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Sex-Specific Acclimation of A2 Noradrenergic Neuron Dopamine- β -Hydroxylase and Estrogen Receptor Variant Protein and 5'-AMP-Activated Protein Kinase Reactivity to Recurring Hypoglycemia in Rat

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Abstract

Hindbrain estrogen receptors (ER) impose sex-dimorphic control of counter-regulatory hormone and hypothalamic glucoregulatory transmitter and glycogen metabolic responses to hypoglycemia. A2 noradrenergic neurons are estradiol- and metabolic-sensitive. Estradiol controls dopamine-beta-hydroxylase (DBH) protein habituation to recurrent insulin-induced hypoglycemia (RIIH) in females. Current research investigated the premise that sex-dimorphic patterns of A2 ER variant acclimation to RIIH correlate with differential A2 DBH and 5'-AMP-activated protein kinase (AMPK) adaptation to RIIH. A2 neurons were laser-catapult-microdissected from male and female rats after one or four insulin injections for Western blot analysis. A2 pAMPK and DBH levels were increased in males, but suppressed in females after single insulin dosing. ER-alpha (ER α) and -beta (ER β) protein profiles were unaffected or decreased by acute hypoglycemia in each sex, whereas G protein-linked ER-1 (GPER) reactivity varied by sex. Antecedent hypoglycemia diminished basal A2 ER α /GPER and elevated ER β content in each sex, yet reduced pAMPK and DBH levels in female rats only. Reintroduced hypoglycemia suppressed A2 ER β levels in each sex, but altered DBH ($\downarrow$), ER α ($\downarrow$), and GPER ($\uparrow$) levels in males only. Data document sex differences in A2 DBH adaptation to RIIH, e.g. a shift from positive-to-negative response in males versus loss of negative reactivity in females, as well as attenuated AMPK activation in both sexes. Between hypoglycemic episodes, A2 neurons in each sex likely exhibit diminished sensitivity to ER α /GPER signaling, but heightened receptivity to ER β input. RIIH-induced changes in ER α and GPER expression in male but not female may contribute to DBH suppression (males) versus no change (females) relative to adapted baseline expression.

Keywords

recurrent insulin-induced hypoglycemia; dopamine-beta-hydroxylase; estrogen receptor-alpha; estrogen receptor-beta; G protein-coupled estrogen receptor-1; laser-catapult microdissection; sex differences; AMP

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The authors have no interests to declare.

Introduction:

The catecholamine neurotransmitter norepinephrine (NE) acts within the brain to regulate glucostasis. Hindbrain caudal dorsal vagal complex (DVC A2 noradrenergic neurons monitor availability of the oxidizable energy fuel L-lactate. These cells express Fos in response to monocarboxylate transporter blockade [Patil and Briski, 2005], and exhibit activation of the ultra-sensitive energy sensor 5'-AMP-activated protein kinase (AMPK) in response to hypoglycemia-associated lactoprivation, coincident with L-lactate – reversible hypoglycemic augmentation of hypothalamic NE activity [Shrestha et al., 2014]. A2 nerve cells express estrogen receptor-alpha (ER α) and -beta (ER β) proteins [Ibrahim et al., 2013], and integrate steroid and metabolic stimuli [Ibrahim et al., 2014; Shrestha et al., 2014]. Estradiol is a principal endocrine regulator of glucostasis. ERs are highly expressed in the DVC and other visceral sensory loci in the brain [Simerly et al., 1990]. Estradiol delivery to the caudal fourth ventricle alters blood glycemic profiles in insulin-injected ovariectomized (OVX) female rats [Nedungadi and Briski, 2012]. This hormone regulates metabolic sensor input to the hypothalamus as DVC AMPK activation elicits estradiol-dependent patterns of hypothalamic transcriptional activation [Ibrahim et al., 2013] and phosphoAMPK (pAMPK) and metabolic transmitter protein expression [Alenazi et al., 2014].

Hindbrain ERs impose sex-specific control of hypoglycemic patterns of counter-regulatory hormone secretion and metabolic neurotransmitter signaling and glycogen mobilization in the ventromedial hypothalamic nucleus (VMN), a key element of the brain glucoregulatory network [Ali et al., 2019; Napit et al., 2019]. The VMN gluco-stimulatory signal nitric oxide is up-regulated by DVC ER α - versus ER β -dependent mechanisms in hypoglycemic males or females, respectively, whereas hypoglycemic suppression of gluco-inhibitory γ -aminobutyric acid transmission occurs by ER-independent (male) or ER β -reliant (female) means. During hypoglycemia, VMN glycogen synthase and phosphorylase proteins are correspondingly governed by DVC ER β or ER α in male versus females; ER β signaling attenuates glycogen accumulation in male, but stimulates breakdown in female. During hypoglycemia, ER α and ER β regulate glucagon secretion in the female only, whereas ER β controls corticosterone release in both sexes.

Recurrent insulin-induced hypoglycemia (RIIH) can trigger hypoglycemia-associated autonomic failure, a pathophysiological mal-adaptation that manifests as diminished hypoglycemic awareness and glucose counter-regulatory dysfunction [Cryer et al., 2003; Cryer, 2010]. Clinical studies reveal sex differences in RIIH effects on counter-regulation, as antecedent hypoglycemia (AH) impedes this corrective outflow in men, but not women [Davis et al., 2006]. Experimental models for RIIH that simulate insulin delivery route, frequency of administration, and duration of action in the clinical setting document blunted neuron transactivation in glucoregulatory loci in male, but not female brain, which infers that neural desensitization to this metabolic stress is sex-dimorphic [Paranjape and Briski, 2005; Kale et al., 2006; Nedungadi et al., 2006]. AH-associated attenuation of hypoglycemia-associated nerve cell transactivation in the male rat DVC [Paranjape and Briski 2005] prompts speculation that local metabolic sensory function may be similarly impaired [Smith and Amiel 2002]. Previous work showed that AH did not modify (males) or

decreased (females) basal A2 expression of mRNA encoding the catecholamine biosynthetic enzyme dopamine- β -hydroxylase (D β H), but that in each sex, transcripts declined relative to post-AH baseline during renewed exposure to hypoglycemia [Cherian and Briski, 2011; Cherian and Briski, 2012]. Western blot analyses indicate that AH suppresses basal A2 D β H protein levels in estradiol-, but not oil-implanted OVX female rats, and that RIIH causes a further decline in D β H protein expression in the former animals, but does not affect this protein in the latter group [Tamrakar and Briski, 2017]. Moreover, those studies showed that estradiol attenuated AMPK activation in OVX female rats during RIIH. Corresponding insight on if and how A2 D β H expression in the male may habituate to RIIH is currently lacking. Research here investigated the hypothesis that A2 nerve cell D β H protein responses to acute and/or recurring hypoglycemia vary by sex and correlate with sex-specific patterns of AMPK activation by this metabolic stress.

Estradiol regulates A2 neurons by action on multiple receptors, namely ER α , ER β , and G protein-coupled estrogen receptor-1 (GPER). Studies involving OVX females replaced with peak estrous cycle-like estradiol levels showed that acute hypoglycemia did not alter ER α protein profiles, but up-regulated A2 ER β content [Shrestha et al., 2015]. Research outcomes also implicated ER β signaling in hypoglycemia-associated patterns of D β H expression, as well as hypoglycemic effects on hypothalamic NE activity and pituitary gonadotropin and counter-regulatory hormone secretion [Briski and Shrestha, 2016]. Current work addressed the premise that acute hypoglycemia causes differential adjustments in ER variant protein expression in male versus female rats, and that cellular acclimation to RIIH may reflect sex-specific changes in A2 nerve cell sensitivity to specific ER-mediated estradiol input.

Materials and Methods:

Experimental Design:

Animals: Adult male and female Sprague Dawley were housed in groups (2–3 per cage) by sex, under a 14 hr light/10 hr dark cycle (lights on at 05.00 h), and acclimated to daily handling. Animals had *ad-libitum* access to standard laboratory rat chow and water. Experimental and surgical procedures were performed in compliance with NIH guidelines for laboratory animal care and use, under approval by the ULM Institutional Animal Care and Use Committee.

Surgeries and hormone replacement: On study day 1, animals were anesthetized with ketamine/xylazine (0.1 mL/100 g *bw*; 90 mg ketamine:10 mg xylazine/mL; Henry Schein Inc., Melville, NY, US), and implanted with a PE-20 cannula into the caudal fourth ventricle (CV4) [Singh and Briski, 2005]. Anesthetized females were also bilaterally OVX. After surgery, rats were injected subcutaneously (*sc*) with ketoprofen (1 mg/kg *bw*) and intramuscularly with enrofloxacin (10 mg/0.1 mL), treated by topical application of 0.25% bupivacaine to closed incisions, then transferred to individual cages. Plasma estradiol were standardized in female animals to avoid potential variability caused by variability in endogenous steroid hormone secretion over the estrous cycle. On day 7, female rats were implanted with a *sc* silastic capsule (i.d. 0.062 in./o.d. 0.125 in.; 10 mm/100 g *bw*) filled

with 30 ug 17 β - estradiol-3-benzoate/mL safflower oil, under isoflurane anesthesia (5% - induction; 2.5% - maintenance). This steroid replacement regimen yields approximate plasma estradiol concentrations of 22 pg/ml [Briski et al., 2001], replicating metestrus stage plasma hormone levels in 4-day cycling animals [Butcher et al., 1974]. Rats of each sex were randomly assigned to treatment groups (Table 1).

Induction of acute versus recurring hypoglycemia: On days 14–17, animals were injected *sc* at 9.00 hr (time zero, t_0) with neutral protamine Hagedorn insulin (I; 10.0 U/kg *bw*; Henry Schein, Inc) or vehicle (sterile diluent; V), as follows: 1) VVVV groups: *sc* V injection on days 14–17; n=4 males; n=4 females; 2) VVVI groups: *sc* V injection on days 14–16, followed by *sc* I injection on day 17 (n=4 males; n=4 females; 3) IIIV groups: *sc* I injection on days 14–16, followed by *sc* V injection on day 17 (n=4 males; n=4 females; 4) IIII groups: *sc* I injection on days 14–17 (n=4 males; n=4 females). On day 17, animals were sacrificed by rapid decapitation one hour after injections (10.00 hr).

Western Blot Analysis of Laser-Catapult Microdissected DVC A2 Noradrenergic Nerve Cell Protein Expression:

Hindbrains were cut into serial 10 μ m-thick frozen hindbrain sections between 14.36 to 14.86 mm posterior to *bregma*, and mounted on polyethylene naphthalate membrane-covered slides (Carl Zeiss MicroImaging LLC, Thornwood, NY, US). After acetone fixation and blocking with 5% normal horse serum (Vectastain Elite ABC mouse IgG kit; prod. no. PK-6102; Vector Laboratories, Inc., Burlingame, CA, US), tissues were incubated (24 hr; 4°C) with mouse monoclonal antibodies against tyrosine hydroxylase (TH; prod. no. 22941; 1:1,000; ImmunoStar, Inc., Hudson, WI, US), followed by Vectastain IgG Elite ABC mouse IgG kit biotinylated secondary antibody, ABC reagent, and ImmPACT DAB substrate kit reagents (prod. no. SK-4103; Vector Lab.) to visualize tyrosine hydroxylase (TH)-immunoreactive (-ir) neurons. Individual TH-ir cells exhibiting a visible nucleus and complete labeling of the cytoplasmic compartment were laser-dissected using a Zeiss P.A.L.M. UV-A system (Carl Zeiss MicroImaging), as described [Briski et al., 2009], and collected into lysis buffer [2.0% sodium dodecyl sulfate, 0.05 M dithiothreitol, 10.0% glycerol, 1.0 mM EDTA, 60.0 mM Tris-HCl, pH 7.2]. For each protein of interest, aliquots of A2 cell lysates from individual subjects were pooled within treatment groups (n=50 neurons/treatment group) ahead of separation on Bio-Rad TGX 10–12% stain-free gels (prod. no. 161–0183, Bio-Rad Laboratories Inc., Hercules CA, US) [Ibrahim et al., 2019]; each protein was analyzed in triplicate at minimum. Gels were UV light-activated (1 min) in a Bio-Rad ChemiDoc TM Touch Imaging System prior to overnight protein transblotting (30 V, 4°C) to 0.45- μ m PVDF membranes (ThermoFisherScientific; Waltham, MA, US) [Ibrahim et al., 2019]. Membranes were pretreated with Western blotting signal enhancer (ThermoFisherSci.), blocked with Tris-buffer saline (TBS), pH 7.4, containing 0.1% Tween-20 (Sigma Aldrich, St. Louis, MO, US) and 2% bovine serum albumin (MP Biomedicals, Solon, OH, US), then probed with primary antisera raised in rabbit against AMPK $_{\alpha 1/2}$ (prod. no. 2532; 1:2,000; Cell Signaling Technology, Danvers, MA, US), phosphoAMPK $_{\alpha 1/2}$ -Thr 172 (pAMPK; prod. no. 2535; 1:2,000; Cell Signaling Technol.), D β H (prod. no. sc-15318; 1:1,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, US), ER β /NR3A2 (prod. no. NB120–3577, 1:1,000; Novus Biologicals, LLC, Centennial, CO,

US) or GPER/GPR30 (prod. no. NLS 4271, 1:1,000; Novus Biol.), or raised in mouse against ER α /NR3A1 (prod. no. NB300560, 1:1,000; Novus Biol.). Membranes were incubated (1 hr) with horseradish peroxidase-labeled goat anti-rabbit (prod. no. NEF812001EA, 1:5,000; PerkinElmer, Waltham, MA, US) or goat anti-mouse (prod. no. NEF822001EA, 1:6000; PerkinElmer) secondary antibodies, then exposed to Supersignal West Femto maximum sensitivity chemiluminescent substrate (prod. no. 34096; ThermoFisherSci.). Membrane buffer washes and antibody incubations were performed by Freedom Rocker™ Blotbot® automation (Next Advance, Inc., Troy, NY, US). Chemiluminescence band optical density (O.D.) values obtained in the ChemiDoc MP system were normalized to total in-lane protein with Imagemag software (Image Lab™ 6.0.0; Bio-Rad). Precision plus protein molecular weight dual color standards (prod. no. 161–0374; Bio-Rad) were included in each Western blot analysis. All antisera used in the present work are regularly employed in research performed in our laboratory; these reagents yield single protein bands of consistent molecular weight regardless of sex, experimental treatment, or brain region.

Plasma Glucose Analyses:

Circulating glucose levels was measured with an ACCU-CHECK Aviva Plus glucometer (Roche Diagnostics USA, Indianapolis, IN, US), as described (Kale et al., 2006).

Statistical analyses:

Mean normalized A2 protein O.D., pAMPK/AMPK O.D. ratio, and plasma glucose values were evaluated between treatment groups within each sex by two-way analysis of variance and Student-Newman-Keuls *post-hoc* test. Differences of $p < 0.05$ were considered significant. In each figure, statistical differences between specific pairs of treatment groups are denoted with the following symbols: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Results:

Effects of acute versus recurring hypoglycemia on A2 neuron AMPK total protein expression and activity in male versus female rats.

Figure 1 illustrates high-resolution utility of laser-catapult microdissection for efficacious harvesting of individual TH-immunopositive hindbrain dorsal vagal complex A2 neurons for Western blot analyses. Figure 2 depicts effects of single versus serial insulin dosing on total AMPK and pAMPK protein content in laser-microdissected A2 neurons obtained from male versus female rats. AMPK protein profiles were up-regulated in male [$F_{(3,8)} = 10.90$; $p = 0.0034$], but not female rats [$F_{(3,12)} = 2.19$; $p = 0.141$] during acute hypoglycemia (Figure 2A). After recovery from exposure to IHH on days 14–16, males exhibited elevated baseline AMPK protein content compared to controls [M-IIIIV versus M-VVVV] (Table 2). Renewed exposure to hypoglycemia on day 17 caused a decline in A2 AMPK levels in males compared to M-IIIIV and M-VVVI treatment groups. RIIIH female rats showed no change in AMPK expression compared to F-IIIIV baseline values.

Single insulin dosing correspondingly elevated or diminished A2 pAMPK levels in male [$F_{(3,8)} = 11.06$; $p = 0.0032$] versus female [$F_{(3,8)} = 6.93$; $p = 0.013$] rats (Figure 2B). Baseline

pAMPK expression was suppressed in AH-exposed female rats [F-IIIIV versus F-VVVV]. Induction of a fourth consecutive bout of hypoglycemia on day 17 did not alter pAMPK protein profiles from IIIIV baseline levels in either sex. In male rats, the mean A2 pAMPK/AMPK ratio [$F_{(3,8)} = 32.12$; $p < 0.0001$] was refractory to acute hypoglycemia, but was elevated during RIIH compared to IIIIV and VVVI treatment groups [M-IIIIV versus M-IIIIV; M-IIIIV versus M-VVVI] (Figure 2C). Notably, baseline measures of this ratio were significantly reduced on day 17 in AH-exposed males compared to VVVV controls [M-IIIIV versus M-VVVV]. Conversely, females [$F_{(3,8)} = 1.45$; $p = 1.45$] did not exhibit a change in this ratio versus baseline during acute or renewed IHH. Plasma glucose data presented in Table 3 show that, in both sexes, circulating concentrations were decreased to an equivalent extent after one versus the fourth of four insulin injections.

Effects of single or serial insulin dosing on A2 D β H protein content in each sex.

Acute insulin administration significantly elevated or suppressed this profile in male [$F_{(3,8)} = 14.06$; $p = 0.0015$] and female [$F_{(3,8)} = 7.27$; $p = 0.011$] rats, respectively (Figure 3). On day 17, baseline D β H expression was decreased in AH-exposed female [F-IIIIV versus F-VVVV], but not male rats. During a fourth and final bout of hypoglycemia on day 17, this profile was significantly diminished in male [M-IIIIV versus M-IIIIV], but not female rats.

Effects of acute versus recurring hypoglycemia on A2 nerve cell estrogen receptor variant expression in male versus female rats.

Data in Figure 4 depict patterns of A2 nerve cell ER α (Figure 4A [male: $F_{(3,8)} = 23.59$, $p = 0.0003$; female: $F_{(3,12)} = 4.53$, $p = 0.024$]), ER β (Figure 3B; [male: $F_{(3,8)} = 26.18$, $p = 0.0002$; female: $F_{(3,16)} = 15.35$, $p = 0.0003$]), and GPER (Figure 3C; [male: $F_{(3,8)} = 61.17$, $p < 0.0001$; female: $F_{(3,8)} = 21.56$, $p < 0.0001$]) protein expression in male and female rats during exposure to acute versus recurring hypoglycemia. In each sex, A2 ER α protein content was unaffected by one insulin dosing, whereas ER β expression was elevated under the same conditions. GPER responses to acute hypoglycemia varied according to sex as this protein was increased or decreased in hypoglycemic male versus female rats. Acclimation of each ER variant to AH was similar between sexes. On day 17, male and female rats previously exposed to hypoglycemia exhibited similar adjustments in baseline ER α (decreased), ER β (increased), GPER (decreased) levels compared to VVVV control groups. ER responses to a final episode of hypoglycemia were sex-monomorphic, namely ER β protein profiles were decreased in each sex, as well as -dimorphic, e.g. ER α content was decreased in males, but unchanged in females whereas GPER levels were elevated in the male, but unchanged in the female.

Discussion:

Overview:

Hindbrain DVC A2 neurons integrate estradiol and metabolic cues, and provide critical sensory information to the brain glucoregulatory network. Neural desensitization to hypoglycemia-associated cellular energy imbalance is a causal factor in RIIH-associated neurogenic unawareness and counter-regulatory collapse [Smith and Amiel 2002]. Attenuated DVC nerve cell transactivation in RIIH males infers that local metabolic sensory

function may be impaired [Paranjape and Briski 2005]. Estradiol controls A2 D β H protein adaptation to RIIH in female rats [Tamrakar and Briski, 2017]. Current research addressed the premise that A2 energy sensor habituation to RIIH is sex-specific and is correlated with sex-dimorphic adaptation of noradrenergic transmitter marker protein, e.g. D β H reactivity to hypoglycemia. Data show that acute hypoglycemia increases A2 pAMPK, DBH, and GPER expression in males, but decreases levels of these proteins in females, whereas ER α and ER β levels are impacted similarly in male and female rats. Baseline A2 pAMPK and DBH protein content declines after AH in female, but not male rats. Post-AH adjustments in baseline A2 ER β (increased) and ER α /GPER (decreased) levels in each sex imply that these cells may become more or less sensitive to receptor-specific estradiol input. Sex-dimorphic A2 DBH adaptation to RIIH, e.g. positive-to-negative switch in males versus abolition of negative reactivity in females may be mediated, in part, by sex-contingent ER α and GPER responses to RIIH.

Effects of antecedent hypoglycemia on A2 neuron AMPK total protein and pAMPK expression in male versus female rats:

Male, but not female rats exhibited up-regulated A2 nerve cell total AMPK content after single insulin dosing, a response was accompanied by elevated pAMPK levels; on the other hand, A2 pAMPK content declined in females exposed to acute hypoglycemia. These opposite adjustments in pAMPK expression likely reflect a loss (male) versus gain (female) in A2 cell energy stability; yet, this interpretation remains speculative as technology with requisite sensitivity for quantification of ATP in neurotransmitter-specific nerve cell tissue samples is not currently available. Effects of AH on basal A2 AMPK versus pAMPK protein profiles varied according to sex, as males exhibited up-regulated total AMPK alongside decreased pAMPK, whereas levels of neither protein were different between VVVV versus IIIV groups in the female. Similarities in magnitude of up-regulated AMPK expression in IIIV versus VVVI males infer that this baseline intensification may reflect adaptation to augmented need. The functional relevance of amplified total AMPK protein remains unclear, however, as pAMPK is the active enzyme form. Diminution of baseline A2 pAMPK protein profiles in females in the wake of multiple hypoglycemia exposures thus likely reflects implementation of a state of greater energy stability, mimicking earlier reactivity to hypoglycemia. Previous studies showed that in the presence of estradiol, A2 cells exhibit elevated baseline expression of the rate-limiting glycolytic enzyme PFKL in the aftermath of hypoglycemia, coincident with diminished respiratory chain complex V- α and ATP synthase- α protein profiles, suggesting that acclimation may involve stimulation of glycolysis, alongside suppression of mitochondrial aerobic respiration/energy production by this hormone [Tamarkar and Briski, 2017].

Antecedent hypoglycemia-associated adjustments in A2 neuron AMPK/pAMPK ratio:

Bi-directional adjustments in basal AMPK (increased in males) versus pAMPK (decreased in females) expression due to AH resulted in differential changes in the baseline A2 pAMPK/AMPK ratio in IIIV groups of male and female rats, namely a significant ratio reduction in the former versus no net change in the latter animals. General opinion on whether this ratio provides more or less meaningful insight on cellular energy balance compared to absolute pAMPK expression levels alone is divided. In the present context,

pAMPK/AMPK ratio changes due to expanded denominator value indicate a reduced fraction of activated enzyme, corresponding to diminished enzyme specific activity and possible reduction in this regulatory brake on acetyl Co-A carboxylase (ACC) enzyme conversion of acetyl Co-A to malonyl Co-A. It remains unclear if augmented AMPK expression here reflects a change in A2 cell energy state. Alternatively, amplified total AMPK protein could, depending upon incremental increase, eventually expand enzyme mass available for activation by phosphorylation. It is speculated that AMPK phosphorylation, which is a rapid post-translational modification, produces an appropriate acute response to hypoglycemia, whereas altered total AMPK protein expression may possibly serve as a more protracted adaptive response.

Effects of hypoglycemia re-introduction on A2 AMPK and pAMPK protein content:

RIIH did not alter A2 pAMPK protein expression compared to day 17 basal values in either sex, but caused a significant decline in total AMPK levels compared to the IIIV treatment group. These data infer that sex-specific pAMPK reactivity to acute hypoglycemia, e.g. up-regulation in males versus down-regulation in females is eliminated as a result of recurring exposure to this metabolic stress. Thus, A2 cells may likely acquire capabilities to maintain energy stability during renewed threat of imbalance. RIIH-associated amplification of the pAMPK/AMPK ratio in males, in the absence of change in pAMPK expression, may be intended to limit numbers of enzyme molecules available for additional phosphorylation, which could thus check that response. Again, it is not known if this male-specific AMPK inhibitory response to RIIH is mediated by cellular metabolic status or other stimulus.

A2 D β H protein expression in acute hypoglycemic male versus female rat:

Data here show that acute hypoglycemia up- or down-regulated expression of the catecholamine biosynthetic enzyme D β H protein in male and female rat A2 neurons, respectively; observations involving the latter sex confirm previous studies from our laboratory [Tamarkar and Briski, 2017]. These divergent D β H responses directly parallel hypoglycemia-associated alterations in cellular pAMPK content, inferring that acute A2 signaling may convey divergent, e.g. negative versus positive changes in metabolic stability in the two sexes. Parallel adjustments in A2 D β H and pAMPK content were also observed in AH-exposed female rats, where baseline expression of both proteins was reduced in acclimation to earlier exposure to hypoglycemia. Again, it is plausible that in one sex only, these neurons communicate an adaptive enhancement of energy state between bouts of this metabolic stress. A2 neurons regulate a wide range of physiological and behavior functions, including responsiveness to various stressors. Findings here raise the question, for the female, if and how habituated baseline noradrenergic activity may affect metabolic and non-metabolic bodily functions, including reactivity to non-metabolic stressors in the aftermath of hypoglycemia.

A2 D β H protein acclimation to RIIH: In the current experimental paradigm, a final episode of hypoglycemia elicited sex-dimorphic A2 D β H responses as this protein profile declined from IIIV baseline levels in males, but was refractory to renewed hypoglycemia in females. These acclimated reactions signify a shift from positive-to-negative in males versus loss of negative reactivity in the female. As RIIH-associated patterns of D β H and pAMPK

expression in the male do not coincide, reductions in the former profile may not solely reflect A2 energy state, which may be considered mal-adaptive in terms of neural regulation of systemic energy balance. Further effort is required to determine if metabolic cues other than cellular AMP/ATP ratio, which regulates AMPK activity, or feedback signals from non-metabolic noradrenergic targets, impact A2 D β H expression in RIIH males. Ongoing studies are needed to investigate whether habituated A2 noradrenergic signaling is involved in counter-regulatory collapse associated with RIIH in males.

Effects of acute hypoglycemia on ER variant protein expression in male versus female: Current data document nuclear (ER α , ER β) and membrane (GPER) ER protein expression in A2 neurons. ER α and - β protein responses to acute hypoglycemia were sex-monomorphic, whereas GPER protein levels were altered in a sex-contingent manner. Observations of hypoglycemic up-regulation of A2 ER β content extends prior findings in females [Shrestha et al., 2015] with new evidence for similar positive reactivity in OVX animals replaced with estradiol at levels that mimic tonic secretion as well as in males. Earlier work showed that ER β signaling was critical for acute hypoglycemic regulation of D β H expression in OVX females exhibiting proestrus-like circulating estradiol levels [Briski and Shrestha, 2016]. It remains to be determined for the male and female rat models used here, e.g. testes-intact and OVX plus metestrus-stage estradiol replacement, respectively, whether up-regulated ER β -mediated estradiol input elicits divergent D β H responses described here. It is intriguing to speculate whether acute hypoglycemia-associated up-versus down-regulation of GPER expression in male versus female rats contributes, in some way, to differences in energy sensor and/or transmitter marker protein responses to this stress between sexes.

A2 nerve cell ER variant protein acclimation to RIIH:

Current results show that A2 neuron ER protein content acclimated to AH in a sex-similar manner, e.g. down-regulated ER α and GPER and up-regulated ER β , indicating that adaptation involves more or less cellular sensitivity to receptor-specific estradiol input. While these findings argue against involvement of these ERs in differential adjustments in baseline D β H expression between sexes, the possibility of sex differences in post-receptor signal processing, resulting in dissimilar receptor-mediated estradiol impact on A2 neurons cannot be discounted. RIIH reduced A2 ER β content relative to IIIV baseline in both sexes, which represents an adaptive reversal of augmented protein expression during acute hypoglycemia. Only male rats showed ER α (down-regulated) and GPER (up-regulated) reactivity to recurring hypoglycemia, which constitute a gain of negative response and ongoing positive response, respectively. There remains a clear need to identify mechanisms that underlie patterns of ER protein habituation to RIIH unique to each sex, and to examine the respective roles of ERs in divergent adjustments in A2 cell function during ongoing hypoglycemia.

Summary:

Outcomes document acclimated baseline A2 pAMPK and D β H expression in AH-exposed female, but not male rats, changes that mirror protein responses to initial hypoglycemia. In RIIH females, loss of negative pAMPK and D β H reactivity to renewed hypoglycemia may

reflect a state of enhanced A2 metabolic stability between hypoglycemia exposures. Alternatively, male rats exhibit an adaptive positive-to-negative switch in D β H protein reactivity to hypoglycemia, alongside loss of amplification of pAMPK expression; thus, non-metabolic-sensory cues may drive acclimated noradrenergic signaling in this sex. During RIIH, ER α and GPER protein expression is decreased in male, but not female A2 cells, inferring that sex differences in estradiol input mediated by these ER variants may contribute to corresponding attenuation or unaffected noradrenergic transmission in male versus female, respectively. Outcomes bolster the need to investigate the impact of habituated A2 noradrenergic signaling in glucose counter-regulatory dysfunction in RIIH males.

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Abbreviations:

AH	antecedent hypoglycemia
AMPK	5'-AMP-activated protein kinase
DVC	dorsal vagal complex
ERα	estrogen receptor-alpha
ERβ	estrogen receptor-beta
GPER	G protein-coupled estrogen receptor-1
NE	norepinephrine
OVX	ovariectomy
pAMPK	phosphoAMPK

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Figure 1. Laser-Catapult Microdissection of Immunocytochemically-Characterized Hindbrain Dorsal Vagal Complex A2 Noradrenergic Neurons.

A2 neurons located between 14.36 to 14.86 mm posterior to *bregma* were identified *in situ* by tyrosine hydroxylase (TH) immunoreactivity (-ir). The orange rectangle in the coronal brain map *at top* illustrates the location of these cells within the dorsomedial hindbrain; immunostained A2 neurons are indicated by orange stars within the enlarged rectangle. In Figure 1A, pre-dissected TH-ir-positive neurons in Panel 1A are indicated by blue arrows. The area shown in Figure 1A was re-photographed after positioning of a continuous laser track (depicted in green) around individual TH-ir neurons [Figure 1B] and subsequent ejection of each cell by laser pulse [Figure 1C]. Note that this microdissection technique causes negligible destruction of surrounding tissue and minimal inclusion of adjacent tissue. *Abbreviations:* GR, gracile nucleus; NTSco, l, m, nucleus of the solitary tract, commissural, lateral, and medial parts; DMX, dorsal motor nucleus vagus nerve; C, central canal; XII, hypoglossal nerve. Scale bar at bottom right of Figures 1A-1C: 100 μ m.

Figure 2. Effects of Single versus Serial Exposure to Insulin-Induced Hypoglycemia on A2 Noradrenergic Neuron 5'-AMP-Activated Protein Kinase (AMPK) and Phospho-AMPK (pAMPK) Protein Expression in Male and Female Rats.

Groups of testes-intact and ovariectomized, estradiol-implanted female rats were injected subcutaneously (*sc*) with vehicle (V) or insulin (I; 10.0 U/kg *bw*) on four consecutive days as follows: *sc* V injection on days 1–4 (VVVV; solid white bars); *sc* V injection on days 1–3, I injection on day 4 (VVVI; diagonal-striped white bars); *sc* I injection on days 1–3, V injection on day 4 (IIIV; solid gray bars); *sc* I injection on days 1–4 (IIII; diagonal-striped gray bars). Data depict mean normalized A2 cell AMPK (Figure 2A) or pAMPK (Figure 2B) protein optical density (O.D.) values $\pm$ S.E.M. for male (*at left*) and female (*at right*) treatment groups. Figure 2C depicts mean pAMPK/AMPK ratio values for VVVV, VVVI, IIIV, and IIII groups of male and female rats. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Figure 3. Effects of Antecedent Insulin-Induced Hypoglycemia on Baseline and Hypoglycemic Patterns of A2 Nerve Cell Dopamine-Beta-Hydroxylase (D β H) Protein Expression in Male and Female Rats.

Graphs *at left* and *right* depict mean normalized D β H O.D. values $\pm$ S.E.M. for A2 neurons collected from VVVV, VVVI, IIIV, and IIII groups of male or female rats, respectively.

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Figure 4. Impact of Single versus Repeated Induction of Insulin-Induced Hypoglycemia on Estrogen Receptor-Alpha (ER α), Estrogen Receptor-Beta (ER β), and G-Protein-Coupled Estrogen Receptor-1 (GPER) Protein Profiles in Male and Female Rat A2 Noradrenergic Neurons.

Figures 4A (ER α), 4B (ER β), and 4C (GPER) show mean normalized ER variant O.D. values $\pm$ S.E.M. for A2 neurons obtained from male (*at left*) and female (*at right*) VVVV, VVVI, IIIV, and IIII treatment groups. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Table 1.

Experimental Design

Subjects by Sex		Four-Day Treatment Schedule				Treatment Group Identifiers
		Day 14	Day 15	Day 16	Day 17	
Male; n=4	V ^a	V	V	V	V	M-VVVV
Female; n=4	V	V	V	V	V	F-VVVV
Male; n=4	V	V	V	V	I ^b	M-VVVI
Female; n=4	V	V	V	V	I	F-VVVI
Male; n=4	I	I	I	I	V	M-IIIV
Female; n=4	I	I	I	I	V	F-IIIV
Male; n=4	I	I	I	I	I	M-IIII
Female; n=4	I	I	I	I	I	F-IIII

^a sterile diluent; 100 µL/100 g bw

^b 10.0 U neutral protamine Hagedorn insulin/kg bw

Summary

Acute versus Recurring Insulin-Induced Hypoglycemia Effects on A2 Noradrenergic Neuron 5'-AMP-Activated Protein Kinase (AMPK) Activation and Dopamine-β-Hydroxylase (DβH), and Estrogen Receptor Variant Protein Expression in Male and Female Rats:

Table 2.

	MALE				FEMALE			
	VVVV ^a	VVVV ^b	III ^c	III ^d	VVVV	VVVV	III ^e	III ^f
AMPK	↑ vs VVVV	↑ vs VVVV	↑ vs VVVV	↓ vs IIIV	N.D. ^e vs VVVV	N.D. vs VVVV	N.D. vs IIIV	N.D. vs IIIV
pAMPK ^f	↑ vs VVVV	↑ vs VVVV	N.D. vs VVVV	N.D. vs IIIV	↓ vs VVVV	↓ vs VVVV	N.D. vs IIIV	N.D. vs IIIV
DβH	↑ vs VVVV	↑ vs VVVV	N.D. vs VVVV	↓ vs IIIV	↓ vs VVVV	↓ vs VVVV	N.D. vs IIIV	N.D. vs IIIV
ERα ^g	N.D.	↑ vs VVVV	↓ vs VVVV	↓ vs IIIV	N.D. vs VVVV	↓ vs VVVV	N.D. vs IIIV	N.D. vs IIIV
ERβ ^h	↑ vs VVVV	↑ vs VVVV	↑ vs VVVV	↓ vs IIIV	↑ vs VVVV	↑ vs VVVV	↓ vs IIIV	↓ vs IIIV
GPER ⁱ	↑ vs VVVV	↑ vs VVVV	↓ vs VVVV	↑ vs IIIV	↓ vs VVVV	↓ vs VVVV	N.D. vs IIIV	N.D. vs IIIV

^a subcutaneous (sc) injection of vehicle (V; sterile diluent) on days 14–17

^b sc V injection on days 14–16, followed by sc injection of neutral protamine Hagedorn insulin (I; 10.0 U/kg bw) on day 17

^c sc I injection on days 14–16, followed by sc V injection on day 17

^d sc I injection on days 14–17

^e not different

^f phosphoAMPK

^g estrogen receptor-alpha

^h estrogen receptor-beta

ⁱ G protein-coupled estrogen receptor-1

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Table 3.
Effects of Single versus Serial Neutral Insulin Injection on Plasma Glucose Levels in Male and Female Rats

Sex	Treatment Groups			
	VVVV ^a	VVVI ^b	IIIV ^c	IIII ^d
Male	109.5 ± 3.2	40.8 ± 1.8 [*]	120.8 ± 2.8	40.1 ± 2.8 [*]
Female	124.7 ± 3.6	46.3 ± 3.0 [*]	116.0 ± 4.1	52.3 ± 1.9 [*]

^a subcutaneous (sc) injection of vehicle (V; sterile diluent) on days 14–17

^b sc V injection on days 14–16; sc injection of neutral protamine Hagedorn insulin (I; 10.0 U/kg bw) on day 17

^c sc I injection on days 14–16; sc V injection on day 17

^d sc I injection on days 14–17

^{*} p<0.05 compared to control, e.g. VVVI versus VVVV, IIII versus IIIV.