Norepinephrine Regulation of Adrenergic Receptor Expression, 5' AMP-Activated Protein Kinase Activity, and Glycogen Metabolism and Mass in Male Versus Female Hypothalamic Primary Astrocyte Cultures

Mostafa M. H. Ibrahim (ID)**, Khaggeswar Bheemanapally,
Paul W. Sylvester and Karen P. Briski** (ID)

Abstract

Norepinephrine (NE) control of hypothalamic gluco-regulation involves astrocyte-derived energy fuel supply. In male rats, exogenous NE regulates astrocyte glycogen metabolic enzyme expression *in vivo* through 5'-AMP-activated protein kinase (AMPK)-dependent mechanisms. Current research utilized a rat hypothalamic astrocyte primary culture model to investigate the premise that NE imposes sex-specific direct control of AMPK activity and glycogen mass and metabolism in these glia. In male rats, NE down-regulation of pAMPK correlates with decreased CaMMKB and increased PP1 expression, whereas noradrenergic augmentation of female astrocyte pAMPK may not involve these upstream regulators. NE concentration is a critical determinant of control of hypothalamic astrocyte glycogen enzyme expression, but efficacy varies between sexes. Data show sex variations in glycogen synthase expression and glycogen phosphorylase-brain and –muscle type dose-responsiveness to NE. Narrow dose-dependent NE augmentation of astrocyte glycogen content during energy homeostasis infers that NE maintains, over a broad exposure range, constancy of glycogen content despite possible changes in turnover. In male rats, $beta_1$- and $beta_2$-adrenergic receptor (AR) profiles displayed bi-directional responses to increasing NE doses; female astrocytes exhibited diminished $beta_1$-AR content at low dose exposure, but enhanced $beta_2$-AR expression at high NE dosages. Thus, graded variations in noradrenergic stimulation may modulate astrocyte receptivity to NE *in vivo*. Sex dimorphic NE regulation of hypothalamic astrocyte AMPK activation and glycogen metabolism may be mediated, in part, by one or more ARs characterized here by divergent sensitivity to this transmitter.

Keywords

norepinephrine, glycogen, glycogen phosphorylase, AMPK, adrenergic receptor, sex differences

Brain astrocytes sustain nerve cell function by multiple mechanisms, including provision of oxidizable substrate fuel (Stobart and Anderson, 2013; Hertz et al., 2015). Glucose, the primary energy source to the brain, is acquired from the circulation into this cell compartment, where it is either stored as the complex polymer glycogen or converted to L-lactate for trafficking to neurons (Wiesinger et al., 1997; Laming et al., 2000). The brain

School of Basic Pharmaceutical and Toxicological Sciences, College of Pharmacy, University of Louisiana Monroe, Monroe, United States

Corresponding Author:
Karen P. Briski, School of Basic Pharmaceutical and Toxicological Sciences, College of Pharmacy, University of Louisiana Monroe, 356 Bienville Building, 1800 Bienville Drive, Monroe, LA 71201, United States.
Email: briski@ulm.edu

glycogen depot undergoes active turnover during normal brain activity and metabolic homeostasis, and is a vital source of lactate equivalents during amplified neural activity or glucoprivation (Hertz and Chen, 2018; Dienel, 2019; Dienel & Carlson, 2019; DiNuzzo et al., 2019; Duran et al., 2019). In the brain and elsewhere, glycogen metabolism is governed by opposing actions of glycogen synthase and glycogen phosphorylase, which control glucose assembly into or liberation from glycogen, respectively. Diversion of glucose through the astrocyte glycogen shunt, prior to entry into the glycolytic pathway, accounts for a significant fraction of glucose catabolism in these glia (Walls et al., 2009; Schousboe et al., 2010). Multiple GP isoforms expressed in brain (Pfeiffer-Guglielmi et al., 2000) differ with respect to cell-type localization and regulation by phosphorylation versus AMP (Nadeau et al., 2018). GP-muscle type (GPmm) and GP-brain type (GPbb) are both expressed in astrocytes, whereas GPbb is also present in neurons. Phosphorylation elicits complete versus partial activation of GPmm or GPbb, whereas GPbb exhibits greater affinity for and sensitivity to AMP activation relative to GPmm, and requires AMP binding for optimal enzyme function and Km. Cortical astrocyte *in vitro* cell culture models show that the catecholamine neurotransmitter norepinephrine (NE) potently stimulates glycogenolysis in these cells (Fillenz et al., 1999; Dong et al., 2012), and that GPmm activity is required for that action (Müller et al., 2015),

Hypothalamic glucose-regulatory loci receive vital metabolic sensory input from hindbrain energy sensors in the form of NE signaling. Dorsal vagal complex (DVC) A2 noradrenergic neurons are a likely source of such input as these cells express hypoglycemia-sensitive metabolic sensory biomarkers, e.g. glucokinase, K_{ATP}, and the ultra-sensitive energy gauge 5'-AMP-activated protein kinase (AMPK) (Briski et al., 2009; Cherian and Briski, 2011). In the hypothalamus, NE regulates expression of marker proteins for gluco-stimulatory and -inhibitory neurotransmitters in part by control of astrocyte-derived metabolic fuel supply (Mahmood et al., 2019). Exogenous NE delivery to the hypothalamus alters GS and combined GP variant expression *in vivo* (Ibrahim et al., 2019), actions that likely involve direct action on astrocytes as these cells express alpha$_1$ (α1)-, alpha$_2$ (α2)-, and beta$_1$ (β1)-adrenergic receptor (AR) variant proteins (Ibrahim et al., 2019; Mahmood et al., 2019). NE is implicated in regulation of hypothalamic GP variant expression *in vivo*. In an experimental model for hindbrain energy imbalance, homeostatic versus lactoprivic-driven (e.g. elevated) patterns of NE input to the hypothalamus impose distinctive control of GPmm and GPbb protein profiles in male rats

(Briski and Mandal, 2020). Noradrenergic regulation of hypothalamic astrocyte glycogen mass is unclear for either sex. Current research utilized a characterized hypothalamic astrocyte primary cell culture model (Ibrahim et al., 2020) to investigate the premise that NE exerts concentration-dependent effects on astrocyte GS and GP variant protein expression and glycogen accumulation that are unique to each sex, and that sex-dimorphic NE regulation of astrocyte glycogen may correlate with differential patterns of expression of the ARs described above and/or β2-AR.

AMPK is activated via phosphorylation in response to cellular metabolic deficits. Phosphorylated AMPK (pAMPK) inhibits ATP-consuming biosynthetic pathways, while stimulating ATP-producing metabolic pathways such as fatty acid oxidation (Hardie, 2003; Kahn et al., 2005). AMPK monitors both dynamic energy state and energy reserves, signified by AMP/ATP ratio and glycogen mass, respectively (McBride et al., 2009; Jensen and Richter, 2015; Li et al., 2015). Glucoprivic activation of AMPK is correlated with decreased GS activity and reductions in glycogen mass (Halse et al., 2003). Hypothalamic astrocytes express the ultrasensitive energy gauge 5'-AMP-activated protein kinase (AMPK) (Tamrakar and Briski, 2015). In male rats, NE regulation of hypothalamic astrocyte glycogen metabolic enzyme protein expression *in vivo* is AMPK-dependent (Ibrahim et al., 2019). A corollary objective of this work sought to examine the premise that NE controls astrocyte total AMPK protein expression and activity, and that such action may involve regulation of upstream stimulatory kinase (calcium/calmodulin-dependent protein kinase kinase-β) and inhibitory phosphatase (protein phosphatase-1) protein expression in one or both sexes.

Recent studies document sex-dimorphic regulation of hypothalamic astrocyte glycogen metabolism and mass by the ovarian steroid estradiol (Ibrahim et al., 2020a). This hormone increases astrocyte glycogen content in female, but not male hypothalamic astrocyte primary cultures during glucostasis, but correspondingly depletes or augments this energy reserve in each sex during glucoprivation (Ibrahim et al., 2020b). Metabolic state-specific control of glycogen accumulation by estradiol may likely involve adjustments in estrogen receptor-specific signaling as glucoprivation down-regulates estrogen receptor-alpha (ERα) and G-protein coupled estrogen receptor-1 (GPER) expression in each sex, but enhances estrogen receptor-beta (ERβ) profiles in female only. To gain insight on if or how NE regulates hypothalamic astrocyte ER variant proteins in either sex, effects of graded NE concentrations on expression profiles were investigated in primary cultures obtained from male or female rats.

Materials and Methods

Primary Astrocyte Cell Culture

Adult male and female Sprague-Dawley rats (3–4 months of age) were housed under a 14 hr light:10 hr dark lighting schedule (lights on at 05.00 hr), and allowed free access to standard laboratory rat chow (Harlan Teklad LM-485; Harlan Industries, Madison, WI) and tap water. Acclimation of rats to daily handling was performed over a 7 day period prior to experimentation. All animal protocols were conducted in accordance with NIH guidelines for care and use of laboratory animals, and approved by the ULM Institutional Animal Care and Use Committee (IACUC). After sacrifice, the hypothalamus was dissected as a single block from each brain at the following boundaries: anterior: rostral border of optic chiasm; posterior: rostral margin of mammillary bodies; lateral: lateral border of tuber cinereum; dorsal: dorsum of third ventricle (Kuo et al., 2010). Highly-purified primary astrocyte cultures were obtained by modification of methods reported by Schildge et al. (2013), as previously described (Ibrahim et al., 2020a). Briefly, hypothalamus tissue was enzymatically digested in 2.5 mL 2.5% trypsin (prod. no. 15090-046; ThermoFisherScientific; Waltham, MA) and 22.5 mL Hanks' balanced salt solution (HBSS; prod. no. H2387, Sigma Aldrich; St. Louis, MO), incubated (30 min) in a 37 °C water bath with shaking every 10 min, then centrifuged (5 min) at 300×g to obtain supernatant by careful decantation. Tissue was dissociated into a single-cell suspension by addition of 10 mL astrocyte plating media [DMEM high glucose media (prod. no. 12800-017; ThermoFisherSci.) supplemented with 10.0% heat-inactivated fetal bovine serum (FBS; GE Healthcare Bio-Sciences, Pittsburgh, PA) and 1.0% penicillin/streptomycin (prod. no. 15140-122, ThermoFisherSci.)] and vigorous pipetting (20–30 times) with a 10 mL pipette. Efficacy of cell isolation was confirmed by cell counting with a hemocytometer. For each astrocyte collection, cells from three hypothalami were pooled by suspension in an adjusted media volume of 20 mL astrocyte plating media, plated in Poly-D-lysine (prod. no. A-003-E, MilliporeSigma, Burlington, MA) – coated T75 culture flasks at a concentration of 50 µg/mL, and incubated at 37 °C in a humidified environment in the presence of 5% CO2 with media changes every 2–3 days post-plating. Approximately 7–8 days after plating, microglia were removed from cultures by aspiration after shaking at 180 rpm (30 min). Cells were re-suspended in fresh media (20 mL) and shaken first on an orbital platform at 240 rpm (6–8 hr), then vigorously by hand (1 min) before aspiration to discard oligodendrocyte precursor cells (OPC). Confluent astrocytes were washed twice with 10 mM phosphate buffered saline, pH 7.4 (PBS), then incubated at 37 °C (7–10 min) with 5.0 mL 0.05% trypsin-EDTA (prod. no. 25300-062, Thermo FisherSci.). Detached astrocytes were centrifuged at 180×g (5 min) and re-suspended in 40 mL fresh media. Cells derived from each confluent layer were plated in two T75 culture flasks, and incubated at 37 °C for 12–14 days with media changes at 2–3 day intervals. Astrocytes were counted, plated, and grown for two weeks prior to experimentation. Astrocyte culture purity was evaluated by immunofluorescence staining of GFAP-immunoreactivity (-ir), as described (Ibrahim et al., 2020b), and was determined to exceed 95%. Relative abundance of astrocyte glial fibrillary acid protein (GFAP; 1:1,000, prod. no. 3670 D; Cell Signaling Technology, Danvers, MA), microglial (AIF-1/Iba1; prod. no. NBP100-1028, 1:1,000; Novus Biologicals, LLC, Littleton, CO) and oligodendrocyte (O4 marker; 1:1,000, prod. no. MAB345; MilliporeSigma, Burlington, MA) protein marker expression was determined by Western blot (Ibrahim et al., 2020b).

Western Blot Analysis

After initial plating at a density of 1×10^6 cells/100 mm^2 in poly-D-lysine – coated culture dishes, astrocytes were maintained for 3 days in high glucose DMEM media until approximate 70% confluency was achieved. Cells were subsequently steroid-starved for 18 hr by incubation in high glucose media containing 5.0% charcoal-stripped FBS (prod. no. 12676029; ThermoFisherSci.) (Micevych et al., 2007; Kuo et al., 2010; Chen et al., 2014). Astrocyte cultures were then incubated for four hr with HBSS media supplemented with varying doses of NE: 0.1 nM, 1 nM, 10 nM, 100 nM and 1000 nM (prod. no. A7257, SigmaAldrich; St. Louis, MO) dissolved in dimethyl sulfoxide (DMSO). DMSO was administered in an equal volume to all treatment groups to result in a media concentration of 0.01%. Controls were incubated with HBSS media containing DMSO only in volume equal to treatment groups. Astrocytes were detached from plates with 0.05% trypsin EDTA (prod. no. 25300-062; ThermoFisherSci.), washed in PBS, pelleted in lysis buffer [2.0% sodium dodecyl sulfate, 10.0% glycerol, 5% β-mercaptoethanol, 1 mM sodium orthovanadate (protease inhibitor), 60 mM Tris-HCl, pH 6.8], heat-denatured (10 min, 95°C), sonicated, centrifuged, and diluted with 2x Laemmli buffer (Bio-Rad, Hercules, CA). Cell lysate protein concentrations were determined by NanoDrop spectrophotometry (prod. no. ND-ONE-W, ThermoFisherSci.). Sample aliquots of equivalent protein mass was loaded for separation in Bio-Rad TGX 10–12% Stain-Free gels; gels were UV light-activated (1 min) in a Bio-Rad ChemiDoc TM Touch

Imaging System after electrophoresis. Proteins were transblotted overnight in Towbin buffer (30 V, 4 °C) to 0.45-μm PVDF membranes (ThermoFisherSci.). Membrane buffer washes and antibody incubations were carried out by Freedom Rocker™ Blotbot® automation (Next Advance, Inc., Troy NY). Membranes were blocked (2 hr) with Tris-buffer saline, pH 7.4, containing 0.1% Tween-20 and 2% bovine serum albumin prior to overnight (4 °C) incubation with primary antisera raised in rabbit against GS (1:1,000, prod. no. 3893S; Cell Signal. Technol.; RRID: AB_2279563); phosphoGS (pGS; 1:1,000, prod. no. 3891S; Cell Signal. Technol.; RRID: AB_2116390); Gbb (1:1,000, prod. no. NBP1-32799; Novus Biologicals, LLC, Littleton, CO; RRID: AB_2253353); GPmm (1:1,000, prod. no. NBP2-16689; Novus Biol.); $AMPK_{\alpha1/2}$ (1:1,000, prod. no. 2532 L; Cell Signal. Technol.; RRID: AB_330331); $phosphoAMPK_{\alpha1/2}$ (pAMPK; 1:1,000, prod. no. 2535 L; Cell Signal. Technol.; RRID: AB_331250); CaMKKβ (1:1,000, prod. no. 16810S; Cell Signal. Technol.; RRID: AB_2798771); PP1 (1:1,000, prod. no. 2582S; Cell Signal. Technol.; RRID: AB_330822); ERα (1:1,000, prod. no. NB100-91756; Novus Biol.; RRID: AB_1216673); ERβ (1:1,000, prod. no. NB120-3577; Novus Biol.; RRID: AB_2102252); GPER/GPR30 (1:1,000, prod. no. NLS-4271; Novus Biol.; RRID: AB_2263521); aromatase (1:1,000, prod. no. NB100-1596; Novus Biol.; RRID: AB_10000919); α1-AR (1:1,000, prod. no. PA1-047; ThermoFisherScientific; Waltham, MA; RRID: AB_2273801); α2-AR (1:1,000, prod. no. PA1-048; ThermoFisherSci.; RRID: AB_2225243); β1-AR (1:1,000, prod. no. NBP1-59007; Novus Biol.; RRID: AB_11025923); or β2-AR (1:1,000, prod. no. NBP2-15564; Novus Biol.). Membranes were next incubated (1 hr) with horseradish peroxidase-conjugated goat anti-rabbit antibodies (1:5,000; prod. no. NEF812001EA; PerkinElmer, Billerica, MA; RRID: AB_2571640), prior to exposure to Supersignal West Femto chemiluminescent substrate (prod. no. 34095; ThermoFisherSci.). Protein optical density (O. D.) signals were detected and quantified in the ChemiDoc MP System using Image Lab™ 6.0.0 software, build 25, 2017, and normalized to total in-lane protein. Precision plus protein molecular weight dual color standards (prod. no. 161–0374, Bio-Rad) were included in each Western blot analysis. For each sex, protein analyses were carried out in triplicate independent experiments, involving three separate astrocyte collections.

Astrocyte Glycogen Quantification by uHPLC-Electrospray Ionization-Mass Spectrometry (LC-ESI-MS)

Cell lysate supernatant aliquots (10 μL) were hydrolyzed by incubation (2 hr) with 10 μL 0.5 mg/mL amyloglucosidase and 10 μL 0.1 M sodium acetate (pH 5.0), followed by sequential heating (100 °C, 5 min) and cooling to room temperature. Glycogen was measured in a ThermoFisherScientific Vanquish UHPLC + System equipped with Thermo Scientific™ Dionex™ Chromeleon™ 7 Chromatography Data System software, as described (Bheemanapally et al., 2020). Column and autosampler temperatures were 35 °C and 15 °C, respectively. The auto-sampler needle was washed with 10% (v/v) methanol (10 s). Hydrolyzed and non-hydrolyzed samples were derivatized with 100 μL 0.5 M 1-phenyl-3-methyl-5-pyrazolone (PMP) reagent supplemented with 0.3 M NaOH. After acidification with 400 μL 0.75% formic acid, derivatized samples were extracted with chloroform to remove excess PMP, 400 μL of supernatant was vacuum concentrated to remove organic solvents, frozen at −80°C, and lyophilized. Lyophilization product was diluted to 1.0 mL with 10 mM ammonium acetate, bath-sonicated (30 s), and centrifuged. Supernatant aliquots (250 μL) were transferred to 350 μL inserts, which were placed into 2 mL Surestop vials in an autosampler tray. D-(+)-Glucose-PMP was resolved using the Shodex™ Asahipak™ NH2P-40 3E column with a mobile phase (75:25 v/v), acetonitrile:10 mM ammonium acetate (0.2 mL/min). D-(+)-Glucose-PMP ion chromatograms were extracted from Total Ion Current (TIC) at m/z 510.2 to generate area-under-the curve data. Critical LC-ESI-MS parameters such as sheath gas pressure (SGP; 25 psig), auxiliary gas pressure (AGP; 4.6 psig), sweep gas pressure (SWGP; 0.5 psig), vaporizer temperature (VT; 150 °C), ion transfer tube temperature (ITT; 150 °C), source voltage (−2000 V), foreline pressure (1.76 Torr; auto-set by instrument- and variable), source gas (nitrogen; Genius NM32LA 110 V, 10–6520; Peak Scientific, Inchinnan, Scotland), and mass peak area detection algorithm (ICIS/Genesis) were maintained at optimum.

Statistics

Mean normalized protein O.D. and glycogen measures were evaluated between treatment groups within each sex by one-way analysis of variance and Student Newman Keuls post-hoc test. Differences of $p < 0.05$ were considered significant.

Results

Male and female hypothalamic astrocyte cultures were incubated with concentrations of NE over a concentration range of 0.1–1000 nM to determine if this neurochemical exerts dose-dependent effects on total (Figure 1A and B) and/or inactive (Figure 1C and D) GS protein expression. In male astrocytes, GS protein levels were significantly decreased by exposure to NE at
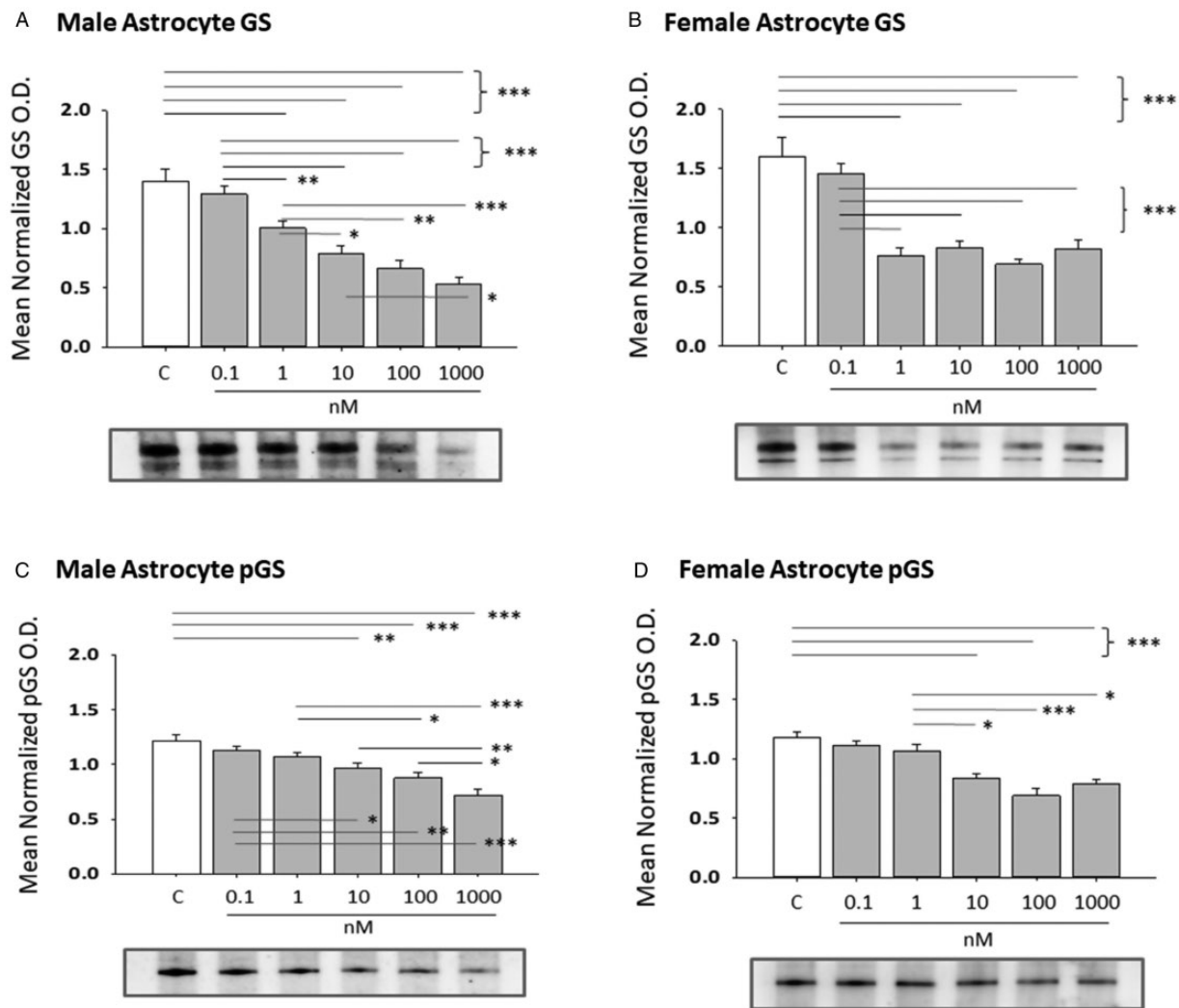
Figure 1. Effects of Graded Norepinephrine (NE) Concentrations on Glycogen Synthase (GS) and PhosphoGS (pGS) Protein Expression in Male and Female Hypothalamic Primary Astrocyte Cultures. Male and female rat hypothalamic astrocytes were plated at a density of 1×10^6 cells/100 mm^2 in poly-D-lysine – coated culture dishes and maintained until 70% confluent. After steroid-starvation for 18 hr, cultures were incubated with media containing O (white bars) or 0.1, 1.0 10.0, 100.0 or 1000.0 nM NE dissolved in dimethyl sulfoxide (DMSO) (gray bars). Final media DMSO concentration for each group was 0.01%. Data indicate depict mean normalized GS [male, Figure 1A; female, Figure 1B] or pGS [male, Figure 1C; female, Figure 1D] protein optical density (O.D.) measures ± S.E.M. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

doses ranging from 1.0 to 1000 nM; higher dosages (10–1000 nM) caused a greater decline in GS protein expression compared to 1.0 nM [$F_{(5,12)} = 22.22$; $p < 0.0001$]. In contrast, female astrocyte GS protein profiles were suppressed to an equivalent extent by 1.0 – 1000 nM dosages [$F_{(5,12)} = 21.94$; $p < 0.0001$]. In each sex, hypothalamic astrocyte pGS protein profiles were refractory to NE concentrations less than 10 nM. In the male, astrocytes incubated with 1000 nM NE showed significantly greater diminution of pGS expression relative to lower, e.g. 10 or 100 nM dosages [$F_{(5,12)} = 13.61$; $p < 0.0001$]. On the other hand, cultured astrocytes from female rats exhibited equivalent-sized decrements in pGS content

after incubation with 10 – 1000 nM NE [$F_{(5,12)} = 17.54$; $p < 0.0001$].

Effects of NE on hypothalamic astrocyte GPbb versus GPmm protein expression were investigated in each sex. Data presented in Figure 2A indicate that male astrocyte GPbb profiles were significantly inhibited after incubation with high, e.g. 100 or 1000 nM doses of NE [$F_{(5,12)} = 12.56$; $p < 0.0001$]. Female hypothalamic astrocytes were more sensitive to NE suppression of this protein as reductions in GPbb content occurred at a low dose of 1.0 nM; the magnitude of NE suppression of this protein was significantly greater after treatment with 10, 100, or 1000 doses (Figure 2B) [$F_{(5,12)} = 17.25$;
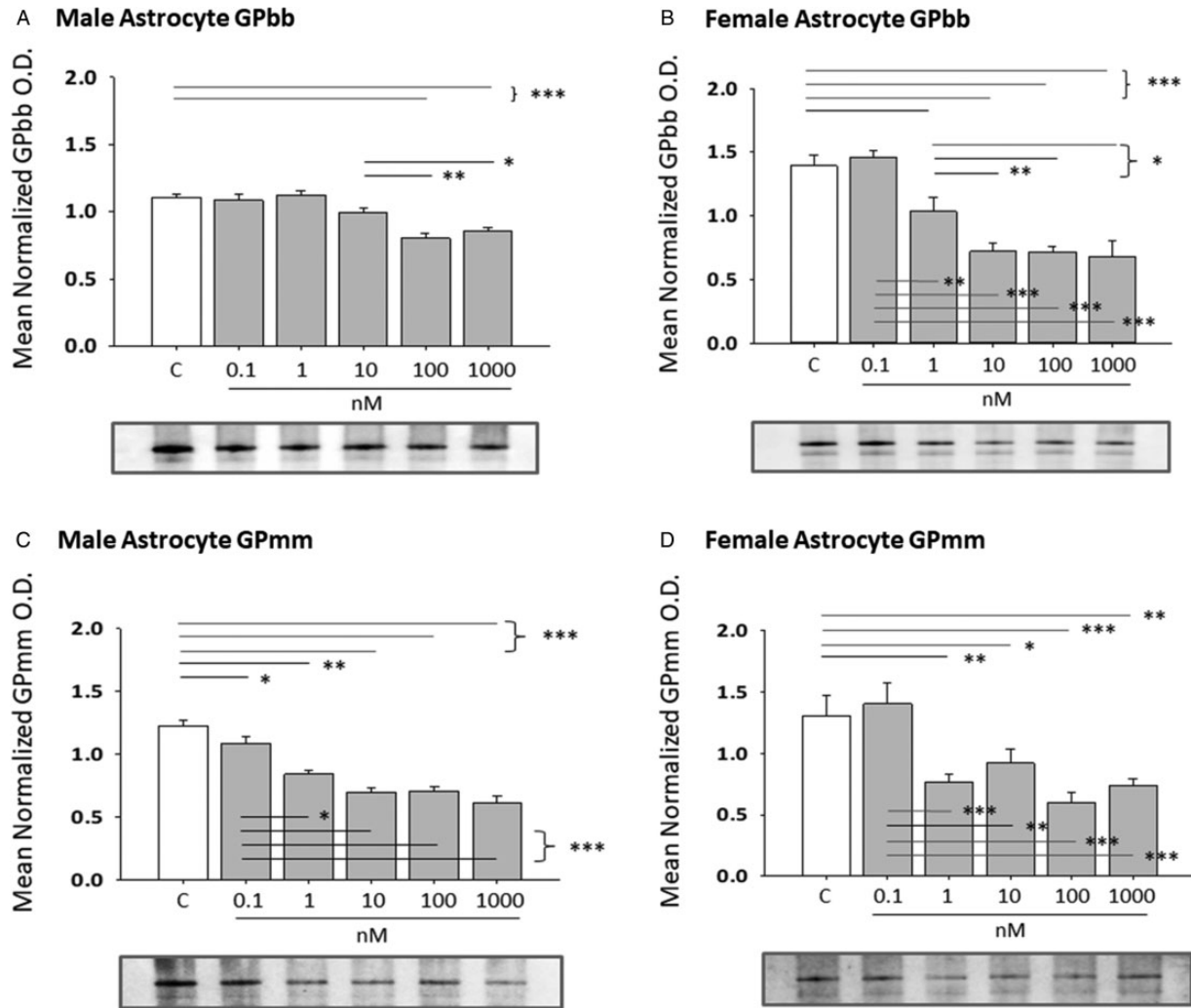
Figure 2. NE Regulation of Glycogen Phosphorylase-Brain Type (GPbb) and GP-Muscle Type (GPmm) Protein Expression in Male versus Female Astrocytes. Bars denote mean normalized GPbb [male, Figure 2A; female, Figure 2B] or GPmm [male, Figure 2C; female, Figure 2D] astrocyte protein O.D. measures $\pm$ S.E.M. after treatment with O (white bars) versus 0.1–1000 nM NE (gray bars). $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

$p < 0.0001$]. GPmm expression in male astrocytes was inhibited at each NE exposure level; this decline was relatively higher after treatment with 1.0–1000 versus 0.1 nM NE (Figure 2C) [$F_{(5,12)} = 29.85$; $p < 0.0001$]. Conversely, female astrocyte GPmm content was equally suppressed by NE dosages ranging from 1–1000 nM (Figure 2D) [$F_{(5,12)} = 8.88$; $p < 0.0001$].

LC-ESI-MS was used to measure glycogen content of primary astrocyte cultures from each sex after incubation with 0.1–1000 nM NE. Data show that in each sex (male, Figure 3A; female, Figure 3B), astrocyte glycogen mass was significantly increased relative to control levels at only a single exposure level, e.g. 10 nM NE. Glycogen content was unaffected by lower or higher NE dosage.

Data in Figure 4 depict effects of graded NE concentrations on male versus female hypothalamic astrocyte AMPK (Figure 4A and B) and pAMPK (Figure 4C

and D) protein expression. In male, (Figure 4A) NE caused dose-proportionate inhibition of male astrocyte AMPK content over the current dosage range [$F_{(5,12)} = 56.75$; $p < 0.0001$]. While female astrocytes also exhibited decreased AMPK levels in response to NE (Figure 4B) [$F_{(5,12)} = 25.49$; $p < 0.0001$], this effect was achieved over a smaller dosage range compared to male (1.0–1000 nM NE). Unique to that sex, AMPK expression was significantly higher after treatment with 1000 nM versus 1.0–100 nM dosages. Astrocyte pAMPK content was significantly diminished or elevated in male [$F_{(5,12)} = 24.83$; $p < 0.0001$] (Figure 4C) versus female [$F_{(5,12)} = 501.3$; $p < 0.0001$] (Figure 4D), respectively, by 0.1–10 nM doses of NE. Interestingly, higher NE concentrations (100 or 1000 nM) either increased (male) or suppressed (female) this protein profile compared to controls. NE effects on expression patterns of
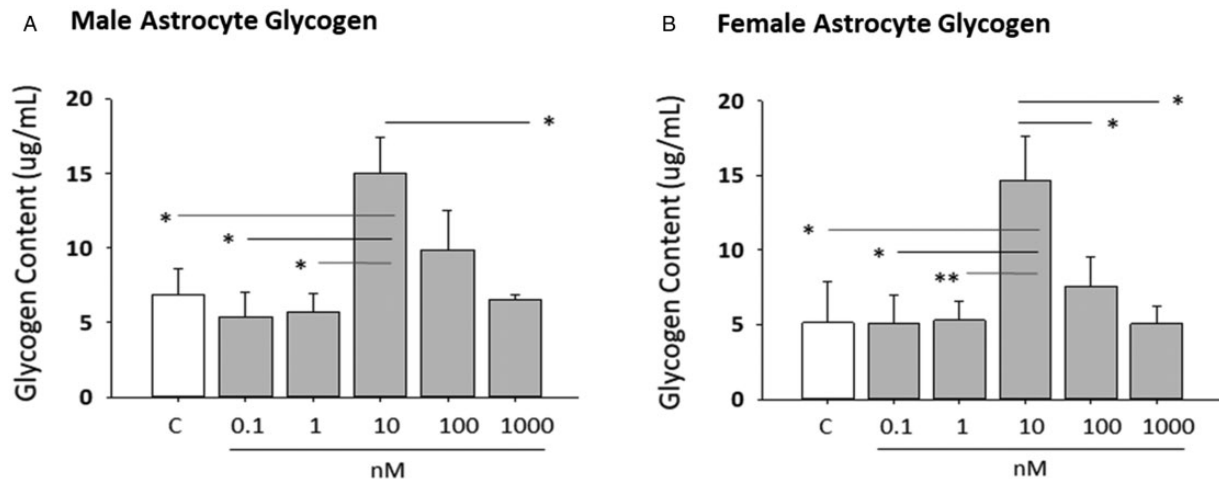
Figure 3. Noradrenergic Regulation of Hypothalamic Astrocyte Glycogen Content in Male versus Female Rats. Data show mean glycogen content ± S.E.M. of male [Figure 3A] and female [Figure 2B] cultured astrocytes after incubation in the absence (white bars) or presence (gray bars; 0.1 – 1000 nM) of NE. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

the upstream kinase CaMKKβ (Figure 5A and B) and phosphatase PP1 (Figure 5C and D) were investigated in male versus female astrocytes. Noradrenergic stimulation caused dose-proportionate reductions in CaMKKβ in the male [$F_{(5,12)} = 32.12$; $p < 0.0001$], but did not alter expression of this protein in the female [$F_{(5,12)} = 2.37$; $p = 0.103$]. In male astrocytes, PP1 protein levels were elevated or decreased by low (0.1 or 1,0 nM) versus high (100 or 1000 nM) NE dosages [$F_{(5,12)} = 46.80$; $p < 0.0001$], while female astrocytes showed only a decline in PP1 expression in response to this neurotransmitter, which occurred at exposure levels that inhibited this protein in the male [$F_{(5,12)} = 4.66$; $p = 0.0009$].

NE regulation of astrocyte AR variant protein content was examined in each sex. Data in Figure 6 illustrate patterns of α1-AR (Figure 6A and B), β1-AR (Figure 6C and D), and β2-AR (Figure 6E and F) protein expression after incubation with graded NE concentrations. Male astrocytes showed bi-directional adjustments in α1-AR protein content as this profile was increased (1.0 nM) or decreased (1000 nM) at distinctive dose NE levels [$F_{(5,12)} = 13.92$; $p < 0.0001$], whereas female astrocytes showed only decreased α1-AR profiles at a single NE concentration, e.g. 0.1 nM [$F_{(5,12)} = 8.69$; $p < 0.0001$]. NE also up- or down-regulated β1-AR expression in male astrocytes, depending upon dosage strength; this protein was increased over a 0.1 – 10 nM dosage range, but declined after exposure to 1000 nM NE [$F_{(5,12)} = 24.50$; $p < 0.0001$]. In contrast, female astrocytes exhibited only a decline in β1-AR levels upon incubation with 1.0 nM NE [$F_{(5,12)} = 45.89$; $p < 0.0001$]. Male astrocyte β2-AR protein profiles were also augmented (1.0 or 10 nM) or inhibited (1000 nM) by NE in a dose-dependent manner [$F_{(5,12)} = 12.69$; $p < 0.0001$]. However, NE elevated β2-AR expression in female

astrocytes at either 100 or 1000 nM concentrations [$F_{(5,12)} = 8.25$; $p < 0.0001$].

Effects of NE on astrocyte ER variant protein expression were investigated in each sex. As shown Figure 7A and B, ERα levels were refractory to all but a single NE concentration (1.0 nM), which stimulated or suppressed this protein in male [$F_{(5,12)} = 5.57$; $p = 0.005$] versus female [$F_{(5,12)} = 4.12$; $p = 0.0025$]. In male astrocytes, NE caused dose-proportionate inhibition of ERβ expression over the entire dosage range (Figure 7C) [$F_{(5,12)} = 27590$ $p < 0.0001$], whereas female astrocytes showed uniform reductions in ERβ content after incubation with 1 – 100 nM NE (Figure 7D) [$F_{(5,12)} = 6.26$; $p < 0.0001$]. GPER protein levels were decreased at only the highest NE concentration (1000 nM) in the male (Figure 7E) [$F_{(5,12)} = 8.44$; $p < 0.0001$], but were diminished in female astrocytes after exposure to 10–1000 nM dosages (Figure 7F) [$F_{(5,12)} = 15.98$; $p < 0.0001$]. Figure 8A and B show that NE suppressed astrocyte aromatase enzyme protein content in male and females, respectively. Noradrenergic inhibition of this protein profile was dose-proportionate in the former [$F_{(5,12)} = 17.73$; $p < 0.0001$], but was dose-independent instead in the latter sex [$F_{(5,12)} = 3.33$; $p = 0.0085$].

Discussion

Current research utilized a rat hypothalamic astrocyte primary culture model to address the premise that NE regulates AMPK activity, glycogen metabolism and mass, and AR and ER variant protein expression in these glia in a sex-contingent manner. Data show that NE causes divergent changes in astrocyte AMPK activity in male versus female glia, which likely involve upstream kinase/phosphatase - dependent versus -independent
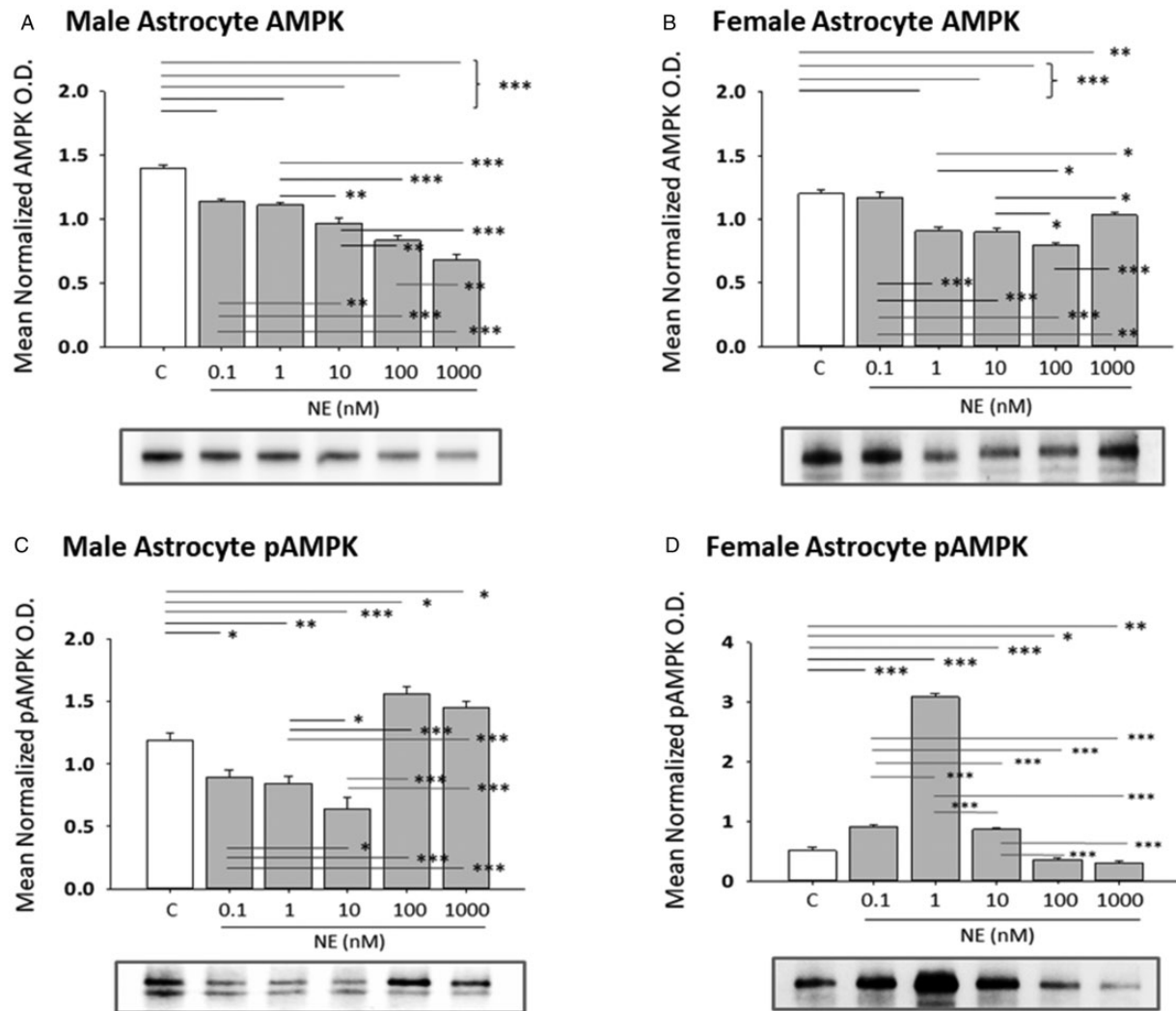
Figure 4. Patterns of Hypothalamic Astrocyte 5'-AMP-Activated Protein Kinase (AMPK) and PhosphoAMPK (pAMPK) Protein Expression in Male and Female Rats: Effects of NE Stimulation. Data depict mean normalized AMPK or pAMPK protein O.D. measures $\pm$ S. E.M. for vehicle- (white bars) or NE-treated (gray bars) male [Figure 4A and C] and female [Figure 4B and D] cultured astrocytes. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

mechanisms, respectively. Sex differences in noradrenergic regulation of glycogen metabolic enzyme expression include fine- versus coarse-control of GS expression and differential sensitivity of GPbb versus GPmm to increasing NE dosages. NE increased astrocyte glycogen content over a narrow segment of the current dose range, suggesting that in presence of glucose, NE may have negligible effects on the net ratio of glycogen synthesis versus breakdown at most exposure levels. Sex differences in noradrenergic control of astrocyte ARs and ERs concern dosage efficacy and direction of regulatory action. Outcomes indicate NE signal volume is an important determinant of hypothalamic astrocyte receptivity to noradrenergic and estradiol input in each sex. Sex dimorphic NE regulation of hypothalamic astrocyte AMPK activation and glycogen metabolism may be mediated, in part,

by one or more ARs exhibiting differential sensitivity according to sex.

NE inhibited each astrocyte glycogen metabolic enzyme protein over all or part of the current dosage range, albeit with sex-dependent dosage efficacy. NE imposition of discriminative, i.e. dose-specific regulation of GS and pGS protein profiles in astrocytes from male, but not female infers that noradrenergic control of glycogen assembly is tighter in the former sex. Low NE dose suppression of total GS expression alongside absence of effect on pGS levels could conceivably diminish the proportion of active enzyme among less abundant GS molecules, thus exacerbating noradrenergic inhibition of glycogen expansion. Brain GPmm and -bb variants exhibit dissimilar sensitivity to phosphorylation versus AMP, thereby facilitating discriminative glycogen
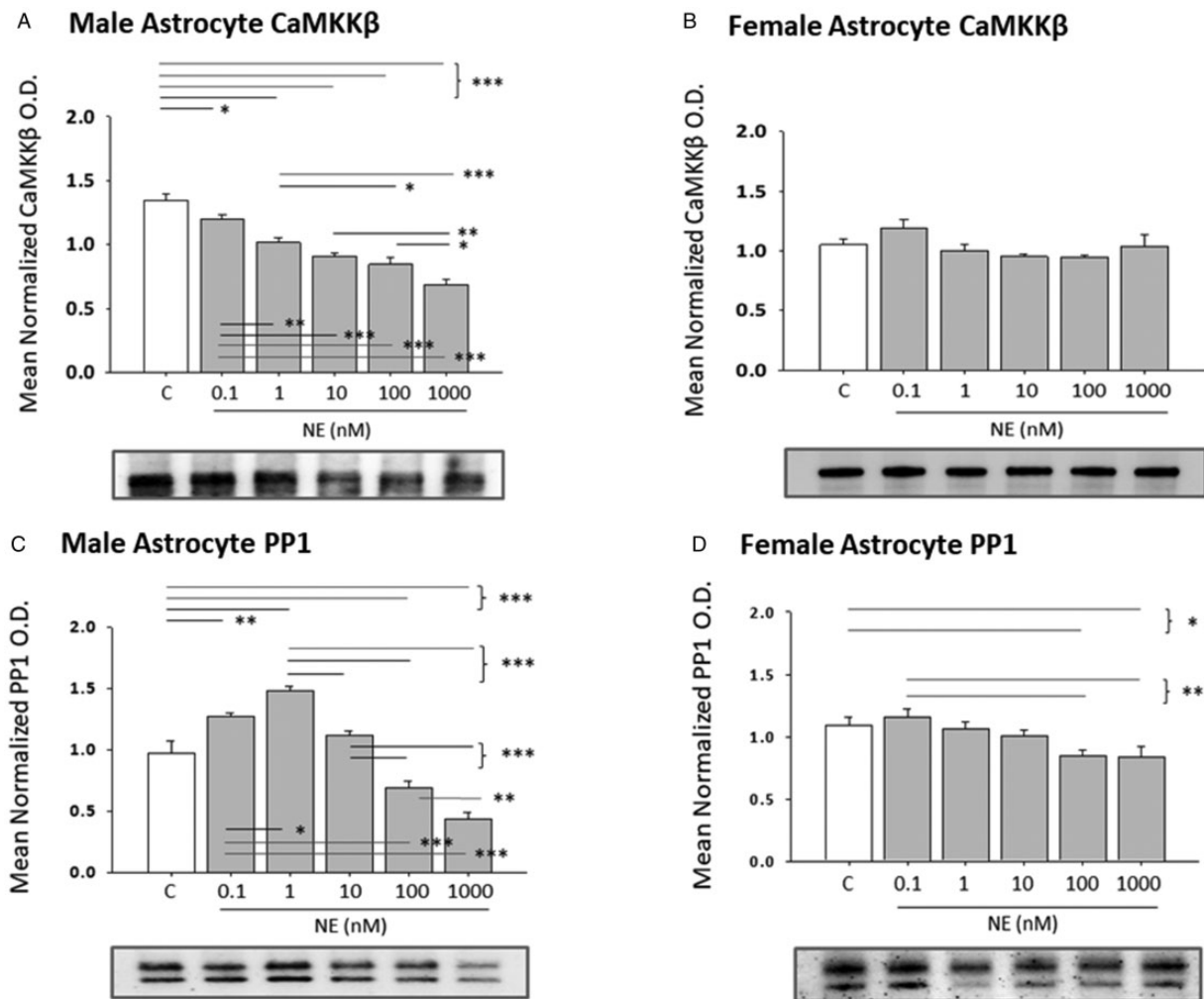
Figure 5. Hypothalamic Astrocyte Calcium/Calmodulin-Dependent Protein Kinase Kinase-β (CaMKK β) and Protein Phosphatase-1 (PP1) Protein Expression in NE-Treated Male versus Female Rats. Data illustrate mean normalized CaMKK β or PP1 protein O.D. measures ± S.E.M. for vehicle- (white bars) or NE-treated (gray bars) male [Figure 5A and C] and female [Figure 5B and D] cultured astrocytes. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

reactivity to these distinctive regulatory stimuli (Müller et al., 2015). Here, the AMP-sensitive GP variant GPbb was down-regulated at lower NE concentrations in female versus male hypothalamic astrocytes, whereas phosphorylation-regulated GPmm was inhibited by the least NE dosage in male, but not female. Dissimilar GP variant sensitivity to NE suggests that transmitter regulation of physiological stimulus-specific glycogen breakdown likely differs between sexes. Results document, for the male rat, a notable divergence in minimum NE dose required to inhibit GPmm versus GPbb protein expression, e.g. 0.1 versus 100 nM, as well as dose-proportionate effects of lower NE dosages on GPmm content. Thus, in this sex, low NE concentration exposure may cause graded suppression of phosphorylation-mediated glycogen breakdown, in the absence of effect on glycogen sensitivity to AMP. Meanwhile, female

astrocytes exhibited down-regulated expression of GPmm and -bb over a similar NE dose range (1.0–1000 nM), but inhibition was either uniform (GPmm) or graded (GPbb). NE may thus exert finer control of energy deficit versus phosphorylation regulation of female astrocyte glycogen disassembly. It should be noted that total GP protein content measures do not fully elucidate how NE may affect glycogen disassembly in the current model as phosphorylated GP (pGP) is the active enzyme form. Since analytical tools for assessment of pGP expression are not currently available, speculation on potential change in net specific activity of this enzyme under current experimental conditions remains to be verified. In the event that NE decreases net phosphorylation alongside diminution of total GPmm or GPbb protein mass, a plausible outcome would be a reduction in glycogen breakdown by their respective
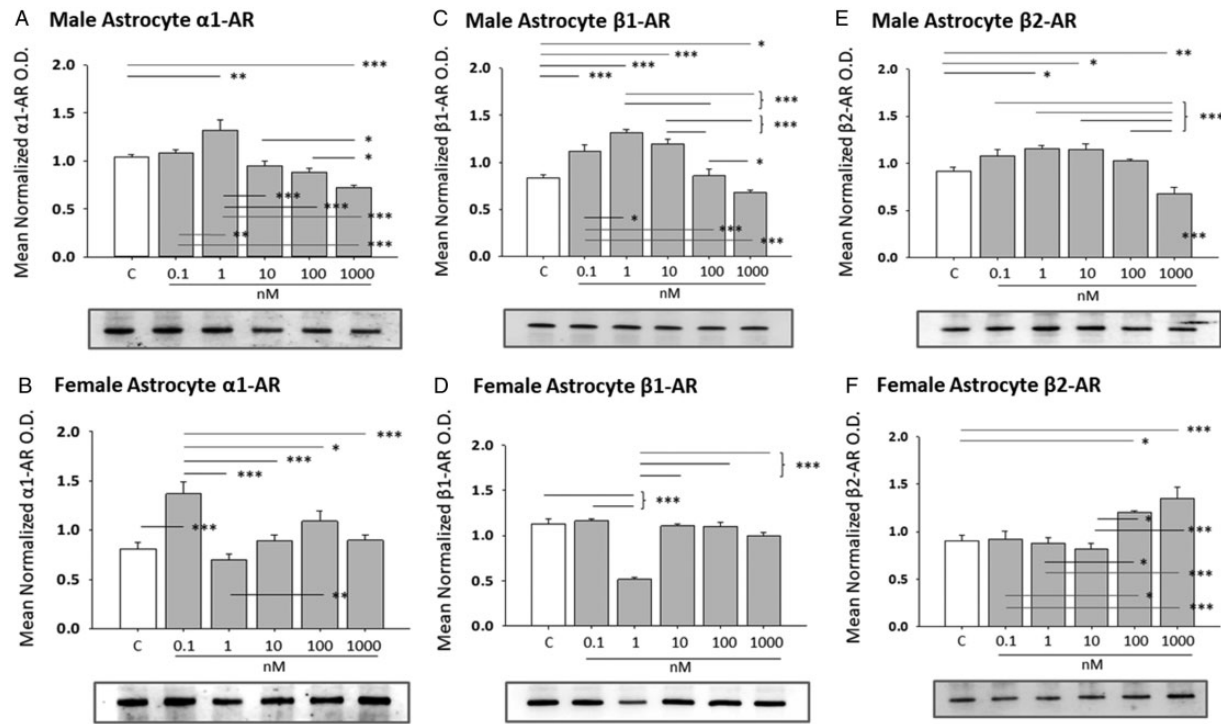
Figure 6. Noradrenergic Regulation of Alpha1 (α1)-, Beta1 (β1)-, and Beta1 (β2)-Adrenergic Receptor (AR) Protein Expression in Male versus Female Rat Hypothalamic Astrocytes. Data show mean normalized α1-AR [Figure 6A (male) and B (female)], β1-AR [Figure 6C (male) and D (female)], or β2-AR [Figure 6E (male) and F (female)] protein O.D. measures ± S.E.M. for vehicle- (white bars) or NE-treated (gray bars) male and female cultured astrocytes. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.
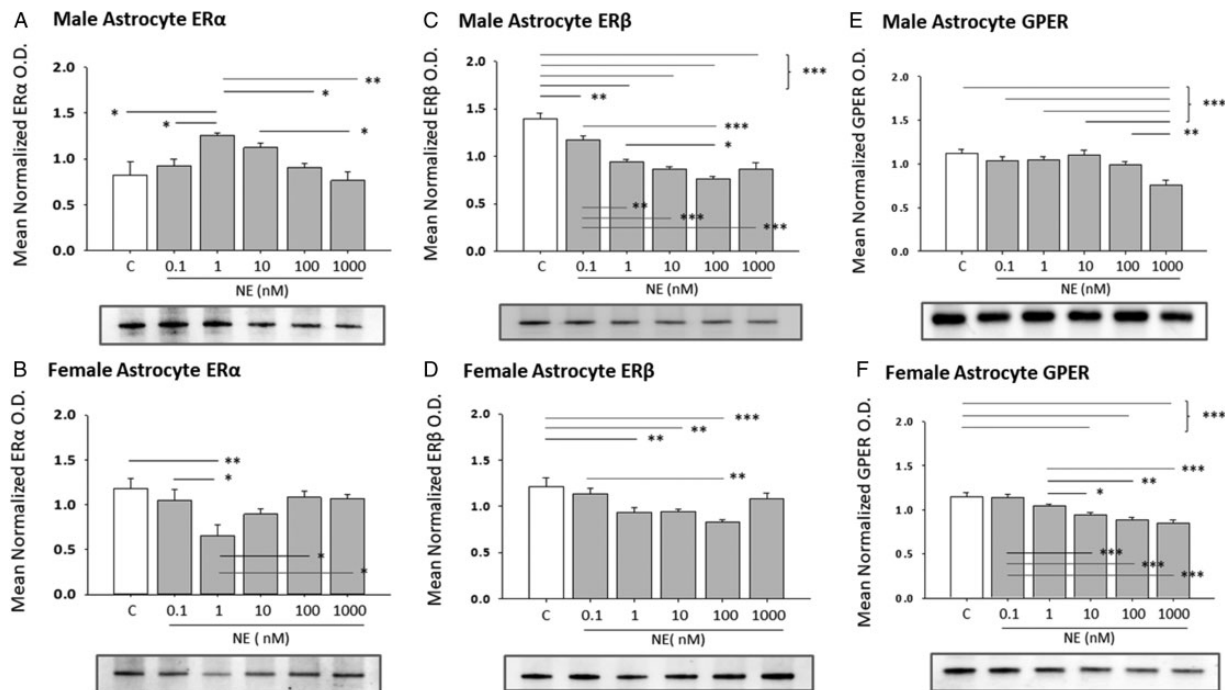

Figure 7. Noradrenergic Regulation of Estrogen Receptor-Alpha (ERα), Estrogen Receptor-Beta (ERβ), and G-Protein Coupled Estrogen Receptor-1 (GPER) Protein Expression in Male versus Female Rat Hypothalamic Astrocytes. Data show mean normalized ERα [Figure 7A (male) and B (female)], ERβ [Figure 7C (male) and D (female)], or GPER [Figure 7E (male) and F (female)] protein O.D. measures ± S.E.M. for vehicle- (white bars) or NE-treated (gray bars) male and female cultured astrocytes. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.
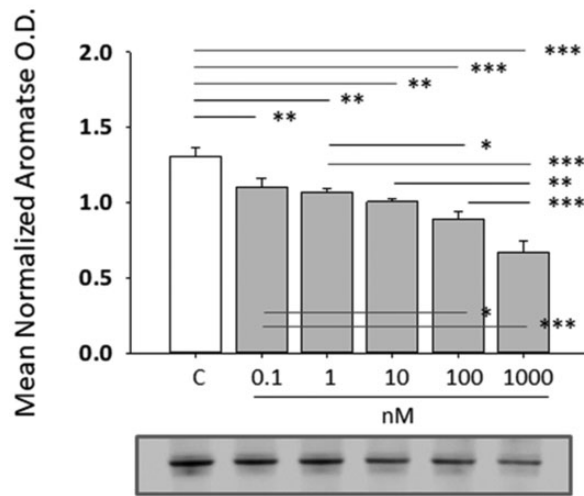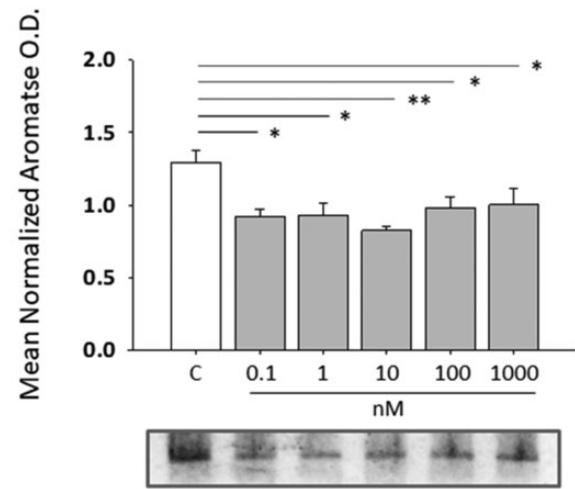
Figure 8. Effects of NE Stimulation on Hypothalamic Astrocyte Aromatase Protein Expression in Male versus Female Rats. Data show mean normalized aromatase protein O.D. measures ± S.E.M. for vehicle- (white bars) or NE-treated (gray bars) male [Figure 8A] and female [Figure 8B] cultured astrocytes. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

action. Reduced phosphorylation of GPmm would likely attenuate active glycogen mobilization under current circumstances, while decreased pGPbb would diminish that process in response to energy deficiency. Alternatively, NE-associated enhancement of phosphorylation of less abundant GP protein would presumably increase enzyme specific activity, and in doing so, may amplify glycogen disassembly by that GP variant.

Current outcomes emphasize evident variability of NE control of astrocyte glycogen metabolism between brain regions. In light of ample evidence for noradrenergic stimulation of cortical astrocyte glycogenolysis *in vitro* (Walls et al., 2009; Hertz et al., 2015; Cogga et al., 2018), our working hypothesis here was that in one or both sexes, hypothalamic astrocyte glycogen content would be significantly diminished upon exposure to NE. Instead, LC-ESI-MS analyses show that this neurotransmitter promotes concentration (10 nM) - dependent glycogen accumulation, but did not diminish this mass in either sex at other lesser or greater levels of exposure. Thus, during energy homeostasis, NE may have negligible net impact on the ratio of glucose assimilation into versus release from glycogen in hypothalamic astrocytes except at a very narrow concentration range. However, it should be noted that current work did not seek to generate experimental confirmation of direct correlations between net changes in glycogen enzyme protein expression versus activity due to NE. As the 10 nM NE dose down-regulated GS and one or both GP variant protein profiles, measurable increases in astrocyte glycogen mass after this treatment may reflect, in part, an augmented ratio of GS versus GP specific activities and resultant net

escalation of net glucose monomer incorporation into versus liberation from glycogen, such that glycogen expansion occurs despite a possible net decline in turnover rate. It would be informative for future research to characterize the *in vivo* hypothalamic noradrenergic activity patterns that are replicated here *in vitro* by exposure to 10 nM NE versus smaller or higher transmitter concentrations.

NE caused dose-dependent decrements in astrocyte AMPK total protein expression in each sex. However, male astrocytes were more sensitive to this suppressive action as AMPK content was inhibited at 0.1 versus 1.0 nM NE concentrations, respectively, in that sex versus the female. Interestingly, this transmitter caused divergent changes in astrocyte pAMPK expression between the two sexes at each level of the current dosage range. In each sex, the direction of NE effect, e.g. stimulation versus inhibition of the activated enzyme form was reversed as dosage levels increased, in an opposite trend in each sex. Up- or down-regulated pAMPK expression is generally interpreted as a likely indicator of negative versus positive change in cellular energy stability. However, this interpretation remains hypothetical here as measurements of NE treatment effects on astrocyte ATP concentrations, in the presence of sustained glucose provision, were not performed. In male astrocytes, evidence for bi-directional noradrenergic regulation of PP1 protein expression supports the prospect that observed pAMPK protein decrements at low NE dosages, but elevated expression at higher concentrations may owe principally to upstream inhibitory kinase activity, and diminished CaMKKβ stimulatory function

at low NE dosages, rather than deviations from cellular metabolic balance. Yet, the latter possibility as a contributing factor should not be entirely discounted as potential NE regulation of intracellular energy metabolic, e.g. namely glycolytic, tricarboxylic, and oxidative respiration pathways, may alter AMP/ATP ratios and amplify AMPK activity via phosphorylation despite glucose availability. In contrast, in female astrocytes, up- versus down-regulation of pAMPK expression at low versus high NE concentrations coincided with minimal change in either CaMKKβ or PP1 protein profiles. These data suggest that noradrenergic control of astrocyte sensor activity in this sex may involve other stimulatory or inhibitory kinases, such as kinases liver kinase B1 (LKB1) and TGFβ-activated kinase 1 (TAK1) and phosphatases protein phosphatase 2 A; protein phosphatase 2 C, and Mg^{2+}-/Mn^{2+}-dependent protein phosphatase 1E [], and/or predominant allosteric regulation of AMPK activity due to amplification of the AMP/ATP ratio (Jeon, 2016). AMPK monitors glycogen mass; glycogen binding to the AMPK beta subunit diminishes phosphorylation of the enzyme's catalytic subunit (Janzen et al., 2018). Here, astrocytes from each sex exhibited discordant astrocyte pAMPK expression and glycogen responses to NE at most exposure levels, outcomes that argue against glycogen modulation of sensor activity in the presence of NE. Previous *in vivo* studies in male rats showed that AMPK activity was required for exogenous NE regulation of hypothalamic GS and GP protein expression (Ibrahim et al., 2019). Opposite sex-specific noradrenergic effects on pAMPK expression observed at each exposure level could contribute to differential GS, GPbb, and/or GPbb protein responses to individual NE doses. Ongoing research aims to determine if and how NE concentration-dependent AMPK activity impacts astrocyte glycogen turnover and mass and other energy metabolic functions, namely substrate fuel uptake and catabolism in these cells. Here, NE enhancement of astrocyte glycogen content coincided with down- versus up-regulated pAMPK expression in male and female rats, respectively.

The present study provides unique proof that hypothalamic astrocytes are direct targets for sex dimorphic noradrenergic regulation of AR variant protein expression. In male astrocytes, α1-, β1-, and β2-AR protein profiles were each stimulated at lower NE doses, but were refractory to the 100 nM dose; moreover, each protein was inhibited at the highest exposure level (1000 nM). Moreover, α1- (1 nM), β1- (0.1 – 10 nM), and β2-AR (1 – 10 nM) proteins were up-regulated over different segments of the current dose range. Meanwhile, female astrocytes exhibited augmented (α1-AR, 0.1 nM; β2-AR, 100 – 1000 nM) or diminished β1-AR (1.0 nM) protein expression at more restricted points along the same dose range. Outcomes emphasize that uniquely in

each sex, NE signal strength is a critical determinant of astrocyte receptivity to this stimulus. Graded variations in physiological patterns of NE activity *in vivo* may result in neurotransmitter volume-dependent patterns of AR signaling and post-receptor signal transduction regulatory mechanisms. Sex-specific noradrenergic regulation of hypothalamic astrocyte AMPK activity state and glycogen metabolic enzyme protein expression may be mediated, in part, by one or more ARs characterized here by discriminative sensitivity to NE.

Estradiol imposes sex-dimorphic control of hypothalamic astrocyte AMPK activity and glycogen mass through regulation of ER variant expression (Ibrahim et al., 2020a, 2020b). This project provides novel evidence that astrocyte receptivity to estradiol is also controlled by NE. Here, NE caused opposite changes, e.g. up- versus down-regulation of ERα content in male versus female rats at a single exposure level (1.0 nM), but had no effect on this profile at other concentrations. Meanwhile, ERβ protein was decreased after treatment with 0.1 – 100 nM NE doses in each sex. Female astrocytes were more sensitive to NE regulation of the membrane ER GPER than male, as this protein level was decreased over a 10 – 1000 nM dosage range in female, but only after exposure to 1000 nM in males. Observed NE treatment effects on expression patterns of astrocyte ERs and other proteins of interest did not reflect exogenous estradiol signaling via noradrenergic-sensitive ERs as NE was administered in charcoal-stripped incubation media. In the hypothalamus and elsewhere in the brain, ERs are stimulated by hormone of dual origin, as estradiol is acquired from the circulation as well as produced locally by aromatase conversion of testosterone to estradiol. The cellular sources (s) of neuroestradiol produced in the hypothalamus are unclear, yet both neurons and astrocytes in other neural structures express aromatase (Azcoitia et al., 2011; Fester et al., 2016). Current work study provides unique evidence for aromatase expression in hypothalamic astrocytes in each sex, and shows, moreover, that this protein is inhibited by NE over the current concentration exposure range. NE evidently imposes tighter control of aromatase expression in male astrocytes, as this protein was suppressed in a dose-proportionate manner, as opposed to uniform inhibition by NE irrespective of dosage in the female. These results support speculation that endogenous estradiol generated within cultured astrocytes is likely not involved in NE treatment effects on target proteins. However, as intrinsic estradiol production by non-NE- versus NE-exposed cultured astrocytes was not evaluated here, the extent to which *de novo* synthesis of this hormone may be diminished by NE under current experimental circumstances remains to be determined.

Current research aimed to evaluate, for each sex, concentration-dependent NE effects on astrocyte target
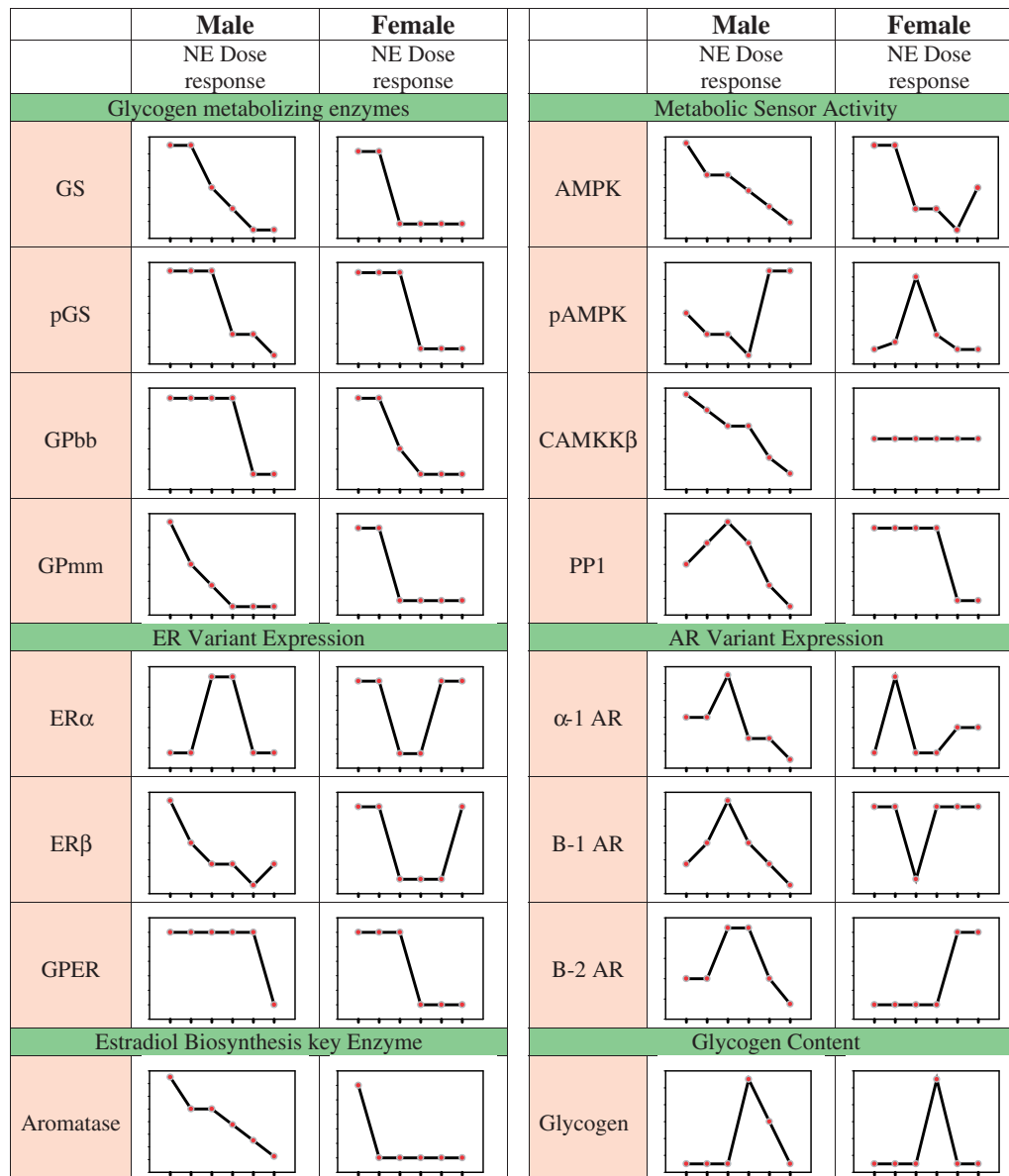
Figure 9. Sex-Specific NE Regulation of Hypothalamic Astrocyte Glycogen Metabolic Enzyme and Adrenergic and Estrogen Receptor Variant Protein Expression and Glycogen Content. Noradrenergic inhibition of male astrocyte pAMPK expression is likely mediated by CaMMKB and PP1 actions, but intensification of sensor activity in the female by equivalent NE doses may involve alternative mechanisms. NE concentration governs hypothalamic astrocyte glycogen enzyme reactivity, but dosage efficacy varies between sexes. Outcomes reveal sex differences in glycogen synthase expression and glycogen phosphorylase-brain and –muscle type dose-responsiveness to NE. Narrow dose-dependent NE augmentation of astrocyte glycogen content during energy homeostasis infers that NE maintains, over a broad exposure range, constancy of glycogen content despite possible changes in turnover. In male rats, beta$_1$- and beta$_2$-AR profiles displayed bi-directional responses to increasing NE doses; female astrocytes exhibited diminished beta$_1$-AR content at low dose exposure, but enhanced beta$_2$-AR expression at high NE dosages. Thus, graded variations in noradrenergic stimulation may modulate astrocyte receptivity to NE *in vivo*. Sex dimorphic NE regulation of hypothalamic astrocyte AMPK activation and glycogen metabolism may be mediated, in part, by one or more ARs characterized here by divergent sensitivity to this transmitter.

protein and glycogen content. As Western blot analyses were carried out separately for each sex, e.g. in different gels due to the large number of treatment groups for each sex, we refrained from direct comparison of data obtained in females versus males. Thus, the prospect exists that baseline expression of one or more

AR variants may vary between male and female astrocytes, and that resultant differential sensitivity to NE over all or part of the current dosage range may contribute, in part, to divergent patterns of exogenous NE effects on target proteins examined here, including AR profiles.

In summary, current research shows that NE exerts sex-specific direct control of AMPK activity and glycogen mass and metabolism in hypothalamic astrocytes (Figure 9). Noradrenergic inhibition of male astrocyte pAMPK expression is likely mediated by CaMMKB and PP1 actions, but intensification of sensor activity in the female by equivalent NE doses involves other mechanisms. NE concentration controls hypothalamic astrocyte glycogen enzyme reactivity, but dosage efficacy differs between sexes. Data show sex variations in glycogen synthase expression and glycogen phosphorylase-brain and –muscle type dose-responsiveness to NE. Narrow dose-dependent NE augmentation of astrocyte glycogen content during energy homeostasis infers that NE maintains, over a broad exposure range, constancy of glycogen content despite possible changes in turnover. In male rats, beta$_1$- and beta$_2$-AR profiles displayed bi-directional responses to increasing NE doses; female astrocytes exhibited diminished beta$_1$-AR content at low dose exposure, but enhanced beta$_2$-AR expression at high NE dosages. Thus, graded variations in noradrenergic stimulation may modulate astrocyte receptivity to NE *in vivo*. Sex dimorphic NE regulation of hypothalamic astrocyte AMPK activation and glycogen metabolism may be mediated, in part, by one or more ARs characterized here by divergent sensitivity to this transmitter.

Declaration of Conflicting Interests

Funding

ORCID iDs

Mostafa M. H. Ibrahim 🆔 https://orcid.org/0000-0003-2038-9933
Karen P. Briski 🆔 https://orcid.org/0000-0002-7648-1395

References

Azcoitia, I., Yague, J. G., & Garcia-Segura, L. M. (2011). Estradiol synthesis within the human brain. *Neuroscience*, *191*, 139–147.

Bheemanapally, K., Ibrahim, M. M. H., & Briski, K. P. (2020). Combinatory high-resolution microdissection/ultra performance liquid chromatographic–mass spectrometry approach for small tissue volume analysis of rat brain glycogen. *Journal of Pharmaceutical and Biomedical Analysis*, *178*, 112884. https://doi.org/10.1016/j.jpba.2019.112884

Briski, K. P., & Mandal, S. K. (2020). Hindbrain metabolic deficiency regulates ventromedial hypothalamic nucleus glycogen metabolism and glucose-regulatory signaling. *Acta Neurobiol. Exper*, *80*, 57–65.

Briski, K. P., Cherian, A. K., Genabai, N. K., & Vavaiya, K. V. (2009). *In situ* coexpression of glucose and monocarboxylate transporter mRNAs in metabolic-sensitive dorsal vagal complex catecholaminergic neurons: Transcriptional reactivity to insulin-induced hypoglycemia and caudal hindbrain glucose or lactate repletion during insulin-induced hypoglycemia. *Neuroscience*, *164*(3), 1152–1160.

Chen, C., Kuo, J., Wong, A., & Micevych, P. (2014). Estradiol modulates translocator protein (tspo) and steroid acute regulatory protein (StAR) via protein kinase A (PKA) signaling in hypothalamic astrocytes. *Endocrinology*, *155*(8), 2976–2985.

Cherian, A., & Briski, K. P. (2011). Quantitative RT PCR and immunoblot analyses reveal acclimated A2 noradrenergic neuron substrate fuel transporter, glucokinase, phospho-AMPK, and dopamine-beta-hydroxylase responses to hypoglycemia. *Journal of Neuroscience Research*, *89*(7), 1114–1124.

Cogga, J. S., Keller, D., Calì, C., Lehväslaiho, H., Markram, H., Schürmann, F., & Magistretti, P. J. (2018). Norepinephrine stimulates glycogenolysis in astrocytes to fuel neurons with lactate. *PLoS Computational Biology*, *14*(8), e1006392.

Dienel, G. A. (2019). Brain glucose metabolism: Integration of energetics with function. *Physiological Reviews*, *99*(1), 949–1045.

Dienel, G. A., & Carlson, G. M. (2019). Major advances in brain glycogen research: Understanding of the roles of glycogen have evolved from emergency fuel reserve to dynamic, regulated participant in diverse brain functions. *Advances in Neurobiology*, *23*, 1–16.

DiNuzzo, M., Walls, A. B., Öz, G., Seaquist, E. R., Waagepetersen, H. S., Bak, L. K., Nedergaard, M., & Schousboe, A. (2019). State-dependent changes in brain glycogen metabolism. *Advances in Neurobiology*, *23*, 269–309.

Dong, J. H., Chen, X., Cui, M., Yu, X., Pang, Q., & Sun, J. P. (2012). B2-adrenergic receptor and astrocyte glucose metabolism. *Journal of Molecular Neuroscience*, *48*(2), 456–463.

Duran, J., Gruart, A., López-Ramos, J. C., Delgado-García, J. M., & Guinovart, J. J. (2019). Glucogen in astrocytes and neurons: Physiological and pathological aspects. *Advances in Neurobiology*, *23*, 311–329.

Fester, L., Brandt, N., Windhorst, S., Pröls, F., Bläute, C., & Rune, G. M. (2016). Control of aromatase in hippocampal neurons. *The Journal of Steroid Biochemistry and Molecular Biology*, *160*, 9–14.

Fillenz, M., Lowry, J. P., Boutelle, M. G., & Fray, A. E. (1999). The role of astrocytes and noradrenaline in neuronal glucose metabolism. *Acta Physiologica Scandinavica*, *167*(4), 275–284.

Halse, R., Fryer, L. G., McCormack, J. G., Carling, D., & Yeaman, S. J. (2003). Regulation of glycogen synthase by glucose and glycogen: A possible role for AMP-activated protein kinase. *Diabetes*, *52*(1), 9–15.

Hardie, D. G. (2003). The AMP-activated protein kinase cascade: the key sensor of cellular energy status. *Endocrinology*, *144*, 5179–5183.

Hertz, L., & Chen, Y. (2018). Glycogenolysis, an astrocyte-specific reaction, is essential for both astrocytic and neuronal activities involved in learning. *Neuroscience*, *370*, 27–36.

Hertz, L., Xu, J., Song, D., Du, T., Li, B., Yan, E., & Peng, L. (2015). Astrocytic glycogenolysis: Mechanisms and functions. *Metabolic Brain Disease*, *30*(1), 317–333.

Ibrahim, M. M. H., Alhamami, H. N., & Briski, K. P. (2019). Norepinephrine regulation of ventromedial hypothalamic nucleus metabolic transmitter biomarker and astrocyte enzyme and receptor expression: Impact of 5' AMP-activated protein kinase. *Brain Research*, *1711*, 48–57.

Ibrahim, M. M. H., Bheemanapally, K., Sylvester, P. W., & Briski, K. P. (2020a). Sex-specific estrogen regulation of hypothalamic astrocyte estrogen receptor expression and glycogen metabolism in rats. *Molecular and Cellular Endocrinology*, *504*, 110703.

Ibrahim, M. M. H., Bheemanapally, K., Sylvester, P. W., & Briski, K. P. (2020b). Sex differences in glucoprivic regulation of glycogen metabolism in hypothalamic primary astrocyte cultures: Role of estrogen receptor signaling. *Molecular and Cellular Endocrinology*, *518*, 111000. https://doi.org/10.1016/j.mce.2020.111000

Janzen, N. R., Whitfield, J., & Hoffman, N. J. (2018). Interactive roles for AMPK and glycogen from cellular energy sensing to exercise. Intl. *International Journal of Molecular Sciences*, *19*(11), 3344. https://doi.org/10.3390/ijms, 19113344

Jeon, S. M. (2016). Regulation and function of AMPK in physiology and diseases. *Experimental & Molecular Medicine*, *48*(7), e245. https://doi.org/10.1038/emm.2016.81

Kahn, B. B., Alquier, T., Carling, D., & Hardie, D. G. (2005). AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. *Cell Metabol*, *1*, 15–25.

Kuo, J., Hamid, N., Bondar, G., Prossnitz, E. R., & Micevych, P. (2010). Membrane estrogen receptors stimulate intracellular calcium release and progesterone synthesis in hypothalamic astrocytes. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *30*(39), 12950–12957.

Laming, P. R., Kimelberg, H., Robinson, S., Salm, A., Hawrylak, N., Muller, C., Roots, B., & Ng, K. (2000). Neuronal-glial interactions and behavior. *Neuroscience and Biobehavioral Reviews*, *24*(3), 295–340.

Li, X., Wang, L., Zhou, E. X., Ke, J., de Waal, P. W., Gu, X., Tan, M. H. E., Wang, D., Wu, D., Xu, E. H., & Melcher, K. (2015). Structural basis of AMPK regulation by adenosine nucleotides and glycogen. *Cell Res*, *25*, 50–66.

Mahmood, A. S. M. H., Mandal, S. K., Bheemanapally, K., Ibrahim, M. M. H., & Briski, K. P. (2019). Norepinephrine control of ventromedial hypothalamic nucleus glucoregulatory neurotransmitter expression in the female rat: Role monocarboxylate transporter function. *Molecular and Cellular Neuroscience*, *95*, 51–58.

McBride, A., Ghilagaher, S., Nikolaev, A., & Hardie, D. G. (2009). The glycogen-binding domain on the AMPK beta subunit allows the kinase to act as a glycogen sensor. *Cell Metabol*, *9*, 23–34.

Micevych, P., Chaban, V., Ogi, J., Dewing, P., Lu, J. K. H., & Sinchak, K. (2007). Estradiol stimulates progesterone synthesis in hypothalamic astrocyte cultures. *Endocrinology*, *148*(2), 782–789.

Müller, M. S., Pedersen, S. E., Walls, A. B., Waagepetersen, H. S., & Bak, L. K. (2015). Isoform-selective regulation of glycogen phosphorylase by energy deprivation and phosphorylation in astrocytes. *Glia*, *63*(1), 154–162.

Nadeau, O. W., Fontes, J. D., & Carlson, G. M. (2018). The regulation of glycogenolysis in the brain. *The Journal of Biological Chemistry*, *293*(19), 7099–7109.

Pfeiffer-Guglielmi, B., Bröer, S., Bröer, A., & Hamprecht, B. (2000). Isozyme pattern of glycogen phosphorylase in the rat nervous system and rat astroglia-rich primary cultures: Electrophoretic and polymerase chain reaction studies. *Neurochemical Research*, *25*(11), 1485–1491.

Schildge, S., Bohner, C., Beck, K., & Schachtrup, C. (2013). Isolation and culture of mouse cortical astrocytes. *J Vis Exp*, (71), 50079. https://doi.org/10.3791/50079

Schousboe, A., Sickmann, H. M., Walls, A. B., Bak, L. K., & Waagepetersen, H. S. (2010). Functional importance of the astrocytic glycogen-shunt and glycolysis for maintenance of an intact intra/extracellular glutamate gradient. *Neurotoxicity Research*, *18*(1), 94–99.

Stobart, J. L., & Anderson, C. M. (2013). Multifunctional role of astrocytes as gatekeepers of neuronal energy supply. Front. *Cell Neurosci*, *7*, 38. https://doi.org/10.3389/fncel.2013.00038

Tamrakar, P., & Briski, K. P. (2015). Estradiol regulates hypothalamic astrocyte adenosine 5′-monophosphate-activated protein kinase activation by hypoglycemia: role of hindbrain catecholaminergic signaling. *Brain Res Bull*, *110*, 47–53.

Walls, A. B., Heimbürger, C. M., Bouman, S. D., Schousboe, A., & Waagepetersen, H. S. (2009). Robust glycogen shunt activity in astrocytes: Effects of glutamatergic and adrenergic agents. *Neuroscience*, *158*(1), 284–292.

Wiesinger, H., Hamprecht, B., & Dringen, R. (1997). Metabolic pathways for glucose in astrocytes. *Glia*, *21*(1), 22–34.