to NE. GS [$F_{(2,6)} = 8.02$; $p = 0.02$] and GP [$F_{(2,6)} = 13.25$; $p = 0.006$] profiles were each significantly decreased by NE; Cc pretreatment prevented these inhibitory responses.

Figure 3 depicts effects of Cc pretreatment on VMN astrocyte α_1 A-R (Panel A), α_2 A-R (Panel B), and β_1 A-R (Panel C) protein responses to NE. Results show that α_2 A-R [$F_{(2,6)} = 18.31$; $p = 0.009$] but not α_1 A-R [$F_{(2,6)} = 4.14$; $p = 0.04$] profiles were diminished by NE.

Combinatory Cc plus NE treatment elicited a significant reduction in α_1 A-R expression relative to controls (NE/Cc versus V/V), but did not modify α_2 A-R levels compared to the V plus NE group. Astrocyte β_1 A-R protein was significantly increased after NE administration [$F_{(2,6)} = 12.67$; $p=0.0001$], a response that was reversed by Cc.

Data in Figure 4 show VMN astrocyte ER α (Panel A), ER β (Panel B), and GPR30 (Panel C) protein responses to NE. ER α [$F_{(2,6)} = 7.58$; $p=0.014$] expression was unaffected, whereas ER β levels were significantly reduced by NE [$F_{(2,6)} = 6.16$; $p=0.009$]. Pretreatment with Cc augmented ER α protein compared to V/V controls, but had no significant impact on ER β expression relative to NE-treated rats (NE/Cc versus NE/V). NE caused a significant elevation in astrocyte GPR30 profiles NE [$F_{(2,6)} = 4.85$; $p=0.02$]; this response was prevented by Cc.

Results in Figure 5 illustrate effects of NE with or without Cc pretreatment on VMN astrocyte PFKL (Panel A) and MCT1 (Panel B) protein expression. Both profiles were refractory to NE alone, but were each significantly increased in response to combinatory Cc plus NE treatment [PFKL: $F_{(2,6)} = 10.07$; $p=0.01$; PFKL: $F_{(2,6)} = 8.68$; $p=0.02$].

Remaining polyethylene naphthalate membrane-mounted 10 μ m-thick VMN sections from V controls were immunolabeled to identify GAD_{65/67}- (Figure 6, right-hand bars) or nNOS-expressing (Figure 6, left-hand bars) neurons for laser-catapult microdissection. Data show that α_1 A-R (Panel A), α_2 A-R (Panel B), and β_1 A-R (Panel C) proteins each occur in VMN GABAergic and nitrergic neurons. Whereas α_1 A-R and β_1 A-R are expressed in equivalent amounts in these two cell populations, α_2 A-R protein is present at a higher level in GAD_{65/67}-positive nerve cells.

Figure 7 depicts effects of intra-VMN administration of NE, with or without prior delivery with Cc, on circulating glucose (Panel A), free fatty acids (FFA; Panel B), glucagon (Panel C), and corticosterone (Panel D). Glucose [$F_{(2,12)} = 1.02$; $p=0.904$], glucagon [$F_{(2,12)} = 1.61$; $p=0.252$], and corticosterone [$F_{(2,12)} = 0.894$; $p=0.451$] levels did not differ between NE/V or NE/Cc animals versus V/V controls. However, NE elicited a significant decline in plasma free fatty acid concentrations [$F_{(2,12)} = 8.325$; $p=0.007$]; this response was refractory to Cc.

Discussion:

The current study research addressed the premise that NE governs VMN metabolic transmitters that inhibit or stimulate glucose counter-regulation via AMPK-dependent mechanisms. Results provide unique evidence for noradrenergic stimulation versus suppression of VMN GAD and nNOS protein expression, while demonstrating a role for AMPK in the latter response. VMN nitrergic neurons are functionally stimulated by pharmacological inhibition of glycogen breakdown [Alhamami et al., 2018]. Here, we investigated whether local glycogen metabolic enzyme protein expression is subject to noradrenergic control, and if VMN astrocytes are directly receptive to this neurochemical signal. Evidence for Cc-reversible NE down-regulation of GS and GP proteins implies diminution of astrocyte glycogen shunt activity, which may be mediated, in part, by AMPK-dependent amplification of astrocyte β_1 A-R and GPR-30 protein profiles. Observed NE

effects on VMN gluco-regulatory neurotransmitter marker proteins support the likelihood that this neurochemical enhances local energy stability, which may be a consequence, to some degree, of altered volume of neuro-glial metabolic coupling due to attenuated passage of glucose through glycogen prior to entry into the glycolytic pathway. Alternatively, NE downregulation of VMN GS and GP protein profiles may reflect, at this post-acute time point after catecholamine exposure, decreased neuronal reliance on astrocyte substrate fuel provision because of persistent positive energy balance owing to direct noradrenergic neuroprotective action on those cells. Results moreover implicate the VMN as a substrate for NE regulation of circulating FFA levels.

Ventromedial hypothalamic NE activity is intensified in response to IHH [Beverly et al., 2001; de Vries et al., 2005], and is implicated in augmented local GABA signaling [Beverly et al., 2000]. Outcomes here reinforce the notion of a positive causal relationship between NE and GABA transmission in this site. Concurrent noradrenergic stimulation and repression of corresponding VMN gluco-inhibitory and gluco-stimulatory marker proteins may likely be indicative of metabolic effector transmitter signaling of NE-induced net positive change in resident cell energy status. This interpretation remains speculative as technology that support assessment of single-cell ATP concentrations is currently unavailable; thus, definitive proof that NE effects on VMN GABA- and nitrergic neurotransmission reflect, in totality or in part, enhanced cell energetic stability remains elusive. While current data support the possibility that astrocytes may mediate at least some noradrenergic actions on GABA and/or NO signaling, novel proof acquired here that GABA- and nitrergic neurons are directly receptive to NE regulation owing to multiple adrenoreceptor variant expression implies that these nerve cells may serve as additional targets for noradrenergic stimulation. The energy sensor AMPK functions upon activation to stimulate metabolic pathways that produce and conserve energy and to limit energy expenditure to order to boost energy availability. Proof here of Cc reversal of NE-induced inhibition of nNOS implies that AMPK-triggered energy augmentation or other adaptation that positively shifts energy balance ultimately represses nitrergic signaling. Current data align with reports that ventromedial hypothalamic AMPK is critical for glucoprivic induction of NO gluco-stimulatory signaling [Routh et al., 2014]. Further research will be required to determine if NE-sensitive AMPK is expressed by nitrergic and/or upstream VMN neurons. Data also disclose augmenting effects of Cc pretreatment on GAD expression in NE-treated animals, implying that noradrenergic amplification of GABA transmission does not require AMPK activity, but that input from this sensor attenuates GAD reactivity to this neurochemical signal. Additional effort will be needed to identify the cellular source(s) of AMPK-triggered cues that restrain GABAergic reactivity to NE. It would be informative to learn if those stimuli are intrinsic (e.g. of GABA nerve cell origin) versus extrinsic (derived from other metabolic-sensory, possibly nitrergic neurons). VMN SF-1 protein levels were refractory to NE, but elevated in the NE plus Cc treatment group; these findings suggest that AMPK may impose a suppressive tone on this protein profile as well during noradrenergic stimulation.

The brain glycogen depot exhibits dynamic turnover during normal brain activity and metabolic homeostasis, and is a critical source of lactate equivalents during states of heightened activity or glucose deficiency [Stobart and Anderson, 2013]. The astrocyte

glycogen shunt, involving sequential glucose incorporation into and liberation from glycogen prior to entry into the glycolytic pathway, is a dynamic process accounting for a significant fraction of glucose catabolism in these cells [Walls et al., 2009; Schousboe et al., 1020]. We predicted that NE would stimulate VMN GP protein expression *in vivo* as this neurochemical is reported to enhance cerebral astrocyte glycogenolysis *in vitro* [Harik et al., 1982; Magistretti et al., 1993]; however, current data instead show a Cc-preventable reduction in GS and GP profiles in NE-treated animals at +2 hr, findings that imply that at time points well past catecholamine administration, noradrenergic signaling may restrict glucose entry into the glycogen pool via AMPK-dependent mechanisms, and instead promote its metabolism via glycolysis. As the present project did not evaluate NE effects on local lactate production over the post-treatment interval, it remains speculative whether the current treatment paradigm stimulated astrocyte glycolytic activity and net lactate yield over the entirety or a segment of that interval. Since VMN tissue was analyzed here 2 hours after short-term NE infusion, we cannot discount the possibility that GS and GP proteins exhibit a biphasic pattern of response after brief noradrenergic stimulation, and that the observed decline in these profiles was preceded by their elevated expression, and associated amplification of glycogenolysis and glycogen turnover over some interval between time zero and time of sacrifice. As the NE dosage used here is pharmacological compared to endogenous tissue catecholamine levels [de Vries et al., 2004], the notion that effects on one or more parameters here may differ in direction and/or magnitude at specific time points after exposure compared to physiological-like stimulation. Walls et al. [2009] report that NE stimulates the astrocyte glycogen shunt activity *in vitro*, and that inhibition of this shunt intensifies glycolysis in these cells. Further studies aim to investigate whether NE enhances astrocyte substrate fuel provision to VMN neurons by restricting glucose passage through the glycogen pool, and if that action is preceded by mobilization of available glycogen glycosyl residues. An alternative to the scenario that NE-mediated down-regulation of VMN astrocyte glycogen metabolic enzyme protein expression is a cause of augmented GABAergic and diminished nitrergic neuron signaling is that, instead, astrocyte glycogen turnover and breakdown at +2 hr after catecholamine administration may be an effect of regulatory signals from these nerve cell populations that communicate a positive change in energy status due to direct noradrenergic stimulation.

We previously reported that pharmacological suppression of VMN GP activity increases nNOS protein expression [Alhamami et al., 2018]. Differences between that study and current work include singular manipulation of GP expression versus combination of GS and GP; potential dissimilarities in magnitude of inhibition of GP enzyme activity achieved by inhibitor drug action versus NE; potential as-yet-uncharacterized effects of NE on astrocyte glucose uptake, phosphorylation, and/or entry into and catabolism within the glycolytic pathway. As astrocytes express AMPK [Tamarkar and Briski, 2015], it is very likely that Cc acts, in part, on these cells to reverse inhibitory GS and GP responses to NE.

VMN astrocyte PFKL and MCT1 protein expression did not differ between V- versus NE-treated animals at +2 hr; however, both protein profiles were significantly up-regulated in Cc plus NE rats compared to other treatment groups. As astrocyte PFKL enzyme activity was not evaluated here, definitive evidence for if or how NE regulates net glycolytic pathway output is still lacking. Present outcomes provide novel evidence for AMPK regulation of astrocyte

lactate production and trafficking during exposure to NE. Data imply that AMPK activity levels triggered by NE may lead to stabilization of glycolytic yield and export of lactate at this specific time point after treatment, thereby preventing intensification of these functions at that time. It remains to be determined if production of PFKL and/or MCT1 proteins was increased at some time point prior to sacrifice. Present outcomes raise the interesting prospect that NE may exert differential effects on astrocyte lactate provision according to magnitude of AMPK activation, namely that sensor inactivity may paradoxically amplify yield of this substrate fuel during noradrenergic signaling.

Novel aspects of current results include identification of multiple adrenoreceptor subtypes expressed by hypothalamic astrocytes *in vivo*, as well as characterization of discriminating reactivity of these receptor variants to NE. Outcomes demonstrate NE inhibition or augmentation of VMN astrocyte α_2 A-R and β_1 A-R protein expression, respectively, while documenting AMPK involvement in the latter, but not former regulatory action. Present findings support the possibility that β_1 A-R up-regulation may be crucial for NE regulation of VMN astrocyte glycogen metabolic enzyme expression. Although β_2 A-R were not evaluated here, reports that this receptor enhances counter-regulation [Szepietowska et al., 2011] suggest that it may function to curb or repress noradrenergic promotion of metabolic effector signaling of metabolic stability. Current data in support of VMN astrocyte ER α , ER β , and GPR30 protein expression concur with prior evidence for multi-receptor – mediated estradiol input to hypothalamic astrocytes [Azcoitia et al., 1999; Garcia-Segura et al., 1999; Garcia-Ovejero et al., 2005; Pawlak et al., 2005]. Here, data show that NE exerts opposing effects on astrocyte ER β (inhibitory) versus GPR30 (stimulatory) profiles, but lacks influence on ER α content. The efficacy of Cc to reverse this GPR30, but not ER β response implies AMPK involvement in NE regulation of the former receptor, and suggests, moreover, that this membrane receptor variant may be required for β_1 A-R reactivity to NE, or vice versa.

New evidence that noradrenergic up- and down-regulation of VMN neurons that respectively inhibit or enhance glucose counter-regulation coincides with diminished astrocyte glycogen metabolic enzyme protein expression impelled us to address the question of whether VMN GABA- and/or nitrergic neurons may be direct substrates for noradrenergic stimulation. Employing new methodology for combinatory immunocytochemical identification of these cell types *in situ* and western blot analysis of laser-catapult microdissected GAD_{65/67} or nNOS-expressing neurons, we obtained unique proof-of-principle here that both cell types express α_1 A-R, α_2 A-R, and β_1 A-R proteins expression, and that the former receptor variant may occur at relatively higher levels in GABA- versus nitrergic neurons. Further studies will be necessary to ascertain whether one or more of these receptors mediate direct neuroprotective effects of NE on energy balance.

In the current study, circulating glucose and counter-regulatory hormone (e.g. glucagon and corticosterone) levels were equivalent between intra-VMN NE- versus V-treated groups at a time point where NE-associated up- (GABA) and down- (NO) regulation of VMN gluco-regulatory neurotransmitter signaling was detected. A plausible interpretation of these results is that NE actions confined to the VMN are insufficient to cause significant adjustments in systemic glucose availability. As IIH alters whole-hypothalamic NE turnover

[Bellin and Ritter, 1981], it is likely that integrated noradrenergic action on multiple hypothalamic substrates during hypoglycemia is a critical determinant of counter-regulatory reactivity to that condition. Moreover, effects of IHH-associated patterns of NE activity on the VMN may be modulated by other neural, hormonal, or nutrient signals of metabolic insufficiency that are triggered by hypoglycemia, interactions that are not replicated in the current experimental design. Alternatively, we do not discount the probability that glucose and/or counter-regulatory hormone levels differed from vehicle controls between time zero and +2 hours, and that values associated with that post-treatment time point reflect restoration of the normal range. Interestingly, plasma FFA levels were significantly decreased at +2 hours in the NE/V group versus controls, indicating that noradrenergic input to the VMN can suppress systemic availability of this major lipid fuel. This NE action is evidently mediated by non-AMPK mechanisms as this observed FFA decline was refractory to Cc. As noradrenergic stimulation of the VMN counter-regulation inhibitor GABA is likewise unrelated to AMPK activity, GABA may function within local circuits that govern FFA.

Conclusion:

In summary, present research addressed the premise that NE causes AMPK-dependent changes in VMN astrocyte glycogen metabolic enzyme and gluco-regulatory neuron biomarker protein expression, coincident with altered systemic glucose, FFA, and counter-regulatory hormone concentrations in adult male rats. Outcomes implicate AMPK in noradrenergic diminution of VMN nitrenergic metabolic-deficit signaling and astrocyte glycogen shunt activity, but show that NE-associated GABA signal up-regulation does not involve this sensor. These data imply that short-term exposure to NE promotes persistent improvement in VMN nerve cell energy stability. Concurrent down-regulated astrocyte GS and GP protein expression and altered metabolic effector transmitter signaling suggests that altered glycogen metabolism may be either a cause or, alternatively, a consequence of noradrenergic neuroprotection of neuronal metabolic balance. Data document AMPK-independent down-regulation of VMN astrocyte α_2 -, alongside Cc-reversible augmentation of β_1 -adrenergic receptor protein profiles due to NE. NE also caused divergent adjustments in astrocyte ER β (AMPK-unrelated reduction) and GPR-30 (Ccrevocable increase) protein expression. Discriminative effects of noradrenergic stimulation on local VMN adrenoreceptor and estrogen receptor variants support the likelihood that NE control of local glycogen metabolism may be mediated, in part, by one or more receptors characterized here by sensitivity to this neurochemical signal.

Experimental Procedures:

Animals and Experimental Design:

Adult male Sprague-Dawley rats (3–4 months of age) were maintained under a 14 h light:10 h dark lighting schedule (light on at 05.00 h), and allowed free access to standard laboratory rat chow (Harlan Teklad LM-485; Harlan Industries, Madison, WI) and tap water. Rats were acclimated to daily handling over a 7-day period prior to experimentation. All animal 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. On study day 1, rats were anesthetized with ketamine/xylazine (0.1 mL/100 g *bw ip*, 90 mg ketamine: 10 mg xylazine/mL; Butler Schein Inc., Melville, NY) prior to stereotactic implantation of bilateral 26-gauge stainless-steel cannula guides (C235G-1.2/SPC; Plastics One, Inc., Roanoke, VA) aimed dorsal to the VMN [coordinates: anteriorposterior: -2.85 mm posterior to *bregma*; lateral: 0.6 mm lateral to midline; dorsal-ventral: 9.0 mm below skull surface], by motorized computer-controlled stereotactic positioning in all three orthogonal axes guided by state-of-the-art software (Stoelting Co., Wood Dale, IL). Animals were transferred to individual cages after surgery. At 09.00 hr on day 7, rats were divided into 3 treatment groups (n = 5/group) and injected into the VMN via bilateral 33-gauge internal injection cannulas (C235I-1.2/SPC; Plastics One; 0.6 mm projection beyond guide) with vehicle [V; dimethyl sulfoxide (DMSO); groups 1 and 2] or the AMPK inhibitor Compound c (Cc; 1.0 µg/0.5 µL DMSO; group 3), over 2 min, using a Genie Touch syringe pump (Lucca Technologies, Harwinton, CT). Internal cannulas were left in place for subsequent intra-VMN administration, at 09.15 hr, of vehicle (V; sterile saline; group 1) or NE [5.91 mM (McCarren et al., 2014); groups 2 and 3], in a 0.5 µL total volume, which was infused at a rate of 0.25 µL/min over 2 min. Rats were sacrificed at 11.00 hr on day 7 for brain tissue and trunk blood collection. Dissected brains were immediately snap-frozen in liquid nitrogen-cooled isopentane and stored in -80 °C. Plasma was stored at -20 °C. Accuracy of cannula targeting of the VMN was verified by visual examination of consecutive frozen tissue sections cut through that structure for micropunch or laser dissection. As potential NE diffusion outward from the confines of the VMN was not evaluated here, the prospect that observed effects of exogenous catecholamine administration on one or more parameters may involve, to an-as-yet-undetermined extent, action of this neurotransmitter on adrenergic receptors located outside the VMN cannot be overlooked.

Western Blot Analyses of VMN Metabolic Neurotransmitter and Astrocyte Glycogen Metabolic Enzyme Protein Expression:

Forebrains were cut into alternating series of 100 µm- or 10 µm-thick frozen sections over the length of the VMN, over repeating distances of 200 µm (2 x 100 µm sections) and 100 µm (10 x 10 µm thick sections), respectively. VMN tissue was micropunch-dissected from 100 µm sections using 0.50 mm-diameter hollow needles (Stoelting, Inc., and collected into lysis buffer [2.0% sodium dodecyl sulfate (SDS), 0.05 M dithiothreitol, 10.0% glycerol, 1.0 mM EDTA, 60 mM Tris-HCl, pH 7.2] for heat denaturation. For each protein of interest, three separate tissue aliquot pools were created for each treatment group ahead of separation in BioRad TGX 10–12% stain-free gels [Shakya et al., 2018]. After electrophoresis, gels were activated for 1 min by UV light in a BioRad ChemiDoc TM Touch Imaging System prior to protein transfer (30 V, overnight at 4°C; Towbin buffer) to 0.45-µm PVDF membranes (ThermoFisherScientific; Waltham, MA). Membranes were blocked for 2 hr with Tris-buffer saline (TBS), pH 7.4, containing 0.1% Tween-20 and 2% bovine serum albumin prior to overnight (4°C) incubation with primary antibody: rabbit anti-neuronal nitric oxide synthase (nNOS; 1:1000; sc-648; Santa Cruz Biotechnology, Inc., Santa Cruz, CA); rabbit anti-steroidogenic factor-1 (SF-1; 1:500; sc-2874; Santa Cruz Biotechnol.), goat anti-glutamate decarboxylase_{65/67} (GAD_{65/67}; 1:1000; sc-7513; Santa Cruz Biotechnol.), rabbit anti-glycogen synthase (GS; (1:1000; 3893S; Cell Signaling Technology, Danvers,

MA); goat anti-glycogen phosphorylase (GP; PYGB/L/M; 1:1000; sc-46347; Santa Cruz Biotechnol.). Membranes were next exposed for 1 hr to peroxidase-conjugated secondary antisera [goat anti-rabbit (1:5000; NEF812001EA; PerkinElmer, Billerica, MA) or donkey anti-goat (1:5000; sc-2020; Santa Cruz Biotechnol.) prior to incubation with Supersignal West Femto chemiluminescent substrate (34095; ThermoFisherScientific, Rockford, IL). Optical density (O.D.) signals were detected and quantified in a Bio-Rad (Hercules, CA) ChemiDoc MP Imaging System equipped with Image Lab software, and normalized per individual lane by Bio-Rad Stain-Free Imaging Technology.

Western Blot Analysis of Laser-Catapult Microdissected VMN Astrocytes:

10 μ m-thick frozen VMN tissue sections were mounted on polyethylene naphthalate membrane slides (Carl Zeiss MicroImaging, Inc., Thornwood, NY), fixed with acetone, blocked with 5% normal horse serum (Vectastain Elite ABC mouse IgG kit; PK-4002; Vector Laboratories, Inc., Burlingame, CA), then incubated (24 hr; 4°C) with a mouse anti-glial fibrillary acidic protein (GFAP) antiserum (1:200; Cell Signal. Technol.). Sections were sequentially incubated with Elite ABC mouse IgG (biotinylated secondary antibody, ABC reagent), and Vector DAB Peroxidase (HRP) substrate kit reagents (SK-4100; Vector Laboratories) for astrocyte visualization. Individual astrocytes exhibiting complete labeling of the cytoplasmic compartment were harvested using a Zeiss P.A.L.M. UV-A microlaser (Carl Zeiss MicroImaging). Each protein of interest was analyzed by Western blot in separate triplicate pools of n=60 astrocyte lysates for each treatment group. Astrocyte protein targets were detected with Novus Biologicals (Littleton, CO) primary antisera raised in rabbit against α_1 A adrenergic receptor/ADRA1A (α_1 A-R; 1:1000; NB100-78585), α_2 A adrenergic receptor/ADRA3A (α_2 A-R; 1:1000; NBP1-67832), β_1 adrenergic receptor/ADRB1 (β_1 A-R; 1:1000; NBP1-59007)ER-alpha/NR3A1 (ER α ; 1:1000; NB100-91756), ER-beta/NR3A2 (ER β ; 1:1000; NB120-3577), or the G protein-coupled membrane estrogen receptor GPER/GPR30 (1:1000; NLS4271), or with rabbit antisera against the rate-limiting glycolytic enzyme phosphofructokinase/liver (PFKL; 1:1500; 30-117; ProSci, Poway CA) or MCT1 (1:1500; AB3540P; Millipore Sigma, Burlington, MA). Protein O.D.s generated from chemiluminescent substrate were detected and processed by Bio-Rad Stain-Free Imaging Technology, as described above.

Western Blot Analysis of Laser Catapult-Microdissected VMN GAD_{65/67} and nNOS Neurons:

Remaining polyethylene naphthalate membrane-mounted 10 μ m VMN tissue sections from V-infused controls were immunolabeled for GAD_{65/67} or nNOS prior to laser-microdissection, using rabbit primary antisera against GAD_{65/67} (prod. no. ABN904, 1:2000; MilliporeSigma, Burlington, MA) or nNOS (prod. no. NBP1-39681, 1:500; Novus Biologicals, LLC, Littleton, CO), respectively. For each harvested neuron population, triplicate pools of n=50 heat-denatured cell lysates were created for western blot analysis of α_1 A-R, α_2 A-R, or β_1 A-R proteins, as described in the previous section.

Glucose and Counter-Regulatory Hormone Measurements:

Blood glucose levels were determined using an ACCU-CHECK Aviva plus glucometer (Roche Diagnostic Corporation, Indianapolis, IN) [Kale et al., 2006]. Plasma free fatty acid

concentrations using Free Fatty Acid Quantitation Kit reagents (MAK044; Sigma Aldrich, St. Louis, Mo) [Briski et al., 2017]. Plasma corticosterone (ADI-900–097; Enzo Life Sciences, Inc., Farmingdale, NY) and glucagon (EZGLU-30K, EMD Millipore, Billerica, MA) concentrations were determined using commercial ELISA kit reagents, as described [Alhamami et al., 2018].

Statistical analyses:

Mean glucose, free fatty acid, corticosterone, glucagon, and normalized Western blot protein O.D. values were evaluated between treatment groups by two-way analysis of variance and Student Newman Keuls post-hoc test. Differences of $p < 0.05$ were considered significant.

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Abbreviations:

α_1A-R	alpha ₁ adrenergic receptor
α_2A-R	alpha ₂ adrenergic receptor
β_1A-R	beta ₁ adrenergic receptor
AMPK	5'-AMP-activated protein kinase
E	estradiol
ERα	estrogen receptor-alpha
ERβ	estrogen receptor-beta
GABA	γ -aminobutyric acid
GAD	glutamate decarboxylase _{65/67}
GP	glycogen phosphorylase
GPα-/GPR-30	G protein-coupled estrogen receptor
GS	glycogen synthase
IHH	insulin-induced hypoglycemia
MCT1	monocarboxylate transporter-1
MCT2	monocarboxylate transporter-2
NE	norepinephrine
NO	nitric oxide
nNOS	neuronal nitric oxide synthase
SF-1	steroidogenic factor-1

VMN

ventromedial hypothalamic nucleus

References:

- Alhamami HN, Alshamrani A, Briski KP. Effects of the glycogen phosphorylase inhibitor 1,4-dideoxy-1,4-imino-D-arabinitol on ventromedial hypothalamic nucleus 5'-adenosine monophosphate-activated protein kinase activity and metabolic neurotransmitter biosynthetic enzyme protein expression in eu- versus hypoglycemic male rats. *Physiol. Reports* 2018; 103: 236–249.
- Ashford MLJ, Boden PR, Treherne JM. Glucose-induced excitation of hypothalamic neurons is mediated by ATP-sensitive K⁺ channels. *Pfugers Arch.* 415: 479–483, 1990.
- Arevalo MA, Santos-Galindo M, Bellini MJ, Azcoitia I, Garcia-Segura LM. Actions of estrogens on glial cells: Implications for neuroprotection. *Biochim. Biophys. Acta* 2010; 1800: 1106–1112. [PubMed: 19818384]
- Azcoitia I, Sierra A, Garcia-Segura LM. Localization of estrogen receptor beta-immunoreactivity in astrocytes of the adult rat brain. *Glia* 1999; 26: 260–267. [PubMed: 10340766]
- Barros LF. Metabolic signaling by lactate in the brain. *Trends Neurosci.* 36: 396–404, 2013. [PubMed: 23639382]
- Bellin SI, Ritter S. Insulin-induced elevation of hypothalamic norepinephrine turnover persists after glucose restoration unless feeding occurs. *Brain Res.* 1981; 217: 327–337. [PubMed: 7018642]
- Beverly JL, de Vries MG, Beverly MF, Arseneau LM. Norepinephrine mediates glucoprivic-induced increase in GABA in the ventromedial hypothalamus of rats. *Amer. J. Physiol. Regul. Integr. Comp. Physiol* 2000; 279: R990–R996. [PubMed: 10956258]
- Beverly JL, de Vries MG, Bouman SD, Arseneau LM. Noradrenergic and GABAergic systems in the medial hypothalamus are activated during hypoglycemia. *Amer. J. Physiol. Regul. Integr. Comp. Physiol* 2001; 280: R563–R569. [PubMed: 11208588]
- Borg MA, Tamborlane WV, Shulman GI, Sherwin RS. Local lactate perfusion of the ventromedial hypothalamus suppresses hypoglycemic counterregulation. *Diabetes* 2003; 52: 663–666. [PubMed: 12606506]
- Borg MA, Sherwin RS, Borg WP, Tamborlane WV, Shulman GI. Local ventromedial hypothalamus glucose perfusion blocks counterregulation during systemic hypoglycemia in awake rats. *J. Clin. Invest* 1997; 99: 361–365. [PubMed: 9006005]
- Briski KP, Alenazi FSH, Shakya M, Sylvester PW. Estradiol regulates hindbrain A2 noradrenergic neuron adenosine 5'-monophosphate-activated protein kinase (AMPK) activation, upstream kinase/phosphatase protein expression, and receptivity to hormone and fuel reporters of short-term food deprivation in the ovariectomized female rat. *J. Neurosci. Res* 2017; 95: 1427–1437. [PubMed: 27618227]
- Chafetz MD, Parko K, Diaz S, Leibowitz SF. Relationships between medial hypothalamic α 2-receptor binding, norepinephrine, and circulating glucose. *Brain Res.* 1986; 384: 404–408. [PubMed: 3022870]
- Dhandapani KM, Brann DW. Role of astrocytes in estrogen-mediated neuroprotection. *Exp. Gerontol* 2007; 42: 70–75. [PubMed: 16872778]
- de Vries MG, Lawson MA, Beverly JL. Dissociation of hypothalamic noradrenergic activity and sympathoadrenal responses to recurrent hypoglycemia. *Amer. J. Physiol. Regul. Integr. Comp. Physiol* 2004; 286: R910–R915. [PubMed: 14726425]
- de Vries MG, Lawson MA, Beverly JL. Hypoglycemia-induced noradrenergic activation in the VMH is a result of decreased ambient glucose. *Amer. J. Physiol. Regul. Integr. Comp. Physiol* 2005; 289: R977–R981. [PubMed: 16183631]
- Dong JH, Chen X, Cui M, Yu X, Pang Q, Sun JP. B2-adrenergic receptor and astrocyte glucose metabolism. *J. Mol. Neurosci* 2012; 48: 456–463. [PubMed: 22399228]
- Fan X, Ding Y, Brown S, Zhou L, Shaw M, Vella MC, Cheng H, McNay EC, Sherwin RS, McCrimmon RJ. Hypothalamic AMP-activated protein kinase activation with AICAR amplifies counterregulatory responses to hypoglycemia in a rodent model of type 1 diabetes. *Amer. J. Physiol. Regul. Integr. Comp. Physiol* 2009; 296: R1702–R1708. [PubMed: 19357294]

- Fillenz M, Lowry JP, Boutelle MG, Fray AE. The role of astrocytes and noradrenaline in neuronal glucose metabolism. *Acta Physiol. Scand* 1999; 167: 275–284. [PubMed: 10632627]
- Fioramonti X, Marsollier N, Song Z, Fakira KA, Patel RM, Brown S, Duparc T, Pica-Mendez A, Sanders NM, Knauf C, Valet P, McCrimmon RJ, Beuve A, Magnan C, Routh VH. Ventromedial hypothalamic nitric oxide production is necessary for hypoglycemia detection and counterregulation. *Diabetes* 2010; 59: 519–528. [PubMed: 19934009]
- Garcia-Ovejero D, Azcoitia I, DonCarlos LL, Melcangi RC, Garcia-Segura LM. Glia-neuron cross-talk in the neuroprotective mechanisms of sex steroid hormone. *Brain Res. Rev* 2005; 48: 273–286. [PubMed: 15850667]
- Garcia-Segura LM, Naftolin F, Hutchison JB, Azcoitia I, Chowen JA. Role of astroglia in estrogen regulation of synaptic plasticity and brain repair. *J. Neurobiol* 1999; 40: 574–584. [PubMed: 10453057]
- Garfield AS, Shah BP, Madara JC, Burke LK, Patterson CM, Flak J, Neve RL, Evans ML, Lowell BB, Myers MG, and Heisler LK. A parabrachial-hypothalamic cholecystokinin neurocircuit controls counterregulatory responses to hypoglycemia. *Cell Metab.* 2014; 20: 1030–1037. [PubMed: 25470549]
- Han SM, Namkoong C, Jang PG, Park IS, Hong SW, Katakami H, Chun S, Kim SW, Park JY, Lee KU, Kim MS. Hypothalamic AMP-activated protein kinase mediates counter-regulatory responses to hypoglycaemia in rats. *Diabetologia* 2005; 48: 2170–2178. [PubMed: 16132951]
- Harik SI, Busto R, Martinez E. Norepinephrine regulation of cerebral glycogen utilization during seizures and ischemia. *J. Neurosci* 1982; 2: 409–414. [PubMed: 6279795]
- Hertz L, Lovatt D, Goldman SA, Nedergaard M. Adrenoreceptors in brain: cellular gene expression and effects on astrocytic metabolism and $[Ca^{2+}]$. *Neurochem. Int* 2010; 57: 411–420. [PubMed: 20380860]
- Koizumi J. Glycogen in the central nervous system. *Prog. Histochem. Cytochem* 1974; 6: 1–37.
- Magistretti PJ, Sorg O, Yu N, Martin JL, Pellerin L. Neurotransmitters regulate energy metabolism in astrocytes: implications for the metabolic trafficking between neural cells. *Dev. Neurosci* 1993; 15: 306–312. [PubMed: 7805583]
- McCarren HS, Chalifax MR, Han B, Moore JT, Meng QC, Baron-Hionis N, Sedigh-Sarvestani M, Contreras D, Beck SG, Kelz MB. α 2-Adrenergic stimulation of the ventrolateral preoptic nucleus destabilizes the anesthetic state. *J. Neurosci* 2014; 34: 16385–16396. [PubMed: 25471576]
- McCrimmon RJ, Shaw M, Fan X, Cheng H, Ding Y, Vella MC, Zhou L, McNay EC, Sherwin RS. Key role for AMP-activated protein kinase in the ventromedial hypothalamus in regulating counterregulatory hormone responses to acute hypoglycemia. *Diabetes* 2008; 57: 444–450. [PubMed: 17977955]
- Meek TM, Nelson JT, Matsen ME, Dorfman MD, Guyenet SJ, Damian V, Allison MB, Scarlett JM, Nguyen HT, Thaler JP, Olson DP, Myers MG, Schwartz MW, Morton GJ. Functional identification of a neurocircuit regulating blood glucose. *Proc. Natl. Acad. Sci* 2016; 113: E2073–E2082. [PubMed: 27001850]
- Nedungadi TP, Briski KP. Site-specific effects of intracranial estradiol administration on recurrent insulin-induced hypoglycemia in ovariectomized female rats. *Neuroendocrinology* 2012; 96: 311–323. [PubMed: 22572755]
- Oomura Y, Ono H, Ooyama H, Wayner MJ. Glucose and osmosensitive neurons of the rat hypothalamus. *Nature* 222: 282–284, 1969. [PubMed: 5778398]
- Pawlak J, Karolczak M, Krust A, Chambon P, Beyer C. Estrogen receptor-alpha is associated with the plasma membrane of astrocytes and coupled to the MAP/Src-kinase pathway. *Glia* 2005; 50: 270–275. [PubMed: 15712205]
- Pellerin L, Pellegri G, Bittar PG, Charnay Y, Bouras C, Martin JL. Evidence supporting the existence of an activity-dependent astrocyte-neuron lactate shuttle. *Dev. Neurosci* 20: 291–299, 1998. [PubMed: 9778565]
- Phelps CH. Barbiturate-induced glycogen accumulation in brain. An electron microscopic study. *Brain Res.* 39: 225–234, 1972. [PubMed: 5025645]
- Routh VH, Hao L, Santiago AM, Sheng Z, Zhou C. Hypothalamic glucose sensing: making ends meet. *Front. Syst. Neurosci* 2014; 8:236. doi: 10.3389/fnsys.2014.00236. [PubMed: 25540613]

- Schousboe A, Sickmann HM, Walls AB, Bak LK, Waagepetersen HS. Functional importance of the astrocytic glycogen-shunt and glycolysis for maintenance of an intact intra/extracellular glutamate gradient. *Neurotox. Res* 18: 94–99, 2010. [PubMed: 20306167]
- Shakya M, Shrestha PK, Briski KP. Hindbrain 5'-monophosphate-activated protein kinase mediates short-term food deprivation inhibition of the gonadotropin-releasing hormone-luteinizing hormone axis: role of nitric oxide. *Neuroscience* 2018; in press. 10.1016/j.neuroscience.2018.04.040
- Silver IA, Erecska M. Glucose-induced intracellular ion changes in sugar-sensitive hypothalamic neurons. *J. Neurophysiol* 79: 1733–1745, 1998. [PubMed: 9535943]
- Smythe GA, Edwards SR. A role for central postsynaptic α 2-adrenoceptors in glucoregulation. *Brain Res.* 1991; 562: 225–229. [PubMed: 1663413]
- Smythe GA, Edwards SR. Suppression of central noradrenergic neuronal activity inhibits hyperglycemia. *Amer. J. Physiol. Endocrinol. Metab* 1992; 263: E823–827.
- Smythe GA, Grunstein HS, Bradshaw JE, Nicholson MV, Compton PJ. Relationships between brain noradrenergic activity and blood glucose. *Nature* 1984; 308: 65–67. [PubMed: 6322003]
- Sorg O, Magistretti PJ. Vasoactive intestinal peptide and noradrenaline exert long-term control on glycogen levels in astrocytes: blockade by protein synthesis inhibition. *J. Neurosci* 1992; 12: 4923–4931. [PubMed: 1334506]
- Steffens AB, Scheurink AJ, Luiten PG, Bohus B. Hypothalamic food intake regulating areas are involved in the homeostasis of blood glucose and plasma FFA levels. *Physiol. Behav* 1988; 44: 581–589. [PubMed: 3070585]
- Stobart JL, Anderson CM. Multifunctional role of astrocytes as gatekeepers of neuronal energy supply. *Cell. Neurosci* 7: 1–21, 2013.
- Szepietowska B, Zhu W, Chan O, Horblitt A, Dziura J, Sherwin RS. Modulation of β -adrenergic receptors in the ventromedial hypothalamus influences counterregulatory responses to hypoglycemia. *Diabetes* 2011; 60: 3154–3158. [PubMed: 22013013]
- Tamrakar P, Briski KP. Estradiol regulates hypothalamic astrocyte adenosine 5'-monophosphate-activated protein kinase activation by hypoglycemia: role of hindbrain catecholamine signaling. *Brain Res. Bull* 2015; 110: 47–53. [PubMed: 25497905]
- Tamrakar P, Shrestha PK, Briski KP. Dorsomedial hindbrain catecholamine regulation of hypothalamic astrocyte glycogen metabolic enzyme protein expression: Impact of estradiol. *Neuroscience* 2015; 292: 34–45. [PubMed: 25701713]
- Walls AB, Heimbürger CM, Bouman SD, Schousboe A, Waagepetersen HS. Robust glycogen shunt activity in astrocytes: Effects of glutamatergic and adrenergic agents. *Neuroscience* 158: 284–292, 2009. [PubMed: 19000744]

Highlights:

- Ventromedial hypothalamic nucleus (VMN) 'fuel-inhibited' gluco-regulatory neurons monitor astrocyte substrate fuel provision.
- Norepinephrine (NE) suppression of VMN neuronal nitric oxide synthase protein requires 5'-AMPactivated protein kinase (AMPK).
- The AMPK inhibitor Compound C (Cc) normalizes VMN glycogen synthase and glycogen phosphorylase profiles in NE-treated rats.
- NE caused Cc-reversible augmentation of astrocyte β_1 -adrenergic and GPR-30 receptor protein expression.
- AMPK mediates noradrenergic diminution of VMN nitrenergic metabolic-deficit signaling and astrocyte glycogen shunt activity.

Figure 1. Effects of the 5'-AMP-Activated Protein Kinase (AMPK) Inhibitor Compound C (Cc) on Norepinephrine (NE) Regulation of Ventromedial Hypothalamic Nucleus (VMN) Glutamate Decarboxylase_{65/67} (GAD_{65/67}), Neuronal Nitric Oxide Synthase (nNOS), and Steroidogenic Factor-1 (SF-1) Protein Expression in Adult Male Rats.

Groups of rats ($n=5$ /treatment group) were pretreated by bilateral intra-VMN delivery of vehicle (NE/V; solid gray bars) or Cc (NE/Cc; diagonal-striped gray bars) prior to NE administration. Controls were given vehicle only (V/V; white bars) into the VMN. For each treatment group, three separate aliquot pools of micropunched VMN tissue were analyzed by Western blot for each of the following proteins: GAD_{65/67} (Panel A), nNOS (Panel B), or SF-1 (Panel C). Bars depict mean normalized protein optical density (O.D.) measures $\pm$ S.E.M. ** $p<0.01$; *** $p<0.001$.

Figure 2. Effects of NE on VMN Glycogen Synthase (GS) and Glycogen Phosphorylase (GP) Protein Expression; Impact of Cc Pretreatment.

Bars depict mean normalized GS (Panel A) or GP (Panel B) protein O.D. measures $\pm$ S.E.M. for V/V (white bars), NE/V (solid gray bars), and NE/Cc (diagonal-striped gray bars) treatment groups. * $p<0.05$; ** $p<0.01$.

Figure 3. VMN Astrocyte Adrenoreceptor Protein Expression in NE-Treated Male Rats. Astrocytes immunolabeled for the marker protein glial fibrillary acidic protein (GFAP) were harvested by laser-catapult microdissection from 10 μ m-thick fresh frozen sections cut through the VMN. Alpha₁ adrenergic (α_1 A-R; Panel A), alpha₂ adrenergic (α_2 A-R; Panel B), and beta₁ adrenergic (β_1 A-R; Panel C) receptor proteins were each analyzed by Western blot in triplicate pools of n=60 astrocyte lysates for each treatment group. Data depict mean normalized protein O.D. values $\pm$ S.E.M. for V/V (white bars), NE/V (solid gray bars), and NE/Cc (diagonal-striped gray bars) treatment groups. * p <0.05; *** p <0.001.

Figure 4. NE Regulation of VMN Astrocyte Estrogen Receptor Protein Expression in Cc- Versus Vehicle-Pretreated Rats.

GFAP-immunopositive astrocyte lysates pools (n=60 cells per pool) were analyzed in triplicate, for each treatment group, for estrogen receptor-alpha (ER α ; Panel A), estrogen receptor-beta (ER β ; Panel B), or GRP30 (Panel C). Data depict mean normalized protein O.D. values $\pm$ S.E.M. for V/V (white bars), NE/V (solid gray bars), and NE/Cc (diagonal-striped gray bars) treatment groups. * p <0.05; ** p <0.01.

Figure 5. Effects of Cc on Noradrenergic Regulation of VMN Astrocyte Phosphofructokinase/Liver (PFKL) and Monocarboxylate Transporter-1 (MCT1) Protein Expression.

GFAP-immunopositive astrocyte lysates pools (n=60 cells per pool) were analyzed in triplicate, for each treatment group, for PFKL (Panel A) and MCT1 (Panel B). Data depict mean normalized protein O.D. values $\pm$ S.E.M. for V/V (white bars), NE/V (solid gray bars), and NE/Cc (diagonal-striped gray bars) treatment groups. * p <0.05; ** p <0.01.

Figure 6. Western Blot Analysis of VMN GAD_{65/67} and nNOS Nerve Cell Adrenergic Receptor Protein Expression.

VMN neurons labeled for GAD_{65/67}- or nNOS-immunoreactivity were individually removed by laser-catapult microdissection from 10 μ m-thick sections cut from vehicle-injected control brains. For each nerve cell population, the presence of α_1A-R , α_2A-R , or β_1A-R protein was determined by Western blot analysis of separate triplicate pools of n=50 heat-denatured cell lysates. * p <0.05.

Figure 7. PHTPP Alters Glucose, Luteinizing Hormone (LH), Glucagon, and Corticosterone Responses to Insulin-Induced Hypoglycemia.

Data depict mean glucose (Panel A), free fatty acid (Panel B), plasma glucagon (Panel C), and corticosterone (Panel D) levels $\pm$ S.E.M. for V/V (white bars), NE/V (solid gray bars), and NE/Cc (diagonal-striped gray bars) treatment groups. * $p<0.05$; ** $p<0.01$.