

Impact of recurrent hypoglycemic stress on hindbrain A2 nerve cell energy metabolism and catecholamine biosynthesis: modulation by estradiol

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It is unclear if habituation of hindbrain A2 metabolo-sensory neurons to recurrent insulin-induced hypoglycemia (RIIH) correlates with estradiol-dependent adjustments in energy metabolism that favor positive energy balance. Laser-microdissected A2 cells from estradiol- or oil-implanted ovariectomized female rats were analyzed by Western blot to assess effects of three prior daily insulin injections on basal and hypoglycemic patterns of catecholamine biosynthetic enzyme dopamine-beta-hydroxylase (DβH) and rate-limiting energy pathway enzyme protein expression. Precedent hypoglycemia respectively decreased or increased baseline DβH expression in estradiol- (E) vs. oil (O)-treated rats; this protein profile was further suppressed or augmented in those animals at 2 hr after re-induction of hypoglycemia. These data suggest that estradiol may curtail A2 noradrenergic-controlled functions both *in the midst of* and *between* hypoglycemic bouts. Results also show that prior hypoglycemia exposure upregulated A2 neuron glycolytic enzyme protein levels when E was present, and exerted differential effects on basal and hypoglycemia-associated respiratory chain and fatty acid synthetic pathway enzyme expression. E may thus accordingly amplify glycolysis-derived metabolites/energy, coupled with reduced reliance on oxidative phosphorylation, and activate the fatty acid synthetic pathway during RIIH. E may also be of benefit by preventing maladaptive reductions in A2 neuron Krebs cycle/electron transport enzyme expression during re-exposure to hypoglycemia. Augmentation of negative energy balance during this recurring metabolic stress in the absence of E is a likely impetus for augmented vs. decreased A2 signaling of energy imbalance by DβH in O vs. E rats during RIIH.

Key words: ATP synthase-alpha, dopamine-beta-hydroxylase, estradiol, laser-catapult microdissection, recurrent insulin-induced hypoglycemia, Western blot

INTRODUCTION

Hindbrain noradrenergic neurons, including the A2 cell group located in the caudal dorsal vagal complex (cDVC), are involved in neural regulation of a wide array of physiological, behavioral and cognitive functions that maintain homeostasis, including control of emotional, endocrine, and autonomic responses to stress (Rinaman 2011). A2 noradrenergic neurons are implicated in metabolo-sensory function by characteristic biomarker expression, including glucokinase (Briski et al. 2009), the ATP-dependent potassium channel, K_{ATP} (Briski et al. 2009), and the ultra-sensitive energy gauge adenosine 5'-monophosphate-activated protein kinase (AMPK) (Cherian and Briski 2011, 2012). Evidence for glucose antimetabolite-induced increases in Fos protein (Briski and Marshall 2000) and dopamine-β-hydroxylase (DβH) mRNA (Li et al. 2006) expression by A2 neurons provides proof of functional susceptibility to glucoprivation.

A2 cells communicate hypoglycemia-associated cell energy imbalance to the brain gluco-regulatory network as insulin-induced hypoglycemia-associated patterns of hypothalamic metabolo-sensory neuron AMPK activation, hypothalamic metabolic effector transmitter expression, and hypoglycemic hyperphagia are normalized by substrate fuel repletion of the cDVC (Gujar et al. 2014). Indeed, recent studies show that lactoprovic signaling from hindbrain to hypothalamus is selectively performed by A2 neurons (Shrestha et al. 2014). Insulin-dependent diabetes mellitus management is complicated by unremitting iatrogenic hypoglycemia and associated risk of brain dysfunction and injury. Evidence that hypoglycemia-induced transcriptional activation in the male rat DVC is diminished by precedent exposure (Paranjape and Briski 2005) prompts speculation that metabolo-sensory function in that site may be vulnerable to maladaptive desensitization to hypoglycemia (Smith and Amiel 2002).

The ovarian hormone estradiol is a potent stimulus for energy metabolism, enhancing substrate catabolism and oxidative respiration in the brain during bio-energetic instability due to stroke (Chen et al. 2009). Estradiol is neuroprotective against singular bio-energetic insults, but the impact of this hormone on neuron energy metabolism during recurring hypoglycemia is unclear. Estradiol delivery to the ovariectomized (OVX) female rat caudal hindbrain sustains uniformity of glycemic responses to repeated insulin dosing while vehicle controls exhibit delayed recovery from recurring hypoglycemia (Nedungadi and Briski 2012). Moreover, estradiol prevents habituation of hypoglycemia-associated nerve cell Fos immunostaining in the DVC (Nedungadi et al. 2006). Estradiol may thus prevent cellular energy state adjustments over serial hypoglycemic bouts or, alternatively, may mask acquired cellular energy instability by promoting signals of energy balance. A2 neurons are likely direct targets for estradiol regulatory actions as these cells express estrogen receptor- α and - β proteins (Ibrahim et al. 2013). The current studies investigated the hypothesis that estradiol regulation of recurring hypoglycemia on A2 catecholamine neurotransmitter synthesis correlates with neuro-protective effects on energy metabolic pathway function during and between bouts of hypoglycemia. This work had two aims: 1) to determine how precedent hypoglycemia influences between-hypoglycemia baseline expression of the catecholamine biosynthetic enzyme D β H and key metabolic enzyme proteins and 2) to ascertain how habituated basal profiles of these proteins react to re-induction of hypoglycemia. Individual A2 neurons were identified *in situ* by immunocytochemistry and laser-catapult microdissected from brains of estradiol- vs. vehicle-implanted OVX rats after recovery from serial bouts of insulin-induced hypoglycemia or during re-exposure to hypoglycemia. Pooled A2 cells were evaluated by high-sensitivity Western blotting to determine expression of rate-limiting metabolic, e.g. glycolytic, tricarboxylic acid cycle, respiratory chain, and oxidative phosphorylation, acetyl-CoA carboxylase-AMPK-fatty acid synthase pathway, and D β H enzyme protein expression between and during bouts of recurring hypoglycemia.

METHODS AND MATERIALS

Animals

Adult female Sprague Dawley rats (225–250 g *bw*) were housed in groups of 2–3 per cage under a 14 hr light: 10 hr dark schedule (lights on at 05.00 hr), and gentled by daily handling. Rats were obtained from the ULM School of Pharmacy Vivarium in-house breeding colony; breeders were purchased from Harlan Laboratories, Inc. Rats had unrestricted access to standard laboratory chow and water.

All animal protocols were conducted in accordance with National Institutes of Health guidelines for care and use of laboratory rodents, and approved by the ULM Institutional Animal Care and Use Committee. On day 1, rats were anesthetized with ketamine/xylazine (0.1 mL/100 g *bw sc*; 90 mg ketamine: 10 mg xylazine/mL; Henry Schein Inc., Melville, NY), then bilaterally OVX and implanted with a subcutaneous (*sc*) capsule (*i.d.* 1.58 mm, *o.d.* 3.18 mm; 10 mm/100 g *bw*) filled with 17 β estradiol-3-benzoate (E; 30 μ g/mL safflower oil) or oil (O) (Briski et al. 2001). This hormone replacement strategy results in circulating plasma estradiol concentrations that mimic endogenous hormone concentrations measured on metestrus in 4-day (Butcher et al. 1974) or on diestrus day-2 in 5-day cycling rats (Goodman 1978). After surgery, rats were transferred to individual cages.

Experimental design

At 11.00 hr on days 6–8, groups of estradiol- and oil-implanted OVX rats were injected *sc* with neutral protamine Hagedorn insulin (I; 12.5 U/kg/mL *sc*; Henry Schein Inc.; n=8 E-implanted; n=8 O-implanted) or vehicle (V; n=4 E-implanted; n=4 O-implanted). At 11.00 hr on day 9, unfasted rats previously injected with V, one dose per day, on three previous days, received a final/fourth injection of V [n=4 E-implanted (E-V⁴); n=4 O-implanted (O-V⁴)] at the same time, rats treated with 3 prior daily I injections were injected with V [n=4 E-implanted (E-I³V); n=4 O-implanted (O-I³V)] or I [n=4 E-implanted (E-I⁴); n=4 O-implanted (O-I⁴)] (Fig. 1). E-V⁴ and O-V⁴ groups were included in the design in order to assess effects of precedent hypoglycemia on baseline patterns of protein expression. Trunk blood and brain tissue were collected at 13.00 hr, at time point that coincides with the glucose nadir achieved by this insulin treatment paradigm (Paranjape and Briski 2005, Nedungadi et al. 2006). Rats were killed by rapid decapitation, a method approved by American Veterinary Medical Association Guidelines on Euthanasia (AVMA 2013). Dissected hindbrains were snap-frozen in liquid nitrogen-cooled isopentane.

Western blot analysis of A2 nerve cell and cDVC metabolic and neurotransmitter biosynthetic enzyme protein expression

Serial 10 μ m frozen sections of the cDVC (–14.36 to –14.86 mm posterior to bregma) were cut in a cryostat and processed for tyrosine hydroxylase (TH)-immunoreactivity (–ir) (Ibrahim et al. 2013). Only TH-ir-positive neurons characterized by an intact, visible nucleus and complete staining of the cytoplasmic compartment were individually removed from tissue sections using a P.A.L.M. UV-A

microlaser (Carl Zeiss Microimaging, Inc., Thornwood, NY). The cDVC was bilaterally removed from serial 200 μm frozen hindbrain sections obtained from additional rats ($n=4/\text{treatment combination}$), using calibrated hollow micropunch needles (Stoelting, Inc., Kiel, WI); micropunched tissue was pooled for each rat. For each protein of interest, $n=50$ TH-ir cells per treatment group (12–13 cells/protein/rat; $n=4$ rats/treatment group) were collected in lysis buffer for immunoblotting; each protein was analyzed in triplicate in separate lysate pools. cDVC aliquots from each rat were pooled within each treatment group; pyruvate dehydrogenase (PDH), succinate dehydrogenase (SDH), and oxidative phosphorylation enzyme complexes were independently evaluated in triplicate in separate sample pools. Protein separation and transblotting were performed as described (Cherian and Briski 2011, 2012). The following primary antisera were used to analyze A2 proteins: phosphofructokinase (PFKL; 30-17/12108-1101, ProSci., Inc., Poway, CA; rabbit polyclonal, 1:5000); pyruvate dehydrogenase (PDH E2/E3pb; MSP02/D0959-ab110416, MitoSciences, Eugene, OR; mouse monoclonal, 1:5000); isocitrate dehydrogenase (IDH2; 26-297/12108-1101, ProSci.; rabbit polyclonal, 1:5000); alpha ketoglutarate dehydrogenase (OGDH; MS202/HCESHC-ab110410, MitoSci.;

mouse monoclonal, 1:5000); succinate dehydrogenase (SDH, Complex II-C-V-alpha; MS202/HCESHC-ab110410, MitoSci., mouse monoclonal, 1:5000); oxidative phosphorylation (ATP synthase- α ; MS604/D1848-ab110413, MitoSci., mouse monoclonal, 1:5000); Ca^{++} /calmodulin-dependent protein kinase beta (CaMM $\kappa\beta$ L-19, sc-9629, Santa Cruz Biotechnol., Inc, Santa Cruz, CA; goat polyclonal, 1:5000); protein phosphatase 2A (PP2A-C α/β L-19; sc-81601/J1410; Santa Cruz Biotechnol.; mouse monoclonal, 1:5000); AMPK α 1/2 (sc-25792, Santa Cruz Biotechnol.; rabbit polyclonal, 1:5000); phosphoAMPK α 1/2 (pAMPK) (sc-33524, Santa Cruz Biotechnol., rabbit polyclonal, 1:5000); acetyl CoA carboxylase (ACC) (3662S/4, Cell Signaling Technol., Danvers, MA; rabbit polyclonal, 1:5000); phosphoACC (pACC S79; 3661S/8, Cell Signaling; rabbit polyclonal, 1:5000); fatty acid synthase (FAS) (C2065; 3180S/2, Cell Signaling; rabbit polyclonal, 1:5000); and D β H (sc-15318, Santa Cruz Biotechnol.; rabbit polyclonal, 1:500). DVC tissue was probed with PDH, SDH, and oxidative phosphorylation protein complex antisera to detect the following proteins: PDH: PDH-E2 (72 kDa); PDH-E2/E3bp (55 kDa); and PDH-E1-alpha (42 kDa); SDH: C-V-alpha (F1-alpha; 53 kDa); C-II-30 (SDHB; 30 kDa); and C-II-70 (SDHA; 70 kDa); oxidative phosphorylation: ATP synthase (Complex V) subunit alpha (CV-alpha; 53 kDa);

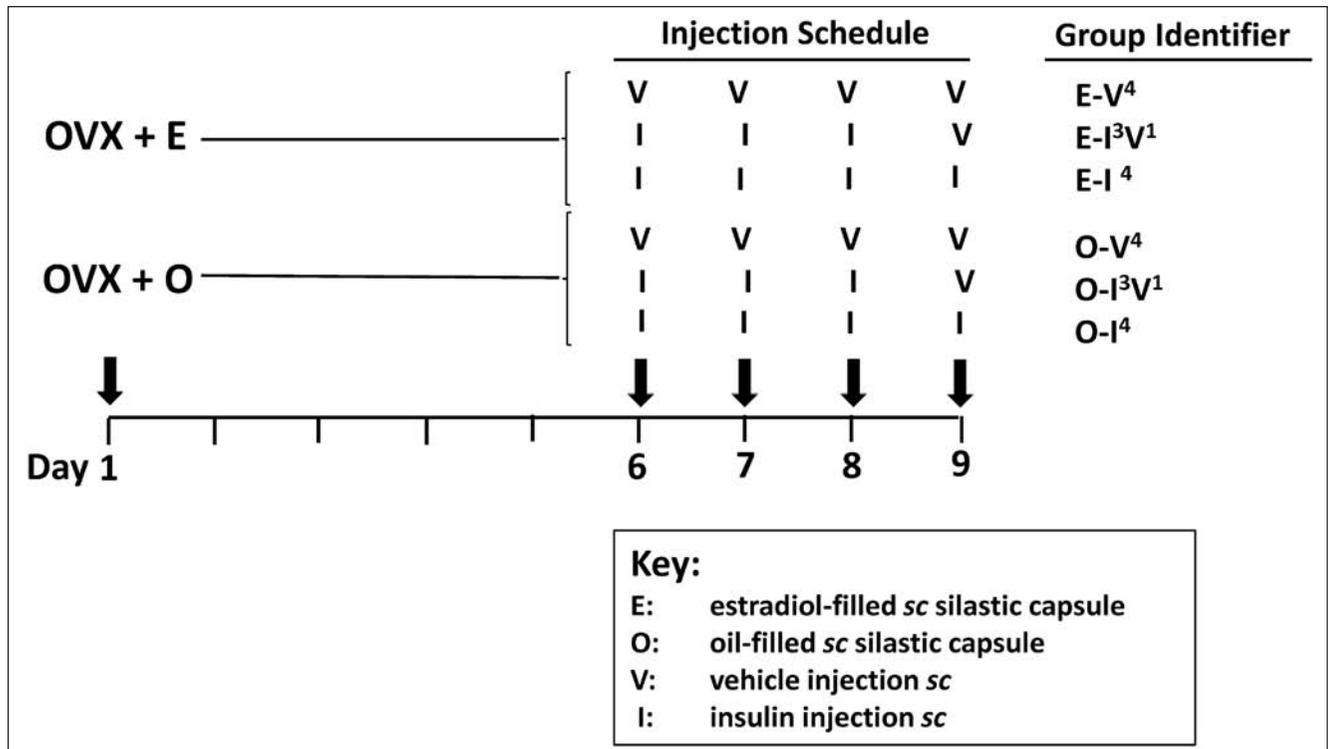


Fig. 1. Outline of experiment schedule and treatment group identification. Adult female rats were bilaterally ovariectomized (OVX) and implanted with a subcutaneous capsule (*i.d.* 1.58 mm, *o.d.* 3.18 mm; 10 mm/100 g *bw*) filled with 17 β estradiol-3-benzoate (30 $\mu\text{g}/\text{mL}$ safflower oil) or oil on day 1 of the study. On experiment days 6–8, rats were injected at 11.00 hr with vehicle (V) or neutral protamine Hagedorn insulin (I; 12.5 U/kg *bw*), one dose per day. On day 9, rats previously injected with V were given a final injection of V [$n=4$ estradiol-implanted (E-V⁴); $n=4$ oil-implanted (O-V⁴)]. At the same time, rats previously injected with I were given a V injection [$n=4$ estradiol-implanted (E-I³V¹); $n=4$ oil-implanted (O-I³V¹)] or a fourth I dose [$n=4$ estradiol-implanted (E-I⁴); $n=4$ oil-implanted (O-I⁴)].

Q-cytochrome c oxidoreductase (Complex III) subunit Core 2 (CIII-core 2; 47 kDa); Cytochrome c oxidase (Complex IV) subunit I (CIV-I; 39 kDa); Succinate Dehydrogenase (Complex II) subunit 30 kDa (CII-30; 30 kDa); and NADH-coenzyme Q oxidoreductase (Complex I) subunit NDUFB8 (CI-20; 20 kDa). The loading control, α -tubulin, was probed with mouse monoclonal antibodies (CP06; Calbiochem, Gibbstown, NJ; 1:2000). Membranes were incubated with peroxidase-conjugated goat anti-mouse (NEF822001EA; 1:5000; Perkin Elmer, Boston, MA); goat anti-rabbit (NEF812001EA; 1:5000; Perkin Elmer, Waltham, MA); or donkey anti-goat (sc-2020; 1:5000; Santa Cruz Biotechnol.) secondary antibodies, then exposed to Supersignal West Femto Maximum Sensitivity substrate (Thermo Fisher Scientific, Waltham, MA). Chemiluminescent signal was visualized in a Syngene G:box Chemi (Syngene USA, Frederick, MD). Immunoblot densities were quantified with AlphaImager HP V 5.0.1 software (Cell Biosciences; Santa Clara, CA); optical density (O.D.) values were normalized to α -tubulin. Protein MW markers were included in each blot.

Blood analyte measurements

Blood glucose concentrations were measured with an AccuCheck Advantage glucometer (Roche diagnostics, Indianapolis, IN), as described (Kale et al. 2006). Plasma estradiol concentrations were determined by radioimmunoassay, as reported (Briski et al. 2001); assay sensitivity is approximately 1.5 pg/mL, with intra- and inter-assay variabilities of 4.2 and 6.9%.

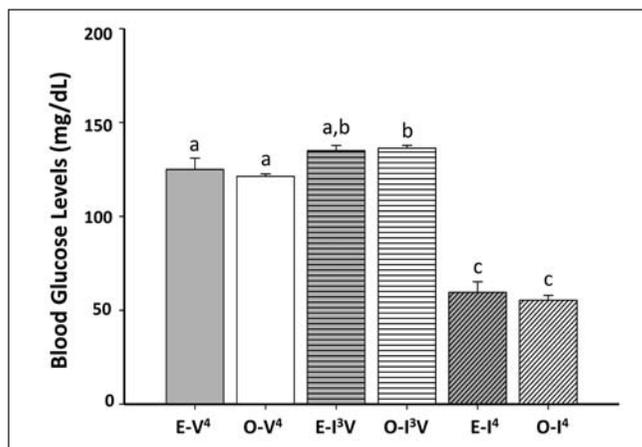


Fig. 2. Effects of precedent insulin (I) - induced hypoglycemia on blood glucose concentrations after I or vehicle (V) injection of estradiol- or oil-implanted ovariectomized (OVX) female rats. Groups of OVX+estradiol (E) and OVX+oil (O) groups were injected as follows: 1) four doses of V, on as many days [n=4 E-V⁴; n=4 O-V⁴]; 2) three daily doses of I (12.5U neutral protamine Hagedorn insulin/kg *bw sc*), followed by V on day four [n=4 E-I³V; n=4 O-I³V]; or 3) four doses of I, one per day [n=4 E-I⁴; n=4 O-I⁴]. Bars depict mean blood glucose concentrations \pm SEM at +2 hr after final treatments on day four. Data labeled with dissimilar letters differ statistically.

Statistics

Mean circulating glucose and estradiol concentrations and normalized protein O.D. measures were evaluated by two-way ANOVA and Student-Newman-Keuls *post-hoc* test. Differences of $p < 0.05$ were considered significant.

RESULTS

Fig. 2 shows that estradiol- and oil-implanted OVX rats injected with vehicle (V) over the four day experiment exhibited comparable blood glucose levels [E-V⁴ vs. O-V⁴] ($F_{5,18}=92.4$, $p < 0.0001$; insulin effect: $F=230.9$, $p < 0.0001$). Injection of insulin (I) on study days 1–3 did not modify baseline glucose levels on day 4 in E-I³V (vs. E-V⁴), but glucose was elevated in O-I³V compared to O-V⁴. Treatment with a fourth of four doses of insulin suppressed blood glucose in E-I⁴ and O-I⁴ to an equivalent extent. Sc E-filled capsule implantation resulted in plasma hormone concentrations of 21.4 \pm 0.3 (E-V⁴), 21.9 \pm 0.4 (E-I³V), and 21.7 \pm 0.4 (E-I⁴) pg/mL; circulating estradiol was undetectable in groups of OVX+oil rats.

Acclimation of rate-limiting glycolytic, tricarboxylic acid cycle, and respiratory chain enzyme protein expression to hypoglycemia in A2 neurons: impact of estradiol

Fig. 3 illustrates effects of serial insulin injections on basal and hypoglycemic expression profiles for the glycolytic enzyme PFKL (Fig. 3A); the PDH subunit protein, E2/E3pb (Fig. 3B); tricarboxylic acid cycle enzymes, IDH2 (Fig. 3C) and OGDH (Fig. 3D); the respiratory chain Complex II protein subunit, C-V-alpha (Fig. 3E), and ATP synthase- α (Fig. 3F) in cDVC A2 neurons. Data show that these proteins, excepting OGDH, were increased in E-V⁴ vs. O-V⁴. Following insulin treatment on days 1–3, A2 PFKL protein levels on day 4 (Fig. 3A) were elevated in E-I³V, but not O-I³V vs. respective V⁴ controls [$F_{5,12}=29.9$, $p < 0.0001$; estradiol effect: $F=105.0$, $p < 0.0001$; insulin effect: $F=20.0$, $p=0.001$]. Day 4 Insulin injection did not alter this profile in E-I⁴ or O-I⁴. Insulin dosing on days 1–3 did not alter PDH E2/E3 pb (Fig. 3B) protein expression on day 4 (E-I³V vs. E-V⁴; O-I³V vs. O-V⁴) [$F_{5,12}=11.4$, $p=0.0003$; estradiol effect: $F=36.9$, $p < 0.0001$; insulin effect: $F=9.97$, $p=0.003$]. Yet, insulin injection on day 4 reduced PDH E2/E3 pb protein expression in E-I⁴ and Oil-I⁴. Figs 3C and 3D indicate that A2 IDH2 [$F_{5,12}=11.2$, $p=0.003$; estradiol effect: $F=51.3$, $p < 0.0001$] and OGDH [$F_{5,12}=19.7$, $p < 0.0001$; estradiol effect: $F=62.8$, $p < 0.0001$; insulin effect: $F=4.82$, $p=0.03$; estradiol and insulin interaction: $F=13.0$, $p=0.001$] proteins were unaltered in E-I³V or O-I³V vs. V⁴ controls. Insulin injection on day 4 did

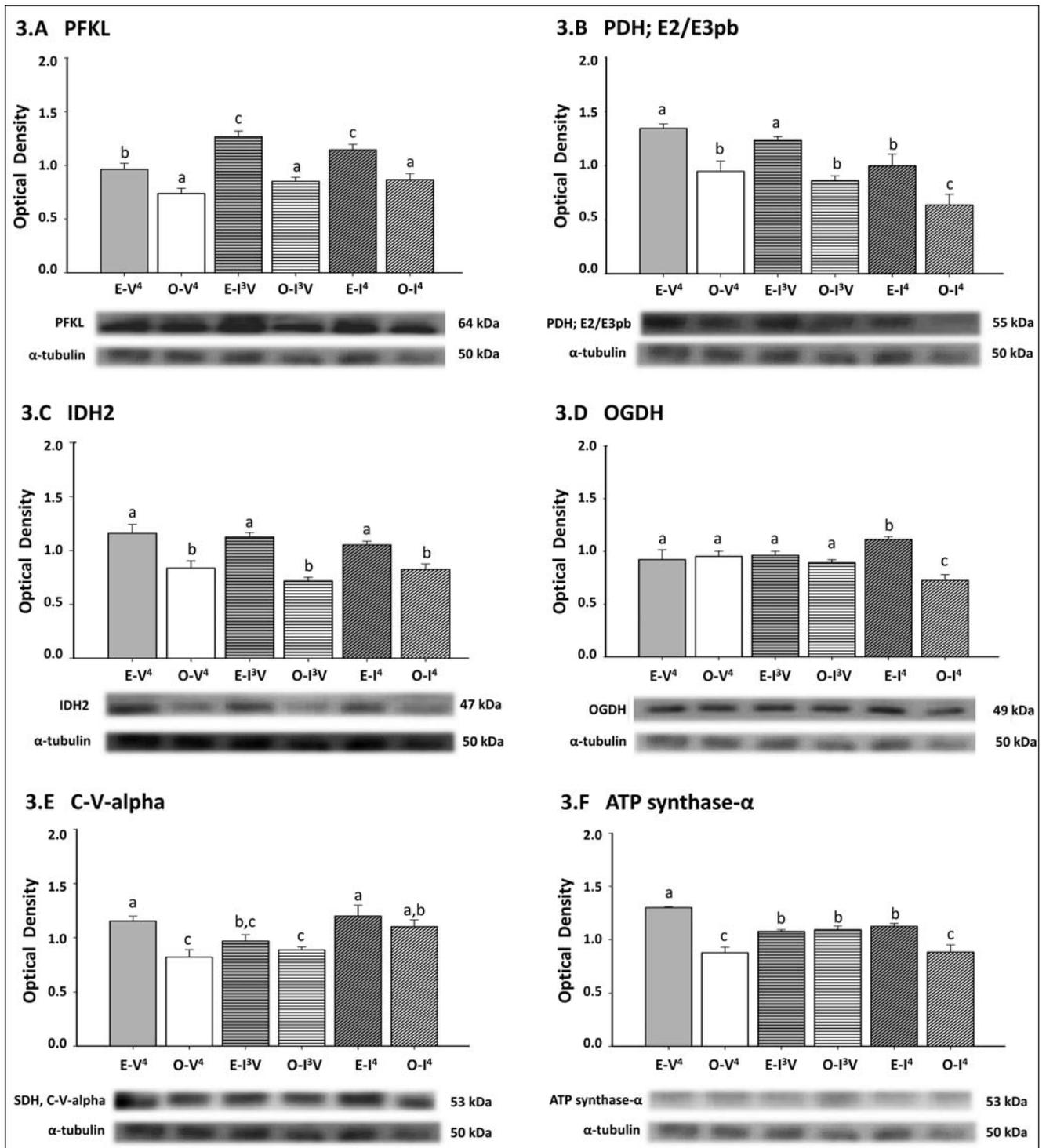


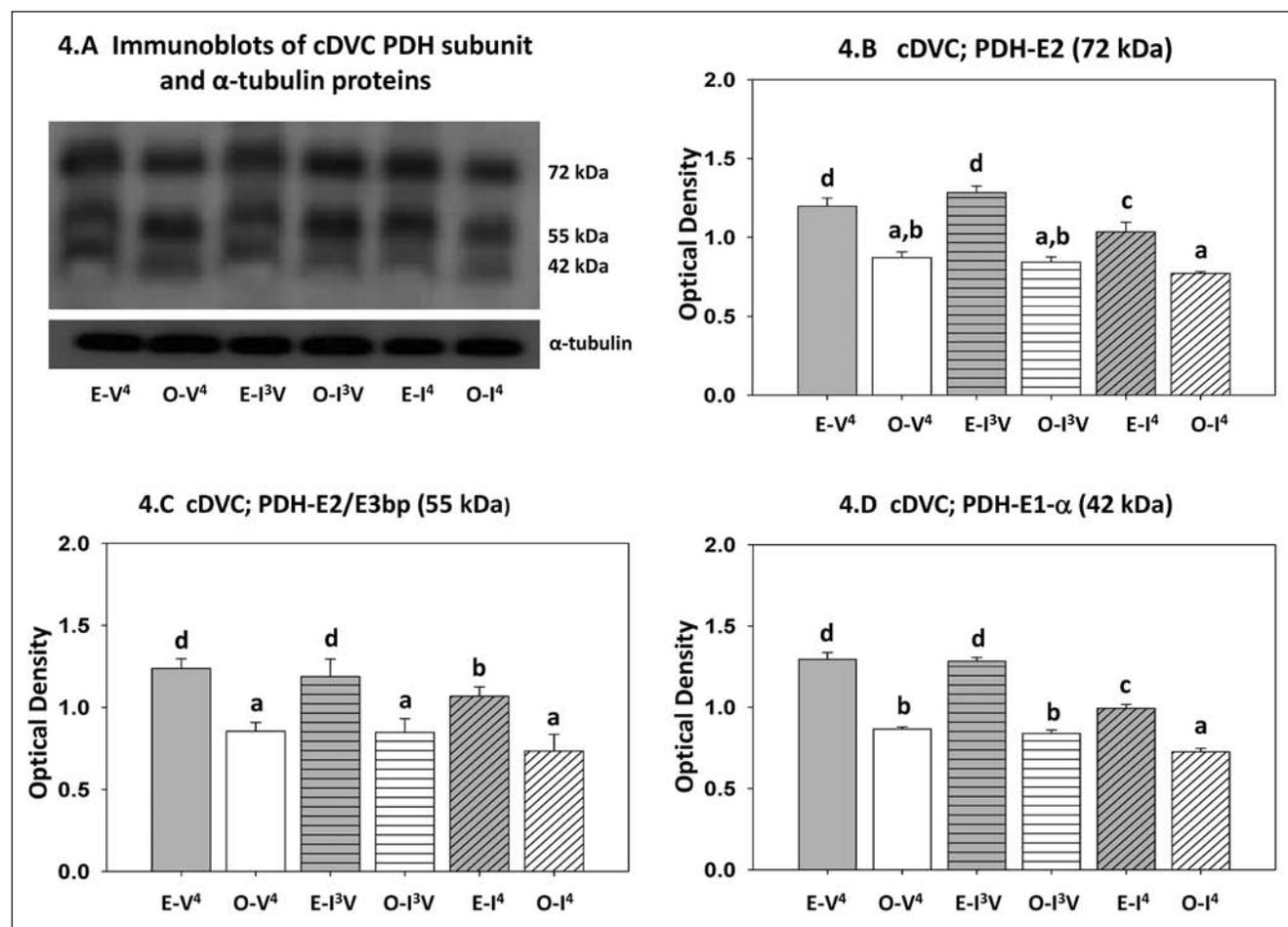
Fig. 3. Effects of precedent hypoglycemia on basal and hypoglycemic patterns of A2 nerve cell energy metabolism enzyme protein expression in ovariectomized OVX+E vs. OVX+O female rats. Tyrosine hydroxylase (TH)-immunolabeled A2 neurons from the caudal dorsal vagal complex of E-V⁴ [solid grey bars], O-V⁴ [solid white bars], E-I^{3V} [horizontal striped grey bars], O-I^{3V} [horizontal striped white bars], E-I⁴ [diagonal striped grey bars], and O-I⁴ [diagonal striped white bars] treatment groups (n=4 rats/group). Separate pooled lysates of n=50 A2 cells/group (n=12–13 neurons/rat) were analyzed by Western blot for the rate-limiting glycolytic enzyme, phosphofruktokinase (PFKL) (A); the pyruvate dehydrogenase complex subunit protein E2/E3 pb (B); the rate-limiting tricarboxylic acid cycle enzymes, isocitrate dehydrogenase (IDH2) (C) and alpha ketoglutarate dehydrogenase (OGDH) (D); the respiratory chain complex II protein subunit, C-V-alpha (E), or ATP synthase-α (F). Each protein analysis was carried out on triplicate lysate pools. Protein band optical densities (O.D.) were quantified with Alphasampler HP V 5.0.1 software, and expressed relative to the loading control, α-tubulin. Graphs depict mean normalized O.D. values ±SEM for each protein for each treatment group. Representative immunoblots are shown below each graph. Data labeled with dissimilar letters differ statistically.

not modify IDH2 expression, but respectively increased or decreased OGDH in E-I⁴ or O-I⁴. Fig. 3E shows that C-V-alpha protein was lower in E-I^{3V} vs. E-V⁴, but equivalent in O-I^{3V} vs. O-V⁴; insulin dosing on day 4 elevated this profile in both E-I⁴ and O-I⁴ [$F_{5,12}=7.80$, $p=0.002$; estradiol effect: $F=20.6$, $p=0.0007$; insulin effect: $F=6.48$, $p=0.012$]. Data in Fig. 3F show respective inhibitory or stimulatory effects of precedent insulin dosing on A2 ATP synthase- α protein [$F_{5,12}=22.2$, $p<0.0001$]. Insulin injection on day 4 did not alter (E-I⁴) or inhibited (O-I⁴) this profile [estradiol effect: $F=92.6$, $p<0.0001$; insulin effect: $F=7.03$, $p=0.009$].

Acclimation of caudal dorsal vagal complex pyruvate dehydrogenase, complex II, and respiratory chain complex I-V enzyme protein profiles to hypoglycemia: impact of estradiol

Figs 4A–4D illustrates precedent insulin dosing effects on cDVC PDH subunit protein expression in estradiol- vs. oil-implanted OVX rats. Fig. 4A depicts representative immunoblots of PDH-E2 (72 kDa), PDH-E2/E3bp (55 kDa), PDH-E1- α (42 kDa), and α -tubulin proteins in E-V⁴; O-V⁴;

E-I^{3V}; O-I^{3V}; Est/I⁴; and Oil/I⁴ cDVC tissue. Fig. 4B [PDH-E2: $F_{5,12}=24.1$, $p<0.0001$; estradiol effect: $F=99.3$, $p<0.0001$; insulin effect: $F=8.27$, $p=0.0055$], Fig. 4C [PDH-E2/E3bp: $F_{5,12}=87.3$, $p<0.0001$; estradiol effect: $F=33.0$, $p<0.0001$; insulin effect: $F=45.1$, $p<0.0001$]; estradiol and insulin interaction: $F=7.45$, $p=0.008$], and 4.D [PDH-E1- α : $F_{5,12}=5.40$, $p=0.008$; estradiol effect: $F=22.7$, $p=0.0005$] show that each subunit was elevated in E-V⁴ vs. O-V⁴. Precedent insulin dosing did not modify baseline protein subunit expression, but PDH-E2 and PDH-E1- α proteins were diminished in E-I⁴ but not O-I⁴. Fig. 4E depicts representative immunoblots of complex II subunit C-II-70 (SDHA; 70 kDa), C-V-alpha (F1-alpha; 53 kDa), C-II-30 (SDHB; 30 kDa), and α -tubulin in cDVC tissue. Data in Panels 4.F [C-II-70: $F_{5,12}=4.94$, $p=0.011$; estradiol effect: $F=19.7$, $p=0.001$], 4.G [C-V-alpha: $F_{5,12}=7.986$, $p=0.002$; estradiol effect: $F=24.7$, $p=0.0003$; insulin effect: $F=4.19$, $p=0.042$], and 4.H [C-II-30: $F_{5,12}=5.16$, $p=0.009$; estradiol effect: $F=14.0$, $p=0.003$; insulin effect: $F=5.18$, $p=0.023$] show that subunit levels were elevated in E-V⁴ vs. O-V⁴. Precedent hypoglycemia did not alter baseline or hypoglycemia-induced patterns of SDH subunit expression. Panel 4.I depicts representative immunoblots of Complex V (CV) ATP synthase- α (53 kDa), Complex III (CIII) subunit Core 2 (CIII-core 2; 47 kDa), Complex IV (CIV) subunit I (CIV-I; 39 kDa),

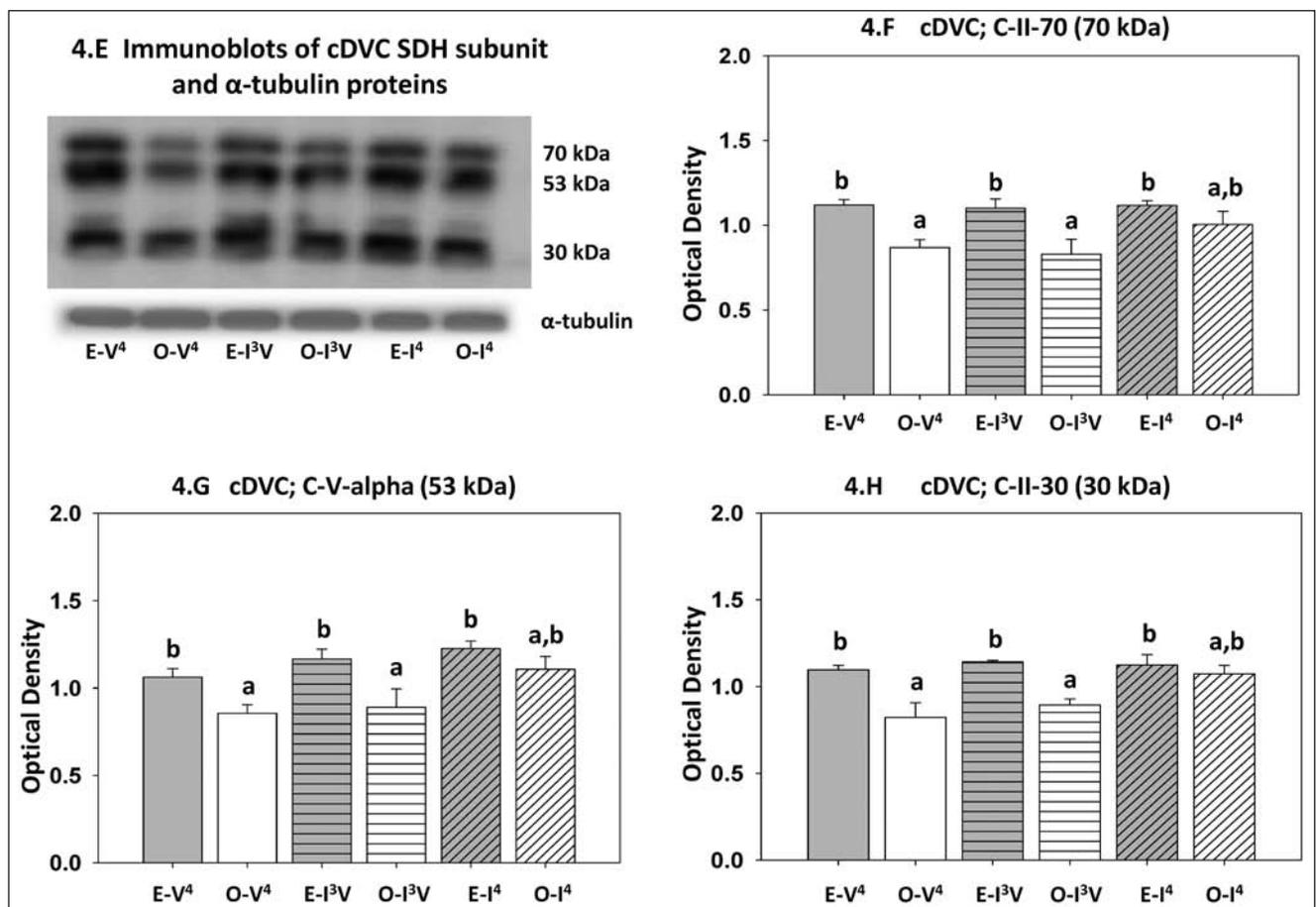


Complex II (CII) subunit 30 kDa (CII-30; 30 kDa), and Complex I (CI) subunit NDUFB8, CI-20 (20 kDa) proteins detected in cDVC tissue from estradiol- and oil-implanted OVX rats. Quantitative data show that cDVC ATP synthase-subunit alpha [Fig. 4J; $F_{5,12}=4.51$, $p=0.015$; estradiol effect: $F=16.3$, $p=0.002$], CIII-core [Fig. 4K; $F_{5,12}=34.4$, $p<0.0001$; estradiol effect: $F=81.4$, $p<0.0001$], CIV [Fig. 4L; $F_{5,12}=17.1$, $p<0.0001$; insulin effect: $F=27.1$, $p<0.0001$; estradiol and insulin interaction: $F=5.92$, $p=0.016$], CII-30 [Fig. 4M; $F_{5,12}=3.91$, $p=0.025$; insulin effect: $F=9.34$, $p=0.004$], and CI [Fig. 4N; $F_{5,12}=11.7$, $p=0.0003$; estradiol effect: $F=6.63$, $p=0.024$; insulin effect: $F=24.5$, $p<0.0001$] levels were higher in E-V⁴ vs. O-V⁴. Precedent hypoglycemia elevated CIV-I expression in O-I³V, reduced CIII levels in E-I³V, and increased ATP synthase- α in O-I³V. Insulin injection on day 4 decreased CIV-I, CII-30, and CI-20 profiles in E- and O-I⁴, but only reduced CIII-core 2 and ATP synthase- α proteins in the latter.

Acclimation of A2 neuron AMPK and upstream kinase and phosphatase protein expression to hypoglycemia: impact of estradiol

Figs 5A and 5B depict effects of daily insulin dosing on A2 neuron AMPK and pAMPK proteins. Data show

that precedent insulin dosing did not modify AMPK [$F_{5,12}=29.742$, $p<0.0001$; estradiol effect: $F=5.99$, $p=0.031$; insulin effect: $F=64.9$, $p<0.001$; estradiol and insulin interaction: $F=6.51$, $p=0.012$] or pAMPK [$F_{5,12}=15.7$, $p<0.0001$; estradiol effect: $F=16.5$, $p=0.023$; insulin effect: $F=35.4$, $p<0.0001$; estradiol and insulin interaction: $F=9.23$, $p=0.002$] levels. A fourth insulin injection significantly augmented both proteins, albeit to a greater extent in O-I⁴ vs. E-I⁴. Data in Figs 5C and 5D show that the upstream kinase, CaMM κ β [$F_{5,12}=25.305$, $p<0.0001$; estradiol effect: $F=19.1$, $p<0.0001$] and upstream phosphatase, protein phosphatase 2A (PP2A) [$F_{5,12}=23.9$, $p<0.0001$; estradiol effect: $F=87.1$, $p<0.0001$; insulin effect: $F=15.3$, $p=0.0005$] are correspondingly elevated or diminished in O-V⁴ vs. E-V⁴. Insulin dosing on days 1–3 did not modify either profile. Insulin injection on day 4 did not change CaMM κ β expression, but suppressed PP2A expression in E-I⁴. Figs 5E and 5F show that precedent hypoglycemia had no effect on ACC [$F_{5,12}=19.0$, $p<0.0001$; estradiol effect: $F=28.3$, $p=0.0002$; insulin effect: $F=32.3$, $p<0.0001$] or pACC [$F_{5,12}=10.5$, $p=0.0005$; estradiol effect: $F=30.865$, $p=0.0001$; estradiol and insulin interaction: $F=8.41$, $p=0.005$] expression in E-I³V (vs. E-V⁴), but correspondingly increased and decreased these proteins in O-I³V vs.



O-V⁴. Insulin injection on day 4 increased ACC and pACC profiles in E-I⁴, but not O-I⁴. Data in Fig. 5G show that FAS protein levels [$F_{5,12}=6.69$, $p=0.003$; estradiol effect: $F=17.6$, $p=0.001$; insulin effect: $F=4.75$, $p=0.03$] were refractory to precedent insulin administration, but were augmented by insulin dosing on day 4 in E-I⁴, but not in Oil-I⁴.

Acclimation of A2 neuron D β H enzyme protein expression to hypoglycemia: impact of estradiol

Fig. 6 shows that D β H protein expression was higher in E-V⁴ vs. O-V⁴ [$F_{5,12}=36.2$, $p<0.0001$; insulin effect: $F=7.33$, $p=0.008$; estradiol and insulin interaction: $F=82.8$, $p<0.0001$].

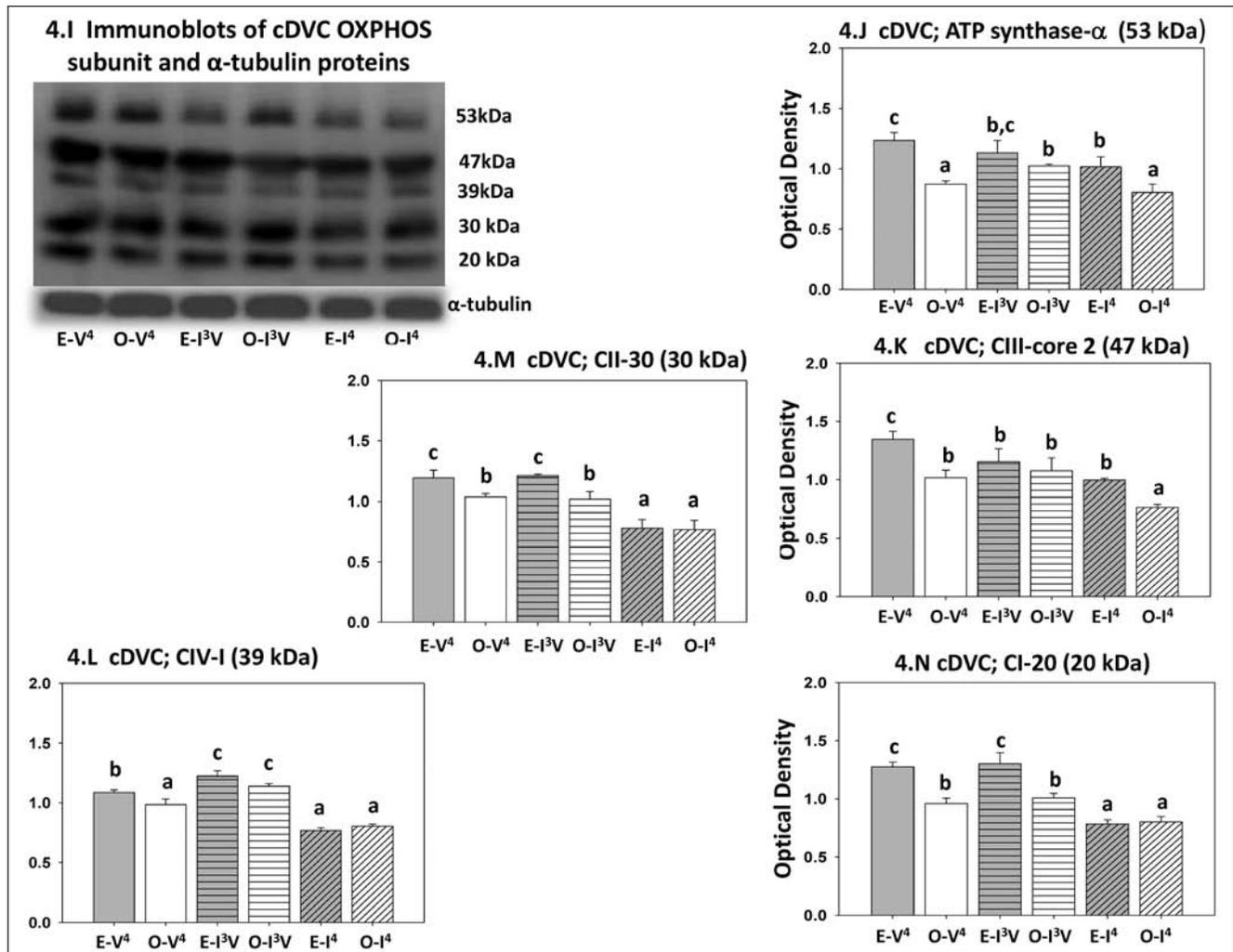


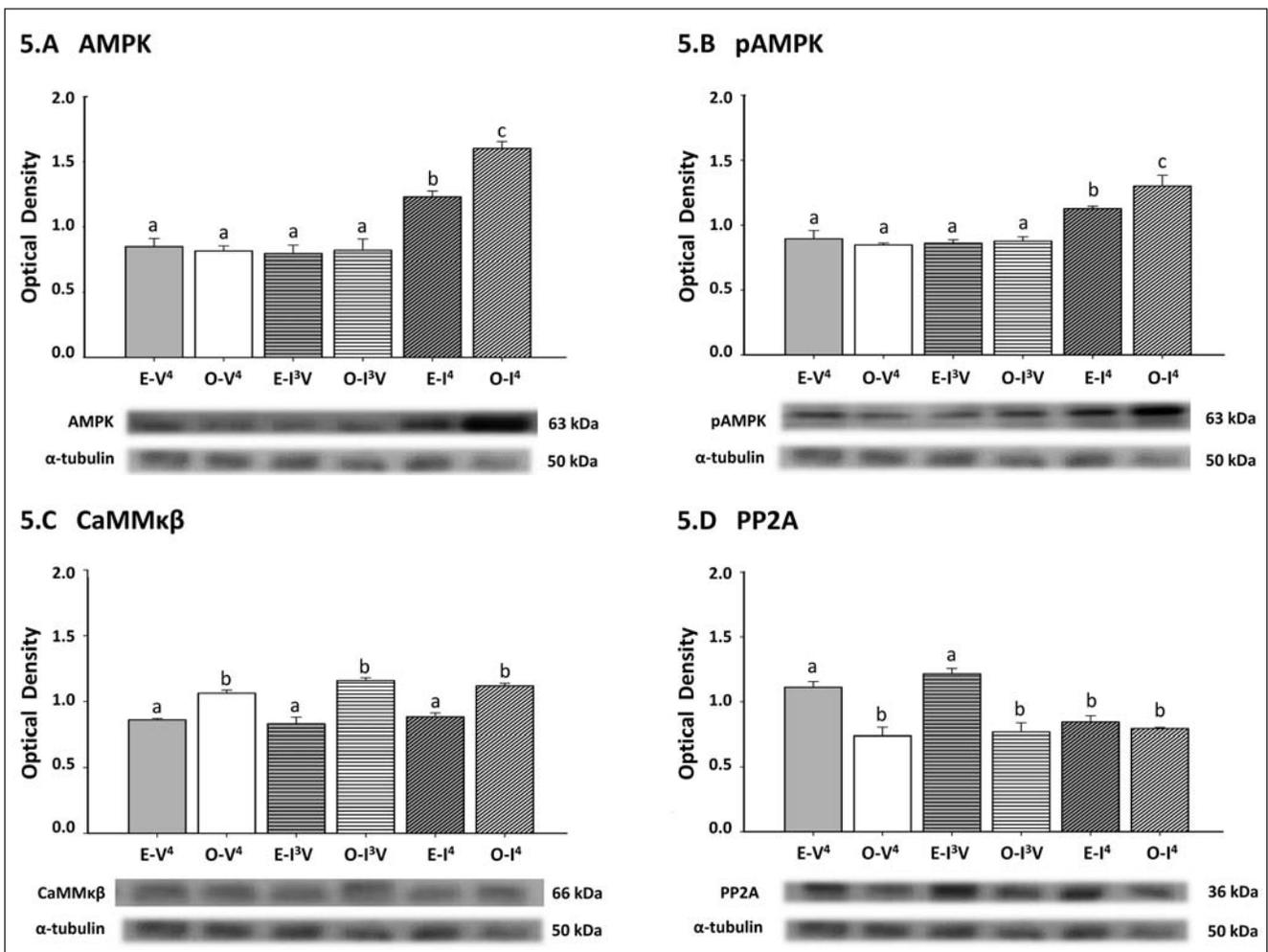
Fig. 4. Effects of precedent hypoglycemia on basal and hypoglycemic patterns of caudal dorsal vagal complex (cDVC) pyruvate dehydrogenase (PDH), succinate dehydrogenase (SDH), and respiratory chain complex subunit protein expression in ovariectomized OVX+estradiol vs. OVX+oil female rats. cDVC aliquots for each treatment group ($n=4$ rats/group) were evaluated for PDH complex proteins; analyzes were performed on separate triplicate aliquots. Representative immunoblots in Fig. 4A show that PDH-E2, PDH-E2/E3bp, and PDH-E1-alpha subunits were detected in each treatment group. Bar graphs show mean normalized O.D. values \pm SEM ($n=4$ /group) for cDVC 72 kDa (B), 55 kDa (C), and 42 kDa (D) PDH subunit expression for the following treatment groups: E-V⁴ [solid grey bars], O-V⁴ [solid white bars], E-I^{3V} [horizontal striped grey bars], O-I^{3V} [horizontal striped white bars], E-I⁴ [diagonal striped grey bars], and O-I⁴ [diagonal striped white bars]; data labeled with dissimilar letters show statistical difference. The SDH subunit immunoblot in Fig. 4E shows that C-V-alpha (53 kDa), C-II-30 (30 kDa), and C-II-70 (70 kDa) proteins were detectable in cDVC tissue from OVX+estradiol and OVX+oil rats. Bar graphs in Figs 4F–4H depict mean normalized O.D. values \pm SEM for cDVC 70 kDa, 53 kDa, and 30 kDa subunit proteins, respectively. The immunoblot in Fig. 4I shows that Complex V (CV) ATP synthase-subunit alpha (53 kDa), Complex III (CIII) subunit Core 2 (CIII-core 2; 47 kDa), Complex IV (CIV) subunit I (CIV-I; 39 kDa), Complex II (CII) subunit 30 kDa (CII-30; 30 kDa), and Complex I (CI) subunit NDUFB8, CI-20 (20 kDa) were each detected in cDVC tissue from estradiol- or oil-implanted OVX rats. Mean O.D. values \pm SEM for cDVC Complex I-V protein subunit expression are presented in Fig. 4J (ATP synthase-subunit alpha), Fig. 4K (cDVC CIII-core), Fig. 4L (cDVC CIV-I), Fig. 4M (cDVC CII-30), and Fig. 4N (cDVC CI-20). Data show that PDH enzyme subunits were down-regulated in E-V⁴ vs. O-V⁴. Baseline protein profiles were refractory to prior hypoglycemia, but PDH-E2 and PDH-E1-alpha proteins were reduced in E-I⁴ but not O-I⁴ in response to a final bout of hypoglycemia. Prior hypoglycemia did not modify baseline or hypoglycemia-induced patterns of SDH subunit expression. Moreover, precedent hypoglycemia elevated basal CIV-I expression in O-I^{3V}; decreased CIII protein levels in E-I^{3V}; and elevated ATP synthase profiles in both E- and O-I^{3V}. A final hypoglycemic bout decreased CIV-I, CII-30, and CI-20 in both E- and O-I⁴, but only reduced CIII-core 2 and ATP synthase in O-I⁴.

Insulin dosing on days 1–3 correspondingly increased or decreased this protein in E-I³V or O-I³V. Insulin injection on day 4 further inhibited or augmented this profile in E-I⁴ and O-I⁴, respectively.

DISCUSSION

The current studies investigated the hypothesis that estradiol regulation of recurring hypoglycemia on A2 catecholamine neurotransmitter synthesis correlates with neuro-protective effects of this hormone on energy metabolic pathway function during and between bouts of hypoglycemia. Precedent exposure to serial bouts of hypoglycemia caused opposite changes in basal A2 neuron D β H protein expression in estradiol- vs. oil-implanted OVX female rats, plus exacerbation of these divergent adjustments by renewed hypoglycemia. As these cells communicate hypoglycemia-associated cell energy imbalance signaling (Gujar et al. 2014; Shrestha et al. 2014), estradiol-dependent adaptive down-regulation of noradrenergic neurotransmission by A2 neurons implies that this hormone may promote a positive energy state during this

recurring metabolic stress. Estradiol also regulates effects of recurrent hypoglycemia on enzyme proteins involved in energy metabolism and fatty acid synthesis. As A2 cells exhibit elevated baseline expression of the rate-limiting glycolytic enzyme PFKL in the aftermath of hypoglycemia, coincident with diminished C-V-alpha and ATP synthase- α protein profiles, estradiol may stimulate glycolysis, yet suppress mitochondrial aerobic respiration/energy production. During the fourth of four daily hypoglycemic episodes, estradiol permitted OGDH up-regulation and prevented down-regulation of ATP synthase- α , implying that it may beneficially alleviate energy state negativity in A2 cells during re-exposure to this stress. This view is bolstered by evidence that A2 AMPK activation is greater in O vs. E rats in response to recurring hypoglycemia. Taken together, current data suggest that estradiol-mediated reductions in A2 noradrenergic signaling may reflect, in part, energetic resilience of these cells to recurring hypoglycemia. Further research is needed to determine effects of adaptive adjustments in between-hypoglycemia A2 neurotransmission on glucostatic and non-glucostatic functions governed by this cell group.



Acclimation of rate-limiting glycolytic, tricarboxylic acid cycle, and respiratory chain enzyme protein expression to hypoglycemia in A2 neurons: impact of estradiol

Precedent hypoglycemia exerts divergent, e.g. stimulatory or inhibitory effects on A2 PFKL vs. C-V-alpha and ATP synthase- α protein profiles in the E-I³V treatment group. Adaptive augmentation of baseline PFKL expression reported here is accompanied by a loss of this positive reaction to hypoglycemia in E-I⁴ (Tamrakar et al. 2015). These results suggest that estradiol may enhance glycolytic yield of ATP and NADH, while diminishing mitochondrial fuel catabolism and aerobic respiration in the aftermath of this stress. In contrast, augmentation of basal ATP synthase- α protein expression in post-hypoglycemic

OVX+oil rats implies that, in the absence of estradiol, increased energy output may occur via oxidative phosphorylation as A2 cells recover from hypoglycemia. In Est/I⁴ group, renewed exposure to hypoglycemia, e.g. induction of a fourth daily bout, did not further modify A2 PFKL expression relative to the heightened day four baseline, suggesting that the glycolytic pathway may adopt a heightened steady-state that is refractory to recurring hypoglycemic episodes. Re-exposure to hypoglycemia on day four suppressed the 55 kDa PDH complex subunit protein E2/E3 pb below baseline in A2 cells from both estradiol- and oil-implanted OVX rats, an acquired negative response that is estrogen-independent. A2 neurons may thus develop a lesser need for glycolysis-derived substrate for mitochondrial aerobic respiration during recurring hypoglycemia. A fourth hypoglycemic bout did not elevate A2 ATP synthase- α protein levels relative to now-diminished basal profiles in E-I⁴, but suppressed this protein below baseline in O-I⁴, inferring that mitochondrial energy production may adaptively down-regulate in the latter treatment group. Since acute hypoglycemia did not modify ATP synthase- α expression in E or O (Tamrakar et al. 2015), current results infer continuation of this lack of response in E-I⁴ and development of negative reactivity in O-I⁴. Results also demonstrate sustained hypoglycemic up-regulation of the tricarboxylic acid cycle enzyme OGDH in E-I⁴ rats, but inhibition of this protein profile by recurring hypoglycemia in O-I⁴, amounting to gain of negative response. These findings suggest that estradiol may promote an ongoing need for non-energy derivative Krebs cycle products. It should be noted that up- or down-regulation of A2 enzyme protein expression is not automatic proof of corresponding increases and decreases in enzyme activity; however, prior evidence for correlated increases in metabolic enzyme expression and activity due to estradiol treatment supports the plausibility of that outcome (Irwin et al. 2008). Affirmation of effects of recurring hypoglycemia on basal and hypoglycemic patterns of A2 enzyme activity awaits the development

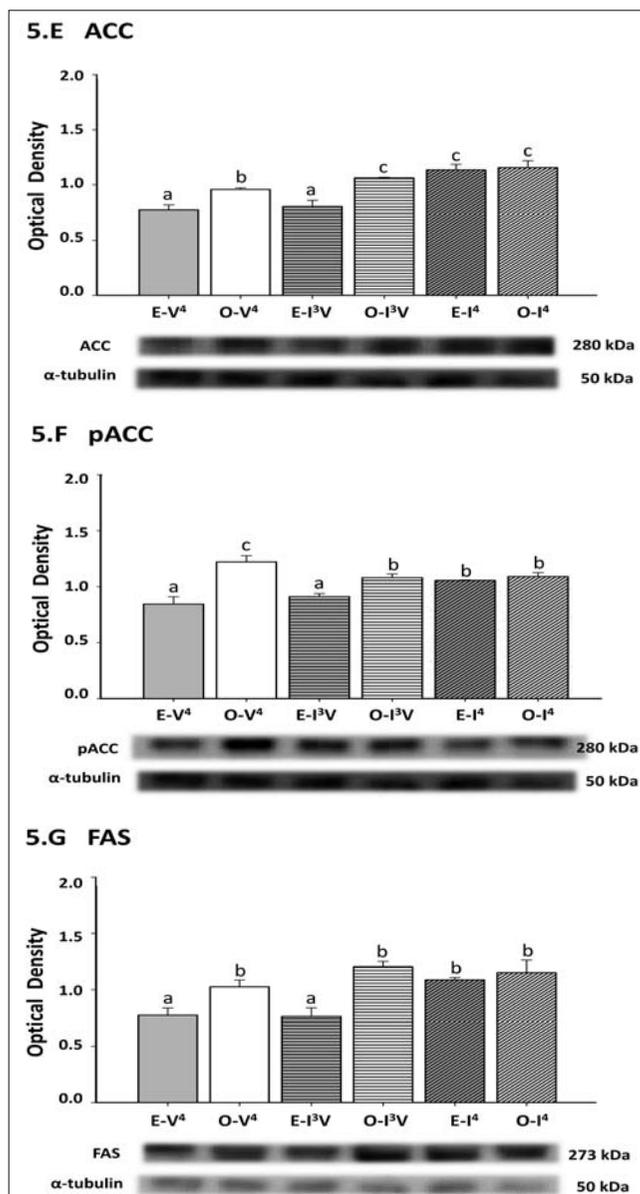


Fig. 5. Effects of precedent hypoglycemia on basal and hypoglycemic patterns of A2 nerve cell adenine 5'-monophosphate-activated protein kinase (AMPK), phosphoAMPK (pAMPK), Ca⁺⁺/calmodulin-dependent protein kinase beta (CaMMk β), protein phosphatase-2A (PP2A), acetyl CoA carboxylase (ACC), phosphoACC (pACC), and fatty acid synthase (FAS) protein expression in estradiol- vs. oil-implanted ovariectomized female rats. Separate pooled lysates of n=50 A2 cells/treatment group (n=12–13 neurons/rat) were analyzed by Western blot for AMPK (A); pAMPK (B); CaMMk β (C), PP2A (D), ACC (E), pACC (F), or FAS (G). Each protein analysis was performed on triplicate lysate pools. Graphs depict mean normalized protein O.D. values \pm SEM for E-V⁴ [solid grey bars], O-V⁴ [solid white bars], E-I³V [horizontal striped grey bars], O-I³V [horizontal striped white bars], E-I⁴ [diagonal striped grey bars], and O-I⁴ [diagonal striped white bars] treatment groups. Representative immunoblots are shown below each graph. Data labeled with dissimilar letters show statistical difference.

of analytical methods of requisite sensitivity for analysis of small-size neuron pool samples equivalent to those evaluated here.

Acclimation of A2 neuron AMPK and upstream kinase and phosphatase protein expression to hypoglycemia: impact of estradiol

Precedent hypoglycemia did not modify day four baseline pAMPK protein profiles in A2 neurons from estradiol- or oil-implanted OVX rats. In the absence of correlative information on post-hypoglycemic A2 cell ATP content, we speculate that steady-state energy

levels in these cells may be preserved despite adaptive changes in energy pathway enzyme expression. ATP stores are likely to remain unchanged if net increases or reductions in ATP output are matched by parallel up- or downsizing of energy utilization. During the fourth of four hypoglycemia bouts, A2 pAMPK protein levels were elevated in both E-I⁴ and O-I⁴, albeit to a significantly greater extent in the latter. These results suggest that precedent hypoglycemia may render A2 neurons vulnerable to energy instability during re-exposure, but that extent of energy deficiency may be attenuated by estradiol. We previously reported that estradiol promotes deactivation of AMPK during chronic hypoglycemia (Cherian and Briski 2012). A critical

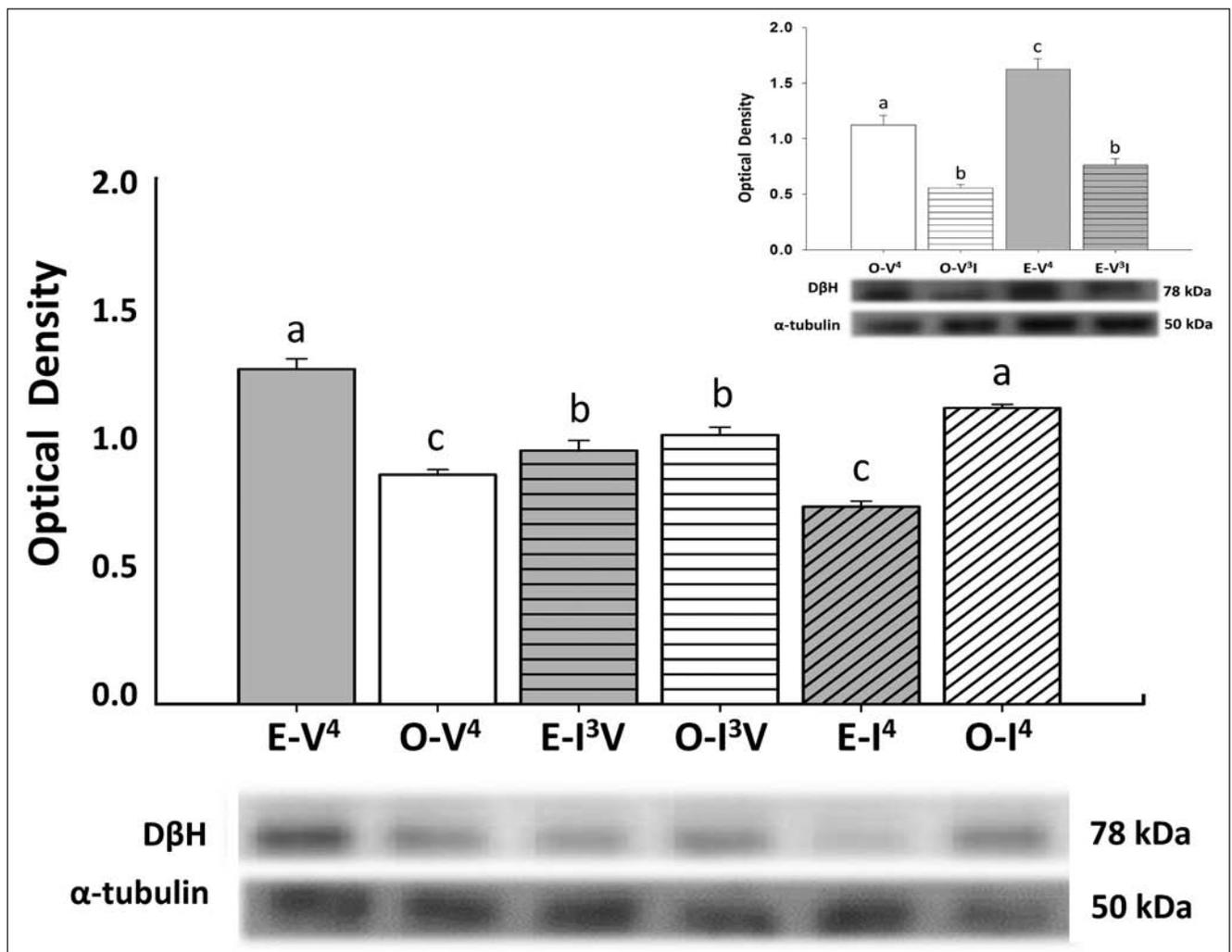


Fig. 6. Effects of precedent hypoglycemia on basal and hypoglycemic patterns of A2 nerve cell dopamineβ-hydroxylase (DβH) protein expression in estradiol- vs. oil-implanted ovariectomized female rats. For each treatment group, pooled lysates of n=50 A2 neurons (n=12–13 neurons/rat) were analyzed by Western blot for DβH in triplicate. Data show mean normalized DβH O.D. values ±SEM for E-V⁴ [solid grey bars], O-V⁴ [solid white bars], E-I^{3V} [horizontal striped grey bars], O-I^{3V} [horizontal striped white bars], E-I⁴ [diagonal striped grey bars], and O-I⁴ [diagonal striped white bars] groups. Representative immunoblots are presented below the graph. Data labeled with dissimilar letters differ statistically. The insert to Fig. 6 shows the effect of acute insulin-induced hypoglycemia on A2 neuron DβH protein levels. Bars show mean O.D. values ±SEM for oil and estradiol groups given four consecutive daily vehicle injections [n=4 E-V⁴ (grey bar); n=4 O-V⁴ (white bar)] or treated with vehicle on days 1–3, then injected with I on day four [n=4 E-V^{3I} (horizontal-stripe grey bar); n=4 O-V^{3I} (horizontal-striped white bar)]. Data show that a single hypoglycemic episode decreased mean DβH protein in both estradiol- and oil-implanted rats.

difference between that study and the present work studies is improved sensitivity of immunocytochemical methodology for identification of cDVC TH-ir neurons in hindbrain cryosections, as reflected by higher numbers of collectable A2 cells per individual animal, resulting in sufficient numbers of pools for quantitative protein analysis in the present research. Thus, prior results may reflect, in part, the distinctive reactivity of a subset of A2 cells characterized by relatively high neurotransmitter content.

Estradiol may function to prevent adaptation of upstream AMPK-regulating protein kinase and phosphatase enzymes to recurring hypoglycemia. A fourth of four hypoglycemic bouts did not modify A2 CaMK β protein content in E-I⁴ or O-I⁴, but diminished PP2A levels only in the former group. As a single hypoglycemic episode was previously observed to increase CaMK β and reduce PP2A protein profiles in OVX+oil rats (Tamrakar et al. 2015), current data suggest that when estradiol is absent, precedent exposure eliminates these responses. As we did not directly compare incremental changes in AMPK activity in acute vs. chronic hypoglycemic rats, we do not know if precedent hypoglycemia alters the magnitude of A2 energy debilitation in OVX+oil rats. In light of aforementioned adaptive loss of facilitative kinase and inhibitory phosphatase protein responses to renewed hypoglycemia in O-I⁴ rats, cellular reductions in ATP may play a relatively greater role in AMPK activation during recurring vs. acute hypoglycemia in that group. Precedent hypoglycemia did not alter basal A2 ACC or pACC protein profiles in E-I^{3V}, but respectively increased or decreased these proteins in O-I^{3V}. These data imply that when estradiol is absent, ACC activity may be enhanced amidst bouts of repeated hypoglycemia; the possibility that this outcome coincides with augmented cellular malonyl CoA accumulation and decreased fatty acid oxidation awaits experimental verification. Renewed hypoglycemia on day four elevated both profiles in E-I⁴ without effect in O-I⁴, suggesting that in the latter this enzyme acquires a new state of heightened activity that is refractory to recurring hypoglycemia. A potential ensuing lack of change in malonyl CoA levels may impede fatty acid utilization for energy. Acute hypoglycemia had no effect on ACC, pACC, or FAS protein profiles in estradiol- or oil-implanted OVX rats (Tamrakar et al. 2015); current observations of concurrent augmentation of ACC and FAS protein profiles in the E-I⁴ group imply a gain in positive reactivity of these proteins in this group.

As the DVC is comprised of multiple neuron neurotransmitter phenotypes, analyses of mitochondrial complex enzyme protein subunit expression in whole DVC samples shed light on whether patterns of A2 nerve cell protein adaptation to recurring hypoglycemia are representative or uncommon in this neural structure.

For example, DVC PDH-E2 and PDH-E1- α subunit protein profiles acquire negative reactivity in E-I⁴ but not O-I⁴; yet, A2 PDH-E2 protein is down-regulated by acclimation in both E-I⁴ and O-I⁴, inferring that negative A2 adaptation in the absence of estradiol is atypical of the DVC. DVC and A2 neuron samples also showed differential habituation of the Complex II subunit C-V- α , as baseline expression of this protein was respectively unchanged or decreased in these samples from E-I^{3V} rats, and recurring hypoglycemia increases protein levels in A2 neurons but not whole DVC in O-I⁴. Precedent hypoglycemia elevated synthase- α protein in the O-I^{3V} DVC and O-I^{3V} A2 neurons, but decreased this protein in E-I^{3VA2} cells.

Observations of estradiol-mediated augmentation of basal D β H protein expression concur with evidence that estrogen stimulates D β H mRNA expression in rat brain (Senova et al. 2002). Modifications in A2 D β H protein levels in estradiol- vs. oil-treated rats one day following serial exposure to hypoglycemia reflect a persistent impact of that prior experience on baseline catecholamine biosynthesis despite recovery from hypoglycemia. Data demonstrating a decline vs. increase in this protein profile in E- vs. O-I^{3V} groups concur with previous reports of divergent changes in A2 D β H mRNA after precedent hypoglycemia (Cherian and Briski 2012), implying that neurotransmitter production may be reduced or augmented between bouts of recurring hypoglycemia, at least within post-recovery time frame examined here. It should be noted that protein analyses were performed 2 hr after initiation of the final hypoglycemic episode; thus, the possibility (particularly in the presence of estrogen) that D β H levels measured at this time point may reflect rebound from a preceding stimulatory response that occurred between time zero and sampling, while this protein profile remains elevated in the absence of estradiol cannot be discounted. Further research is needed to evaluate ramifications of estradiol-dependent acclimation of between-hypoglycemia A2 nerve cell signaling on neural functions controlled by this cell group. Exacerbation of adjustments in baseline D β H protein expression by a fourth daily hypoglycemic episode implies that potential deviations in A2 noradrenergic signaling may be corresponding heightened during renewed exposure to this metabolic stress. Hypoglycemia poses a significant risk of brain injury as a continuous glucose supply is needed to provide sufficient energy to maintain neuron transmembrane ion gradients. Rigorous glycemic control is crucial for ideal management of insulin-dependent diabetes mellitus (IDDM), but is complicated by recurring iatrogenic insulin-induced hypoglycemia (Cryer 2008, 2011). In IDDM patients, recurring hypoglycemia elicits hypoglycemia-associated autonomic failure, which results in severe, unrelieved hypoglycemia due to maladaptive diminishment of glucose counter-regulatory outflow and neurogenic unawareness (Cryer 2005). Current results

bolster the need for additional research to investigate effects of adaptive down- vs. up-regulation of A2 nerve cell signaling in the presence vs. absence of estradiol on metabolic as well as non-metabolic functions that rely upon input from this cell group between and during bouts of ongoing hypoglycemia in patients prone to this therapy complication.

Acclimation of A2 neuron D β H enzyme protein expression to hypoglycemia: impact of estradiol

This study did not affirm our prediction of parallelism of adaptation of A2 nerve cell D β H protein expression and DVC Fos immunolabeling (Nedungadi et al. 2006) in both estradiol- and oil-implanted OVX rats. Rather, evidence for divergence of these two parameters, independent of estradiol status, detracts from our working assumption that hypoglycemia-induced DVC Fos immunoreactivity is a reliable indicator of metabolo-sensory neuron signaling. Further work is needed to identify AP-1 – controlled nerve cell transcriptional activities that habituate in an estradiol-dependent manner to precedent hypoglycemia and consequent effects on A2 function.

The female lifespan includes transition between different reproductive states, e.g. juvenile quiescence, fecundity, and senescence, typified by unique patterns of ovarian estrogen secretion. Current results emphasize the need to understand how dissimilar physiological patterns of estradiol output, including variations in hormone secretion that occur over estrous cycle and pregnancy, as well as states of low estrogen output before and after fecundity may influence A2 neuron energetic habituation to recurring hypoglycemia.

CONCLUSIONS

The present studies demonstrate that estradiol permits amplification of A2 nerve cell expression of key glycolytic, Krebs cycle, oxidative phosphorylation, and acetyl CoA-FAS pathway enzyme proteins during recurring hypoglycemia. Between hypoglycemic bouts, A2 cells may acquire, when estradiol is present, heightened needs for glycolytic metabolites or derived energy, coincident with decreased reliance upon oxidative phosphorylation. Estrogen-mediated prevention of maladaptive decline in OGDH and ATP synthase- α expression during recurring hypoglycemia may contribute to energetic resilience to this persistent metabolic insult. Opposite direction of D β H habituation to recurring hypoglycemia in estradiol- vs. non-steroid-replaced OVX female rats implies that potential acclimation of A2 signaling to this stress is hormone-dependent.

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