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Hypoglycemic and post-hypoglycemic patterns of glycogen phosphorylase isoform expression in the ventrolateral ventromedial hypothalamic nucleus: impact of sex and estradiol

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Glycogen metabolism shapes ventromedial hypothalamic nucleus (VMN) control of glucose homeostasis. Brain glycogen mass undergoes compensatory expansion post-recovery from insulin-induced hypoglycemia (IIH). Current research utilized combinatory high-resolution microdissection/high-sensitivity Western blotting to investigate whether IIH causes residual adjustments in glycogen metabolism within the metabolic-sensory ventrolateral VMN (VMNvl). Micropunch-dissected tissue was collected from rostral, middle, and caudal levels of the VMNvl in each sex for analysis of glycogen synthase (GS) and glycogen phosphorylase (GP)-muscle type (GPmm; norepinephrine-sensitive) and GP-brain type (GPbb; glucoprivic-sensitive) isoform expression during and after IIH. Hypoglycemic suppression of VMNvl GS levels in males disappeared or continued after reestablishment of euglycemia, according to sampled segment. Yet, reductions in female VMNvl GS persisted after IIH. Males exhibited reductions in GPmm content in select rostro-caudal VMNvl segments, but this protein declined in each segment post-hypoglycemia. Females, rather, showed augmented or diminished GPmm levels during IIH, but no residual effects of IIH on this protein. In each sex, region-specific up- or down-regulation of VMNvl GPbb profiles during glucose decrements were undetected post-recovery from IIH. Results provide novel proof of estradiol-dependent sex-dimorphic patterns of VMNvl GP variant expression at specific rostro-caudal levels of this critical gluco-regulatory structure. Sex differences in persistence of IIH-associated GS and GPmm patterns of expression after restoration of euglycemia infer that VMNvl recovery from this metabolic stress may involve dissimilar glycogen accumulation in male *versus* female.

Key words: insulin-induced hypoglycemia, letrozole; micropunch dissection, ventrolateral ventromedial hypothalamic nucleus, glycogen phosphorylase-muscle type

INTRODUCTION

The brain consumes a disproportionate fraction of total body energy to sustain vital high energy-demand nerve cell activities. Iatrogenic insulin (INS)-induced hypoglycemia (IIH) is an unremitting complication of obligatory glycemic management practiced by type I diabetes mellitus patients (Cryer, 2013; 2014). Hypoglycemic neuro-glucopenia poses a significant risk of neurological impairment (Auer and Siesjö, 1988; 1993; Auer, 2004). Glucoprivation elicits neural-driven counter-regulatory autonomic, neuroendocrine, and behavioral outflow that increases circulating glucose levels. Cellular energy stability is continuously monitored in the ventromedial hypothalamic nucleus (VMN) and other select brain loci by dedicated metabolic-sensory neurons that adjust synaptic transmission in response to excessive or diminished substrate fuel supply (Oomura et al., 1969; Ashford et al., 1990; Adachi et al., 1995, 1997; Silver and Erecińska, 1998). This dynamic

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readout, together with hormonal indicators of peripheral energy reserves, provides critical input for VMN regulation of glucose homeostasis. The ventrolateral VMN (VMNvl), one of a unique set of sex-dimorphic structures in the brain (Simerly, 2002), contains metabolic-sensory neurons (Cotero and Routh, 2009; Santiago et al., 2016) that participate in gluco-regulation (He et al., 2020).

Brain astrocytes store the complex carbohydrate glycogen as an energy reserve (Stobart and Anderson, 2013). Glycogen metabolism is controlled by the enzymes glycogen synthase (GS) and glycogen phosphorylase (GP), which respectively catalyze glycogen assembly or breakdown. Norepinephrine-sensitive GP-muscle (GPmm) and glucoprivic sensitive GP-brain (GPbb) type isoforms (Nadeau et al., 2018) confer stimulus-specific regulation of brain glycogen. Pharmacological inhibition of VMN glycogen disassembly up-regulates marker protein expression for neurotransmission of signal energy deficiency, which infers that local metabolic-sensory neurons gauge glycogen turnover and associated substrate fuel liberation (Alhamami et al., 2017; Briski et al., 2021). The prospect that hypoglycemia may produce sex-specific adjustments in glycogen metabolism within the sex-dimorphic VMNvl has not been addressed. Current studies utilized a combinatory high-resolution microdissection/high-sensitivity Western blot approach to investigate the premise that basal and/or hypoglycemic patterns of VMNvl glycogen metabolic enzyme expression differ between sexes. The VMNvl exhibits high estrogen receptor (ER) content compared to dorsomedial and central divisions (Yang and Shah, 2014). Thus, a corollary objective of this research was to determine if estradiol mediates sex-contingent GS and/or GP protein responses to hypoglycemia in one or both sexes. Here, target proteins were analyzed after VMNvl micropunch dissection from subcutaneous (sc) letrozole (Lz)- or vehicle-injected male and sc estradiol- or oil-implanted ovariectomized (OVX) female rats after INS or vehicle injection for analysis.

Transitory exposure to physiological conditions leading to energy imbalance, e.g., hypoxia, glucoprivation, and sleep deprivation, purportedly causes adaptive post-exposure augmentation of whole-brain glycogen content (Brucklacher et al., 2002; Kong et al., 2002; Choi et al., 2003). There has been speculation that post-hypoglycemic expansion of the glycogen reserve may adversely attenuate counter-regulation (Herzog et al., 2008; Duarte et al., 2017; Öz et al., 2017). This view has remained controversial, in part, due to a lack of methods of requisite neuroanatomical resolution for analysis of glycogen metabolism within sites where metabolic sensory neurons reside. The current project afforded the novel opportunity to examine whether regulatory effects of IIH on VMNvl glycogen metabolic enzyme protein expression coincide with plasma glucose decrements and/or are demonstrable following reestablishment of euglycemia in one or both sexes.

METHODS

Adult male and female Sprague Dawley were housed in groups by sex (2-3 per cage), under a 14 h light/10 h dark cycle (lights on at 05: 00 h). Animals were allowed unrestricted access to standard laboratory rat chow and water, and were acclimated to daily handling. Five days before the study, female rats were bilaterally OVX under ketamine/xylazine (0.1 mL/100 g bw; 90 mg ketamine: 10 mg xylazine/mL; Henry Schein Inc., Melville, NY) anesthesia. 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. On day 1 of the experiment, randomly-assigned female rats were anesthetized with isoflurane for sc implantation of a estradiol (30 ug 17β estradiol-3-benzoate/mL safflower oil)- or oil-filled silastic capsule (i.d. 0.062 in./o.d. 0.125 in.; 10 mm/100 g bw). This validated hormone replacement paradigm was used to standardize plasma estradiol levels at metestrus-like levels to avoid between estrous cycle-associated variations in endogenous steroid concentrations in ovary-intact animals (Briski et al., 2001). On days 1-5, testes-intact male rats were randomly injected *sc* with the aromatase inhibitor letrozole (Lz; 1 mg/kg bw) or the vehicle 0.9% saline (SAL) containing 5.0% Tween-30 and 5.0% ethanol, as described (Bhatnagar et al., 1993). At 09: 00 h on day 6, animals of each sex were injected with neutral protamine Hagedorn insulin (INS; 10.0 U/kg bw) or sterile diluent (V), then sacrificed by microwave fixation (1.45 sec; In Vivo Microwave Fixation System, 5kW; Stoelting Co., Wood Dale, IL) at the following time points (Fig. 1A): V-injected controls: time zero (n=4 V- and n=4 Lz-pretreated males; n=4 estradiol- and n=4 oil-implanted females); INS-injected animals: +1.0 h (n=4 V- and n=4 Lz-pretreated males; n=4 estradiol- and n=4 oil-implanted females), +3.5 h (n=4 V- and n=4 Lz-pretreated males; n=4 estradiol- and n=4 oil-implanted females), or +7.0 h (n=4 V- and n=4 Lz-pretreated males; n=4 estradiol- and n=4 oil-implanted females). Individual dissected brains were snap-frozen in liquid nitrogen-cooled isopentane and stored at -80°C. Plasma was kept at –20°C for glucose measurement.

All surgical and experimental protocols were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Ani-

mals, 8th Edition, under approval by the ULM Institutional Animal Care and Use Committee.

VMN tissue micropunch dissection and Western blot analysis

Each brain was cut into consecutive coronal 10 µm-thick frozen sections over the length of VMN. For each animal, bilateral micropunches of VMNvl tissue were taken using a calibrated 0.5 mm hollow punch tool (prod. no. 57401; Stoelting Co., Kiel, WI; Fig. 1B) from sections cut at rostral (-1.8 to -2.3 mm), middle (-2.3 to -2.8), and caudal (-2.8 to -3.3 mm) levels of the VMNvl, and combined within each segment in lysis buffer (2.0% sodium dodecyl sulfate, 0.05M dithiothreitol, 10.0% glycerol, 1.0 mM EDTA) for Western blot analysis. Regional micro-dissection of VMNvl tissue was performed here as recent studies show that INS-induced hypoglycemia effects on whole-VMN glycogen content vary along the longitudinal axis of the VMN (Ibrahim et al., 2020b). Accuracy of use of micropunch methodology for collection of distinctive hypothalamic loci of interest, including the VMN, as indicated by marker protein expression, has been validated (Mandal et al., 2017; 2018). In each treatment group, tissue lysate aliquots from individual subjects were pooled from rostral middle, or caudal VMNvl segments to create for each target protein, at minimum, triplicate sample pools per level. Pooled tissue samples were separated in Bio-Rad TGX 10% stain-free gels (Bio-Rad, Hercules, CA); after electrophoresis, gels were UV light-activated (1 min) in a Bio-Rad ChemiDoc TM Touch Imaging System (Ibrahim et al., 2019) to quantify total protein content in each lane. After protein transblotting, 0.45-µm PVDF-Plus membranes (prod. no. 121639; Data Support Co., Panorama City, CA) were blocked with Tris-buffer saline containing 0.1% Tween-20 and 2.0% bovine serum albumin, then incubated between 36-42 h (4°C) with primary rabbit polyclonal antisera raised against GS (1:2,000; prod. no. 3893S; Cell Signaling Technology, Danvers, MA), GPmm (1:2,000; prod. No. NBP2-16689;

A

	Subcutaneous *(sc)* Injection			
		Insulin[b] (INS)		
Pretreatments	**Vehicle[a] (V)**	**+1 hr**	**+ 3.5 hr**	**+7.0 hr**
Male				
sc vehicle (V) injections[c]	n=4	n=4	n=4	n=4
sc letrozole (Lz) injections[d]	n=4	n=4	n=4	n=4
Female				
OVX[e], *sc* estradiol implant[f]	n=4	n=4	n=4	n=4
OVX, *sc* vehicle implant[g]	n=4	n=4	n=4	n=4

a sterile diluent, 100 uL/100 g *bw*, administered at 09.00 hr on study day 6; sacrifice at time zero
b 10.0 U neutral protamine Hagedorn insulin/kg *bw*, administered at 09.00 hr on study day 6; post-injection sacrifice at 1, 3.5 or 7 hr after time zero.
c 0.9% saline
d 1 mg/kg *bw* administered daily on study days 1-5
e bilateral ovariectomy
f silastic capsule (0.062 in. *i.d*, 0.125 in. *o.d.*; 10 mm/100 g *bw*) containing 30 ug 17β-estradiol-3-benzoate/mL safflower oil, implanted on study day 1
g silastic capsule filled with safflower oil alone

B

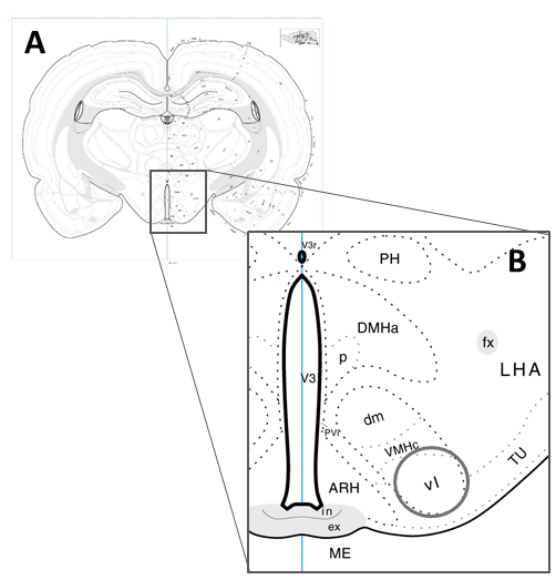

Fig. 1. Experimental design and ventromedial hypothalamic nucleus-ventrolateral part (VMNvl) micropunch dissection. (A) groups of male were pretreated on study days 1-5 by serial once-per-day subcutaneous (sc) injection of vehicle (V) or letrozole (Lz), while ovariectomized (OVX) female rats were implanted with a *sc* silastic capsule containing estradiol or the vehicle safflower oil over the same time interval. Groups of male V- or Lz-pretreated rats and groups of female estradiol- or oil-implanted OVX animals were injected *sc* at time zero (t_o) with vehicle (V; sterile diluent; sacrificed at t_o) or insulin (INS; 10.0 U neutral protamine Hagedorn insulin/kg *bw*; sacrificed at 1.0, 3.5, or 7.0 h after injection). For each sex, each final treatment group consisted of n=4 rats. (B) the rectangle the rectangle in the Panel A brain map (-2.85 mm posterior to bregma) depicts the VMN within the mediobasal hypothalamus, and is enlarged (Panel B) to illustrate the location of VMN in that region. The blue circle denotes positioning of a 0.50 mm diameter circular micropunch tool over the VMNvl for selective harvesting of this substructure. ARH: arcuate hypothalamic n.; DMHa,p: anterior, posterior dorsomedial hypothalamic n.; fx: fornix; LHA: lateral hypothalamic area; ME: median eminence; PVi: intermediate periventricular hypothalamic n.; VMHc,dm,vl: central, dorsomedial, ventrolateral ventromedial hypothalamic n.; TU: tuberal n.; V3: third ventricle.

Novus Biologicals, Littleton, CO), or GPbb (1:2,000; prod. no. NBP1-32799; Novus Biol.). Membranes were sequentially exposed to goat anti-rabbit horseradish peroxidase-labeled secondary antibodies (1:5,000; prod. no. NEF812001EA; PerkinElmer, Waltham, MA) and SuperSignal West Femto maximum sensitivity chemiluminescence substrate (prod. no. 34096; ThermoFisherScientific, Waltham, MA). Automated membrane buffer washes and blocking and antibody incubations were performed in a Freedom Rocker™ Blotbot. Protein band optical density (OD) measures were normalized to total in-lane protein using Image Lab™ 6.0.0 software (Bio-Rad). Precision plus protein molecular weight dual color standards (prod. no. 161-0374, Bio-Rad) were included in each Western blot analysis.

Statistics

Mean normalized VMNvl segmental protein OD and plasma glucose measures were evaluated 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 as follows: *$p<0.05$; **$p<0.01$; ***$p<0.001$; ****$p<0.0001$.

RESULTS

Current research investigated the premise that insulin-induced hypoglycemia effects on VMNvl glycogen metabolic enzyme expression occur by estradiol-dependent mechanisms in one or both sexes, and that altered expression of one or both GP isoforms may persist in distinctive rostro-caudal segments of the VMNvl after recovery from hypoglycemia. Here, combinatory high-resolution microdissection/high-sensitivity Western blotting was used to measure GP, GPmm, and GPbb protein expression profiles in rostral, middle, and caudal regions of the VMNvl in male rats pretreated with Lz or V, as well as OVX female rats implanted with estradiol or oil.

Data presented in Table I show that INS-injected male and female rats exhibited reductions in plasma glucose concentrations compared to V-injected controls at both +1 and +3.5 h post-treatment. In each sex, restoration of glucose profiles to the normal range occurred between +3.5 and +7 h. INS effects on circulating glucose levels were not different between groups of males that were pretreated with Lz *versus* V, or between groups of estradiol- *versus* oil-implanted OVX female rats.

Rostral, middle, and caudal VMNvl tissue was collected from Lz- or V-pretreated male (Figs 2A, 2C, 2E) and estradiol- or oil-implanted OVX female (Figs 2B, 2D, 2F) rats after INS (patterned bars) or V (solid bars) injection for Western blot for GS protein expression. Lz administration to euglycemic males decreased baseline GS expression in the rostral (Fig. 2A) and caudal (Fig. 2E) VMNvl. INS injection of V-pretreated males (right-side; white bars) caused significant reduction of rostral (+3.5 h), middle (+7 h; Fig. 2C), and caudal (+1, 3.5, 7 h) VMNvl GS content. Lz pretreatment (left-side; gray bars) resulted in either augmentation (rostral VMNvl; +1, 3.5, 7 h) or no change (middle and caudal VMNvl) in this protein profile after INS treatment. In non-INS-injected female controls, VMNvl GS expression was significantly higher in estradiol- *versus* oil-implanted animals in both rostral (Fig. 2B) and caudal (Fig. 2F) segments of the VMNvl. In the presence of estradiol (left-side; gray bars), INS decreased GS content in the rostral (+3.5, 7 h) and caudal (+1, 3.5, 7 h) VMNvl. However, this protein profile was diminished by INS treatment only in the middle VMNvl in OVX rats (Fig. 2D; right-side; white bars). Results show that in each sex, estradiol-dependent mechanisms suppress GS protein expression in specif-

Table I. Effects of insulin (INS) injection on plasma glucose levels in vehicle- *versus* letrozole (Lz)-pretreated male and ovariectomized (OVX), estradiol- *versus* oil-implanted female rats.

		Post-INS Injection Time Point		
Treatment Group Sex/Pretreatment	Time Zero	+1 h	+3.5 h	+7 h
Male/Vehicle	130.3 ± 2.2	74.6 ± 4.4*	62.2 ± 6.8*	122.2 ± 6.7
Male/Lz	189.6 ± 7.6	85.4 ± 2.6*	82.0 ± 3.7*	130.3 ± 13.4
Female/OVX-Estradiol	124.6 ± 7.1	55.3 ± 3.3*	67.0 ± 4.8*	103.5 ± 5.9
Female/OVX-Oil	116.5 ± 5.8	46.9 ± 3.5*	62.8 ± 4.6*	125.0 ± 8.2

* $p<0.05$ compared to Time Zero.

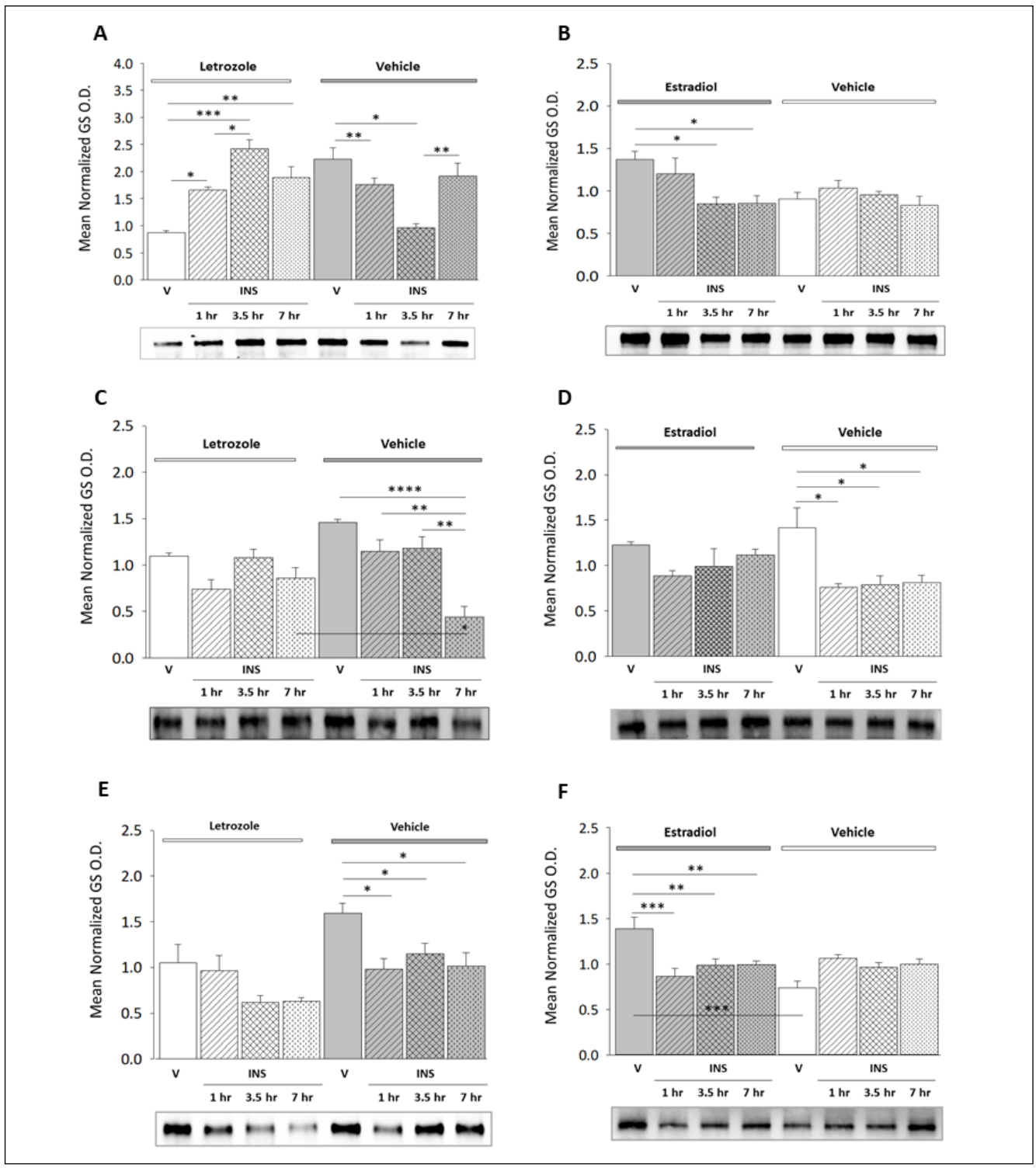

Fig. 2. Effects of sc INS injection on ventrolateral ventromedial hypothalamic nucleus (VMNvl) glycogen synthase (GS) protein expression in V- or Lz-pretreated male and estradiol- or oil-Implanted OVX female rats. VMNvl tissue was bilaterally micropunch-dissected over predetermined rostro-caudal levels from groups of male and female rats after sc vehicle (V; sterile diluent) or INS (10.0 U neutral protamine Hagedorn insulin/kg bw) injection. Data depict mean normalized rostral ((A) male, (B) female), middle ((C) male, (D) female), and caudal ((E) male, (F) female) VMNvl GS protein optical density (OD) measures + S.E.M. for groups of male (left-hand column) and female (right-hand column) rats. Groups of male rats (n=4/group) were pretreated by serial daily sc injection of V- (gray bars) or Lz (white bars) prior to sacrifice after V injection (solid bars) or at 1.0 (diagonal-striped bars), 3.5 (cross-hatched bars), or 7.0 (stippled bars) h after INS injection. Groups of OVX estradiol- (gray bars) or oil (white bars)-implanted female rats (n=4/group) were sacrificed at identical time points after V or INS treatment. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

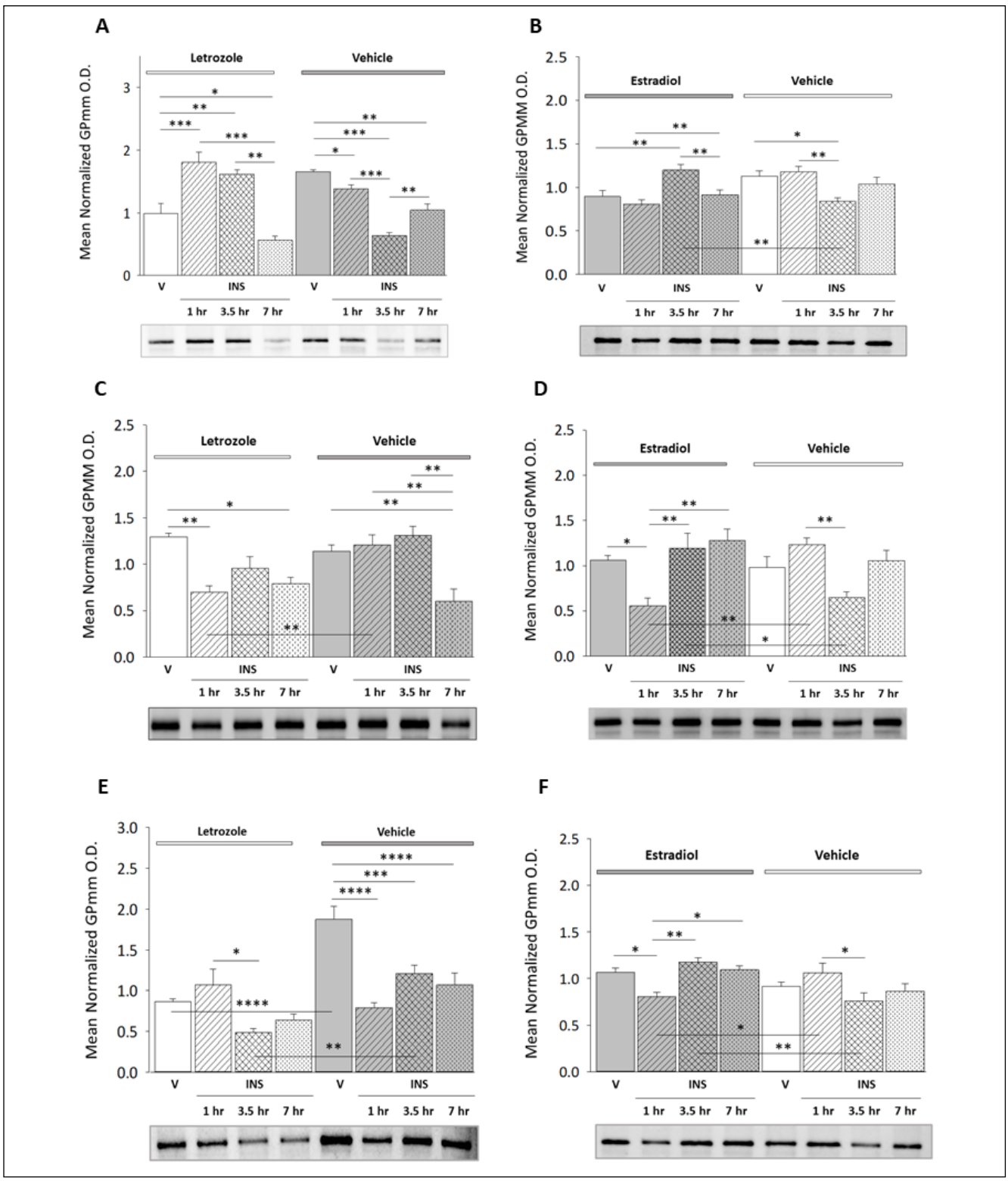

Fig. 3. VMNvl rostrocaudal segment-specific patterns of glycogen phosphorylase-muscle type (GPmm) Expression after V- *versus* INS-injection to male and female rats. Data show mean normalized rostral ((A) male, (B) female), middle ((C) male, (D) female), and caudal ((E) male, (F) female) VMNvl GPmm O.D. measures + S.E.M. for groups of male (left-hand column) and female (right-hand column) rats. Groups of male rats (n=4/group) were pretreated by serial daily sc injection of V- (gray bars) or Lz (white bars) prior to sacrifice after V injection (solid bars) or at 1.0 (diagonal-striped bars), 3.5 (cross-hatched bars), or 7.0 (stippled bars) hrs after INS injection. Groups of OVX estradiol- (gray bars) or oil (white bars)-implanted female rats (n=4/group) were sacrificed at identical time points after V or INS treatment. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

ic rostro-caudal levels of the VMNvl during hypoglycemia. Compared to euglycemic controls, post-hypoglycemic patterns of GS expression were diminished throughout the male VMNvl as well as in the female rostral and caudal VMNvl.

Fig. 3 depicts effects of INS injection on VMNvl GPmm protein expression during and after diminution of plasma glucose concentrations. Euglycemic male rats exhibited a reduction in VMNvl GPmm profiles in the rostral (Fig. 3A) and caudal (Fig. 3F) VMNvl following Lz administration. In this sex, INS significantly inhibited rostral and caudal VMNvl GPmm content both during and after glucose decrements. While INS-injected males showed no change in middle VMNvl GPmm levels during hypoglycemia, this protein profile was subsequently reduced after reestablishment of plasma glucose levels to the normal range. Lz pretreatment prevented hypoglycemia-associated decline in rostral VMNvl GPmm content, but did not prevent diminution of this protein profile at +7 h. Post-hypoglycemic decrements in the male rat middle VMNvl GPmm were also refractory to Lz. Yet, Lz-pretreated males had modified patterns of GPmm expression in the caudal VMNvl during and after hypoglycemia. Data illustrated in Figs 3B, 3D, and 3F reveal divergent INS effects on VMNvl GPmm levels in estradiol-implanted OVX female rats, as this protein was up-regulated in the rostral segment (+3.5 h), but down-regulated at middle and caudal levels (+1 h). In contrast, INS injection did not influence VMNvl GPmm profiles in oil-implanted OVX female rats. In the presence of estradiol, post-hypoglycemic GPmm expression patterns over the length of the VMNvl were equivalent to those in non-INS-injected controls. Outcomes document uniform *versus* bi-directional effects of hypoglycemia on VMNvl GPmm content in male *versus* female, respectively, and identify variation of post-hypoglycemic VMNvl GPmm profiles between sexes.

Effects of INS injection on male *versus* female VMNvl GPbb protein content are presented in Fig. 4. Euglycemic males injected with Lz exhibited significant augmentation of caudal (Fig. 4E), but not rostral (Fig. 4A) or middle (Fig. 4C) VMNvl GPbb expression compared to V controls. INS injection of male rats resulted in either stimulation (rostral and middle VMNvl) or suppression (caudal VMNvl) of GPbb content by 1 h post-treatment. These region-based responses were abolished by Lz. In males sacrificed at 7 h after INS injection, GPbb profiles in all VMNvl segments did not differ from controls. Baseline GPbb levels were significantly lower in rostral (Fig. 4B) and middle (Fig. 4D) VMNvl segments of estradiol- *versus* oil-implanted rats. In response to insulin, estradiol-, but not oil-implanted female rats exhibited up- or down-regulated GPbb expression in rostral (+1 and +3.5 h) and middle (+1 h) VMNvl regions, respectively. INS had primarily no impact on post-hypoglycemic VMNvl GPbb content in estradiol- or oil-implanted animals. Data indicate that region-specific up- or down-regulation of VMNvl GPbb expression coinciding with diminished plasma glucose concentrations is discontinued after recovery from hypoglycemia.

DISCUSSION

Glycogen metabolism affects whole-VMN gluco-regulatory transmitter signaling (Alhamami et al., 2017; Briski et al., 2021). Sex-dimorphic VMNvl sensitivity to estradiol, due to developmental hormonal imprinting, manifests as sex-specific control of several behavioral and physiological functions. VMNvl metabolic-sensory neurons influence blood glucose levels (He et al., 2020). Here, VMNvl tissue was micropunch-dissected in segmental fashion to investigate whether estradiol-dependent mechanisms mediate sex-specific patterns of glycogen metabolic enzyme protein expression in distinctive locations over the longitudinal axis of this structure during and/or after hypoglycemia. Outcomes document estradiol-dependent VMNvl region-specific GS inhibition during glucose decrements, a response that persisted post-hypoglycemia in some (male) or all (female) of those segments. INS-associated diminution of GPmm profiles occurred in hypoglycemic animals of each sex, and also in recovered male, but not female rats. VMNvl GPbb profiles were up- or down-regulated according to rostro-caudal segment during, but not after plasma glucose decline. Results infer that during hypoglycemia, AMP-driven glycogen breakdown is prioritized relative to norepinephrine input in discrete regions of the VMNvl. Hypoglycemia may likely impose lingering inhibitory effects on VMNvl glycogen synthesis in each sex and on noradrenergic stimulation of glycogen breakdown in this structure in males. Further effort is warranted to characterize the molecular mechanisms that underlie common *versus* unique patterns of VMNvl glycogen enzyme expression in the two sexes, and to determine how glucoprivic- *versus* neurotransmitter-mediated glycogen disassembly may affect gluco-regulatory function over the length of the VMNvl.

In each sex, gonad-derived estradiol is a positive stimulus for rostral and caudal VMNvl GS expression during euglycemia, but is involved in hypoglycemic inhibition of that protein in those same locations. These findings likely infer that hypoglycemia sup-

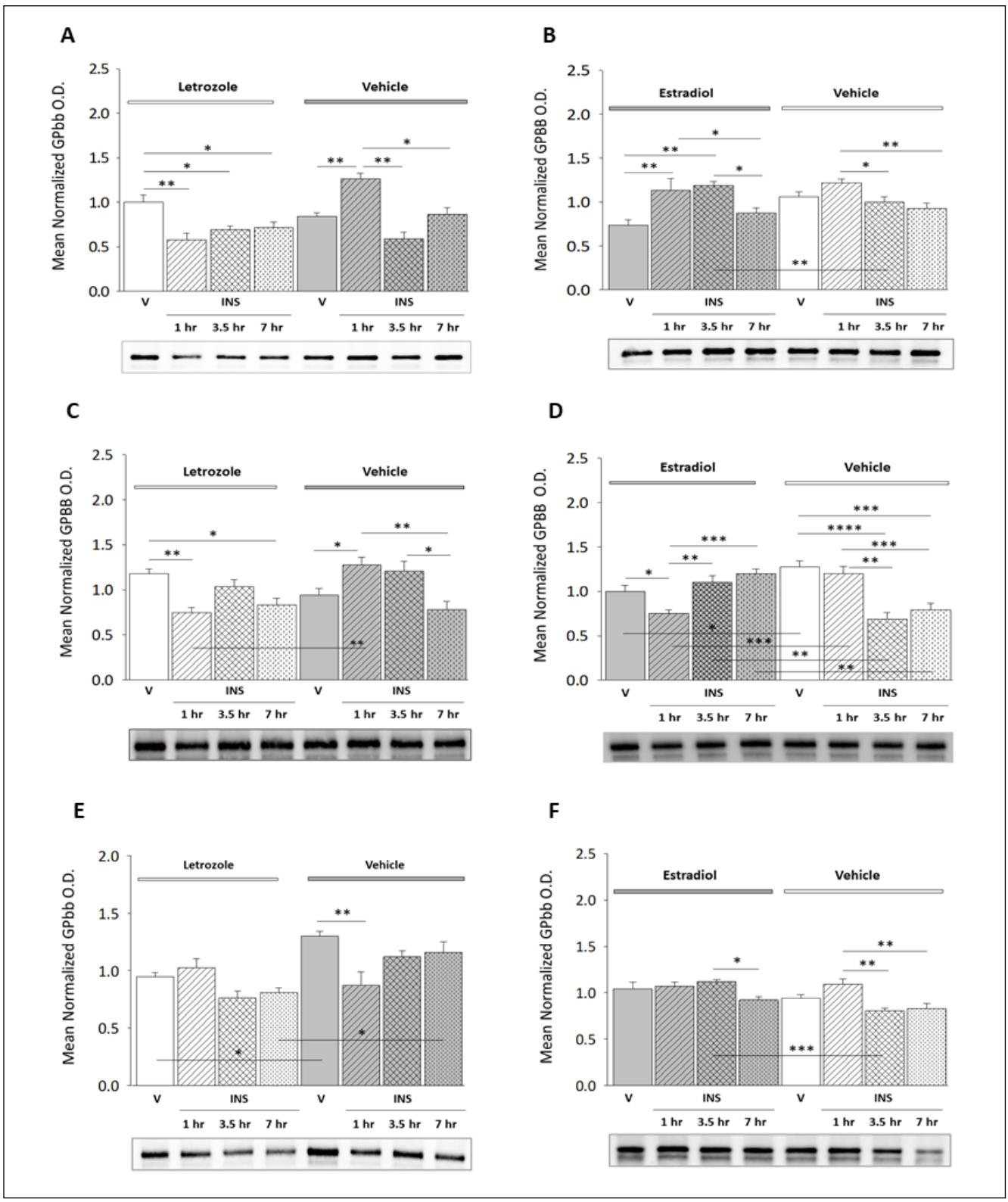

Fig. 4. Effects of INS Injection on VMNvl expression of glycogen phosphorylase-brain type (GPbb) in V- or Lz-pretreated male and estradiol- or oil-implanted OVX female rats. Data show mean normalized rostral ((A) male, (B) female), middle ((C) male, (D) female), and caudal ((E) male, (F) female) VMNvl GPbb O.D. measures + S.E.M. for groups of male (left-hand column) and female (right-hand column) rats. In each sex, bar colors differentiate pretreatments before sc V or INS injection; animals were sacrificed after V injection (solid bars) or at 1.0 (diagonal-striped bars), 3.5 (cross-hatched bars), or 7.0 (stippled bars) hrs after INS injection. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

presses glycogen synthesis at selective levels along the longitudinal axis of the VMNvl. There remains a need to clarify whether this switch in direction of estradiol control of rostral and caudal VMNvl GS may result, in part, from dissimilar astrocyte ER expression due to differences in glucose uptake during normo- *versus* hypoglycemia. Regional differences in VMNvl GS reactivity to hypoglycemia may be contingent, somewhat, upon relative numbers of one or more distinctive nuclear or membrane ER receptor variants. There is also the possibility that control of astrocyte sensitivity to estradiol input by other regulatory signals may vary between rostro-caudal segments. It is notable that in each sex, the onset of hypoglycemia-associated suppression of GS protein diverged between rostral (+3.5 h) *versus* caudal (+1 h) VMNvl. A sizable fraction of glucose acquired by astrocytes from blood is diverted to the glycogen shunt, which involves glucose monomer incorporation into this polymer, then release prior to entry into the glycolytic pathway. Attenuation of glycogen assembly during hypoglycemia is an effective strategy for energy conservation as ATP expenditure is required for glycogen assembly. It is thus possible that latency between onset of hypoglycemia and diminution of glycogen synthesis may be governed, to some extent, by local nerve cell energy requirements. Persistent down-regulation of GS profiles in the male and female caudal VMNvl following recovery from hypoglycemia may similarly reflect continuance of heightened neuronal energy demands in that segment. Further research is required to examine how estradiol signaling may integrate with putative cues on neuronal metabolic stability to control GS expression and glycogen formation.

Current data document sex-specific hypoglycemic regulation of stimulus-specific glycogen disassembly in the VMNvl. The norepinephrine-sensitive GP variant GPmm was inhibited by INS in different segments in each sex, as this protein was decreased at rostral and caudal levels in males, but in middle and caudal segments in the female. Females also showed coincident augmentation of rostral VMN GPmm content. Diminution or augmentation of GPmm expression likely results in decreased or elevated norepinephrine-stimulated glycogen disassembly, respectively. This premise remains speculative as tools for assessment of hypoglycemic effects on phosphorylation, e.g. activation of this GP isoform are currently unavailable. Outcomes also show that, in each sex, hypoglycemia simultaneously augmented or inhibited glucoprivic-sensitive GPbb expression in select VMNvl segments. These results infer that AMP-driven glycogen breakdown may be correspondingly enhanced or reduced in those locations during this metabolic stress. Confirmation of this premise will require region-specific analysis of phosphorylation state of GPbb protein expressed during hypoglycemia. The data summary provided in Table II suggests that in hypoglycemic male rats, select VMNvl regional glycogen stores may be protected from noradrenergic disassembly, and at the same time undergo enhanced (rostral segment) or attenuated (caudal segment) AMP-mediated mobilization. Thus, in the former site, glycogen disassembly may be selectively driven by energy deficiency, whereas glycogen mass may be preserved in the latter region despite neuro-glucopenia. In contrast, the female VMNvl is evidently characterized by regional enhancement (rostral segment) or diminution (middle segment) of glycogen sensitivity to both noradrenergic and glucoprivic breakdown. The possibility that hypoglycemic up- or down-regulation of VMNvl GPbb expression, by segment, may reflect, to some extent, elevated or decreased energy demands needs of local neurons will require further investigation. There is ample justification for additional effort to verify effects of GPbb-mediated acceleration of glucose liberation on metabolic-sensory function. Importantly, outcomes addressed in Table II emphasize estradiol involvement in sex-specific patterns of GP variant expression during hypoglycemia.

Table II. Summary of effects of insulin (INS)-induced hypoglycemia on glycogen synthase (GS) and phosphorylase (GP) isoform protein expression across rostro-caudal segments of male and female rat ventrolateral ventromedial hypothalamic nucleus (VMNvl); role of estradiol.

	Male VMNvl			Female[a] VMNvl		
	Rostral	Middle	Caudal	Rostral	Middle	Caudal
GS	↓ +3.5 h	↓ +7 h	↓ +1,3.5,7 h	↓ +3.5,7 h	N.C.[b]	+1,3.5,7 h
GPmm[c]	↓ +1,3.5,7 h	↓ +7 h	↓ +1,3.5,7 h	↑ +3.5 h	↓ +1 h	+1 h
GPbb[d]	↑ +1 h	↑ +1 h	↓ +1 h	↑ +1,3.5 h	↓ +1 h	N.C.

[a] ovariectomized; implanted with estradiol [30 ug/mL; 10 mm/100g *bw*] – filled silastic capsule *sc*; [b] no change; [c] glycogen phosphorylase-muscle type; norepinephrine-sensitive; [d] glycogen phosphorylase-brain type; AMP- sensitive; +__ h: underlined time points indicate when response to INS was prevented by sc letrozole administration (males) or oil-filled capsule implanted (females).

A possible explanation for these divergent GP responses between sexes may involve, in part, dissimilar of ER expression profiles (Ibrahim et al., 2020).

Current studies provide novel evidence for residual effects of hypoglycemia on VMNvl GPmm, but not GPbb variant expression. Notably, post-hypoglycemic GPmm down-regulation occurred in male, but not female rats; the mechanisms that this sex difference remain unclear. Persistent reductions in this protein would likely attenuate glycogen breakdown in the absence of neuro-glucopenia. Down-regulated GPmm, coincident with normal (rostral VMNvl) or diminished (middle and caudal VMNvl) patterns of GS expression at +7 h after INS injection, plausibly facilitates glycogen enhancement in the former site, yet may stabilize glycogen mass in other regions through attenuation of glycogen shunt activity. Implications of putative post-hypoglycemia glycogen expansion in the male rostral VMNvl on metabolic transmitter signaling require additional consideration. Female rats exhibited suppression of rostral and caudal VMNvl GS expression after recovery from hypoglycemia, alongside reestablishment of GPmm and GPbb profiles to control levels. Post-hypoglycemia glycogen synthesis and glycogen shunt activity are thus likely impeded in those areas. As both GP isoforms were up-regulated in the female rat rostral VMNvl during hypoglycemia, glycogen depletion in that segment occurred may not be reversed within the post-hypoglycemia interval evaluated here. Functional ramifications of possible sex-dimorphic adaptations in rostral VMNvl glycogen accumulation (increased in male, decreased in female) after recovery from hypoglycemia warrant further attention.

In summary, current research offers unique evidence for estradiol-dependent sex-dimorphic VMNvl glycogen metabolic enzyme protein expression during and after exposure to hypoglycemia. Results emphasize, for each sex, rostro-caudal region-specific control of glycogen synthesis and stimulus-specific glycogen mobilization. Further research is needed to investigate potential relationships between local adjustments in glycogen metabolism and nerve cell energy stability during and after hypoglycemia.

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