Symbiosis

www.symbiosisonlinepublishing.com
ISSN Online: 2374-6890

Research Article | Journal of Endocrinology and Diabetes | Open Access

Central Type II Glucocorticoid Receptor Regulation of Ventromedial Hypothalamic Nucleus Glycogen Metabolic Enzyme and Glucoregulatory Neurotransmitter Marker Protein Expression in the Male Rat

Abdulrahman Alhamyani[1], A.S.M. Hasan Mahmood[1], Ayed Alshamrani[1], Mostafa M. H. Ibrahim[1], and Karen P. Briski[1*]

[1]School of Basic Pharmaceutical and Toxicological Sciences, College of Pharmacy, University of Louisiana Monroe, Monroe, LA 71201

Received: December 11, 2020; Accepted: January 5, 2021; Published: January 13, 2021

***Corresponding author :** Dr. Karen P. Briski, Ph.D, Willis-Knighton Endowed Professor of Pharmacy, Director, School of Pharmaceutical and Toxicological Sciences, Professor of Pharmacology and Neuroanatomy, College of Pharmacy ,University of Louisiana at Monroe, 356 Bienville Building, 1800 Bienville Drive, Monroe, LA 71201; TEL:318-342-3283; FAX: 318-342-1737; E-mail: briski@ulm.edu*

Abstract

The ventromedial hypothalamic nucleus (VMN) glucoregulatory neurotransmitters γ-aminobutyric acid (GABA) and nitric oxide (NO) signal adjustments in glycogen mobilization. Glucocorticoids control astrocyte glycogen metabolism in vitro. The classical (type II) glucocorticoid receptor (GR) is expressed in key brain structures that govern glucostasis, including the VMN. Current research addressed the hypothesis that forebrain GR regulation of VMN glycogen synthase (GS) and phosphorylase (GP) protein expression correlates with control of glucoregulatory transmission. Groups of male rats were pretreated by intracerebroventricular (icv) delivery of the GR antagonist RU486 or vehicle prior to insulin-induced hypoglycemia (IIH), or were pretreated icv with dexamethasone (DEX) or vehicle before subcutaneous insulin diluent injection. DEX increased VMN GS and norepinephrine-sensitive GP-muscle type (GPmm), but did not alter metabolic deficit-sensitive GP-brain type (GPbb) expression. RU486 enhanced GS and GPbb profiles during IIH. VMN astrocyte (MCT1) and neuronal (MCT2) monocarboxylate transporter profiles were up-regulated in euglycemic and hypoglycemic animals by DEX or RU486, respectively. Glutamate decarboxylase65/67 and neuronal nitric oxide synthase (nNOS) proteins were both increased by DEX, yet RU486 augmented hypoglycemic nNOS expression patterns. Results show that GR exert divergent effects on VMN GS, MCT1/2, and nNOS proteins during eu- (stimulatory) versus hypoglycemia (inhibitory); these findings imply that up-regulated NO transmission may reflect, in part, augmented glucose incorporation into glycogen and/or increased tissue lactate requirements. Data also provide novel evidence for metabolic state-dependent GR regulation of VMN GPmm and GPbb profiles; thus, GABA signaling of metabolic stability may reflect, in part, stimulus-specific glycogen breakdown during eu- versus hypoglycemia.

Key Words: Glucocorticoid receptor; Ventromedial hypothalamic nucleus; RU486; Dexamethasone; Glycogen phosphorylase; Nitric oxide synthase

Introduction

Insulin-induced hypoglycemia (IIH) is an unrelenting complication of strict glycemic control of type I diabetes mellitus [1, 2]. Hypoglycemic neuro-glucopenia poses a risk of neural dysfunction, as metabolic fuel stream is inadequate to meet nerve cell energy needs [3, 4]. The hypothalamus directs counter-active autonomic, neuroendocrine, and behavioral outflow to attenuate hypoglycemia [5]. The ventromedial hypothalamic nucleus (VMN) utilizes energy substrate, hormonal, and neurotransmitter cues of metabolic status to control glucose counter-regulation [6, 7]. Dedicated VMN metabolic-sensory neurons supply dynamic cellular energy readout by increasing ('fuel-inhibited') or decreasing ('fuel-excited') synaptic firing as tissue energy fuel levels decline [8-10]. Neurochemical signals of ventromedial hypothalamic energy imbalance include ϒ-aminobutyric acid (GABA), which inhibits hypoglycemic patterns of glucagon and epinephrine hypersecretion [11], and nitric oxide (NO), which stimulates counter-regulatory hormone secretion [12, 13].

Brain astrocytes store glycogen as a vital metabolic fuel reserve [13b]. Circulating glucose, the primary energy source to the brain, is taken up into these glia where it is either incorporated into glycogen or converted to the oxidizable substrate fuel L-lactate for trafficking to neurons [14]. Lactate is transferred between these cell compartments by astrocyte (MCT1) - and neuron (MCT2)-specific monocarboxylate transporter function [15]. Ventromedial hypothalamic lactoprivation stimulates counter-regulatory hormone secretion [16] by mechanisms that involve reduced GABA signaling [17]. Glycogen metabolism is controlled by opposing glycogen synthase (GS) and glycogen phosphorylase (GP) enzyme actions that catalyze glycogen synthesis or breakdown, respectively. Evidence that inhibition of VMN GP activity up-regulates the VMN biosynthetic enzyme neuronal nitric oxide synthase (nNOS) in each sex [18, 18b], and suppresses glutamate decarboxylase65/67 (GAD) profiles in females [18b] suggests that diminished astrocyte glycogen mass or turnover may signal energy deficiency to VMN glucoregulatory

neurons.

Adrenal glucocorticoid hormones act within the brain to regulate neural adaptation to recurring IIH [19]. Glucocorticoids modulate norepinephrine (NE) control of astrocyte glycogen synthesis in vitro via classic/type II glucocorticoid receptor (GR) signaling [20]. GR are one of two distinct glucocorticoid receptor populations in the brain, and differ from type I or corticosterone (CORT)-preferring mineral corticoid receptor (CR) concerning neuroanatomical distribution and ligand preferences [21-23]. CR are expressed exclusively in hippocampus and septum, and bind corticosterone with high affinity (Kd » 0.5nM), whereas GR occur throughout the brain, including the VMN and other hypothalamic glucoregulatory loci [24], and exhibit relatively greater affinity for synthetic ligands including dexamethasone (DEX; Kd » 1.5-2.0 nM) versus corticosterone (Kd » 2.5-5.0 nM). Current research utilized pharmacological, high-spatial resolution dissection, and Western blot techniques to investigate the premise that GR govern VMN glycogen metabolism and glucoregulatory transmitter signaling during euglycemia and/or hypoglycemia. Euglycemic adult male rats were injected into the lateral ventricle with DEX, whilst other animals were pretreated by intracerebroventricular (icv) delivery of the GR antagonist RU486 (mifepristone) [25, 26] prior to onset of IIH. VMN tissue obtained by micropunch dissection was analyzed by Western blot for protein markers of GABAergic (GAD) and nitrergic (nNOS) neuron function. Recent studies implicate NE and estradiol in regulation of VMN glycogen metabolism [27, 28]. Our studies show that VMN astrocytes express distinctive adrenoreceptor [alpha1-adrenoreceptor (α1AR), alpha2-AR (α2AR), and beta1-AR (β1AR)] and estrogen receptor [estrogen receptor-alpha (ERα), ER-beta (ERβ), and G protein-coupled estrogen receptor 1 (GPER)] proteins. In the present work, VMN tissue sections were also processed for in situ immunocytochemical identification of glial fibrillary acid protein (GFAP)-reactive astrocytes in advance of laser-catapult micro dissection (LCM) [29]. Pure VMN astrocyte cell samples were analyzed by immunoblotting to examine whether GR regulate expression of one or more AR and ER protein profiles in these glia.

Materials and Methods

Experimental Design

Adult male Sprague Dawley rats (3–4 months of age) were housed in individual cages under a 14 hr light/10 hr dark cycle (lights on at 05.00 h), and allowed *ad-libitum* access to standard laboratory chow (Harlan Teklad LM-485; Harlan industries, Madison, WI) and tap water. All surgical and experimental protocols were conducted in accordance with NIH guidelines for care and use of laboratory animals, under approval by the ULM Institutional Animal Care and Use Committee. On study day 1, animals were implanted with a PE-20 cannula directed to the left lateral ventricle (LV) [coordinates: 0.00 mm posterior to *bregma*, 1.50 mm lateral to midline, 5.0 mm ventral to skull surface; Mahmood et al., 2018] under ketamine/xylazine anesthesia (0.1 ml/100 g bw; 90 mg ketamine:10 mg xylazine/ml; Henry Schein Inc., Melville, NY). After surgery, rats were injected subcutaneously (sc) with ketoprofen (1 mg/kg bw) and intramuscularly with enrofloxacin (10 mg/kg bw), then transferred to individual cages. At 08.45 hr on study day 7, animals were divided into four treatment groups, and infused into the LV over a two minute period with vehicle (V; 2.0 µL; groups 1 and 2, n=4/group), the GR antagonist RU486 [25, 26] (10.0 µg/2.0 µL [30]; group 3, n=4), or dexamethasone (10 µg/2.0 µL [31], n=4). At 9:00 hr, rats in groups 1 and 4 received a sc injection of insulin diluent (V; Eli Lilly & Co., Indianapolis, IN), while groups 2 and 3 were injected sc with neutral protamine Hagedorn insulin (INS; 10.0 U/kg bw; Butler Schein Animal Health, Dublin, OH). Rats were sacrificed at 10:00 hr for brain tissue and trunk blood collection. Brains were snap-frozen in liquid nitrogen-cooled isopentane for storage at -80 °C. Plasma was stored at −20 °C.

VMN Micropunch Dissection and Western Blotting

Between -1.80 and -3-30 mm relative to bregma, alternating series of thin (10 µm-thick) and thick (100 µm-thick) frozen sections werecollected over the length of the VMN, overrepeating distances of 100 µm (n = 10 consecutive thin sections) and 200 µm (n = 2 consecutive thick sections), for LCM or Palkovits micropunch dissection, respectively. For each animal, micro punched VMN tissue samples were bilaterally removed from thick sections using calibrated hollow needle tools (prod. no. 57401; Stoelting Co., Wood Dale, IL; Figure 1), and transferred to lysis buffer (2% sodium dodecyl sulfate, 0.05 M dithiothreitol, 10% glycerol, 1 mM EDTA, 60 mM Tris-HCl, pH 7.2). In each treatment group, tissue lysate aliquots from individual subjects were combined to create three separate sample pools for each protein of interest. Sample proteins were separated in Bio-Rad Stain-Free 10% gradient acrylamide gels (Hercules, CA) after loading of approximately 25 µg protein per individual well, then transblotted to 0.45-µm PVDF-Plus membranes (Osmonics, Gloucester, MA) [32]. Prior to transblotting, gels were UV light-activated (1 min) in a BioRad ChemiDoc TM Touch Imaging system. Membranes were blocked with Tris-buffered saline, pH7.4, containing 0.1% Tween-20 and 2% bovine serum albumin prior to incubation (48 hr) with primary antibodies. Proteins of interest were probed with polyclonal antisera raised in rabbit against glycogen synthase (GS; 1:2000; prod. no. 3893S; Cell Signaling Technology, Danvers, MA), glycogen phosphorylase-muscle type (GPmm; 1:2,000; prod. no NBP2-16689; Novus Biologicals, LLC, Centennial, CO.), glycogen phosphorylase-brain type (GPbb; 1:2,000; prod. no. NBP1-32799; Novus Biol.), MCT1 (1:2,000; prod. no. AB3540P; Millipore Sigma, Burlington, MA); MCT2 (1:2000; prod. no. sc-NBP1-87846; Novus Biol.); nNOS (1:1,000; prod. no. Nbp1-396B1; Novus Biol.), GAD (1:1,000; prod. no. ABN904; EMD Millipore, Billerica, MA), 5'-AMP-activated protein kinaseα1/2 (AMPK; 1:2000; prod. no. 2532S; Cell Signaling Technol.), phosphoAMPK-Thr 172 (pAMPK; 1:2000; prod. no. 2531S; Cell Signaling Technol.) or GR (1:6000, prod. no. 3660, Cell Signaling technol.). Membranes were incubated (1 hr) with horseradish peroxidase-labeled goat anti-rabbit secondary antibodies (1:5,000; prod. no. NEF812001EA; PerkinElmer, Waltham, MA), then exposed to SuperSignal West

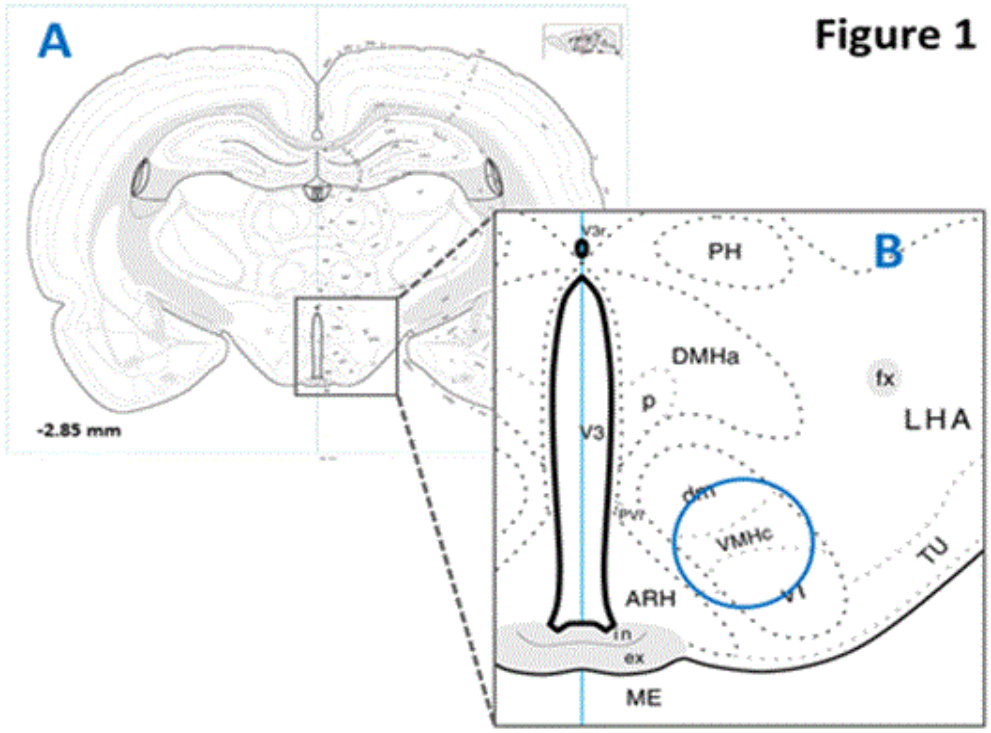

Figure 1: Ventromedial Hypothalamic Nucleus (VMN) Micropunch Dissection. The rectangle at the bottom of the brain map (-2.85 mm posterior to *bregma*) in Panel A depicts the mediobasal hypothalamus wherein the VMN resides. The area within that rectangle is enlarged in Panel B to illustrate neuroanatomical relationships of the VMN with other mediobasal hypothalamic structures. The blue circle denotes the positioning of a 0.50 mm diameter circular micropunch tool over the center of the elliptical-shaped VMN, enabling sampling of tissue from dorsomedial, central, and ventrolateral divisions of the VMN. *Abbreviations in Panel B*: ARH: arcuate hypothalamic n.; DMHap: anterior and posterior part of dorsomedial hypothalamic n.; fx: fornix; LHA: lateral hypothalamic area; ME: median eminence; PVi: intermediate periventricular hypothalamic n.; VMHc,dm,vl: central/dorsomedial/ventormlateral parts of ventromedial hypothalamic n.; TU: tuberal n.; V3: third ventricle.

Femto maximum-sensitivity chemiluminescent substrate (prod. no. 34096; ThermoFisherScientific, Waltham, MA). Protein optical density (O.D.) signals were quantified in the ChemiDoc MP Imaging system, and normalized to total in-lane protein using BioRad Image Lab 6.0.0 software. Bio-Rad precision plus protein dual color standards (prod. no. 161–0374) were included in each Western blot analysis.

VMN Astrocyte Laser-Catapult Microdissection and Western Blotting

Ten µm-thick frozen sections obtained at regular intervals over each VMN were mounted on PEN membrane-coated slides (Carl Zeiss Microscopy, LLC, White Plains, NY), and processed by avidin-biotin peroxidase immunocytochemistry to identify glial fibrillary acid protein (GFAP)-immunoreactive (-ir) astrocytes [29]. Tissues were fixed with acetone (5 min), blocked with 1.5% normal horse serum (prod. no. S-2000, Vector Laboratories, Burlingame, CA), then incubated for 36-48 hr at 4^0C with mouse monoclonal antibodies against GFAP (prod. no. 3670S, Cell Signal. Technol.; 1:500). Sections were next incubated with biotinylated horse anti-mouse IgG secondary antibody, followed by ABC reagent (prod. no. PK-6101; Vector Lab.). GFAP-ir cells were visualized using Vector DAB peroxidase substrate kit reagents (prod. no. SK-4100; Vector Lab.), and collected individually into tissue lysis buffer using a Zeiss P.A.L.M. UV-A microlaser-IV system (Carl Zeiss Microscopy, LLC, Thornwood, NY). Each astrocyte protein of interest was evaluated by immunoblot in separate triplicate pools of n=60 astrocyte lysates for each treatment group. Proteins were detected with primary antisera raised in rabbit against alpha$_{1A}$ AR/ADRA1A (α_1AR; 1:1000; prod. no. NB100-78585; Novus Biol.); alpha$_{2A}$ AR/ADRA3A (α_2AR; 1:1000; prod. no. NBP1-67832, Novus Biol.), beta1 AR/ADRB1 (β_1AR; 1:1000; prod. no. NBP1-59007; Novus Biol.); ER-alpha/NR3A1 (ERα; 1:1000; prod. no. NB100-91756; Novus Biol.); or ER-beta/NR3A2 (ERβ; 1:1000; prod. no. NB120-3577; Novus Biol.). Protein O.D. measures obtained after chemiluminescent substrate incubation were normalized to total in-lane protein using Bio-Rad Stain-Free Imaging Technology, as described above.

Glucose and Counter-Regulatory Hormone Measurements

Plasma glucose levels were determined using an ACCU-CHECK Aviva plus glucometer (Roche Diagnostic Corporation, Indianapolis, IN) [19]. Plasma free fatty acid (FFA) concentrations were measured with Free Fatty Acid Quantitation Kit reagents

(MAK044; Sigma Aldrich, St. Louis, Mo) [33]. Plasma corticosterone (ADI-900-097; Enzo Life Sciences, Inc., Farmingdale, NY) and glucagon (EZGLU-30K, EMD Millipore, Billerica, MA) concentrations were determined using commercial ELISA kit reagents [29b].

Statistical analyses

Mean normalized tissue protein O.D. data and plasma glucose, glucagon, corticosterone, and FFA values were evaluated amongst treatment groups by one-way analysis of variance and Student Newman Keuls post-hoc test. Differences of $p < 0.05$ were considered significant.

Results

Figure 2 depicts effects of icv DEX or sc INS injection with or without icv RU486 pretreatment on VMN glycogen metabolic enzyme and monocarboxylate transporter protein expression in adult male rats. As shown in Panel 2A, VMN GS protein content was elevated following intra-LV administration of DEX [V/DEX versus V/V; $F(3,8) = 49.14$, $p < 0.0001$]. GS profiles were refractory to IIH, but expression levels were significantly increased in RU486- versus V-pretreated INS-injected rats [RU486/INS versus V/INS]. DEX augmented VMN levels of the norepinephrine (NE)-sensitive GP variant GPmm [Müller et al., 2015] [$F(3,8) = 10.53$, $p = 0.004$] (Panel 2B). VMN patterns of GPmm expression did not vary between eu- versus hypoglycemic animals, and were unaffected by RU486 pretreatment prior to INS injection. Data in Panel 2C indicate that DEX [$F(3,8) = 10.39$, $p = 0.004$] did not alter VMN expression of the energy deficit, e.g. AMP-sensitive GP isoform GPbb [Nadeau et al., 2018]. VMN GPbb protein levels were unchanged during IIH, but RU486 pretreatment increased expression of this protein in hypoglycemic rats. DEX administration to euglycemic animals caused significant enhancement

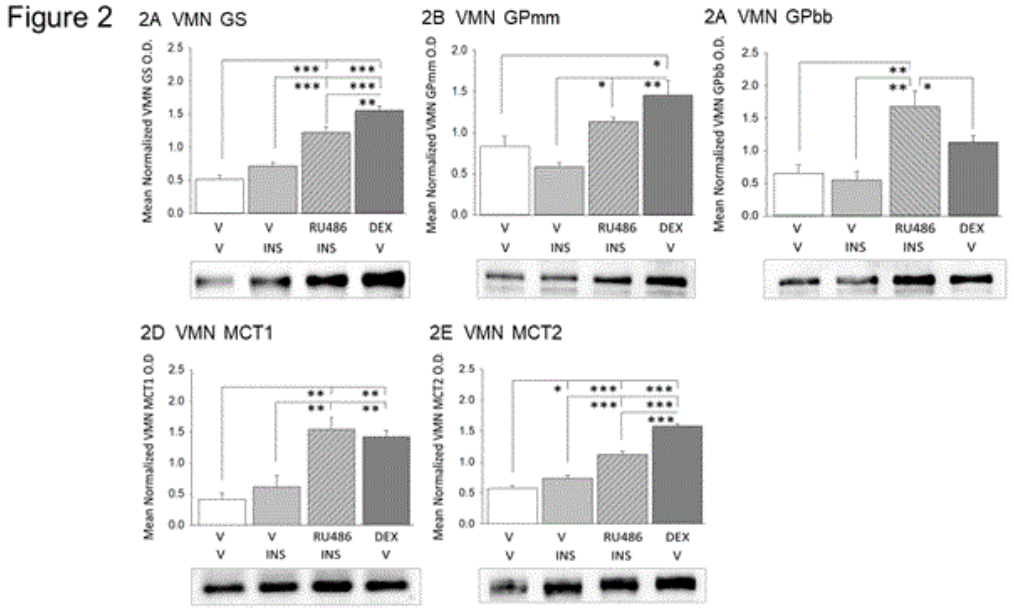

Figure 2: Effects of Intracerebroventricular (icv) Administration of the Glucocorticoid Receptor (GR) Agonist Dexamethasone (DEX) or GR Antagonist RU486 on Ventromedial Hypothalamic Nucleus (VMN) Glycogen Metabolic Enzyme and Monocarboxylate Transporter Protein Expression in the Male Rat. Groups of male rats (n=4) were pretreated by intracerebroventricular (icv) delivery of the GR antagonist RU486 or vehicle (V) prior to subcutaneous (sc) insulin (INS) injection, or were pretreated icv with (DEX) or V before sc insulin diluent (V) injection. For each treatment group, three separate aliquot pools of micropunched VMN tissue were analyzed by Western blot for glycogen synthase (GS; Panel 2A), glycogen phosphorylase (GP)-muscle type (GPmm; Panel 2B), GP-brain type (GPbb; Panel 2C), astrocyte-specific monocarboxylate transporter-1 (MCT1; Panel 2D), or neuron-specific monocarboxylate transporter-2 (MCT2; Panel 2E) protein expression. Bars depict mean normalized protein optical density (O.D.) measures + S.E.M. for the following treatment groups: V/V (solid white bars), V/INS (solid light gray bars), RU486/INS (diagonal-striped light gray bars), and DEX/V (solid dark gray bars) treatment groups. *p<0.05; **p<0.01; ***p<0.001.

of VMN MCT1 and MCT2 content [DEX/V versus V/V] (Panels 2D and 2E). IIH did not modify VMN MCT1 [$F(3,8) = 13.91$, $p = 0.0015$] protein expression, but increased MCT2 [V/INS versus V/V; $F(3,8) = 92.21$, $p < 0.0001$] content. RU486-pretreated, INS-injected rats showed elevated expression of MCT1 and -2 proteins compared to V/INS.

Figure 3 illustrates effects of icv GR agonist or antagonist administration to eu- versus hypoglycemic rats, respectively, on VMN glucoregulatory neurotransmitter biomarker expression and AMPK energy sensor activity. In Panel 3A, results show that VMN GAD content was elevated in response to icv DEX [$F(3,8) = 19.01$, $p = 0.0005$], but was unaffected by IIH with or with RU486 pretreatment. VMN nNOS (Panel 3B) protein profiles

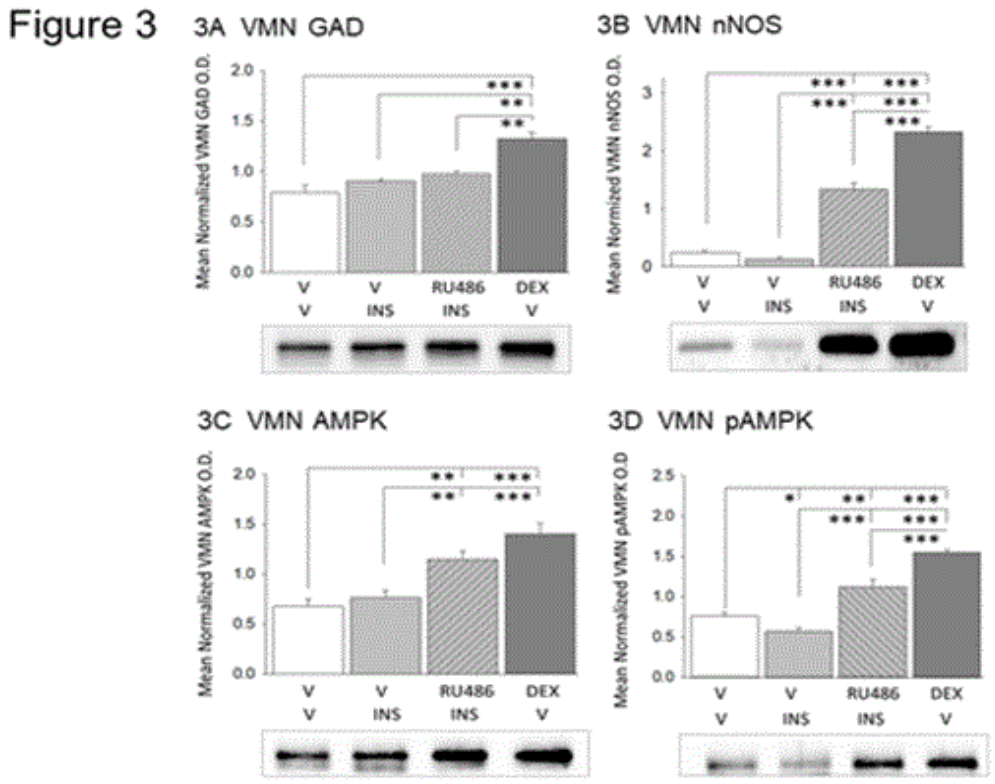

Figure 3: Forebrain GR Regulation of VMN Protein Glucoregulatory Signaling Marker Protein Expression and 5'-AMP-Activated Protein Kinase (AMPK) Activity during Eu- and Hypoglycemia. Bars depict mean normalized VMN glutamate decarboxylase65/67 (GAD; Panel 3A), neuronal nitric oxide synthase nNOS (Panel 3B), AMPK (Panel 3C), or phosphoAMPK (pAMPK) (Panel 3D) protein O.D. measures + S.E.M. for V/V (solid white bars), V/INS (solid light gray bars), RU486/INS (diagonal-striped light gray bars), and DEX/V (solid dark gray bars) treatment groups. *p<0.05; **p<0.01; ***p<0.001.

were elevated in euglycemic and hypoglycemic animals injected with DEX [DEX/V versus V/V] or RU486 [RU486/INS versus V/INS], respectively [F(3,8) = 201.01, p < 0.0001]. VMN total AMPK [F(3,8) = 16.35, p = 0.0002] and pAMPK [F(3,8) = 61.57, p < 0.0001] protein content was increased after DEX administration. IIH did not alter AMPK expression, but suppressed VMN pAMPK content. Hypoglycemic inhibition of pAMPK expression was reversed by RU486 pretreatment.

Data in Figure 4 show patterns of VMN astrocyte AR protein expression in euglycemic rats given vehicle or versus DEX icv and in hypoglycemic animals pretreated by icv administration of vehicle or RU486. Astrocyte α1AR [F(3,8) = 3.68, p = 0.04] and α2AR [F(3,8) = 4.15, p = 0.03] protein profiles were both refractory to DEX or IIH treatment (Panels 4A and 4B). Yet, hypoglycemic patterns of α2AR expression were amplified in RU486-pretreated animals [RU486/INS versus V/INS]. As shown in Panel 3C, DEX, but not IIH stimulated astrocyte β1AR content.

Figure 5 depicts effects of icv DEX or IIH after icv vehicle versus RU486 pretreatment on VMN astrocyte ERα (Panel 5A) and ERβ (Panel 5B) protein content. VMN astrocyte ERβ expression was increased in response to DEX administration [F(3,8) = 10.75, p = 0.001]. Astrocyte ERα expression was elevated in hypoglycemic versus euglycemic controls [V/INS versus V/V]; this profile did not differ between INS-injected groups pretreated with V versus RU486. VMN astrocyte ERβ expression was stimulated by DEX and amplified in hypoglycemic rats pretreated with RU486 [F(3,8) = 24.03, p < 0.0001].

Data in Figure 6 illustrate patterns of VMN GR protein expression after icv GR agonist or antagonist administration. Tissue GR protein content was significantly increased in response to DEX [F(3,8) = 15.02, p = 0.001]. GR profiles were unaffected by IIH, but augmented in RU486- vs. V-pretreated INS-injected rats

Figure 7 presents plasma glucose, glucagon, corticosterone, and FFA responses to icv DEX or sc INS injection with or without icv RU486 pretreatment. As shown in Panel 7A, circulating glucose levels were significantly decreased in response to INS [V/INS versus V/V; F(3,12) = 138.50, p < 0.0001]. Mean glucose measures were not different among vehicle- versus RU486-pretreated hypoglycemic groups. Data also show that Intra-LV DEX administration did not alter plasma glucose levels. Plasma glucagon concentrations (Panel 7B) were significantly elevated in response to INS injection [V/INS versus V/V] [F(3,12) = 6.65, p = 0.009]; this secretory response to IIH was attenuated by RU486 pretreatment. However, plasma glucagon levels were refractory to icv DEX. Circulating corticosterone levels (Panel 7C) were also increased during hypoglycemia, but were unaffected by RU486 pretreatment [F(3,12) = 5.65, p = 0.02]. Icv DEX treatment likewise did not modify plasma corticosterone concentrations.

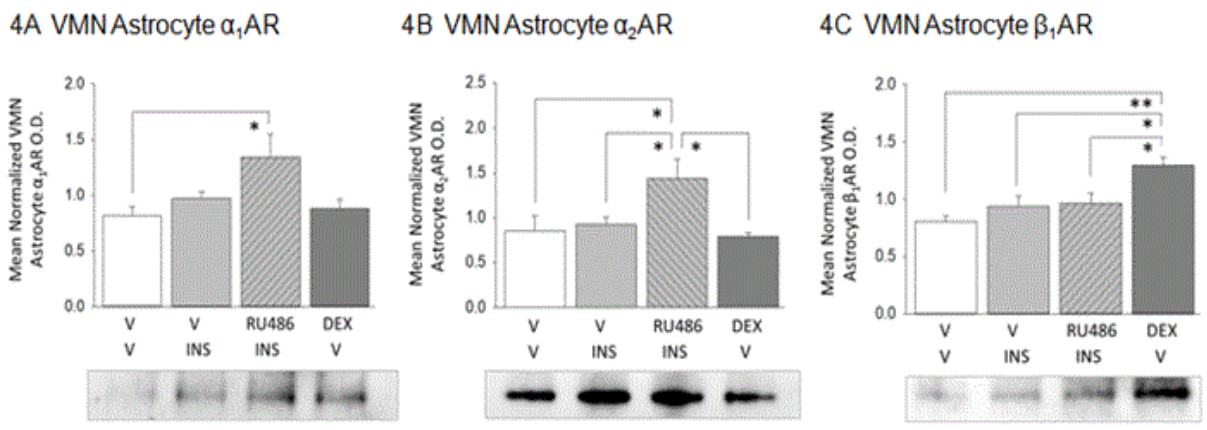

Figure 4: Effects of icv DEX or RU486 Administration on VMN Astrocyte Adrenergic Receptor (AR) Variant Protein Expression in the Male Rat. Pooled lysates of laser-microdissected VMN glial fibrillary acidic protein-immunopositive astrocytes from each treatment group were analyzed in triplicate for alpha1-AR (α1AR; Panel 4A), alpha2-AR (α2AR; Panel 4B), or beta1-adrenergic receptor (β1AR; Panel 4C) protein expression. Data depict mean protein O.D. measures + S.E.M. for V/V (solid white bars), V/INS (solid light gray bars), RU486/INS (diagonal-striped light gray bars), and DEX/V (solid dark gray bars) treatment groups. *p<0.05; **p<0.01; ***p<0.001.

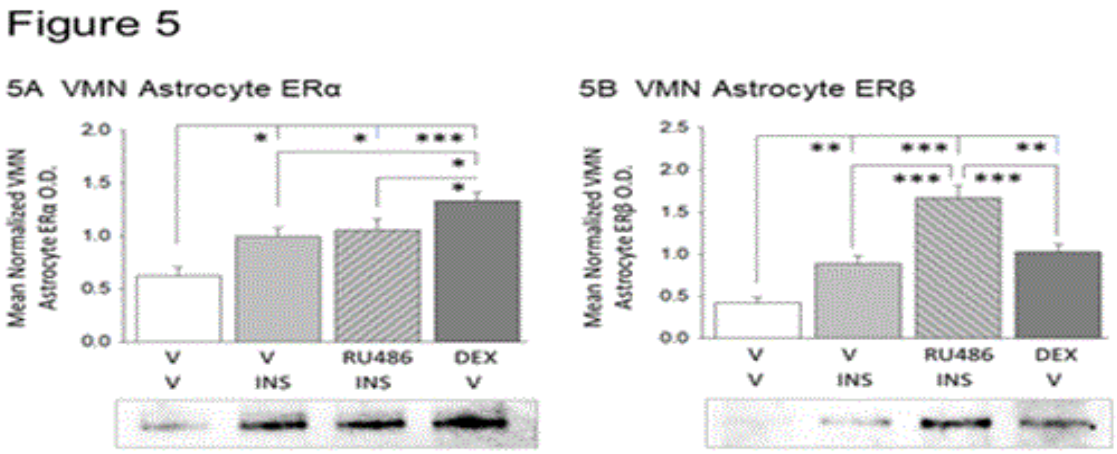

Figure 5: Forebrain GR Regulation of VMN Astrocyte Estrogen Receptor (ER) Variant Protein Expression in the Male Rat. Bars depict mean normalized ER-alpha (ERα) or ER-beta (Eβ) protein optical density (O.D.) values + S.E.M. for V/V (solid white bars), V/INS (solid light gray bars), RU486/INS (diagonal-striped light gray bars), and DEX/V (solid dark gray bars) treatment groups. *p<0.05; **p<0.01; ***p<0.001.

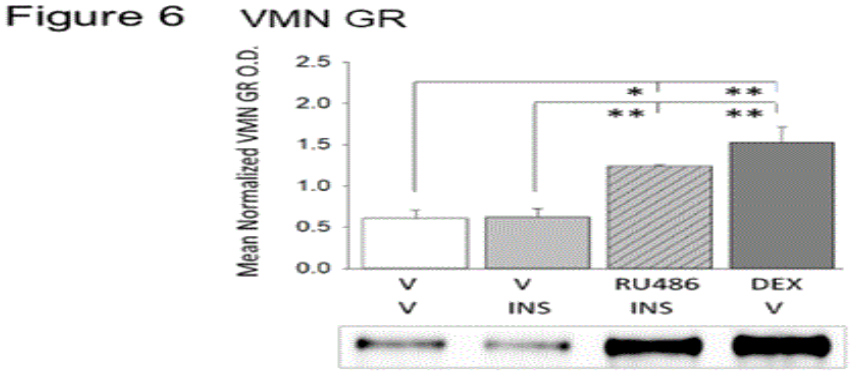

Figure 6: Effects of icv DEX or RU486 Administration on VMN GR Protein Expression in the Male Rat. Bars illustrate mean normalized VMN GR O.D. measures + S.E.M. for V/V (solid white bars), V/INS (solid light gray bars), RU486/INS (diagonal-striped light gray bars), and DEX/V (solid dark gray bars) treatment groups. *p<0.05; **p<0.01; ***p<0.001.

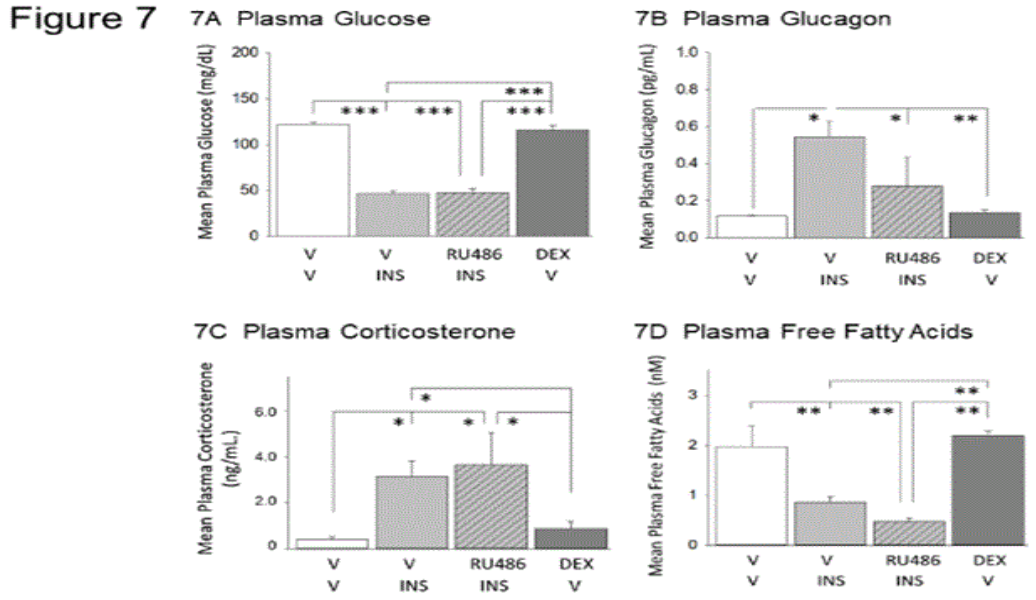

Figure 8: Effects of icv DEX or RU486 Administration on Circulating Glucose, Counter-Regulatory Hormone, and Free Fatty Acid (FFA) Levels in the Male Rat. Data show mean plasma glucose (Panel 7A), glucagon (Panel 7B), corticosterone (Panel 7C), and FFA (Panel 7D) concentrations + S.E.M. for V/V (solid white bars), V/INS (solid light gray bars), RU486/INS (diagonal-striped light gray bars), and DEX/V (solid dark gray bars) treatment groups. *p<0.05; **p<0.01; ***p<0.001.

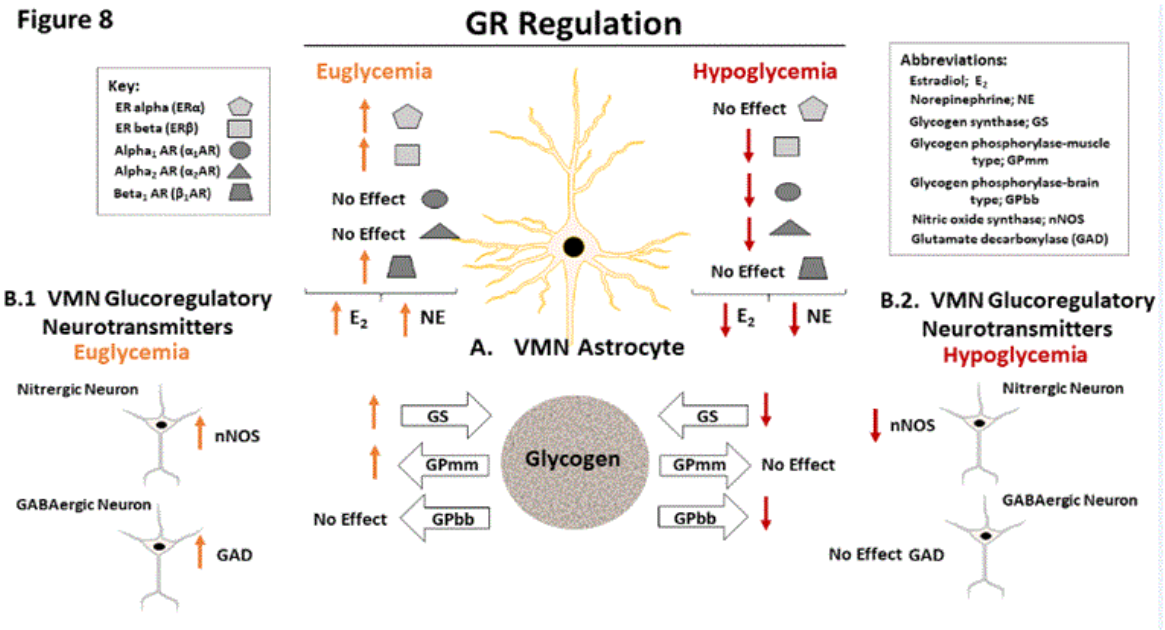

Figure 8: GR Regulation of VMN Astrocyte Receptor and Glycogen Metabolic Enzyme Protein Expression and Glucoregulatory Neuron Marker Protein Profiles during Eu- versus Hypoglycemia. A: Divergent effects of GR signaling on VMN astrocyte estrogen (ERα, ERβ) and adrenergic (α1AR, α2AR, β1AR) receptor protein profiles during eu- versus hypoglycemia [upper middle figure] suggest that GR result in dissimilar volume of estradiol and norepinephrine (NE) input to these glia during glucostasis versus dyshomeostasis. As shown in the lower middle figure, GR elicit metabolic state-dependent changes in astrocyte GS, GPmm, and GPbb levels; opposite changes in GS profiles suggest that GR correspondingly promote or inhibit VMN glycogen accumulation during eu- or hypoglycemia. Differential GR control of NE-sensitive GPmm versus AMP-sensitive GPbb expression may likely result in stimulus-specific VMN glycogen breakdown under distinctive metabolic conditions. Bottom left- (B.1) and right (B.2) -hand figures correspondingly depict effects of GR activation on gluco-stimulatory nitrergic (nNOS) and gluco-inhibitory GABAergic (GAD) neuron marker protein expression during eu- versus hypoglycemia. Outcomes infer that nNOS profiles may reflect, in part, up- or down-regulated glycogen synthesis alongside as-yet-undetermined effects of GR-mediated increases in euglycemic GPmm profiles versus GR suppression of GPbb expression during hypoglycemia. Divergent effects of GR activation on gluco-inhibitory GAD profiles may reflect increased NE-associated glycogen disassembly alone or in relation to glycogen buildup during eu- versus hypoglycemia.

Plasma FFA (Panel 7D) were significantly decreased in response to IIH; this response was not affected by RU486[F(3,12) = 14.88, p = 0.0008]. Icv DEX administration did not modify circulating FFA.

Discussion

Central GR are involved in counter-regulatory mal-adaption and exacerbated glycemic profiles during recurring IIH [19], but neural mechanisms of glucocorticoid hormone control of glucostasis are not known. *In vitro* studies document glucocorticoid modulation of cortical astrocyte glycogen metabolism [20]. Current research used pharmacological, high-resolution microdissection, and Western blot methods to examine the premise that GR regulate signaling by the glycogen-sensitive VMN metabolic transmitters NO and GABA. Results show that GR exert opposite effects on VMN GS and astrocyte- and neuron-specific monocarboxylate transporter proteins during glucose stability (stimulatory) versus insufficiency (inhibitory), and impose differential control of stimulus-specific, e.g. phosphorylation versus AMP-sensitive GP variants under those distinctive metabolic conditions. GR enhance VMN astrocyte ERα and -β and β1AR protein expression during euglycemia, but attenuate ERβ and α1/α2AR profiles during IIH. Outcomes support the possibility that GR-driven gluco-stimulatory NO transmission may signal, in part, augmented glycogen synthesis and/or tissue lactate requirements, while GR-mediated up-regulation of gluco-inhibitory GABA signaling may reflect increased phosphorylation-induced glycogen disassembly alone or in relation to glycogen buildup. It should be noted that outcomes obtained here using a pharmacological approach, i.e. GR agonist or antagonist administration, will require confirmation by additional studies employing corroborative strategies such as genetic manipulation.

Data here show that GR increase VMN GS protein content during euglycemia, but conversely limit GS expression during hypoglycemia, inferring that GR may respectively stimulate or oppose glucose incorporation into VMN glycogen during glucostasis versus glucoprivation. Present outcomes do not clarify how neuro-glucopenia and/or associated cellular energy imbalance may mediate this directional switch in GR control of GS. Evidence that DEX causes concurrent up-regulation of VMN GR and GS supports the need to determine if the latter protein may be augmented, in part, in response to amplified GR signaling; however, the possibility that downstream GR-sensitive signal transduction pathway activity may also be enhanced by DEX should not be discounted. VMN GS protein content was refractory to IIH, but was significantly increased by GR antagonism during IIH. As the current experimental design did not involve icv administration of RU486 to controls, it remains unclear if and to what extent hypoglycemia-associated GR inhibition of GS may differ compared to euglycemia. In the event that ongoing research reveals that IIH augments GR suppression of GS, it could be presumed that this amplified negative stimulus may be counter-balanced by positive inputs of equivalent strength. Since current data show that VMN GR protein content was similar in V/V versus V/INS groups, it is plausible that direction (e.g. inhibition versus stimulation) of GR control of VMN GS expression may be established, in part, by absolute levels of GR expression. At the same time, the likelihood that hypoglycemia may regulate post-receptor signaling to shape GR regulation of GS merits consideration.

GP activity is controlled by serine phosphorylation and/or stimulatory (AMP) and inhibitory (glucose) allosteric effectors [34, 35]. Brain GP isoforms GPmm and GPbb show differential sensitivity to phosphorylation and AMP regulation [36]. Phosphorylation fully activates GPmm, but not GPbb, which requires AMP binding for maximum activation [37]. AMP exhibits greater binding affinity for GPbb versus GPmm, and reduces GPbb Km for glycogen [38]. In brain, GPmm mediates noradrenergic stimulation of cortical astrocyte glycogenolysis in vitro, whereas GPbb mobilizes glycogen breakdown during energy deficiency [39]. Present evidence that GR up-regulate VMN GPmm protein profiles during glucose stability, but impose an inhibitory tone on GPbb expression during glucoprivation suggests that GR may control physiological stimulus-specific effects on VMN glycogen metabolism. Effects of icv drug treatments on VMN GP variant enzyme activities were not assessed here due to lack of requisite analytical sensitivity pertaining to the small tissue sample volumes obtained here. Further studies are required to ascertain proportionate expression of GPmm versus GPbb in the VMN, and to investigate effects of isoform-specific GR regulation on net VMN glycogen mass under discrete metabolic conditions. There also remains the need to determine if DEX-associated up-regulation of VMN MCT1 and MCT2 protein expression is causally related to GR agonist stimulation of glycogen disassembly and associated conversion of liberated glucose to lactate for astrocyte-to-neuron trafficking.

VMN levels of GAD, the marker protein for gluco-inhibitory GABA signaling, were elevated in response to DEX, while hypoglycemic GAD profiles were unaffected by RU486 despite equivalent patterns of VMN GR protein expression in DEX/V versus RU486/INS treatment groups. Potential mechanisms responsible for GABA insensitivity to GR during glucoprivation remain unclear, but could involve GR inactivation and/or suppression of downstream signaling under the latter conditions. Here, VMN GPmm and GAD proteins exhibited parallel up-regulation by DEX yet both proteins were insensitive to combinatory RU486 plus INS treatment, suggesting that GABAergic cues of metabolic stability may be an indicator of extent of stimulus-specific, e.g. NE-driven glycogen degradation. In contrast, VMN nNOS protein expression, an indicator of gluco-stimulatory NO release, was subject either to bi-directional, metabolic state-specific GR control. It would be informative to learn if measurable drug effects on nNOS profiles elicit or are a consequence of GS and/or MCT responses to those treatments. Findings that IIH did not suppress VMN GAD or elevate nNOS profiles concur with previous reports [27]. Here, outcomes here provide unique proof that VMN AMPK activity is decreased one hour after initiation of hypoglycemia, reinforcing the notion that energy stability is maintained within this acute post-treatment interval and that moreover, GR mediate this

reduction in sensor activity. Further studies are required to clarify whether decreased VMN pAMPK expression reflects, in part, reduced partitioning of glucose into the astrocyte glycogen shunt [40, 41] due to down-regulated GS

Recent studies implicate NE and estradiol in regulation of VMN glycogen metabolism [27, 28]. Current data show that GR enhance estradiol signaling to astrocytes via ERα and -β signaling during glucostasis, but attenuate ERβ-mediated input to this glia during glucoprivation. Outcomes also indicate that GR amplify NE input to astrocytes during euglycemia by up-regulating astrocyte β1AR protein content, but restrain noradrenergic stimulation of this glia during hypoglycemia through suppression of astrocyte α1- and α2AR expression. These data support the possibility that divergent GR regulation of VMN GS, MCT 1/2, and nNOS protein profiles during eu- versus hypoglycemia may be mediated by corresponding up-regulation of ERβ and β1AR and down-regulation of ERβ and α1- and α2AR.

Outcomes document up-regulation of VMN GR by the GR agonist DEX, but show that this protein profile is refractory to IIH despite hypoglycemic corticosteronemia and that GR constrain VMN GR expression during IIH. These findings agree with recent reports that stress-associated glucocorticoid inhibition of GR involves diminished AMPK activity [42]. Current studies do not shed light on whether possible differences in GR occupation and activation by DEX compared to that achieved by elevated corticosterone secretion during hypoglycemia may determine self-up- versus down-regulation of VMN GR. The experimental design here utilized an intraventricular route of administration of GR modulators to the brain to facilitate the study objective of activating or inhibiting activity of GR expressed in the VMN as well as other hypothalamic glucoregulatory loci that are functionally interconnected with the VMN. Thus, while observed drug effects on VMN GR protein expression imply that drug action occurs within that structure, there remains a likelihood that extra-VMN GR-sensitive neurotransmitters may mediate, to some degree, observed changes in VMN and systemic experimental endpoints. An objective of the current project was to investigate GR modulator effects on VMN astrocyte GR content. However, this protein was undetectable here in LCM-harvested astrocytes, suggesting that GR expression may fall below limits of analytical sensitivity, or that GR regulation of astrocyte protein expression may be mediated, in part, by chemical stimuli from glucocorticoid-sensitive neurons or other glial cell types. Additional effort is required to determine if VMN GABA and/or nitrergic neurons express GR.

Results indicate that icv DEX administration did not significantly alter plasma glucose or counter-regulatory hormone levels despite observable effects of this treatment paradigm on VMN glucoregulatory transmitter marker protein expression. It is possible that concurrent augmentation of opposing gluco-stimulatory NO and -inhibitory GABA stimuli results in no net change in counter-regulatory outflow. Current evidence that INS suppression of glucose was unaffected by RU486 implies that hypoglycemic hypercorticosteronemia primarily attenuates glucose decrements [43-45] by action on GR that are not accessible to drugs delivered to the cerebral ventricular system. Evidence here that hypoglycemic glucagonemia was partially normalized by icv RU486 administration suggests that hypoglycemic patterns of corticosterone secretion act, in part, on periventricular substrates to limit glucagon outflow. As hypothalamic, specifically paraventricular hypothalamic nucleus GR are implicated in glucocorticoid negative feedback inhibition of the hypothalamic-pituitary-adrenal axis and function to limit the magnitude of corticosterone output during acute stress [46-48], our working premise was that RU486 would enhance corticosterone secretion in INS-injected rats. Yet, current data show that corticosterone levels did not differ between RU485- versus vehicle-pretreated hypoglycemic animals. It is possible that adjusted GR antagonist dosages might more effectively inhibit VMN GR signaling compared to the current treatment paradigm, and/or that analysis of corticosterone release at additional post-INS injection time points may document measurable RU486 effects on hormone secretion. Present evidence that neither DEX nor RU486 altered circulating FFA concentrations suggest that GR involved in regulating this non-glucose substrate fuel are located beyond diffusion range of the cerebroventricular system.

Previous studies in our laboratory utilized immunocytochemical methods to identify GR-immunoreactive neurons that undergo transcriptional activation in response to hypoglycemia [49]. That approach was not used here as immunocytochemistry is an optimum approach for discernment of the cellular distribution of a protein(s) of interest, but relies upon visual discernment of possible treatment-associated differences in enzyme-derived reaction product or fluorophore-associated emitted light between treatment groups. Rather, Western blotting permits a semi-quantitative approach to be implemented for measurement of protein profiles within distinctive cell populations.

A limitation of the present project is the lack of inclusion of an additional treatment group involving RU486 administration to euglycemic animals. Investigation of GR antagonism effects during glucostasis balance could be expected to shed light on relative degree of control imposed by these receptors during glucostasis versus glucoprivation. There also remains a need for insight on how DEX may affect hypoglycemic patterns of protein expression described here. For example, it would be informative to learn if GR activation beyond that achieved by hypoglycemic hypercorticosteronemia. Ongoing studies seek to investigate these unresolved knowledge gaps.

The current experimental design closely adheres to a longstanding paradigm used in our laboratory, involving specific timing of systemic and intracranial drug therapies over a distinct morning time interval to evaluate concerning regulatory associations between hypothalamic neuroendocrine function and energy homeostasis, including GR involvement in neural mechanisms governing glucose counter-regulation [19]. Nonetheless, in light of evidence for diurnal variation in GR activation by endogenous

ligand [50], it should be noted that administration of GR agonists and antagonists at other time points over a 24 hour period may elicit outcomes that diverge from data presented here. Moreover, since only one time between drug delivery and tissue procurement was evaluated here, it is possible that response variables may show measurable differences in magnitude and/or direction at time points before or after that employed here.

In summary, current studies investigated effects of icv delivery of the GR agonist DEX or the GR antagonist RU486 on VMN glycogen metabolic enzyme and glucoregulatory transmitter biomarker protein expression in the male rat (Figure 8). Results show GR exert bi-directional regulatory effects on VMN GS, MCT1/2, and nNOS proteins during eu- (stimulatory) versus hypoglycemia (inhibitory), implying that up-regulated gluco-stimulatory NO transmission may reflect, in part, increased glucose incorporation into glycogen and/or augmented tissue lactate requirements. Data provide unique proof of metabolic state-dependent GR regulation of VMN NE-sensitive GPmm and AMP-sensitive GPbb profiles, and raise the prospect that gluco-inhibitory GABA signaling may reflect, in part, stimulus-specific glycogen breakdown alone or relative to rate of glycogen assembly.

Declarations

Declaration of interest

The authors have no interests to declare.

Source of funding

This work was supported by National Institutes of Health grant DK-109382.

Statement on animal use

All animal experimental was carried out in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, 8th Edition, as stated in the submitted manuscript. Sex of animal used is included, along with discussion of impacts on study outcomes.

Abbreviations

α_1AR: alpha1 adrenergic receptor

α_2AR: alpha2 adrenergic receptor

β_1AR: beta1 adrenergic receptor

DEX: Dexamethasone

ERα: Estrogen receptor-alpha

ERβ: Estrogen receptor-beta

GABA: ϒ-aminobutyric acid

GAD: Glutamate decarboxylase$_{65/67}$

GPbb: Glycogen phosphorylase-brain type

GPmm: Glycogen phosphorylase-muscle type

GPER: G protein-coupled estrogen receptor 1

GR: type II glucocorticoid receptor

GS: Glycogen synthase

Icv: Intracerebroventricular

IIH: Insulin-induced hypoglycemia

INS: Insulin

LV: Lateral ventricle

MCT1: Monocarboxylate transporter-1

MCT2: Monocarboxylate transporter-2

NE: Norepinephrine

NO: Nitric oxide

nNOS: Neuronal nitric oxide synthase

RU486: mifepristone

VMN: Ventromedial hypothalamic nucleus

Reference

1. Cryer PE. Hypoglycemia-associated autonomic failure in diabetes. Handb Clin Neurol. 2013;117:295-307. doi: 10.1016/B978-0-444-53491-0.00023-7.
2. Cryer PE. Glycemic goals in diabetes: trade-off between glycemic control and iatrogenic hypoglycemia. Diabetes. 2014; 63(7): 2188-2195.doi:10.2337/db14-0059.
3. Auer RN. Hypoglycemia brain damage. Forensic Sci Int. 2004; 146(2-3):105-110.doi:10.1016/j.forsciint.2004.08.001.
4. Auer RN, Wieloch T, Olsson Y, Seisjö BK. The distribution of hypoglycemic brain damage. Acta Neuropathol.1984;64(3):177-191. doi: 10.1007/BF00688108.
5. Chan O and Sherwin R. Influence of VMN fuel sensing on hypoglycemic responses. Trends Endocrinol Metab.2013;24(12): 616-624. doi:10.1016/j.tem.2013.08.005.
6. Watts AG and Donovan CM. Sweet talk in the brain: glucosensing, neural networks, and hypoglycemic counterregulation. Front Neuroendocrinol.2010;31(1):32-43.doi: 10.1016/j.yfrne.2009.10.006.
7. Donovan CM and Watts AG. Peripheral and central glucose sensing in hypoglycemic detection. Physiology.2014; 29(5): 314-324. doi:10.1152/physiol.00069.2013.
8. Y Oomura, T Ono, H Ooyama, M J Wayner. Glucose and osmosensitive neurons of the rat hypothalamus. Nature. 1969; 222(5190): 282-284. doi: 10.1038/222282a0.
9. Ashford MLJ, Boden PR, Treherne JM. Glucose-induced excitation of hypothalamic neurons is mediated by ATP-sensitive K+ channels. Pflugers Arch.1990;415(4):479-483.doi: 10.1007/BF00373626.
10. Silver IA and Erecińska M. Glucose-induced intracellular ion changes in sugar-sensitive hypothalamic neurons. J Neurophysiol. 1998;79(4):1733-1745.doi:10.1152/jn.1998.79.4.1733.
11. Owen Chan 1, Wanling Zhu, Yuyan Ding, Rory J McCrimmon, Robert S Sherwin. Blockade of GABA (A) receptors in the ventromedial hypothalamus further stimulates glucagon and sympathoadrenal but not the hypothalamo-pituitary-adrenal response to hypoglycemia.

Diabetes. 2006;55(4):1080-1087. doi:10.2337/diabetes.55.04.06. db05-0958.

12. Xavier Fioramonti, Nicolas Marsollier, Zhentao Song, Kurt A Fakira, Reema M Patel, Stacey Brown, et al. Ventromedial hypothalamic nitric oxide production is necessary for hypoglycemia detection and counterregulation. Diabetes.2010;59(2):519-528.doi:10.233/ db09-0421.
13. Routh VH, Hao L, Santiago AM, Sheng Z, Zhou C. Hypothalamic glucose sensing: making ends meet. Front Syst Neurosci. 2014;8: 236. doi:10.3389/fnsys.2014.00236.

13b. Stobart JL and Anderson CM. Multifunctional role of astrocytes as gatekeepers of neuronal energy supply. Front Cell Neurosci. 2013; 7: 1-21.doi:10.3389/fncel.2013.00038.

14. P R Laming, H Kimelberg, S Robinson, A Salm, N Hawrylak, C Müller, et al. Neuronal-glial interactions and behavior. Neurosci Biobehav Rev. 2000;24(3):295-340.doi:10.1016/s0149-7634(99)00080-9.

15. S Bröer, B Rahman, G Pellegri, L Pellerin, J L Martin, S Verleysdonk, et al. Comparison of lactate transport in astroglial cells and monocarboxylate transporter 1 (MCT 1) expressing Xenopus laevis oocytes. Expression of two different monocarboxylate transporters in astroglial cells and neuronsJ Biol Chem. 1997;272(48):30096-30102. doi: 10.1074/jbc.272.48.30096.
16. Borg MA, Tamborlane WV, Shulman GI, Sherwin RS. Local lactate perfusion of the ventromedial hypothalamus suppresses hypoglycemic counterregulation. Diabetes.2003;52(3):663-666.doi:10.2337/ diabetes.52.3.663.
17. Owen Chan, Sachin A Paranjape, Adam Horblitt, Wanling Zhu, Robert S Sherwin. Lactate-induced release of GABA in the ventromedial hypothalamus contributes to counterregulatory failure in recurrent hypoglycemia and diabetes. Diabetes. 2013; 62(12):4239-4246. doi: 10.2337/db13-0770.
18. Alhamami HN, Alshamrani A, Briski, KP. Inhibition of glycogen phosphorylase stimulates ventromedial hypothalamic nucleus AMP-activated protein kinase. Activity and neuronal nitric oxide synthase protein expression in male rats. Physiol Rep. 2017 Dec; 5(23): e13484. doi: 10.14814/phy2.13484.

18b. Briski KP, Alhamami HN, Alshamrani A, Mandal SK, Shakya M, Ibrahim MMH. Sex differences and role of estradiol in hypoglycemia-associated counter-regulation. Adv Exp Med Biol. 2017;1043:359-383.doi:10.1007/978-3-319-70178-3_17.

19. Kale AY, Paranjape SA, Briski KP. I.c.v. administration of the nonsteroidal glucocorticoid receptor antagonist, CP4-72555, prevents exacerbated hypoglycemia during repeated insulin administration. Neuroscience 2006; 140: 555-565.
20. Allaman I, Pellerin L, Magistretti PJ. Glucocorticoids modulate neurotransmitter-induced glycogen metabolism in cultured cortical astrocytes. J Neurochem. 2004;88(4):900-908.doi:10.1046/j.1471-4159.2003.02235.x.
21. Reul JM, van den Bosch FR, de Kloet ER. Relative occupation of type-I and type-II corticosteroid receptors in rat brain following stress and dexamethasone treatment: functional implications J Endocrinol.1987;115(3):459-467.doi: 0.1677/joe.0.1150459.
22. De Kloet ER, Oitzl MS, Joels M. Functional implications of brain corticosteroid receptor diversity. Cell Mol Neurobiol.1993;13(4): 433-455.doi:10.1007/BF00711582.
23. De Kloet ER, Vreugdenhil E, Oitzl MS, M Joëls. Brain corticosteroid receptor balance in health and disease. Endocr Rev. 1998;19 (3): 269-301. doi: 10.1210/edrv.19.3.0331.
24. Ahima RS and Harlan RE. Charting of type II glucocorticoid receptor-like immunoreactivity in the rat central nervous system. Neuroscience. 1990;39(3):579-604. doi: 10.1016/0306-4522(90)90244-x.
25. Cadepond F, Ulmann A, Baulieu EE. RU486 (mifepristone): Mechanisms of action and clinical uses. Annu Rev Med. 1997;48:129-156. doi:10.1146/annurev.med.48.1.129.
26. Clark RD. Glucocorticoid receptor antagonists. Curr Top Med Chem. 2008; 8(9): 813-838.doi :10.2174/156802608784535011.
27. Mahmood ASMH, Bheemanapally K, Mandal SK, Ibrahim M M H, Briski K P. Norepinephrine control of ventromedial hypothalamic nucleus glucoregulatory neurotransmitter expression in the female rat: role of monocarboxylate transporter function. Mol Cell Neurosci. 2019;95:51-58.doi:10.1016/j.mcn.2019.01.004.
28. Ibrahim MMH, Alhamami HN, Briski KP. Norepinephrine regulation of ventromedial hypothalamic nucleus metabolic transmitter biomarker and astrocyte enzyme and receptor expression: role of 5'-AMP-activated protein kinase. Brain Re.2019;1711:48-57. doi: 10.1016/j.brainres.2019.01.012.
29. Tamrakar P, Shrestha PK, Briski KP. Dorsomedial hindbrain catecholamine regulation of hypothalamic astrocyte glycogen metabolic enzyme protein expression: Impact of estradiol. Neuroscience. 2015;292:34-45.doi:10.1016/j.neuroscience.2015.02.019.

29b. Mahmood ASMH, Uddin MM, Mandal SK, M M H Ibrahim, H N Alhamami, K P Briski. Sex differences in forebrain estrogen receptor regulation of hypoglycemic patterns of counter-regulatory hormone secretion and ventromedial hypothalamic nucleus gluco regu latory neurotransmitter and astrocyte glycogen metabolic enzyme expression. Neuropeptides.2018;72:65-74.doi:10.1016/j.npep. 2018.10.003.

30. Briski KP, Vogel KL, McIntyre AR. The antiglucocorticoid, RU486, attenuates stress-induced decreases in plasma-luteinizing hormone concentrations in male rats. Neuroendocrinology.1995;61(6):638-645. doi:10.1159/000126890.
31. Briski KP, DiPasquale BM, Gillen E. Induction of immediate-early gene expression in preoptic and hypothalamic neurons by the glucocorticoid receptor agonist, dexamethasone. Brain Res. 1997;768(1-2):185-196. doi:10.1016/s0006-8993(97)00642-2.
32. Shakya M, Shrestha PK, Briski KP. Hindbrain 5'-adenosine monophosphate-activated protein kinase mediates short-term food deprivation inhibition of the gonadotropin-releasing hormone-luteinizing hormone axis: role of nitric oxide. Neuroscience.2018; 383: 46-59.doi:10.1016/j.neuroscience.2018.04.040.
33. Briski KP, Alenazi FSH Shakya M, Sylvester PW. Estradiol regulates hindbrain A2 noradrenergic neuron adenosine 5'-monophosphate-activated protein kinase (AMPK) activation, upstream kinase/phosphatase protein expression, and receptivity to hormone and fuel reporters of short-term food deprivation are regulated by estradiol. J Neurosci Res.2017;95(7):1427-1437.doi: 10.1002/jnr.23892.

34. Griffiths JR, Dwek RA, Radda GK. Heterotropic interactions of ligands with phosphorylase B. Eur J Biochem. 1976; 61: 234-251.
35. Barford D and Johnson LN. The allosteric inhibition of glycogen phosphorylase. Nature. 1989; 340: 609-616.
36. Nadeau OW, Fontes JD, Carlson GM. The regulation of glycogenolysis in the brain. J Biol Chem.2018;293(19):7099-7107.doi: 10.1074/jbc. R117.803023.
37. Cécile Mathieu, Ines Li de la Sierra-Gallay, Romain Duval, Ximing Xu, Angélique Cocaign, Thibaut Léger, et al. Insights into brain glycogen metabolism: the structure of human brain glycogen phosphorylase. J Biol Chem.2016;291(35):18072-18083.doi: 10.1074/jbc. M116.738898.
38. Lowry OH, Schulz DW, Passonneau JV. The kinetics of glycogen phosphorylases from brain and muscle. J Biol Chem. 1967; 242(2): 271-280.
39. Müller MS, Pedersen SE, Walls AB, Waagepetersen H S, Bak L K. Isoform-selective regulation of glycogen phosphorylase by energy deprivation and phosphorylation in astrocytes. Glia.2015;63(1):154-162.doi:10.1002/glia.22741.
40. Walls AB, Heimbürger CM, Bouman SD, Schousboe A, Waagepetersen H S. Robust glycogen shunt activity in astrocytes: Effects of glutamatergic and adrenergic agents. Neuroscience.2009;158(1):284-292. doi:10.1016/j.neuroscience.2008.09.058.
41. Schousboe A, Sickmann HM, Walls AB, Bak L K, Waagepetersen H S. Functional importance of the astrocytic glycogen-shunt and glycolysis for maintenance of an intact intra/extracellular glutamate gradient. Neurotox Res.2010;18(1):94-99.doi: 10.1007/s12 640-010-9171-5.
42. Yuan SY, Liu J, Zhou J, Lu W, Zhou H Y, Long L H, et al. AMPK mediates glucocorticoids stress-induced downregulation of the gluco corticoid receptor in cultured rat prefrontal cortical astrocytes. PLoS One. 2016;11(8):e0159513.doi: 10.1371/ journal.pone.015 9513.
43. Khalil Z, Marley PD, Livett BG. Elevation in plasma catecholamines in response to insulin stress is under both neuronal and nonneuronal control. Endocrinology.1986;119(1):159-167.doi:10.1210/endo-119-1-159.
44. Yamaguchi N, Briand R, Gaspo R. Effect of functional adrenalectomy on glucagon secretion and circulating catecholamines during insulin hypoglycemia in the dog. Can J Physiol Pharmacol.1990;68(9):1183-1188.doi:10.1139/y90-177.
45. Komesaroff PA and Funder JW. Differential glucocorticoid effects on catecholamine responses to stress. Am J Physiol. 1994;266(1 Pt 1):E118-E28.doi:10.1152/ajpendo.1994.266.1.E118.
46. Keller-Wood M. Hypothalamic-pituitary-adrenal axis-feedback control. Compr Physiol. 2015;5(3):1161-1182.doi:10.1002/cphy.c1 40065.
47. Laryea G, Schütz G, Muglia LJ. Disrupting hypothalamic glucocorticoid receptors causes HPA axis hyperactivity and excess adiposity. Mol Endocrinol.2013;27(10):1655-1665.doi:10.1210/me.2013-1187.
48. Matia B Solomon, Matthew Loftspring, Annette D de Kloet, Sriparna Ghosal, Ryan Jankord, Jonathan N Flak, et al. Neuroendocrine function after hypothalamic depletion of glucocorticoid receptors in male and female mice. Endocrinology. 2015;156(8):2843-2853.doi:10.1210/en.2015-1276.
49. Kale AY Paranjape SA, Briski KP. Site-specific habituation of insulin-induced hypoglycemic induction of Fos immunoreactivity in glucocorticoid receptor (GR)-immuno-positive neurons in the male rat brain. Exp Brain Res. 2007;176(2):260-266.doi: 10.1007/s 00221-006-0614-4.
50. Spencer RL, Miller AH, Moday H, M Stein, B S McEwen. Diurnal differences in basal and acute stress levels of type I and type II adrenal steroid receptor activation in neural and immune tissues. Endocrinology.1993;133(5):1941-1950.doi:10.1210/endo.133. 5. 8404640.