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Neuroscience. Author manuscript; available in PMC 2024 October 01.

Published in final edited form as:

Neuroscience. 2023 October 01; 529: 73–87. doi:10.1016/j.neuroscience.2023.08.006.

GHRH Neurons from the Ventromedial Hypothalamic Nucleus Provide Dynamic and Sex-Specific Input to the Brain Glucose-Regulatory Network

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Abstract

The ventromedial hypothalamic nucleus (VMN) controls glucose counter-regulation, including pituitary growth hormone (GH) secretion. VMN neurons that express the transcription factor steroidogenic factor-1/NR5A1 (SF-1) participate in glucose homeostasis. Research utilized *in vivo* gene knockdown tools to determine if VMN growth hormone-releasing hormone (Ghrh) regulates hypoglycemic patterns of glucagon, corticosterone, and GH outflow according to sex.

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Declaration of 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 animals used is included, along with discussion of sex impacts on study outcomes.

CRediT Statement: Subash Sapkota: conceptualization, investigation, formal analysis, validation, data curation, visualization, writing – original draft, writing – review and editing, visualization; Md. Haider Ali: conceptualization, methodology; Ayed A, Alshamrani: conceptualization, methodology; Prabhat R. Napit: investigation, formal analysis, validation, data curation; Sagor C. Roy: investigation, formal analysis, validation, data curation; Madhu Babu Pasula: investigation, formal analysis, validation, data curation; Karen P. Briski: conceptualization, writing – original draft, writing – review and editing, supervision, project administration, funding acquisition

Declaration of interest: The authors have no conflicts of interests to declare.

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Intra-VMN Ghrh siRNA administration blunted hypoglycemic hypercorticosteronemia in each sex, but abolished elevated GH release in males only. Single-cell multiplex qPCR shows that dorsomedial VMN (VMNdm) Ghrh neurons express mRNAs encoding Ghrh, SF-1, and protein markers for glucose-inhibitory (γ-aminobutyric acid) or -stimulatory (nitric oxide; glutamate) neurotransmitters. Hypoglycemia decreased glutamate decarboxylase$_{67}$ (GAD_{67}) transcripts in male, not female VMNdm Ghrh/SF-1 neurons, a response that was refractory to Ghrh siRNA. Ghrh gene knockdown prevented, in each sex, hypoglycemic down-regulation of Ghrh/SF-1 nerve cell GAD_{65} transcription. Ghrh siRNA amplified hypoglycemia-associated amplification of Ghrh/SF-1 neuron nitric oxide synthase mRNA in male and female, without affecting glutaminase gene expression. Ghrh gene knockdown altered Ghrh/SF-1 neuron estrogen receptor-alpha (ERα) and ER-beta transcripts in hypoglycemic male, not female rats, but up-regulated GPR81 lactate receptor mRNA in both sexes. Outcomes infer that VMNdm Ghrh/SF-1 neurons are an effector of SF-1 control of counter-regulation, and document Ghrh modulation of hypoglycemic patterns of glucose-regulatory neurotransmitter along with estradiol and lactate receptor gene transcription in these cells. Co-transmission of glucose-inhibitory and - stimulatory neurochemicals of diverse chemical structure, spatial, and temporal profiles may enable VMNdm Ghrh neurons to provide complex dynamic, sex-specific input to the brain glucose-regulatory network.

Keywords

Ghrh; insulin-induced hypoglycemia; GAD65; SF-1; glutaminase; sex differences

Introduction:

Neural regulation of glucose homeostasis involves coordinated neuroendocrine, autonomic, and behavioral outflow to control glucose uptake, utilization, synthesis, and storage; insulin and counter-regulatory hormone secretion; and food intake. Common command of these vital motor functions is enacted by the hypothalamus, the topmost visceral motor center in the brain. The ventromedial hypothalamic nucleus (VMN), a bilateral structure in the medio-basal hypothalamus, is a key sensory and integrative constituent of the widespread glucostatic regulatory network that incorporates fore-, mid-, and hindbrain structures [Watts and Donovan, 2010; Chan and Sherwin, 2013; Tu et al., 2022]. The VMN-specific transcription factor steroidogenic factor-1 (SF-1; NR5A1) is crucial for normal establishment of VMN nerve cell population phenotypes [Davis et al., 2004; McClellan et al., 2006]. SF-1 knockdown impairs VMN cytoarchitectural organization, resulting in metabolic, thermogenic, and reproductive dysfunction [Zhao et al., 2008; Kim et al., 2009, 2010]. In the adult, SF-1 is expressed in neurons located in dorsomedial (VMMdm) and central (VMNc) neuroanatomical divisions of the VMN, but is absent from the ventrolateral VMN (VMNvl) (Cheung et al., 2013; Kim et al.., 2019). SF-1 involvement in neural regulation of systemic energy and glucose homeostasis is well-documented (Dhillon et al., 2008; Kim et al., 2011; Xu et al., 2011; Choi et al., 2013; Garfield et al., 2014; Meek et al., 2016). Systems-level understanding of VMN SF-1 nerve cell function, namely control of downstream glucose-regulatory pathway components, will entail knowledge of mechanisms of neurochemical communication. Beyond evidence that SF-1 neurons express the glutamate marker VGLUT2, which infers that these cells express the excitatory amino acid transmitter

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glutamate [Tong et al., 2007), characterization of cellular neurotransmitter profiles in these cells and investigation of expressed transmitter responsiveness to hypoglycemia *in vivo* has not been carried out.

Growth hormone-releasing hormone (Ghrh; e.g. somatocrinin) is a 44-amino acid hypophysiotropic neuropeptide that is carried by a portal vascular system from the hypothalamic median eminence to the anterior pituitary gland, where it acts on somatotropes to stimulate growth hormone (GH) synthesis and secretion [Frohman, 1988; Grossman et al., 1986; Reichlin, 1989; Vance, 1990; Epelbaum, 1992; Hartman and Veldhuis, 1993]. While median eminence-projecting Ghrh neurons reside primarily in the hypothalamic arcuate nucleus (ARH), non-neuroendocrine Ghrh neurons exist in the VMN and other hypothalamic structures [Steyn, 2018]. Yet, evidence that electrical stimulation of the VMN stimulates GH secretion [Frohman et al., 1968] infers that resident neurons may function upstream of the ARH Ghrh cell population. In lieu of reports that Ghrh neurons are present in the VMNdm [Burgunder, 1991], current studies utilized combinative *in situ* immunocytochemistry-laser catapult microdissection-single-cell multiplex qPCR techniques in conjunction with a validated *in vivo* animal model for insulin-induced hypoglycemia (IIH) [Paranjape and Briski, 2005] to address the premise that VMNdm SF-1 neurons express Ghrh mRNA, and that one or both of these gene profiles are responsive to IIH. To align with the current U.S. National Institutes of Health policy emphasis on evaluation of sex as an important biological variable, the study design implemented here used adult rats of each sex to investigate the corollary notion that baseline and/or hypoglycemia-associated patterns of Ghrh and SF-1 gene expression in VMNdm Ghrh-immunopositive neurons may be sex-dimorphic.

The emerging concept that dual or multiple primary neurochemicals of different structure (amino acids, biogenic amines, neuropeptides, gases, and purines) and disparate release modes may be transmitted by a single nerve cell is a radical departure from the classical Dale principle of ‘one neuron: one neurotransmitter’, yet is bolstered by exponential experimental proof [Vaaga et al., 2014; Svensson et al, 2019; Brunet Avalos et al., 2021]. In the hypothalamus, neuropeptide transmitter co-expression and neuron phenotypic plasticity occur in the ARH [Tamura et al., 2005; Grachev et al., 2014; Yeo and Colledge, 2018; Uenoyama et al., 2019; Garcia-Galiano et al., 2020]. Diverse chemical signals are co-released from axon terminals in the lateral hypothalamic area and hypothalamic paraventricular nucleus [Jo and Role, 2002; Johnson et al., 2018]. Several VMN neurochemicals participate in neural regulation of glucose homeostasis, including amino acid [glutamate (Glu); γ-aminobutyric acid (GABA)] and labile gas [nitric oxide (NO)] transmitters [Chan et al., 2006; Fioramonti et al., 2010; Routh et al., 2014]. Mass spectrometric analysis of amino acid profiles in laser-microdissected VMN neurons shows that Glu and GABA are co-present, albeit at different baseline ratios, in various VMN cell populations, and that Glu:GABA ratios are uniquely affected in individual cell types by pharmacological manipulation of glycogen metabolism [Bheemanapally et al., 2021]. Here, we examined whether VMNdm Ghrh/SF-1 neurons express mRNAs that encode biosynthetic enzyme markers for Glu [glutaminase (GLS)], GABA [glutamate decarboxylase$_{65}$ (GAD$_{65}$); GAD$_{67}$], and NO [neuronal nitric oxide synthase (nNOS)], and if so, whether one or more of these gene profiles exhibit sex-specific responses to IIH.

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Neuropeptides are known to exert modulatory effects within nerve cell circuitries [Nusbaum and Blitz, 2012; Nusbaum et al., 2017]. The current project thus involved pretreatment of animals of each sex by bilateral administration of either Ghrh or scramble siRNA to the VMN prior to IIH to determine if Ghrh imposes neuromodulatory control of co-expressed transmitter mRNA profiles in VMNdm Ghrh/SF-1 neurons, and moreover, to investigate the role of VMN Ghrh signaling in hypoglycemic patterns of counter-regulatory hormone (glucagon, corticosterone, GH) secretion. It was of critical interest to ascertain whether these neurons express Ghrh receptor (Ghrh-R), and whether cell sensitivity to Ghrh input varies between eu- and hypoglycemia in one or both sexes.

Estradiol (E2) imposes control of glucose homeostasis by regulating carbohydrate intake and metabolism, glucose tolerance, and hepatic gluconeogenesis and glycogenesis [Ahmed-Sorour and Bailey, 1980; Bailey and Ahmed-Sorour, 1980; Wurtman and Baum, 1980; Ahmed-Sorour and Bailey, 1981; Lenzen and Bailey, 1984]. Secretion of insulin and the counter-regulatory hormones epinephrine, glucagon, and corticosterone is regulated by E2 [Ahmed-Sorour and Bailey, 1980; Faure et al., 1988; Komesaroff et al., 1988; Adams et al., 2005; Briski and Nedungadi, 2009]. E2 acts on VMN substrates to govern systemic glucose balance as local exogenous hormone administration affects glycemic profiles in insulin-injected ovariectomized (OVX) female rats [Nedungadi and Briski, 2011]. Intracerebroventricular delivery of estrogen receptor-alpha (ERα) or ER-beta (ERβ) antagonists shows that forebrain ERs regulate VMN neurotransmitter marker protein expression [Mahmood et al., 2018]. While there is consensus that VMN ERα expression occurs primarily in the VMNvl, the possibility that one or both nuclear receptors and/or the G protein-coupled plasma membrane ER GPER may facilitate direct control of SF-1 neurons has not been addressed. VMN glucose-regulatory signaling is also subject to control by the monocarboxylate L-lactate, an end-product of astrocyte glycogen metabolism [Mahmood et al., 2019; Bheemanapally et al., 2021]. Lactate likely exerts such effects as a volume transmitter as VMN neurons expressing characterized glucose-regulatory transmitters exhibit the G protein-coupled plasma membrane lactate receptor GPR81/hydroxycarboxylic acid receptor-1 (HCAR1), and VMN GPR81 gene knockdown affects transmitter marker protein expression and counter-regulatory hormone release [Roy et al., 2022]. Research here investigated whether VMNdm Ghrh/SF-1 neurons express mRNAs that transcribe receptors for estradiol or lactate, and if so, whether baseline and/or hypoglycemic transcript profiles vary between the two sexes.

Materials and Methods:

Animals:

Adult Sprague Dawley rats were housed in shoe-box cages (2–3 of one sex per cag), under a 14-hr light: 10-hr dark cycle; lights on at 05.00 hr. Animals were acclimated to handling by daily gentling before onset of the study. Animals had free access to standard laboratory chow and tap water. Study protocols and procedures were performed in conformity with the NIH Guide for Care and Use of Laboratory Animals, 8th Edition, with ULM Institutional Animal Care and Use Committee approval.

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Experimental Design:

On Study Day 1, animals of each sex were randomly assigned to four treatment groups (Table 1; n=8 male and n=8 female rats per treatment group). Rats were anesthetized by intraperitoneal injection of 9.0 mg ketamine/1.0 mg xylazine/0.1 mL/100g *bw* prior to bilateral intra-VMN injection (total volume: 1.0 μL; infusion rate: 3.6 μL/min; coordinates: −2.5 mm posterior to bregma, 0.6 mm lateral to midline, 9.0 mm ventral to skull surface) of Ghrh siRNA (500 pmol; Accell siRNA rat Ghrh, set of 4; prod. no. A-089046–16-0010; Horizon, Cambridge, UK) or scramble (SCR) siRNA (500 pmol; Accell Control Pool Non-Targeting; prod. no. D-001910–10-20; Horizon), as described [Uddin et al., 2021]. Injections were made using a 33 gauge Neuros syringe (prod. no. 53496; Stoelting Co., Wood Dale, IL), using a Neurostar stereotactic Drill and Injection Robot (Neurostar, Tubingen, Germany). In the current study design, circulating plasma estradiol concentrations in female subjects were standardized to minimize variability caused by dissimilar patterns of endogenous estradiol secretion at distinct estrous cycle stages. Thus, anesthetized female rats were bilaterally ovariectomized (OVX) and implanted with a *sc* 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. Mean plasma estradiol levels resulting from this replacement protocol (i.e. 22 pg/ml [Briski et al., 2001]) approximate concentrations measured during metestrus stage in 4-day cycling ovary-intact rats adult rat [Butcher et al., 1974]. After surgery, rats were injected with ketophen (*sc*; Zoetis Inc., Kalamazoo, MI) and enrofloxacin (IM; Bayer HealthCare LLC, Animal Health Division, Shawnee Mission, KS) and treated by topical application of 0.25% bupivacaine to closed incisions, then moved to single-occupancy cages after full recovery from anesthesia. On Study Day 7, male and female rats were injected *sc* at 09.00 hr with vehicle (V; sterile diluent; Eli Lilly & Co., Indianapolis, IN) or neutral protamine Hagedorn insulin (INS; 10.0 U/kg *bw*; Eli Lilly); animals were sacrificed by rapid decapitation one hr post-injection. Individual brains were dissected whole, then snap-frozen by immersion in liquid nitrogen-cooled isopentane for storage at −80 °C.

Laser-Catapult-Microdissection of VMNdm Ghrh Neurons:

Successive individual 10 micron (μm)-thick fresh-frozen sections were collected from the VMN between −1.80 to −2.3 mm posterior to *bregma*, and mounted on polyethylene naphthalate membrane-coated slides (prod. no. 415190–9041-000; Carl Zeiss Microscopy LLC, White Plains, NY). Tissues were fixed with ice-cold acetone (5 min) and blocked with 1.5% normal goat serum (prod. no. S-2000, Vector Laboratories, Burlingame, CA) in Tris-buffered saline, pH 7.4, (TBS), 0.05% Triton X-100 (2 hr), before incubation with a rabbit primary antiserum raised against preproGhrh (prod. No. PA5–102738, 1:2000; Invitrogen, Waltham, MA) (48–72 hr; 4°C). Sections were then incubated with a horseradish peroxidase-labeled goat anti-rabbit secondary antibody (prod. no. PI-1000, 1:1000; Vector Lab.; 1 hr) before exposure to ImmPACT 3,30-diaminobenzidine peroxidase substrate kit reagents (prod. no. SK-4105; Vector Lab.). For each animal, Ghrh-immunoreactive (ir)-positive neurons were detached and propelled from tissue sections using a Zeiss P.A.L.M. UV-A microlaser IV system (Supplementary Figure 1), as described [Uddin et al., 2019; Briski et al., 2020; Mahmood et al., 2020; Bheemanapally et al., 2020, 2021], and collected individually into an adhesive cap (prod. no. 415190–9181-000; Carl Zeiss) containing

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lysis buffer (**4 μL; Single Shot Cell Lysis Kit,** prod. no. 1725080; Bio-Rad Laboratories, Hercules, CA) for multiplex gene expression. Other Ghrh-ir neurons were collected into Western blot lysis buffer for protein analysis (2% sodium dodecyl sulfate [SDS], 0.05 M dithiothreitol, 10.0% glycerol, 1.0 mM EDTA, 60.0 mM Tris-HCl, pH 7.2), as described [Briski et al., 2020; Bheemanapally et al., 2021].

Single-Cell Multiplex Quantitative Reverse Transcription PCR (RT-qPCR) Analysis:

Complementary DNA (cDNA) Synthesis and Amplification: Single-cell lysates were centrifuged (3000 rpm; 4°C), then incubated in an **iCyclerQ RT-PCR Detection System (Bio-Rad) at 25°C (10 min), then 75°C (5 min). A** Bio-Rad Experion gel-based electrophoresis system was used to assess sample RNA integrity, purity, and quantity [Vavaiya and Briski, 2008]. Single-cell mRNA samples were reversetranscribed to cDNA by addition of 1.5 μl cDNA synthesis buffer (iScript™ Advanced cDNA Synthesis Kit. prod. No. 1725038; Bio-Rad) and incubation first at 46°C (20 min), followed by 95°C (1 min), using described methods [Ali et al., 2022a, 2022b; Alshamrani et al., 2022]. A pre-amplification master mix was prepared by combining PrimePCR™ PreAmp for SYBR® Green Assays for Ghrh (prod. no. qRnoCID0007723), SF-1/NR5A1 (prod. no. qRnoCID0001458), GAD_{67}/GAD1 (prod. no. qRnoCID0004554), GAD_{65}/GAD2 (pro. no. qRnoCID0003485), NOS1/nNOS (prod. no. qRnoCED0009301), GLS (prod. no. qRnoCID0007756), ESR1/ERalpha (ERα) (prod. no. qRnoCID0009588), ESR2/ERbeta (ERβ) (prod. no. qRnoCID0008785), GPER (prod. no. qRnoCED0007818), GPR81/HCAR1 (prod. no. qRnoCED0001040) GHRHR (prod. no. qRnoCED0003825), and and GAPDH (prod. no. qRnoCID0057018; Bio-Rad) with SsoAdvanced™ PreAmp Supermix (prod. no. **1725160;** Bio-Rad). **Pre-amplified cDNA was produced by addition of** 9.5 *μL preamplification master mix to individual cDNA samples prior to thermal cycler incubation at* 95°C (3 min), followed by 18 cycles of incubation at 95°C (15 sec), then 58°C (4 min). Pre-amplified cDNA samples were diluted with IDTE (185 μL; prod. No. 11–05-01–05; 1X TE solution; Integrated DNA Technologies, Inc., Coralville, IA). *RT-qPCR Analysis:* PCR samples were prepared by combining Bio-Rad primers [Ghrh (0.5 μL; prod. no. qRnoCID0007723), SF-1/NR5A1 (0.5 μL; prod. no. qRnoCID0001458), GAD_{67} (0.5 μL; prod. no. qRnoCID0004554), GAD_{65} (0.5 μL; prod. no. qRnoCID0003485), GPER(0.5 μL; prod. No. qRnoCED0007818), ESR1 (0.5 μL; prod. no. qRnoCID0009588), ESR2 (0.5 μL; prod. no. qRnoCID0008785), nNOS/NOS1 (0.5 μL; prod. no. qRnoCED0009301), GLS (0.5 μL; prod. no. qRnoCID0007756), HCAR1 (0.5 μL; prod. no. qRnoCED0001040), Ghrh-R (0.5 μL; prod. no. qRnoCED0003825, and GAPDH (0.5 μL; prod. no. qRnoCID0057018)], cDNA sample (*2 μL), and* iTaq™ Universal SYBR® Green Supermix *(*5 *μL,* prod. no. 1725121; Bio-Rad). PCR samples were added to individual wells of hard-shell 384-well PCR plates (prod. no. **HSP3805,** Bio-Rad) for analysis in a CFX384™ Touch Real-Time PCR Detection System *(*Bio-Rad) as follows: initial 30 sec 95°C denaturation, followed by 40 cycles of 1) 3 sec incubation at 95°C and 2) 30 sec incubation at 60°C for GAD_{67}, GPR81/HCAR1 and ESR1; 59.9°C for GAD_{65} and ESR2; 59.8°C for GPER; 59.1°C for SF-1/NR5A1; 58.8°C for GLS; 58.5°C for Ghrh; 58°C for nNOS/NOS1; or 57.3°C for GAPDH, respectively. Melt curve analyses were performed to detect nonspecific products and primer dimers. Data were analyzed by the comparative Ct ($2^{-\Delta\Delta Ct}$) method (Livak and Schmittgen, 2001).

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Western Blot Analysis of Ghrh Protein in Laser-Microdissected Ghrh-ir Neurons:

For each treatment group, triplicate cell lysate pools (n=50 cells/pool/treatment group) were created for Ghrh protein analysis. Sample pool proteins were separated by electrophoresis in Bio-Rad TGX 12% stain-free gels (prod. no. 1610185, Bio-Rad Laboratories Inc., Hercules, CA). Stain-Free imaging technology for total protein measurement was used as the loading control. After separation, gels were activated by UV light (1 min) in a Bio-Rad ChemiDoc MP Imaging System for quantification of individual lane total protein (Supplementary Figure 2). Proteins were transferred to 0.45-μm PVDF-Plus membranes (prod. no. 121639; Data Support Co., Panorama City, CA), for FreedomRocker™ Blotbot® (Next Advance, Inc., Troy, NY) automated wash and antibody incubation processing. Non-specific immunoreagent binding was abated by pretreatment blocking of membranes with Tris-buffer saline, pH 7.4, 10 mM tris hydrochloride, 50 mM sodium chloride (TBS) supplemented with 0.1% Tween-20 and 2% bovine serum albumin. Membranes were incubated for 36–42 h, at 4°C, with a rabbit primary polyclonal antiserum raised against preproGhrh (prod. no. PA5–102738, 1:2000; Invitrogen). Membranes were next incubated with goat anti-rabbit horseradish peroxidase-labeled secondary antibodies (1:5000; prod. no. NEF812001EA; PerkinElmer, Waltham, MA), before exposure to maximum sensitivity SuperSignal WestFemto chemiluminescent substrate (prod. no. 34096; Thermo Fisher Scientific, Rockford, IL). The chemiluminescence optical density (O.D.) value measured for each target protein band was normalized to total protein in that lane using ChemiDoc MP Image Lab™ 6.0.0 software. Bio-Rad Stain-Free gels contain a proprietary trihalo compound that is directly incorporated into the gel chemistry; this compound lacks inherent fluorescence, but renders in-gel proteins fluorescent upon UV photoactivation and thus measurable by O.D. Software sums all individual protein optical densities in a single lane, and relates that total protein O.D. value to target protein O.D. in the same lane, thereby deriving a normalized O.D. value. Each Western blot analysis employed precision plus protein molecular weight dual color standards (prod. no. 161–0374, Bio-Rad). Our figures depict, as Y axis labels denote mean normalized O.D. measures. The formula used for normalization is the ratio of specific target protein O.D./total in-lane protein O.D.

Plasma Glucose and Counter-Regulatory Hormone Analyses:

Plasma glucose concentrations were measured in duplicate for each subject using an ACCU-CHECK Aviva-plus glucometer (Roche Diagnostic Corporation, Indianapolis, IN), as described [Napit et al., 2019]. Circulating corticosterone (prod. no. ADI-900–097; Enzo Life Sciences, Inc., Farmingdale, NY) and glucagon (prod. no. EZGLU-30K, EMD Millipore, Billerica, MA) levels were determined in duplicate using commercial ELISA kit reagents, as reported [Ibrahim et al., 2019]. Serum GH levels were assayed GH Rat ELISA Kit reagents (prod. no. KRC5311; Invitrogen/ThermoFisherScientific, Waltham, MA], per instructions. This assay is capable of detecting less than 0.5 ng rat GH/mL over a range of 0.0–20.0 ng/mL. Intra- and inter-assay variances were less than 3.0%. Briefly, the following reagents were consecutively added, in a 100 uL volume each, to antibody-coated well plates for incubation under rotation at 400 rpm: 1) standard, control, or sample aliquots; rat GH biotin conjugate; streptavidin-horseradish peroxidase solution. Each incubation was followed by aspiration of well contents, then washing (4x). Chromogen incubation (30 min) was terminated by addition of stop solution. Individual well absorbances were read at 450

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nm in a Tecan Infinite M Plex spectrophotometer (Infinite 200 Pro model; Tecan Sales, Austria GmbH).

Statistics:

Mean normalized mRNA profiles were analyzed by three-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: *$p<0.05$; **$p<0.01$; ***$p<0.001$.

Results:

Ghrh neurons reside in VMN neuroanatomical divisions characterized by expression of SF-1, a transcription factor involved in energy and glucose homeostasis. Here, combinatory *in situ* immunocytochemistry/laser-catapult-microdissection/multiplex single-cell qPCR techniques were used to determine if VMNdm SF-1 – expressing neurons contain mRNAs that transcribe Ghrh as well as characterized glucose-regulatory neurotransmitter marker and hormone and nutrient receptor proteins. In the current study design, eu- and hypoglycemia-associated gene transcript profiles were directly compared between adult rats of each sex. Ghrh gene knockdown tools allowed corollary study hypotheses to be addressed, namely that VMN Ghrh transmission may impose neuromodulatory control of Ghrh/SF-1 transmitter and receptor mRNA profiles and is required for optimum counter-regulatory hormone secretion in one or both sexes.

Data shown in Figure 1 illustrate patterns of Ghrh (Figure 1A) and SF-1 (Figure 1B) mRNA expression in individual laser-dissected VMNdm Ghrh-immunoreactive (-ir) neurons from V- or INS-injected male and female rats. Normalized qPCR data for each treatment group are presented in Supplementary Table 1. Outcomes of statistical data analysis are shown in Supplementary Table 2. Results indicate that Ghrh gene transcript levels did not differ between euglycemic animals of either sex, and that in both sexes, these profiles were unaffected by hypoglycemia. In both male and female, pretreatment by delivery of Ghrh siRNA to the VMN significantly decreased Ghrh mRNA levels in Ghrh-ir-positive after V or INS injection. As seen in Figure 1B, SF-1 gene expression was higher in SCR siRNA-pretreated, V-injected female versus male rats [female-SCR siRNA/V (solid white bar) versus male-SCR siRNA/V (solid gray bar)]. Ghrh gene knockdown caused sex-specific changes in this gene profile, as SF-1 transcripts were decreased in female Ghrh siRNA/V (horizontal-striped white bar) versus SCR siRNA/V (solid white bar) treatment groups, but not in male cohorts. Inhibitory effects of IIH on SF-1 mRNA levels in male and female rats [SCR siRNA/INS (diagonal-striped bars) versus SCR siRNA/V (solid bars)] were unaffected by Ghrh knockdown [Ghrh siRNA/INS (cross-hatched bars) versus SCR siRNA/INS (diagonal-striped bars]. Data presented in Figure 1C reveal that Ghrh-R gene expression in V-injected male or female controls was unaffected by Ghrh siRNA. Hypoglycemia elevated Ghrh-R mRNA profiles in male, but not female rats; this male-specific stimulatory response was further amplified by Ghrh gene silencing. Western blot analysis of Ghrh protein in microdissected VMNdm Ghrh-ir neurons showed that Ghrh gene knockdown resulted in significant diminution of cellular neuropeptide content relative

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to SCR siRNA treatment, demonstrating the efficacy of this treatment paradigm for gene product down-regulation (Figure 1D).

GABA and glutamate exert inhibitory or stimulatory effects, respectively, on counter-regulatory hormone outflow. Figure 2 depicts effects of Ghrh siRNA pretreatment on expression profiles in VMNdm Ghrh-ir-positive neurons of mRNAs that encode protein markers for these small molecule neurotransmitters, e.g. the GABA biosynthetic enzyme isoforms GAD1/GAD_{67} (Figure 2A) and GAD2/GAD_{65} (Figure 2B)], and the glutamate biosynthetic enzyme GLS (Figure 2C)]. Data in Figure 2A document dissimilar effects of Ghrh gene knockdown on baseline GAD1 gene expression, as this mRNA was significantly up-regulated by Ghrh gene knockdown in male [Ghrh siRNA/V (solid gray bar) versus SCR siRNA/V (solid gray bar)], but not female rats. IIH inhibited GAD1 transcript profiles in male [SCR siRNA/INS (diagonal-striped gray bar) versus SCR siRNA/V (solid gray bar), but not female rats; this inhibitory response was refractory to Ghrh siRNA pretreatment. GAD1 mRNA expression in VMNdm Ghrh-ir neurons from female rats was unaffected by either Ghrh knockdown or IIH. On the other hand, baseline patterns of GAD2 gene expression in VMNdm Ghrh-ir neurons were refractory to Ghrh siRNA, but this gene profile was down-regulated by hypoglycemia in each sex (Figure 2B). Evidence for GLS mRNA expression in VMNdm Ghrh neurons infers that these cells release both glucose-stimulatory and -inhibitory amino acid transmitters. GLS transcript profiles were augmented by Ghrh; this positive treatment effect was greater in male versus female [Ghrh siRNA/V (gray horizontal-striped bar) versus Ghrh siRNA/V (white horizontal-striped bar). On the other hand, Ghrh gene knockdown did not avert hypoglycemia-associated inhibition of GLS gene expression in either sex.

The gaseous neurotransmitter NO is a positive stimulus for glucose counter-regulatory hormone secretion. As shown in Figure 3, VMNdm Ghrh-ir neurons from male and female rats express mRNA that encodes the NO biosynthetic enzyme nNOS. Data show that Ghrh gene knockdown had sex-specific effects on nNOS gene expression in V-injected controls, as cells from females [Ghrh siRNA/v (horizontal-striped white bar) versus SCR siRNA/V (solid white bar), but not males exhibited up-regulation of this gene profile. IIH resulted in significant intensification of nNOS gene expression in each sex. This stimulatory transcription response was further increased by Ghrh siRNA pretreatment.

Figure 4 illustrates expression patterns of nuclear [ESR1/ERα (Figure 4); ESR1/ERβ (Figure 4B)] and membrane (Figure 4C) ER mRNAs in male versus female VMNdm Ghrh-ir neurons. Data in Figure 4A show that ESR1 mRNA is expressed in cells from each sex, and that baseline transcript profiles are significantly diminished by Ghrh gene silencing in male and female. This ER gene profile was down-regulated by IIH in both sexes. Ghrh siRNA pretreatment had sex-specific effects on this inhibitory response, as ESR1 mRNA content was lower in male, but not female Ghrh siRNA/INS versus SCR/INS treatment groups. Outcomes also document the presence of ESR2 mRNA in male and female VMNdm Ghrh neurons (Figure 4B). This ER variant gene profile is elevated in male versus female V-injected euglycemic controls, and is decreased by Ghrh siRNA in each sex. Effects of IIH on ESR2 transcript content varied between sexes, as male, but not female rats exhibited down-regulation of this mRNA after INS injection. This sex-specific

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transcriptional response was reversed by Ghrh siRNA pretreatment. As shown in Figure 4C, VMNdm Ghrh neurons express mRNA encoding GPER. This plasma membrane ER gene profile was significantly suppressed by Ghrh gene knockdown in each sex. GPER gene transcripts were down-regulated in response to IIH in each sex; this inhibitory response was blunted by Ghrh gene silencing. Data in Figure 4D document the presence of mRNA encoding the enzyme CYP19A1/aromatase, which converts testosterone to neuro-estradiol, in VMNdm Ghrh-ir neurons. Ghrh gene knockdown elevated aromatase transcript profiles cells collected from V-injected male or INS-injected female rats.

Evidence for plasma membrane lactate receptor GPR81 involvement in VMN regulation of counter-regulatory hormone secretion prompted investigation here of whether VMNdm Ghrh/SF-1 neurons may be a potential target for lactate volume transmission input. Data in Figure 5 denote the presence of GPR81 mRNA in these cells, indicating a sex dichotomy, e.g. female > male, of baseline transcription gene rates. Ghrh regulation of this gene profile varies between sexes as Ghrh gene silencing elevated GPR81 transcript profiles in male, but not female rats. INS injection increased GPR81 mRNA expression in males, but had no effect in females. Ghrh siRNA prevented this stimulatory response in hypoglycemic males, but did not alter GPR81 gene expression in hypoglycemic females.

Data presented in Figure 6 depict VMN Ghrh gene silencing effects on eu- and hypoglycemic plasma glucose (Figure 6A), glucagon (Figure 6B), corticosterone (Figure 6C), and GH (Figure 6D) profiles. As shown in Figure 6A, Ghrh siRNA administration to the VMN caused a slight, but significant increase in plasma glucose levels in male, but not female rats. Groups pretreated with Ghrh versus SCR siRNA showed comparable reductions in glucose concentrations one hour-post INS injection. Basal plasma glucagon (Figure 6B) and corticosterone (Figure 6C) concentrations were unaffected by Ghrh gene silencing in either sex. INS injection elevated both glucagon and corticosterone secretion in male and female rats. Ghrh siRNA pretreatment did not modify hypoglycemic patterns of glucagon release in either sex, but abolished or attenuated corticosterone secretion in male or female rats, respectively. Ghrh gene silencing significantly up- or down-regulated plasma GH concentration in male and female rats, respectively (Figure 6D). IIH had sex-specific effects on GH secretion, as this hormone profile was elevated one hour after INS injection in male, but not female rats. Ghrh siRNA pretreatment robustly suppressed GH release in hypoglycemic male animals, but did not affect circulating hormone levels in hypoglycemic females.

Discussion:

The VMN transcription factor SF-1 is crucial for neural regulation of glucostasis. Single-cell multiplex qPCR technology was used here to characterize the neurotransmitter phenotype of VMNdm SF-1 neurons, and to determine if expressed baseline and/or hypoglycemia-associated transmitter mRNA marker profiles differ among sexes. Data document the presence of mRNAs encoding Ghrh, SF-1, as well as GLU, GABA, and NO biosynthetic enzyme proteins in VMNdm Ghrh-immunoreactive neurons. Data show that IIH decreased GAD_{67} transcript levels in males only, but suppressed GAD_{65} and GLS mRNAs or enhanced nNOS gene expression in each sex. Documentation of VMN Ghrh gene knockdown effects

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on GAD_{65} and nNOS gene profiles in hypoglycemic rats infers that Ghrh imposes a neuromodulatory tone on these transcription responses to IIH. Ghrh siRNA pretreatment prevented or lessened hypoglycemic hypercorticosteronemia in male versus female rats, respectively, but abolished IIH-induced GH secretion in the former sex only. Study outcomes support the possibility that VMNdm Ghrh/SF-1 neurons may be an effector of SF-1 control of glucose homeostasis, and that estrogen and nutrient signals may shape that control by direct action on these cells. Further research is warranted to investigate whether individual glucose-inhibitory and - stimulatory Ghrh/SR-1 neuron co-transmitters convey distinctive or redundant information on dynamic aspects of brain cell energy state, metabolic fuel supply, and local/systemic energy reserve capacities. It would also be insightful to understand how integrated signaling over different spatial and temporal domains by these neurons may influence counter-regulation in each sex.

Current outcomes show that in each sex, SF-1 gene expression is down-regulated in VMNdm Ghrh-ir-positive neurons in reaction to IIH. It should be considered that quantitative measures of transcript profile changes do not constitute definitive evidence for parallel modifications in SF-1 protein expression in the same cell population. Present findings do not therefore exclude the prospect that SF-1 protein production may be unaffected by hypoglycemia, in one or both sexes, despite adjustments in gene transcription patterns. Ongoing studies are investigating whether Ghrh/SF-1 neurons directly monitor nutrient and/or energy status by virtue of expression of the characterized biomarkers glucokinase and 5’-AMP-activated protein kinase [Hardie et al.,, 2012; Lopez-Gambero et al., 2019; Matschinsky and Wilson, 2019]. Results here show that SF-1 mRNA is co-expressed with transcripts that encode multiple neurotransmitters of characterized influence on glucose homeostasis (e.g. GABA, glutamate, NO, Ghrh) in VMNdm Ghrh-ir neurons. Ongoing efforts will strive to determine if this transcription factor regulates production of each of these diverse neurochemicals or rather a subset of these co-transmitters. It would also be insightful to know if SF-1 employs a common or unique molecular mechanisms to control individual transmission profiles, to comprehend how such control may be shaped by sex, and to understand how SF-1 regulation of specific transmitters may be affected by systemic glucose imbalance in each sex. Peptide neuromodulators are capable of regulating diverse nerve cell activities, such as presynaptic terminal vesicle trafficking, postsynaptic current strength, receptor dynamics, and gene expression. Current results show that Ghrh signaling imposes sex-specific control of baseline SF-1 transcript profiles (female only), yet has no impact on down-regulated SF-1 gene responses to hypoglycemia in either sex. The mechanisms that mediate metabolic state-specific Ghrh control of SF-1 gene expression in the female are not known at present.

The GAD catalyzes rate-limiting conversion of glutamate to GABA, and is present in the brain as 67 ($GAD1/GAD_{67}$) and 65 ($GAD2/GAD_{65}$) kDa molecular weight variants encoded by distinct genes. Current data confirm that mRNAs that encode these enzyme isoforms are co-expressed at the single-cell level in the VMN [Ali et al., 2022]. These variants are distinguished by differences in amino acid primary structure, nerve cell subcellular localization, and regulation. $GAD1/GAD_{67}$ expression is subject to transcriptional and posttranscriptional control, whereas $GAD2/GAD_{65}$ is controlled by transcriptional and kinetic mechanisms [Behar, 2009]. The presence of $GAD2/GAD_{65}$ protein in axon

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terminals and vesicles compared to GAD1/GAD_{67} localization to cytoplasm raises the prospect of separate vesicular versus cytoplasmic GABA pools and potential GAD2/GAD_{65} involvement in neurotransmission as opposed to GAD1/GAD_{67} participation in cellular metabolic functions [Martin and Barke, 1998; Tavazzani et al., 2014; Schousboe and Waagepetersen, 2017]. Our data disclose sex differences in VMNdm Ghrh nerve cell GAD isoform mRNA expression patterns during hypoglycemia, as GAD1 and GAD2 transcripts were both diminished in males, while only the latter profile is decreased in the female; probable functional implications of this sex-dimorphism warrant further investigation. Intriguingly, this inhibitory GAD2 transcriptional response to IIH is evidently Ghrh-dependent in each sex, whereas down-regulated GAD1 mRNA expression in male Ghrh/SF-1 neurons is resistant to Ghrh signaling. Thus, Ghrh input is likely capable of discriminative modulation of vesicular versus cytoplasmic GABA production and activity in this nerve cell type; the mechanisms that mediate this control will require additional research effort. VMNdm Ghrh/SF-1 neurons also express biosynthetic machinery for production of the glucose-stimulatory amino acid neurotransmitter glutamate. It remains unclear if GABA and glutamate are co-packaged within common synaptic vesicles for simultaneous co-release, or instead exist in separate vesicle populations to under undergo independent exocytosis. Current data show that, in each sex, Ghrh imposes a negative modulatory tone on baseline GLS mRNA expression in these neurons, yet is uninvolved in down-regulated transcription of this gene during hypoglycemia. There remains a need to identify the mechanisms that impose quiescence on this regulatory tone systemic glucose deficiency. Priority aims of future research on this topic will likely include identification of VMNdm Ghrh/SF-1 nerve cell projection sites in the brain, characterization of neurotransmitter phenotype(s) of neurons innervated by this cell population, and elucidation of post-synaptic GABA and glutamate receptor subclasses that transduce these neurochemical signals to affect glucose homeostasis and comparison of receptor expression profiles and post-receptor signal pathway function between the sexes.

The diffusive messenger molecule NO produced by NOS action on the substrate L-arginine principally targets soluble guanylyl cyclase to stimulate cyclic GMP production. In the brain, nNOS imposes long-term control of synaptic transmission [Förstermann and Sessa, 2012]. NO release within the hypothalamus enhances counter-regulatory hormone secretion. Evidence here for nNOS mRNA expression in VMNdm Ghrh/SF-1 neurons from each sex infers that these cells impart information to the neural glucose-regulatory network by non-receptor- as well as receptor-mediated signaling. Data disclose sex-dimorphic Ghrh modulation of basal nNOS gene transcription in these cells, e.g. female only. Yet, Ghrh gene knockdown was found here to amplify hypoglycemic up-regulation of transcript levels in each sex, data that illustrate a gain of Ghrh control of nNOS gene expression in males due to glucose dyshomeostasis. The functional significance of relatively higher levels of nNOS mRNA expression in cells obtained from hypoglycemic males versus females remains to be elucidated. Sex differences in incremental increases in this gene profile due to Ghrh gene knockdown infer that the magnitude of inhibitory tone or ‘brake’ imposed by Ghrh on hypoglycemic patterns of glucose-stimulatory NO release from these neurons may differ between male and female. Similar to the topic discussed above, there remains a need to identify the cellular targets and functional consequences of NO released from VMNdm

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Ghrh/SF-1 neurons, and to determine if NO and co-expressed amino acid transmitters act on common and/or different post-synaptic substrates to affect glucose homeostasis.

Current data document co-expression of nuclear and membrane ER genes in VMNdm Ghrh/SF-1 neurons in each sex. Ghrh signaling is evidently a positive stimulus for ERα, ERβ, and GPER gene transcription during glucose homeostasis, as each ER mRNA profile was diminished by Ghrh gene siRNA. Future effort to characterize the molecular mechanisms whereby which this neuropeptide enhances ER variant-specific sensitivity to estradiol is warranted. Data here show that hypoglycemia suppressed ERα and GPER transcript profiles in each sex, but altered ERβ gene expression in males only. It should be noted that observed changes in ER mRNA expression do not constitute a definitive evidence for corresponding adjustments in receptor protein yield; this notion will remain speculative until analytical methods of requisite sensitivity for quantification of these proteins in single-cell samples becomes available. In the male, Ghrh input was either a positive (ERα) or negative (ERβ, GPER) stimulus for ER mRNA expression. In this sex, hypoglycemia reversed the direction of Ghrh control (e.g. stimulatory to inhibitory) of ERβ and GPER gene expression; current work does not clarify the mechanisms underlying that switch or identify the stimuli that suppress positive ERα. Likewise, in the female, hypoglycemia eliminated Ghrh regulation of ERα and ERβ transcription by as-yet-known mechanisms; identification of regulatory inputs that suppress these gene profiles remains to be accomplished. Further research is needed to investigate how hypoglycemia-associated changes in ER variant-mediated estradiol input to Ghrh/SF-1 neurons affect neurotransmitter protein marker gene expression during this metabolic challenge.

Evidence that GPR81 gene transcription occurs in VMNdm Ghrh/SF-1 neurons of each sex infers that the metabolic fuel L-lactate may act, in part, as a volume transmitter to control function in these cells. The relative impact of receptor-mediated lactate input to these cells is likely sex-dimorphic as basal GPR81 gene transcript profiles were elevated in the female relative to male. Ghrh regulation of baseline lactate receptor mRNA expression is also sex-specific as this gene profile was up-regulated in male, but refractory to Ghrh knockdown in female. Hypoglycemia caused divergent, sex-contingent changes in GPR81 mRNA expression as transcript levels were correspondingly increased or decreased in male versus female Ghrh/SF-1 neurons. This stimulatory transcriptional response in males is Ghrh-dependent, whereas this gene profile is suppressed by Ghrh-independent mechanisms in the female. For each sex, it remains to be determined if lactate receptor signaling may govern the release of all or only distinctive co-expressed VMNdm Ghrh/SF-1 nerve cell transmitters.

Outcomes offer confirmation of VMNdm Ghrh/SF-1 neuron sensitivity to Ghrh signaling, as cells taken from male and female rats express Ghrh-R mRNA. This gene expression profile is evidently controlled by glucose status in the former sex, as it was elevated in hypoglycemic versus euglycemic male rats. In that sex, therefore, differential Ghrh regulation of target gene transcripts in the presence of normal versus decreased systemic glucose levels may involve, in part, adjustments in direct responsiveness to this neuropeptide. An absence of statistically-significant change in Ghrh-R mRNA levels in neurons collected from INS-injected females does not preclude the possibility that

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hypoglycemia may affect post-receptor signal transduction in that sex. An important question that remains unanswered is the cellular source(s) of metabolic sensory cues that control VMNdm Ghrh/SF-1 nerve cell Ghrh-R gene expression in the male.

Application of Ghrh gene knockdown tools *in vivo* enabled investigation of whether this neuropeptide signal of VMN origin regulates hypoglycemic patterns of counter-regulatory hormone secretion according to sex. Data document sex-dimorphic VMN Ghrh control of baseline plasma glucose concentrations as silenced gene expression elevated glycemic profiles in male, but not female; this male-specific control is evidently metabolic state-dependent as glucose levels in INS-injected males were refractory to gene knockdown. Results provide unique evidence that VMN Ghrh regulates corticosterone secretion in hypoglycemic rats of each sex as well as basal and hypoglycemic patterns of GH release in male. Ghrh control of hypocorticosteronemia is evidently sex-dimorphic as this neuropeptide signal is crucial for total or fractional augmentation of corticosterone outflow in male and female rats, respectively. While our working premise included the expectation that hypoglycemia would likely elevate circulating GH levels by sex-contingent increments, the INS treatment paradigm used here resulted in a significant increase in GH 1 hr post-injection of male, but not female rats. Interestingly, while not statistically significant, GH profiles showed a trend toward a decline in SCR siRNA/INS versus SCR siRNA/V female rats. Yet, the probability that GH levels may be elevated relative to baseline in the female at one or multiple time points before or after INS administration injection cannot be overlooked. VMN Ghrh signaling may account, in part, for sex-dimorphic GH outflow during hypoglycemia, as this neuropeptide is essential for this stimulatory hormone response in males, yet has no impact on GH release in the female. It should be noted that in the male, the direction of Ghrh regulation of GH profiles switches is reliant upon systemic glucose status, as this control is inhibitory or stimulatory in eu- versus hypoglycemic animals, respectively. It remains to be determined if the VMN Ghrh signals that impose control of corticosterone and GH secretion emanate solely or partly from VMNdm Ghrh/SF-1 neurons, and if so, whether co-release of other transmitters from these cells is critical for Ghrh regulation of one or both counter-regulatory hormones. It would be insightful to know if these neurons influence hypothalamic-pituitary-adrenal glucocorticoid axis output through direct or indirect innervation of hypothalamic paraventricular nucleus parvicellular corticotropin-releasing hormone neurons, which control pituitary adrenocorticotropin-adrenal corticosterone secretion. Similarly, the prospect that these neurons may affect the function of ARH neuroendocrine Ghrh neurons that regulate anterior pituitary GH warrants investigation.

Current work shows that that VMN Ghrh gene knockdown did not affect hypoglycemic profiles in either sex despite observed treatment effects on plasma corticosterone levels in both sexes and on plasma growth hormone levels in the male. Since circulating glucose was not measured at any time between insulin injection and sacrifice, the possibility that siRNA treatment might have impacted blood glucose over some time interval prior to +1 hour post-insulin injection cannot be overlooked. It is plausible that plasma glucose profiles may exhibit a dynamic flux owing to VMN Ghrh modulation of counter-regulatory hormone release and/or induction of counter-active hepatic gluconeogenic or glycogenolytic functions. Current results may thus provide a snapshot of a temporal phase during which

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plasma glucose levels are normalized after insulin administration as an adaptive reaction to Ghrh-dependent actions that control contra-regulatory outflow.

In summary, outcomes endorse the novel notion that VMNdm Ghrh/SF-1 neurons may be an effector of SF-1 control of glucose homeostasis, that act to shape sex-dimorphic patterns of counter-regulatory hormone secretion. Data provide unique documentation of co-transmission of glucose-inhibitory and -stimulatory neurochemicals of distinctive chemical structure and spatial/temporal release profiles by these neurons, which is presumed to facilitate integrated, dynamic input to the brain glucose-regulatory network (Figure 7). Ghrh imposes sex-specific neuromodulation of critical hormonal (estradiol) and nutrient (L-lactate) regulatory inputs to Ghrh/SF-1 neurons as well as neurochemical transmission patterns; further research is needed to determine if there is a functional link between these outcomes. Ongoing studies seek to determine if ERs and GPR81 regulate co-transmission by SF-1 – dependent and/or -independent mechanisms. Results show that VMN Ghrh signaling is critical for hypoglycemic hypercorticosteronemia in each sex and for the male-only GH stimulatory response to hypoglycemia. There remains a need to identify cellular targets and functional sequelae of individual glucose-regulatory transmitters released by Ghrh/SF-1 neurons, and to determine if NO and co-expressed amino acid transmitters act on common and/or different post-synaptic substrates to affect glucose homeostasis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Funding:

This research was supported by NIH grant DK-109382

Data Availability Statement:

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Abbreviations:

CYP19A1	aromatase
ARH	hypothalamic arcuate nucleus
ERα	estrogen receptor-alpha
ERβ	estrogen receptor-beta
GAD1/GAD$_{67}$	glutamine decarboxylase (GAD)$_{67}$
GAD2/GAD$_{65}$	GAD$_{65}$
GH	growth hormone
Ghrh	growth hormone-releasing hormone

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Ghrh-R	Ghrh receptor
GLS	glutaminase
GPER	G protein-coupled estrogen receptor-1
GPR81/HCAR1	G protein-coupled plasma membrane lactate receptor 81
IIH	insulin-induced hypoglycemia
INS	insulin
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
OVX	ovariectomy
sc	subcutaneous
SF-1/Nr5a1	steroidogenic factor-1

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Highlights:

- The ventromedial hypothalamic nucleus (VMN) controls growth hormone (GH) secretion.
- Growth hormone-releasing hormone (Ghrh) siRNA was delivered to VMN of each sex.
- Gene silencing blunted hypoglycemic corticosterone (both sexes) and GH (male only) output.
- Ghrh neuron glucostatic transmitter expression is revealed by single-cell multiplex qPCR.
- Data infer that Ghrh/SF-1 neurons may be an effector of SF-1 control of counter-regulation.

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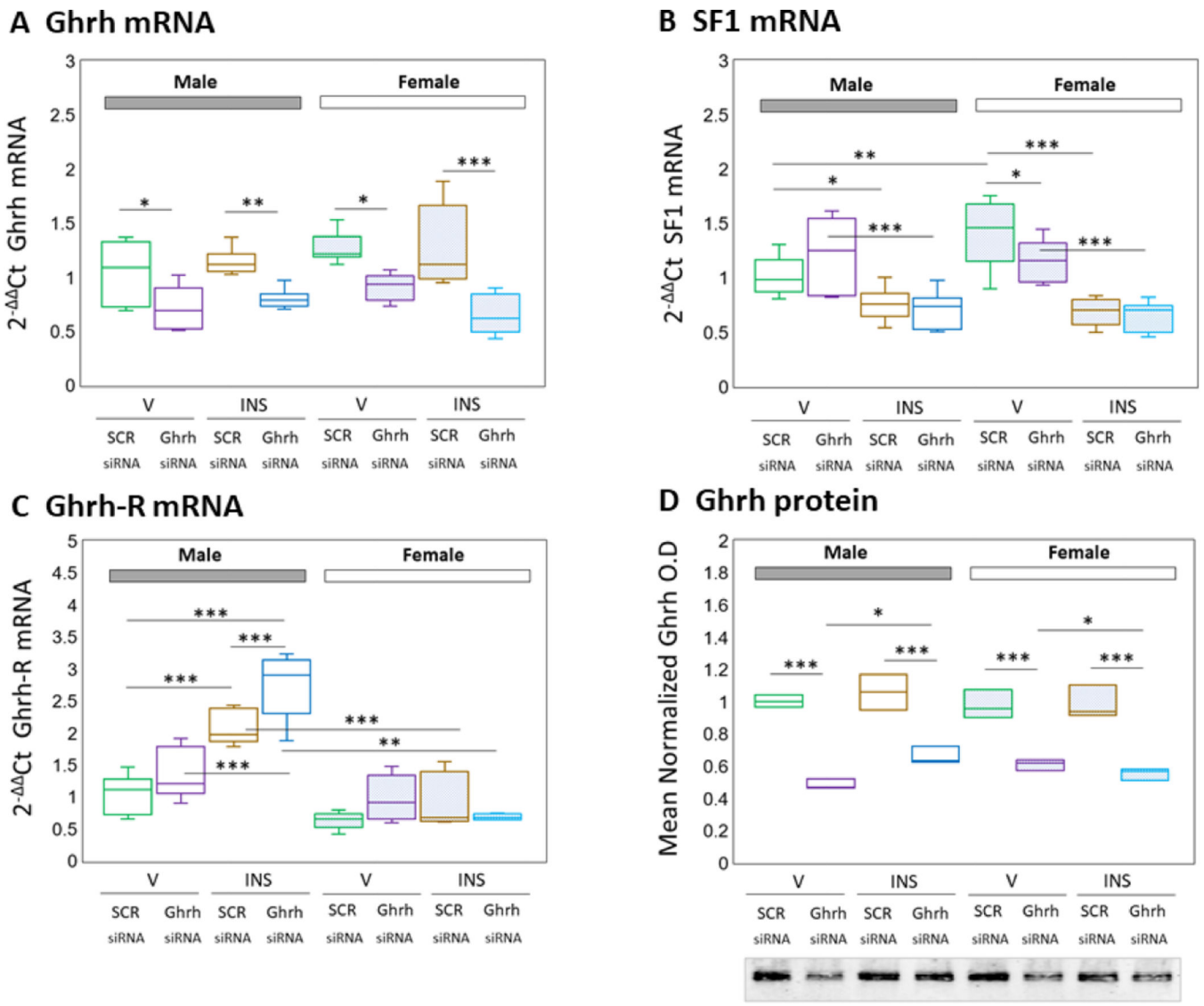

Figure 1. Single-Cell Multiplex qPCR Analysis of Growth Hormone-Releasing Hormone (Ghrh) and Steroidogenic Factor-1 (SF-1) Gene Expression in Male or Female Dorsomedial Ventromedial Hypothalamic Nucleus (VMNdm) Ghrh-Immunopositive Neurons.

Groups of male and female rats (n=8 males and n=8 females per group) were pretreated by bilateral intra-VMN Ghrh or scramble (SCR) siRNA administration seven days prior to subcutaneous (sc) injection of vehicle (V) or neutral protamine Hagedorn insulin (INS; 10.0 U/kg *bw*). Brain dissection was performed one hour post-injection. Individual Ghrh-immunopositive neurons were laser-catapult-microdissected from 10 micron-thick fresh frozen sections cut through the VMNdm for multiplex single-cell qPCR analyses.

Data are presented here in box-and-whisker plot format, which displays the median, lower and upper quartiles, and lower and upper extremes of a data set. Plots depict mean normalized Ghrh (Figure A), SF-1 (Figure B), or Ghrh-R (Figure C) mRNA measures ± S.E.M. for male (four bars, *at left*) and female (four bars, *at right*) rat treatment groups. Treatment groups are identified as follows: SCR siRNA/V (green box-and-whisker plots, male: no fill, n=16; female: stippled fill, n=16); Ghrh siRNA/V (purple box-and-whisker

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plots; male: no fill, n=16; female: stippled fill, n=16); SCR siRNA/INS (brown box-and-whisker plots; male: no fill, n=16; female: stippled fill, n=16); Ghrh siRNA/INS (blue box-and-whisker plots; male: no fill, n=16; female: stippled fill, n=16). mRNA data were normalized to the housekeeping gene GAPDH by the $2^{-\Delta\Delta Ct}$ method [Livak and Schmittgen, 2001]. Aliquots of laser-microdissected VMNdm Ghrh-ir neurons were combined within treatment groups to create triplicate samples for Western blot analysis of Ghrh protein. Figure D depicts mean Ghrh protein O.D. measures ± S.E.M. for the treatments defined above. mRNA and protein data were analyzed by three-way ANOVA and Student-Neuman-Keuls *post-hoc* test using GraphPad Prism, Vol. 8 software. Statistical differences between discrete pairs of treatment groups are denoted as follows: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

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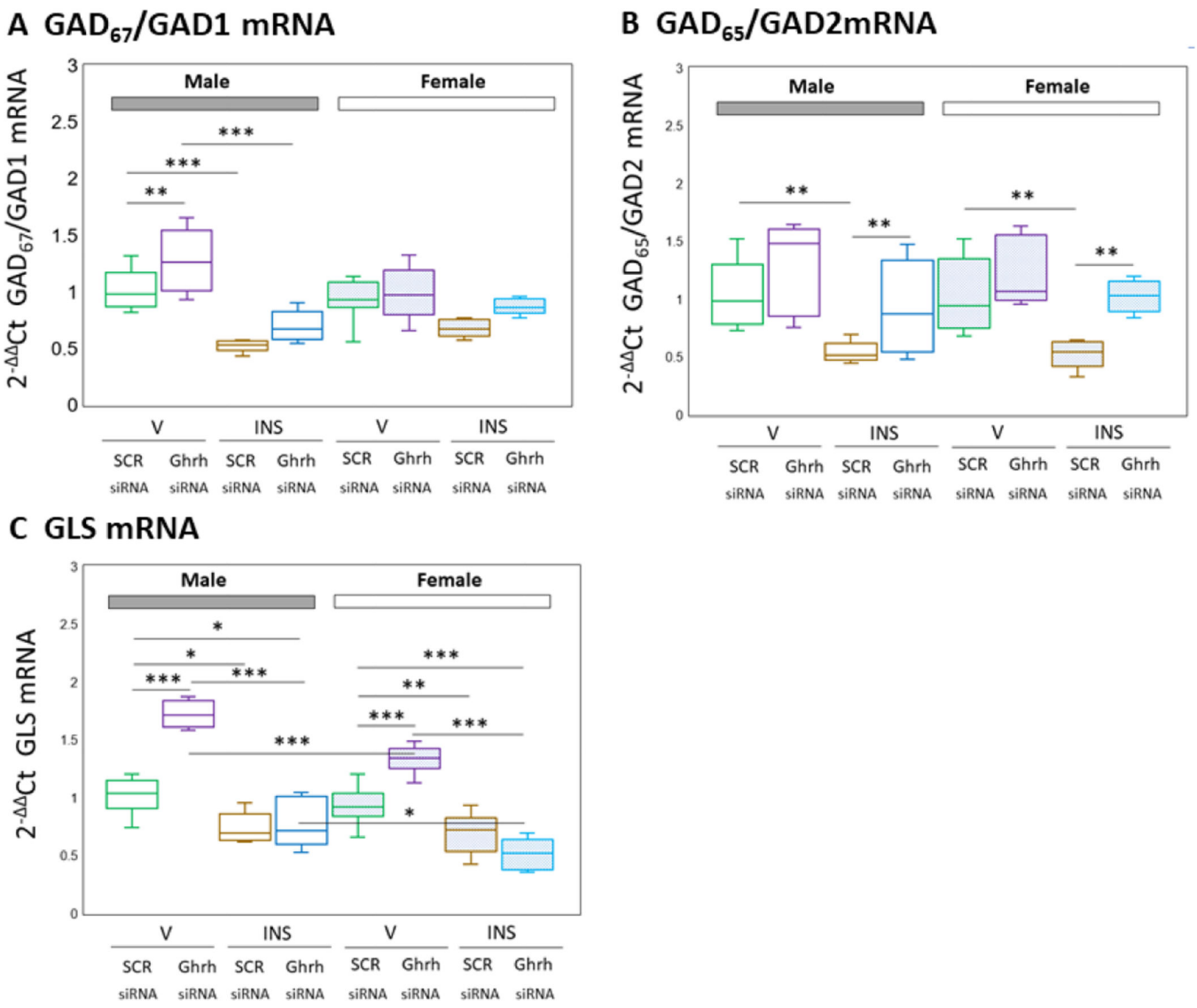

Figure 2. Effects of VMN Ghrh Gene Knockdown on Glutamate Decarboxylase (GAD)-1/ GAD_{67}, GAD2/GAD_{65}, and Glutaminase (GLS) Gene Transcription Profiles in VMNdm Ghrh/ SF-1 Nerve Cell Collected from V- or INS-Injected Male or Female Rats.

Results present mean normalized GAD1 (Figure 2A), GAD2 (Figure 2B), and GLS (Figure 2C) mRNA values ± S.E.M. for male (four bars, *at left*) or female (four bars, *at right*) groups of rats treated as follows: SCR siRNA/V (green box-and-whisker plots; male: no fill, n=16; female: stippled fill, n=16); Ghrh siRNA/V (purple box-and-whisker plots; male: no fill, n=16; female; stipple fill, n=16); SCR siRNA/INS (brown box-and-whisker plots; male: no fill, n=16; female: stippled fill, n=16); Ghrh siRNA/INS (green box-and-whisker plots; male: no fill, n=16; female: stippled fill, n=16). Normalized mRNA data were analyzed by three-way ANOVA and Student-Neuman-Keuls *post-hoc* test using GraphPad Prism, Vol. 8 software. Statistical differences between discrete pairs of treatment groups are denoted as follows: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

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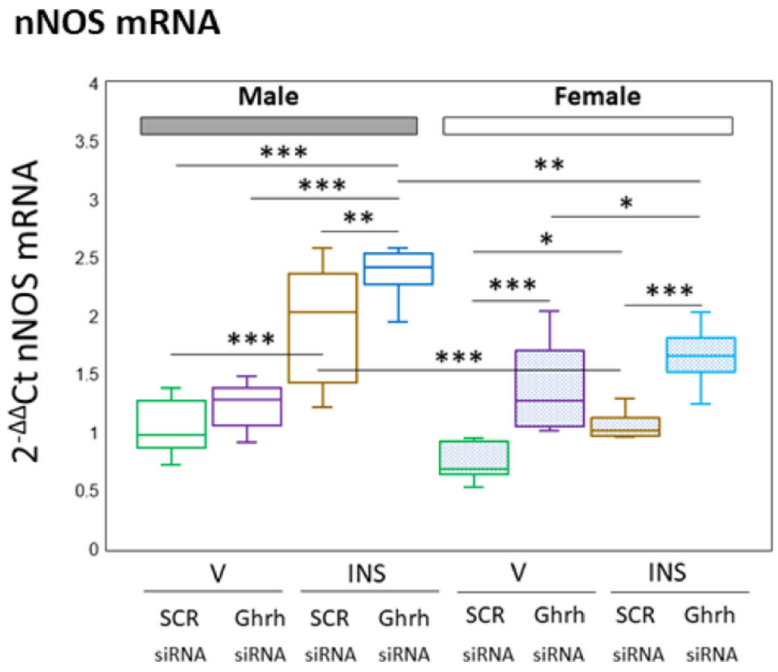

Figure 3. Effects of Insulin-Induced Hypoglycemia (IIH) on Male and Female Rat VMNdm Ghrh/SF-1 Nerve Cell Nitric Oxide Synthase (nNOS) mRNA profiles.

Data show mean nNOS mRNA values ± S.E.M. for the following treatment groups: SCR siRNA/V (male: n=16; female: n=16); Ghrh siRNA/V (male: n=16; female: n=16); SCR siRNA/INS (male: n=16; female: n=16); Ghrh siRNA/INS (male: n=16; female: n=16). Normalized mRNA data were analyzed by three-way ANOVA and Student-Neuman-Keuls *post-hoc* test using GraphPad Prism, Vol. 8 software. Statistical differences between discrete pairs of treatment groups are denoted as follows: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

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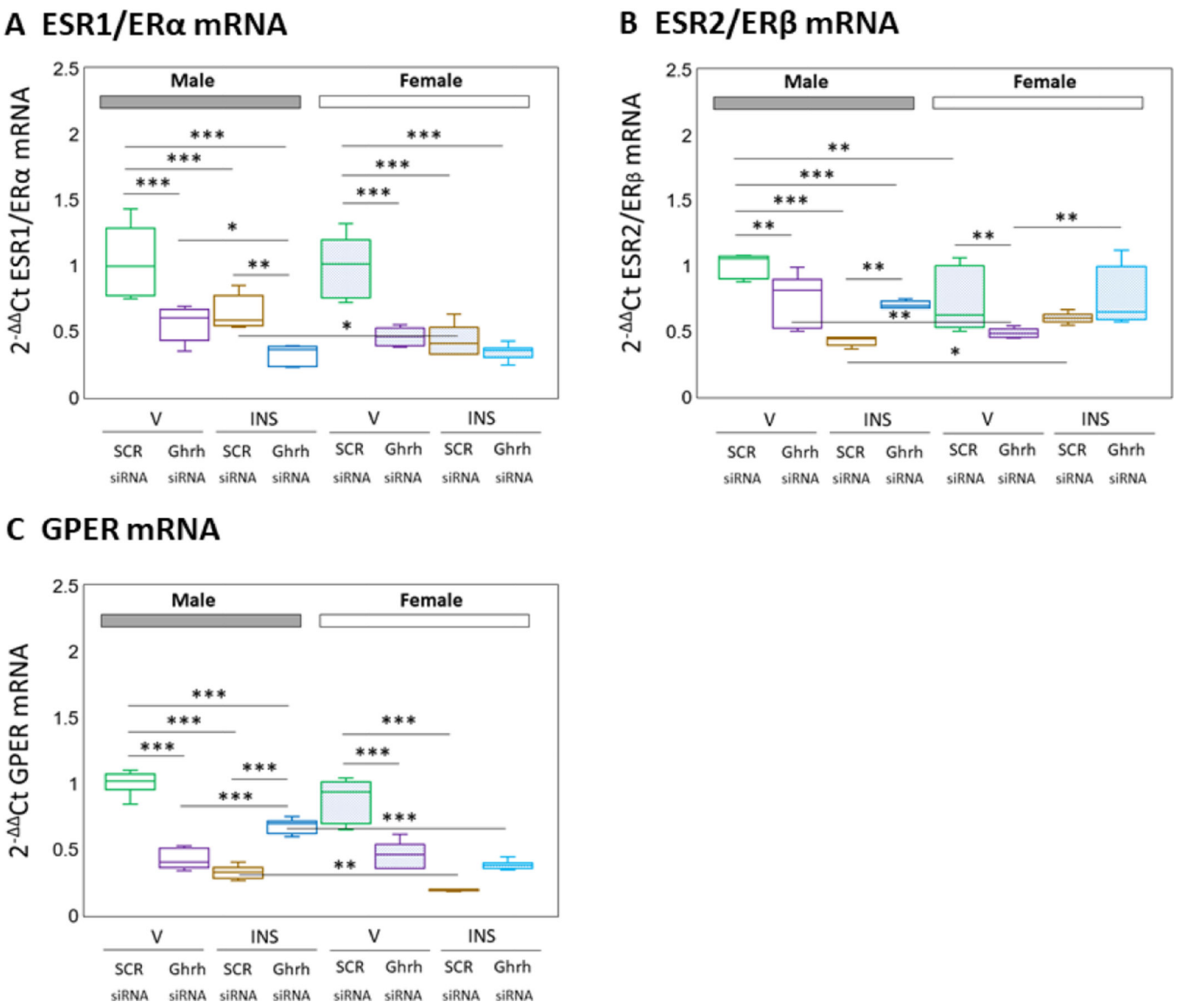

Figure 4. Patterns of VMNdm Ghrh/SF-1 Neuron Estrogen Receptor-Alpha (ESR1/ERα), ER-Beta (ESR2/ERβ), and G Protein-Coupled Membrane Estrogen Receptor-1 (GPER) Gene Expression in Eu- versus Hypoglycemic Male and Female Rats.

Data depict mean nNOS mRNA values ± S.E.M. for ESR1 (Figure 4A), ESR2 (Figure 4B), and GPER (Figure 4c) for the following treatment groups: SCR siRNA/V (male: n=16; female: n=16); Ghrh siRNA/V (male: n=16; female; n=16); SCR siRNA/INS (male: n=16; female: n=16); Ghrh siRNA/INS (male: n=16; female: n=16). Normalized mRNA data were analyzed by three-way ANOVA and Student-Neuman-Keuls *post-hoc* test using GraphPad Prism, Vol. 8 software. Statistical differences between discrete pairs of treatment groups are denoted as follows: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

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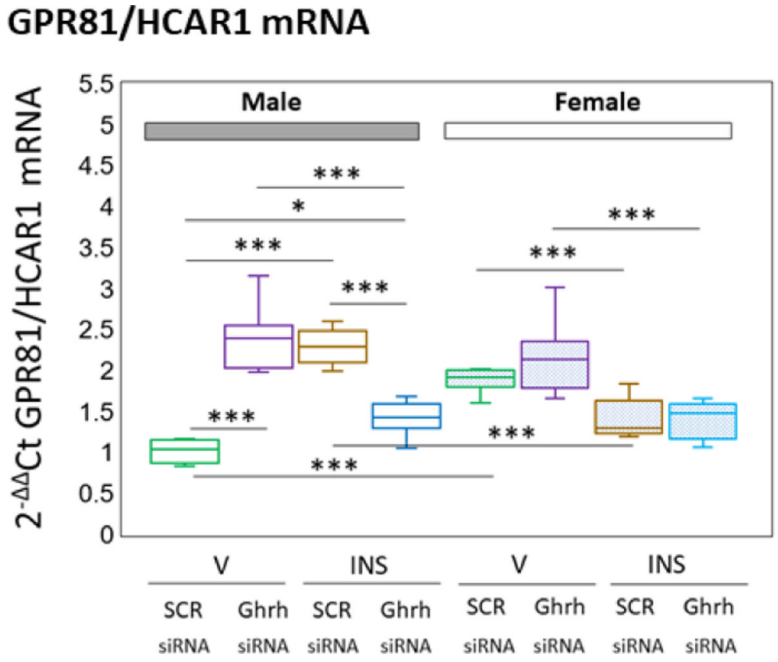

Figure 5. Effects of Ghrh siRNA Pretreatment on VMNdm Ghrh/SF-1 Nerve Cell G Protein-Coupled - 81/HCAR-1 (GPR81) mRNA Expression in Eu- and Hypoglycemic Male and Female Rats.

Data show mean GPR81nNOS mRNA values ± S.E.M. for the following treatment groups: SCR siRNA/V (male: n=16; female: n=16); Ghrh siRNA/V (male: n=16; female: n=16); SCR siRNA/INS (male: n=16; female: n=16); Ghrh siRNA/INS (male: n=16; female: n=16). Normalized mRNA data were analyzed by three-way ANOVA and Student-Neuman-Keuls *post-hoc* test using GraphPad Prism, Vol. 8 software. Statistical differences between discrete pairs of treatment groups are denoted as follows: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

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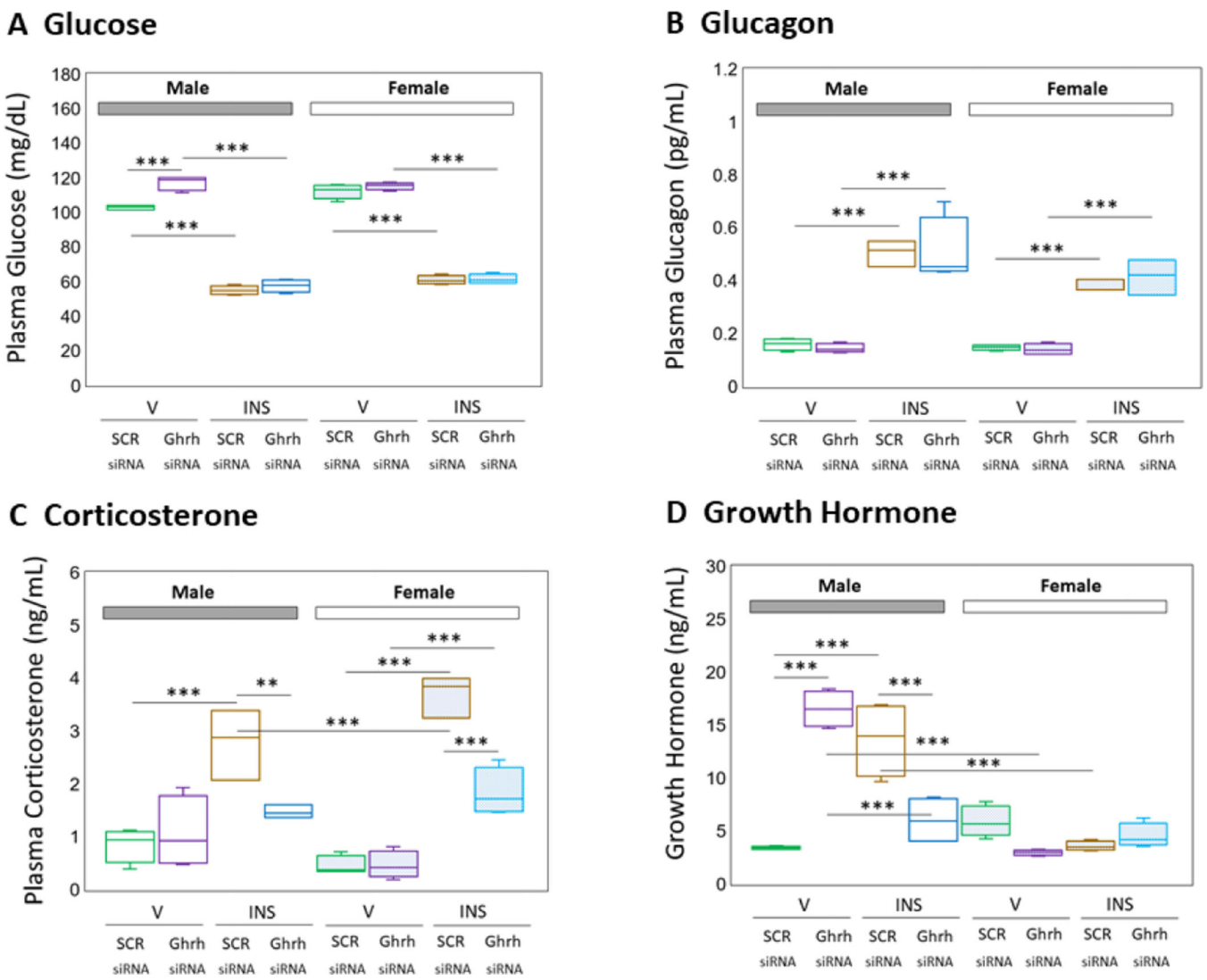

Figure 6. Effects of VMN Ghrh Gene Knockdown on Plasma Glucose and Counter-Regulatory Hormone Profiles in Eu- or Hypoglycemic Male and Female rats.

Plasma samples were obtained from groups of Ghrh or SCR siRNA-pretreated male and female rats one hour after *sc* injection of V or INS, and analyzed for glucose (Figure 6A), glucagon (Figure 6B), corticosterone (Figure 6C), or growth hormone (Figure 6D) concentrations. In each panel, individual treatment group data depict mean plasma concentrations ± S.E.M. for n=8 samples. Data were analyzed by two-way ANOVA and Student-Neuman-Keuls *post-hoc* test, using GraphPad Prism, Vol. 8 software. *p<0.05, **p<0.01, ***p<0.001.

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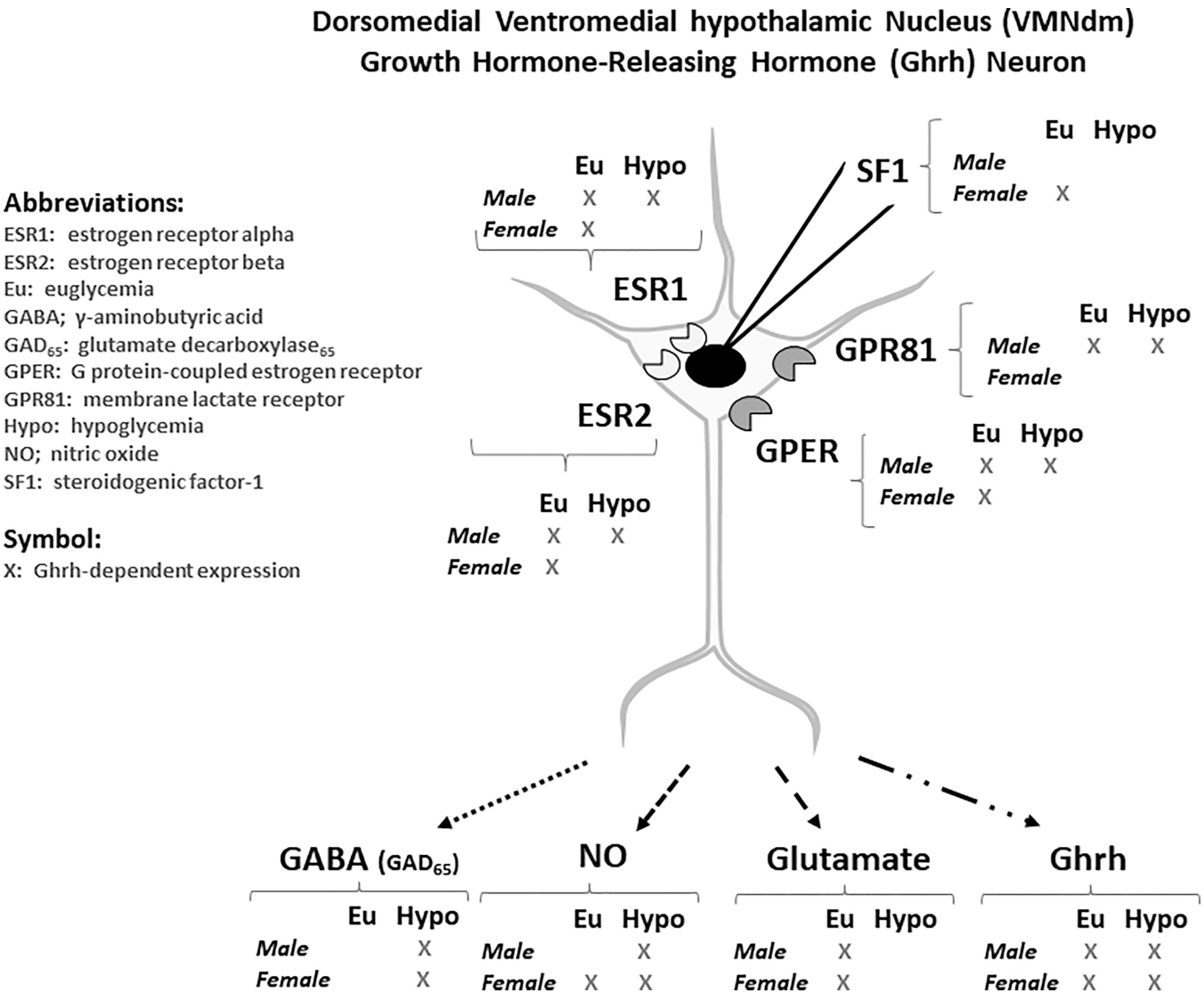

Figure 7. Illustrative Depiction of VMN Ghrh/SF1 Neurons as a Nexus for Hormonal and Nutrient Regulation of Multiple Co-Expressed Glucose-Regulatory Neurochemicals.

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Table 1.

Experimental design

***Sc* Injection; Day 7**	**siRNA Pretreatment; Day 1**	
	SCR siRNA[a]	**Ghrh siRNA[b]**
Vehicle (V)[c]	Male SCR/V; *n* = 8	Male Ghrh/V; *n* = 8
	Female SCR/V; *n* = 8	Female Ghrh/V; *n* = 8
Insulin (INS)[d]	Male SCR/INS; *n* = 8	Male Ghrh/INS; *n* = 8
	Female SCR/INS; *n* = 8	Female Ghrh/INS; *n* = 8

[a] 500 pmol; Accell Control Pool Non-Targeting; prod. no. D-001910-10-20 (Horizon Discovery).

[b] 500 pmol; Accell siRNA Rat Ghrh, set of 4; prod. no. A-089046-16-0010 (Horizon Discovery).

[c] sterile diluent; 100 uL/100 g *bw* subcutaneous (*sc*).

[d] 10.0 U neutral protamine Hagedorn insulin/kg *bw sc*.