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GLUT2 regulation of p38 MAPK isoform protein expression and p38 phosphorylation in male versus female rat hypothalamic primary astrocyte Cultures

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ABSTRACT

Recent studies documented regulation of hypothalamic astrocyte mitogen-activated protein kinase (MAPK) pathways, including p38, by the plasma membrane glucose carrier/sensor glucose transporter-2 (GLUT2). Sex-specific GLUT2 control of p38 phosphorylation was observed, but effects on individual p38 family protein profiles were not investigated. Current research employed an established primary astrocyte culture model, gene knockdown tools, and selective primary antisera against p38-alpha, p38-beta, p38-gamma, and p38-delta isoforms to investigate whether GLUT2 governs expression of one or more of these variants in a glucose-dependent manner. Data show that GLUT2 inhibits baseline expression of each p38 protein in male cultures, yet stimulates p38-delta profiles without affecting other p38 proteins in female. Glucose starvation caused selective up-regulation of p38-delta profiles in male versus p38-alpha and -gamma proteins in female; these positive responses were amplified by GLUT2 siRNA pretreatment. GLUT2 opposes or enhances basal p38 phosphorylation in male versus female, respectively. GLUT2 siRNA pretreatment did not affect glucoprivic patterns of phospho-p38 protein expression in either sex. Outcomes document co-expression of the four principal p38 MAPK family proteins in hypothalamic astrocytes, and implicate GLUT2 in regulation of all (male) versus one (female) variant (s). Glucoprivation up-regulated expression of distinctive p38 isoforms in each sex; these stimulatory responses are evidently blunted by GLUT2. Glucoprivic-associated loss of GLUT2 gene silencing effects on p38 phosphorylation infers either that glucose status determines whether this sensor controls phosphorylation, or that decrements in screened glucose in each instance are of sufficient magnitude to abolish GLUT2 regulation of that function.

Introduction

Intracellular signal transduction pathways transform extracellular regulatory cues to activate appropriate target substrates in order to elicit an efficacious biological response(s). Mitogen-activated protein kinase (MAPK) signaling networks are organized into linear, ordered protein cascades or modules, which collectively regulate a wide range of cell processes, including proliferation, differentiation and stress responses (Seger and Krebs, 1995; Cargnello and Roux, 2011; Morrison, 2012). Sequential actuation of tiered, upstream kinases, i.e. MAPKKK and MAPKK, operating within MAPK cascades triggers MAPK action on effector protein targets. Specificity of MAPK substrate activation is controlled, in part, by distinctive MAPK kinase signaling to MAPK, as well as subcellular MAPK localization, crosstalk with other signaling pathways, and heterogeneity of protein components of each module tier (Keshet and Seger, 2010). For example, characterized MAPK cascades, i.e. extracellular-signal-regulated kinase (ERK), stress-activated protein kinase/Jun amino-terminal kinase (SAPK/JNK), and p38 modules each feature multiple MAPK protein isoforms.

The p38 MAPK cascade is primarily involved in regulation of cellular stress and immune responses, and is activated in response to a diverse array of extracellular stimuli such as heat, osmotic stress, UV light,

Abbreviations: ERK, extracellular-signal-regulated kinase; GLUT2, glucose transporter-2; MAPK, mitogen-activated protein kinase; P38α, p38-alpha MAPK protein; P38β, p38-beta MAPK protein; P38γ, p38-gamma MAPK protein; P38δ, p38-delta MAPK protein; Phospho-p38, phosphorylated p38 Thr180/Tyr182; SAPK/JNK, stress-activated protein kinase/Jun amino-terminal kinase.

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inflammatory cytokines, and growth factors (Zarubin and Han, 2005; Corre et al., 2017). The four principal p38 MAPK protein variants, p38-alpha (p38α; MAPK14), p38-beta (p38β; MAPK11), p38-gamma (p38γ; MAPK12), and p38-delta (p38δ; MAPK13) are encoded by distinct genes, but are each activated by phosphorylation of tyrosine (Tyr) and threonine (Thr) residues in a conserved Thr-X-Tyr motif (Avitzour et al., 2007). These four isoform proteins pair off into two subgroups (p38α and p38β constitute one subgroup, p38γ and p38δ represent another) that exhibit differences in sequence similarity, cellular expression patterns, and substrate specificity (Risco and Cuenda, 2012; Qin et al., 2016; Maik-Rachline et al., 2021). p38α and β exhibit ubiquitous patterns of expression in the body, while the other subgroup is expressed only in specific tissues (Yang et al., 2016). Studies on p38α, the most intensively investigated p38 isoform, reveal that extent of activation of this protein varies according to physiological stimulus and cell type (Avitzour et al., 2007). Although the four p38 gene products phosphorylate similar protein substrates, namely downstream kinases and transcription factors, their unique substrate specificity, due in part to levels of activation and enzyme kinetics, likely imposes differential control of cell function, thereby shaping cell type-/tissue-specific biological responses to a given stimulus (Corre et al., 2017).

The brain engages in dynamic monitoring of its principal metabolic fuel glucose to shape neural control of motor functions that maintain normoglycemia. Brain glucose monitoring occurs at the crucial stages of 1) glucose uptake into the cell and 2) entry into the glycolytic pathway. The integral plasma membrane protein glucose transporter-2 (GLUT2) is a unique member of the major facilitator membrane transporter superfamily (Wood and Trayhurn, 2003; Mueckler and Thorens, 2013; Holman, 2020). GLUT2 is distinguished from other family proteins by its comparatively low affinity for glucose (Km = 17 mM), a feature that presumably allows unimpeded transport of extracellular glucose over an extensive physiological range (Thorens and Mueckler, 2010). Brain astrocytes are neuroglia that play a vital role in neuro-metabolic stability. These glial cells function as the main point of glucose transfer from circulation to brain, and partition this metabolic fuel between the energy reserve glycogen and glycolysis. The former activity involves passage of glucose through the glycogen shunt, whereas the latter process yields L-lactate for trafficking to neurons to support mitochondrial oxidative respiration (Stobart and Anderson, 2013; Argente-Arizón et al., 2017; Douglass et al., 2017; MacDonald et al., 2019; Zhou, 2018). Astrocyte-derived lactate signaling affects hypothalamic neurotransmission that governs glucose counterregulatory hormone secretion (Mahmood et al., 2019; Bheemanapally et al., 2021; Roy et al., 2022). Astrocyte GLUT2 activity in as-yet-identified brain loci is critical for optimal counterregulatory outflow during neuro-glucopenia (Marty et al., 2005). Continuous sensory signals on brain cell glucose status are received by the neural glucostatic network, which is ultimately controlled by the hypothalamus, the primary visceral motor structure in the brain (Watts and Donovan, 2010). Hypothalamic astrocytes express GLUT2, and likely communicate cues from this sensor to glucose-regulatory neurons via sex-specific GLUT2 regulation of astrocyte glucose catabolism and storage and cellular energy status (Pasula et al., 2022).

The expansive GLUT2 intra-cytoplasmic loop domain is implicated in post-membrane transport glucose screening (Guillemain et al., 2000), yet the putative molecular mechanisms whereby GLUT2 may control hypothalamic astrocyte glucose metabolism remain unclear. GLUT2 is reported to regulate patterns of stress-sensitive ERK1/2, JNK/SAPK, and p38 MAPK protein expression and phosphorylation in hypothalamic astrocyte primary cell cultures in a sex-dimorphic manner (Pasula et al., 2023). Prior research sought to acquire proof-of-concept that this transporter/sensor may control total cellular p38 protein content in one or both sexes; thus, a primary antibody that detects a common p38 protein epitope was used. Current work extends those earlier findings by investigating whether and how GLUT2 may control individual p38 isoform expression profiles. Here, a validated hypothalamic astrocyte primary cell culture model was used in conjunction with siRNA gene knockdown reagents, discriminative primary antisera capable of detecting individual p38 protein isoforms, and Stain-Free Western blot analysis to address the premise that GLUT2 may regulate one or more p38 protein variants under conditions of glucose sufficiency and/or deficiency. The current study design included investigation of potential sex differences in GLUT2 regulation of these MAPK proteins to be in accord with current U.S. National Institutes of Health policy emphasis on consideration of sex as a critical biological variable.

Materials and methods

Primary astrocyte cell cultures

High-purity astrocyte primary cultures were prepared from whole-hypothalamus tissue blocks dissected from adult male and female rats (3 months of age), as described (Ibrahim et al., 2020a; Ibrahim et al., 2020b; Alhamyani et al., 2022). Animal use protocols were in accordance with the NIH Guide for the Care and Use of Laboratory Animals, 8th Edition, with approval from the ULM Institutional Animal Care and Use Committee. Briefly, the hypothalamus were dissected from each brain; blocks were trypsin-digested by pipet-dissociation into a single-cell suspension in DMEM high-glucose media (prod. no. 12800–017; ThermoFisherScientific (ThermoFisherSci), Waltham, MA, USA) enriched with 10.0 % heat-inactivated fetal bovine serum (FBS; GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) and 1.0 % penicillin–streptomycin (prod. no. 15140–122, ThermoFisherSci.). Dissociated cells in suspension were incubated (37 °C; 5 % CO_2) in plating media in poly-D-lysine (prod. no. A-003-E; MilliporeSigma, Burlington, MA, USA) - coated T75 culture flasks for two weeks, with media changes every 2–3 days, before removal of microglia and oligodendrocytes (Ibrahim et al., 2020a,b). After further incubation, purified astrocytes were plated (1×10^6 cells/100 mm^2)in poly-D-lysine-coated culture dishes prior to experimentation. Routine detection of the astrocyte marker protein glial fibrillary acidic protein (GFAP) by Western blot and immunofluorescence cytochemical methods verified that culture purity exceeded 95 %.

Experimental design

Cultures grown to approximately 70 % confluency were incubated for 18 h in DMEM high-glucose media supplemented with 5.0 % charcoal-stripped FBS (prod. no. 12676029; ThermoFisherSci.). Astrocytes from each sex were pretreated by 72 h incubation in high-glucose DMEM media containing Accell™ control non-targeting pool (scramble; SCR) siRNA (prod. no. D-001910–10–20, 5.0 nM; Horizon Discovery, Waterbeach, UK) or Accell™ rat GLUT2 siRNA (prod. no. A-099803–14–0010, 5.0 nM; Horizon Disc.) dissolved in siRNA buffer (prod. no. B-002000-UB-100; Horizon Discovery), using reported methods (Pasula et al., 2022). Optimal GLUT2 siRNA dosing was established by Western blot analysis of graded, i.e., 0.2, 1.0, 5.0, or 25.0 nM titrant doses. The knockdown paradigm used here causes an approximate 50 % decrease in gene product expression across all treatment groups (Pasula et al., 2022). SCR or GLUT2 siRNA-pretreated astrocytes were incubated for 8 h with 10 nM 17β-estradiol (28) – supplemented HBSS media containing 5.5 mM ($G_{5.5}$) or 0 mM (G_0) glucose. Cells were detached for suspension in lysis buffer, e.g. 2.0 % sodium dodecyl sulfate, 10.0 % glycerol, 5 % β-mercaptoethanol, 1 mM sodium orthovanadate, 60 mM Tris-HCl, pH 6.8.

Western blot analysis

Astrocyte cell pellets underwent heat-induced denaturation (95 °C; 10 min); lysates were then sonicated, centrifuged, and diluted with 2x Laemmli buffer. Cell lysate protein concentrations were measured by

NanoDrop spectrophotometry (prod. no. ND-ONE-W, ThermoFisher-Sci.). For each target protein, equivalent quantities of protein from each treatment group were separated by electrophoresis in Bio-Rad Stain Free 10 % acrylamide gels (prod. no 161–0183; Bio-Rad, Hercules, CA, USA). After separation, gels were UV-activated (1 min) in a Bio-Rad Chem-iDoc™ Touch Imaging System to utilize total protein measures for individual lanes as loading control, as described (Ibrahim et al., 2019); protein were then transferred to 0.45 μm pore size PVDF-Plus membranes (prod. no. 1212639; Data Support Co., Panorama City, CA, USA). Membranes were blocked (2 h) with Tris-buffered saline, pH 7.4, (TBS) supplemented with 0.2 % Tween-20 (product no. 9005–64–5; VWR, Radnor, PA, USA) and 2 % bovine serum albumin (BSA) (product no. 9048–46–8; VWR), then incubated (24–48 h; 4 °C) with primary antibodies raised in rabbit against GLUT2 (prod. no. PA5–97263; 1:1000; ThermoFisherSci.), p38α MAPK (prod. no. 9218; 1:1000; Cell Signaling Technol., Danvers, MA, USA; RRID: AB_10694846); p38β MAPK (C28C2) (prod. no. 2339; 1:1000; Cell Signal.; RRID:AB_823587); p38γ MAPK (prod. no. 2307; 1:1000; Cell Signal.; RRID:AB_659929); p38δ MAPK (10A8) (prod. no. 2308; 1:1000; Cell Signal.; RRID: AB_10694398); or pPhospho-p38 MAPK (Thr180/Tyr182) (D3F9) (prod. no. 4511; 1:1000; Cell Signal.; RRID:AB_2139682). Membranes were next exposed (1 h) to a horseradish peroxidase-labeled goat anti-rabbit secondary antiserum (product no. NEF812001EA; 1:4000; PerkinElmer, Waltham, MA, USA). Freedom Rocker™ Blotbots were used to perform automated membrane buffer washes and antibody incubations (Next Advance, Inc., Troy, NY, USA). Lastly, membranes were incubated with SuperSignal West Femto maximum sensitivity chemiluminescent substrate (product no. 34096; ThermoFisherSci.). Target protein optical density (O.D.) measures were acquired in a ChemiDoc™ Touch Imaging System. Bio-Rad Stain-Free gels feature direct incorporation of a unique trihalo substance into the gel chemistry, which renders all in-gel proteins fluorescent upon UV photoactivation, thereby quantifiable by O.D. Individual protein O.D.s are summed in each lane to yield total in-lane protein O.D., which is used to derive a normalized value for target protein O.D. measured in the corresponding lane. The Y-axis label of each Western blot figure illustrates this relationship as mean normalized O.D. measures. This superior method for Western blot normalization markedly reduces data variability through improved measurement accuracy and precision (Gilda and Gomes, 2015; Moritz, 2017). Bio-Rad precision plus protein molecular weight dual color standards (prod. no. 161–0374) were included in each Western blot analysis. Three independent experiments, each comprising a minimum of three separate astrocyte collections, were carried out.

Statistical analysis

Mean normalized protein O.D. measures 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

Present studies employed gene silencing tools and a validated hypothalamic primary astrocyte culture model to investigate the premise that GLUT2, a unique membrane transporter that functions as a glucose sensor, may regulate hypothalamic astrocyte p38 isoform protein expression and phosphorylation in a sex-specific manner in the presence and/or absence of glucose. Astrocyte target protein O.D. measures were normalized to total in-lane protein using Stain-Free gel technology; group mean values were analyzed by three-way ANOVA and Student-Newman-Keuls *post-hoc* test.

Results shown in Fig. 1 depict effects of GLUT2 gene knockdown on GLU2 protein expression in hypothalamic astrocyte primary cultures derived from male (at left) versus female (at right) rats. Data show that

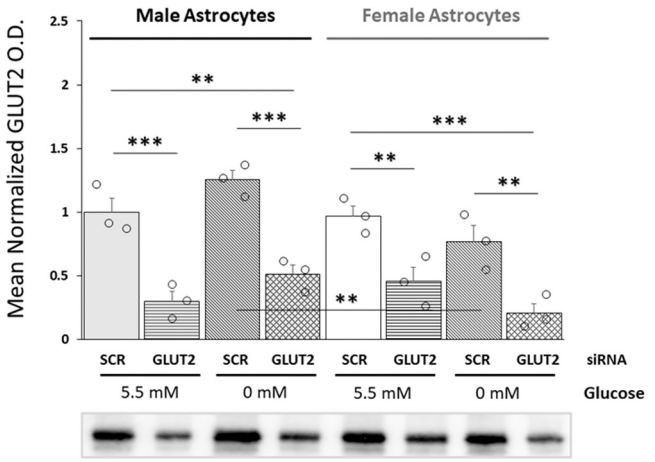

Fig. 1. Efficacy of Glucose Transporter-2 (GLUT2) siRNA Knockdown on Primary Hypothalamic Astrocyte Culture GLUT2 Protein Expression. Confluent male and female rat hypothalamic primary astrocyte cultures were steroid-deprived (18 h) before pretreatment (72 h) with scramble (SCR) or GLUT2 short-interfering RNA (siRNA), then incubated (4 h) with media containing 5.5 or 0 mM glucose. Astrocyte lysate aliquots from each group were analyzed in triplicate by stain-free Western blot for GLUT2 protein content. Target protein optical density (O.D.) measures obtained in a Bio-Rad ChemiDoc™ Touch Imaging System were normalized to aggregate in-lane protein, i.e. entire protein electrophoresed in the individual sample lane, using Bio-Rad Image Lab™ 6.0.0 software. Data show normalized GLUT2 O.D. values + S.E.M. for each treatment group. Data were analyzed by three-way ANOVA and Student-Newman-Keuls *post-hoc* test, using GraphPad Prism (Version 8) software. Statistical differences between discrete pairs of treatment groups are denoted as follows: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

in each sex, GLUT2 protein profiles were significantly reduced in primary cultures pretreated with GLUT2 versus SCR siRNA prior to incubation in the presence of 5.5 mM glucose (GLUT2 siRNA/5.5 mM versus SCR siRNA/5.5 mM) or 0 mM (GLUT2 siRNA/0 mM versus SCR siRNA/0 mM) mM glucose. Outcomes of statistical analysis are as follows: $F_{7,16}= 16.08$, $p<0.001$; Sex main effect: $F_{1,16}= 6.42$, $p=0.022$; Treatment main effect: $F_{1,16}= 0.00$, $p=0.964$; Pretreatment main effect: $F_{1,16}= 91.99$, $p<0.001$; Sex/Treatment interaction: $F_{1,16}= 12.13$, $p=0.003$; Sex/Pretreatment interaction: $F_{1,16}= 1.88$, $p=0.189$; Treatment/Pretreatment interaction: $F_{1,16}= 0.12$, $p=0.730$; Sex/Treatment/Pretreatment: $F_{1,16}= 0.01$, $p=0.974$. These data verify the efficacy of the GLUT2 siRNA tool used here for down-regulation of the gene product GLUT2 in hypothalamic astrocytes of each sex.

Western blot data depicted in Fig. 2 illustrate GLUT2 gene silencing effects on p38α (Fig. 2A) and p38β (Fig. 2B) protein levels in male (bars 1–4; *at left,*) versus female (bars 5–8; *at right,*) hypothalamic astrocyte cultures. ChemiDoc MP in-lane protein detection and full uncropped Western blot images for each protein are presented in Supplementary Figures 1 (p38α) and 2 (p38β). Outcomes of statistical analysis of p38α Western blot data are as follows: $F_{7,16}=19.39$, $p<0.001$; Sex main effect: $F_{1,16}=28.30$, $p<0.001$; siRNA main effect: $F_{1,16}=45.37$, $p<0.001$; Glucoprivic main effect: $F_{1,16}=29.11$, $p<0.001$; Sex/siRNA interaction: $F_{1,16}=1.,97$ $p=0.180$; Sex/glucoprivic interaction: $F_{1,8}=26.74$, $p<0.001$; siRNA/glucoprivic interaction: $F_{1,16}=0.645$, $p=0.434$; Sex/siRNA/glucoprivic interaction: $F_{1,16}=3.56$, $p=0.077$. Data show that p38α protein is expressed at comparable levels in the two sexes in the presence of glucose. Glucose-supplied cells showed increased (male; GLUT2 siRNA/5.5 mM, horizontal-striped gray bar, versus SCR siRNA/5.5 mM, solid gray bar) or no change (female; GLUT2 siRNA/5.5 mM, horizontal-striped white bar, versus SCR siRNA/5.5 mM, solid white bar) in p38α expression in response to GLUT2 gene knockdown. Glucose withdrawal did not affect this protein profile in male astrocytes (SCR siRNA/0 mM, diagonal-striped gray bar, versus SCR siRNA/5.5 mM), but up-regulated

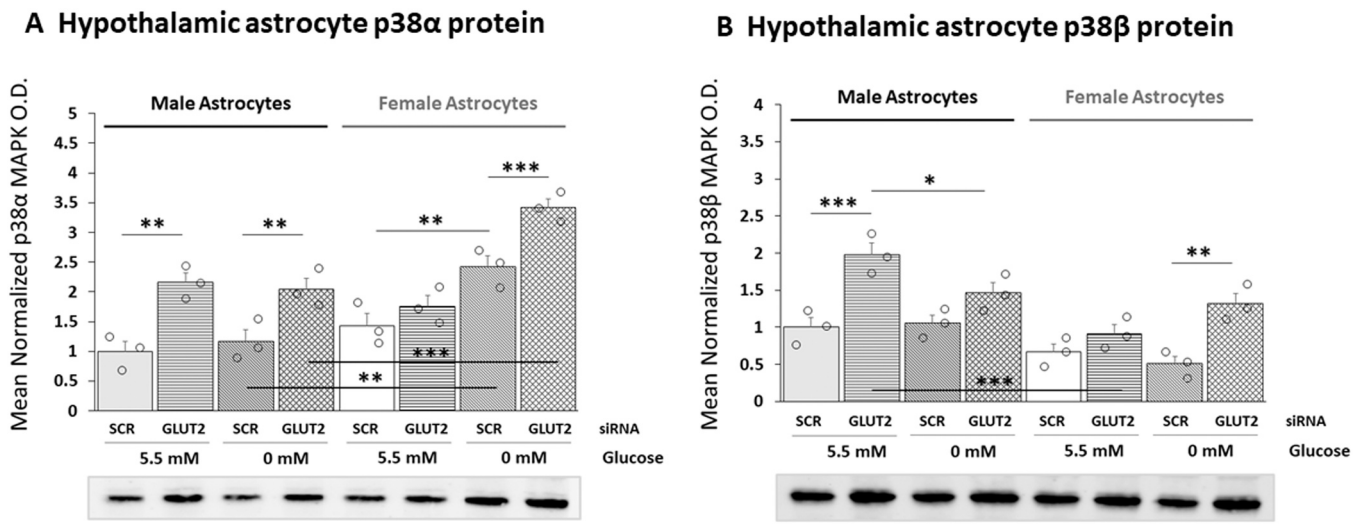

Fig. 2. Effects of GLUT2 gene silencing on Patterns of p38-Alpha (p38α) and p38-Beta (p38β) Protein Expression in Adult Male versus Female Hypothalamic Primary Astrocyte Cultures. Primary astrocyte cultures from each sex were steroid-deprived (18 h) prior to 72 h incubation with scramble (SCR) or GLUT2 short-interfering RNA (siRNA), then incubated (4 h) with media containing 5.5 or 0 mM glucose. Astrocyte cell lysate aliquots of equivalent protein mass were analyzed for each treatment group in triplicate using Bio-Rad StainFree Western blot equipment and software. Target protein optical density (O.D.) was measured in a Bio-Rad ChemiDoc™ Touch Imaging System, and normalized using Bio-Rad Image Lab™ 6.0.0 software to total in-lane protein, e.g. all protein electrophoresed in individual single sample lanes. Data depict mean normalized p38α (Fig. 1A) and p38β (Fig. 1B) protein optical density (O.D.) measures ± S.E.M. for male (*at left*, gray bars) and female (at right, white bars) astrocytes from SCR siRNA/5.5 mM (gray (male) or white (female) solid bars); GLUT2 siRNA/5.5 mM (gray (male) or white (female) horizontal-striped bars); SCR siRNA/0 mM (gray (male) or white (female) diagonal-striped bars); GLUT2 siRNA/0 mM (gray (male) or white (female) cross-hatched bars) treatment groups. Open circles depict individual independent data points. Mean normalized protein O.D. data were analyzed by three-way ANOVA and Student-Newman-Keuls *post-hoc* test, using GraphPad Prism (Volume 8) software. Statistical differences between discrete pairs of treatment groups are denoted as follows: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

p38α levels in the female (SCR siRNA/0 mM, diagonal-striped white bar, versus SCR siRNA/5.5 mM). GLUT2 siRNA pretreatment elevated astrocyte p38α expression in glucose-deprived male (GLUT2 siRNA/0 mM, cross-hatched gray bar) versus SCR siRNA/0 mM) and female (GLUT2 siRNA/0 mM, cross-hatched white bar) versus SCR siRNA/0 mM) cell cultures. Results reveal sensitivity of p38α to glucoprivation is sex-specific, i.e. occurring only in the female. Outcomes show that GLUT2 negatively regulates male hypothalamic astrocyte p38α expression, irrespective of the presence versus absence of glucose, whereas glucose starvation causes gain of GLUT2 inhibitory tone on this p38 isoform, evidently serving to curb up-regulated p38α in this sex

Fig. 2B illustrates effects of GLUT2 gene silencing on hypothalamic astrocyte p38β protein expression in the presence versus absence of glucose ($F_{7,16}$=13.26, p<0.001; Sex main effect: $F_{1,16}$=33.07, p<0.001; siRNA main effect: $F_{1,16}$=45.14, p<0.001; Glucoprivic main effect: $F_{1,16}$=0.37, p=0.552; Sex/siRNA interaction: $F_{1,16}$=0.84, p=0.372; Sex/glucoprivic interaction: $F_{1,8}$=3.72, p=0.072; siRNA/glucoprivic interaction: $F_{1,16}$=0.00, p=0.982; Sex/siRNA/glucoprivic interaction:

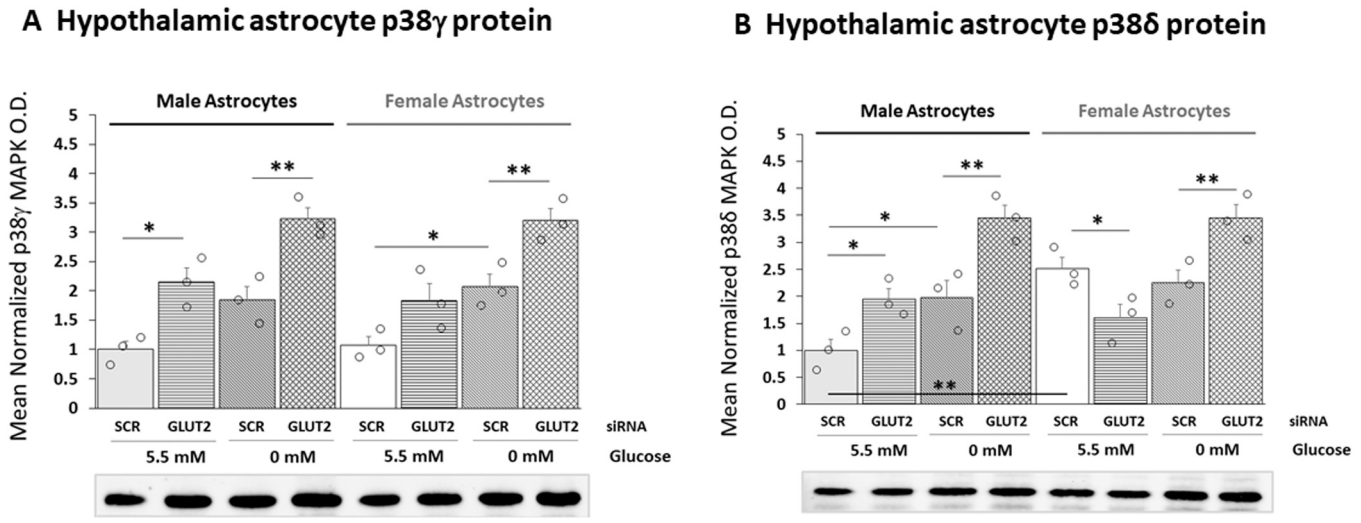

Fig. 3. GLUT2 gene knockdown effects on p38-Gamma (p38γ) and p38-Delta (p38δ) Protein Expression Profiles: Impact of Glucose. Data depict mean normalized p38γ (Fig. 1A) and p38δ (Fig. 2B) protein O.D. measures ± S.E.M. for male (*at left*, gray bars) and female (at right, white bars) astrocytes from SCR siRNA/5.5 mM (gray (male) or white (female) solid bars); GLUT2 siRNA/5.5 mM (gray (male) or white (female) horizontal-striped bars); SCR siRNA/0 mM (gray (male) or white (female) diagonal-striped bars); GLUT2 siRNA/0 mM (gray (male) or white (female) cross-hatched bars) treatment groups. Open circles depict individual independent data points. Mean normalized protein O.D. data were analyzed by three-way ANOVA and Student-Newman-Keuls *post-hoc* test, using GraphPad Prism (Volume 8) software. Statistical differences between discrete pairs of treatment groups are indicated as $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

$F_{1,16}$=9.70, p=0.007). GLUT2 siRNA increased this isoform profile in male, but not female astrocytes. Neither sex exhibited a significant change in p38β content after glucose withdrawal. GLUT2 siRNA pretreatment increased p38β protein expression in glucose-deprived female, but not male astrocyte cultures. Data reveal that sex determines GLUT2 control of baseline hypothalamic astrocyte p38β profiles. Results also show that GLUT2 regulation of this p38β isoform is dependent upon glucose status, as elimination of glucose from culture media abolished GLUT2 repression of this isoform profile in male, yet elicits GLUT2 suppression of p38β expression in the female.

Outcomes of Western blot analyses presented in Fig. 3 depict effects of GLUT2 gene knockdown on p38γ (Fig. 3A) and p38δ (Fig. 3B) protein expression in male versus female hypothalamic astrocyte cultures. ChemiDoc MP in-lane protein detection and full uncropped Western blot images for each protein are presented in Supplementary Figures 3 (p38γ) and 4 (p38δ). Outcomes of statistical analysis of p38γ Western blot data are as follows: $F_{7,16}$=15.46, p<0.001; Sex main effect: $F_{1,16}$=0.00, p=0.946; siRNA main effect: $F_{1,16}$=54.31, p<0.001; Glucoprivic main effect: $F_{1,16}$=51.15, p<0.001; Sex/siRNA interaction: $F_{1,16}$=1.14, p=0.301; Sex/glucoprivic interaction: $F_{1,8}$=0.52, p=0.482; siRNA/glucoprivic interaction: $F_{1,16}$=1.02, p=0.328; Sex/siRNA/glucoprivic interaction: $F_{1,16}$=0.05, p=0.834. GLUT2 gene silencing significantly augmented p38γ protein in male, but not female astrocytes. Glucoprivation up-regulated this p38 protein variant in female, but not male astrocytes. GLUT2 siRNA pretreatment enhanced p38γ expression profiles in male and female astrocytes. Data indicate that GLUT2 regulation of basal p38γ protein is sex, i.e. male-specific. In the female, glucose status evidently imposes an opposite switch in GLUT2 control of p38γ, as regulatory tone changes from undetectable to inhibitory in the presence versus absence of glucose.

Fig. 3B portrays effects of GLUT2 gene silencing on hypothalamic astrocyte p38δ protein expression in the presence versus absence of glucose ($F_{7,16}$=6.01, p<0.001; Sex main effect: $F_{1,16}$=0.74, p=0.402; siRNA main effect: $F_{1,16}$=0.02, p=0.883; Glucoprivic main effect: $F_{1,16}$=31.38, p<0.001; Sex/siRNA interaction: $F_{1,16}$=6.95, p=0.018; Sex/glucoprivic interaction: $F_{1,8}$=1.75, p=0.204; siRNA/glucoprivic interaction: $F_{1,16}$=0.29, p=0.596; Sex/siRNA/glucoprivic interaction: $F_{1,16}$=0.95, p=0.344). Data show that baseline p38δ protein levels were significantly higher in female versus male astrocytes. GLUT2 gene knockdown significantly up-regulated this isoform in male, but not female cultures., which showed diminished protein expression. Glucoprivation augmented p38δ expression in male, but not female. GLUT2 siRNA pretreatment amplified this protein profile in glucose-deprived male and female astrocytes. Results document sex-contingent GLUT2 regulation of basal p38δ protein levels (male only) and p38δ responsiveness to glucoprivation (male only). In the female, glucose withdrawal evidently allows a switch from undetectable to inhibitory GLUT2 control of this isoform profile

Results shown in Fig. 4 illustrate effects of GLUT2 gene knockdown on phospho-p38 protein expression in male versus female hypothalamic astrocyte cultures. ChemiDoc MP in-lane protein detection and full uncropped Western blot images for phospho-p38 protein are presented in Supplementary Figure 5. Outcomes of statistical analysis are as follows: $F_{7,16}$=13.51, p<0.001; Sex main effect: $F_{1,16}$=4.57, p=0.048; siRNA main effect: $F_{1,16}$=0.45, p=0.511; Glucoprivic main effect: $F_{1,16}$=0.19, p=0.736; Sex/siRNA interaction: $F_{1,16}$=73.41, p<0.001; Sex/glucoprivic interaction: $F_{1,8}$=5.61, p=0.031; siRNA/glucoprivic interaction: $F_{1,16}$=5.93, p=0.027; Sex/siRNA/glucoprivic interaction: $F_{1,16}$=4.45, p=0.051. Data document stimulatory versus inhibitory effects on phospho-p38 protein in male versus female astrocytes, respectively. Glucoprivation caused significant up-regulation of this protein profile in the former, but not latter sex. GLUT2 siRNA pretreatment did not modify patterns of phospho-p38 expression in glucose-deprived male or female cultures. Outcomes indicate that GLUT2 imposes contrary regulatory effects on baseline p38 activation, i.e. inhibitory in male versus stimulatory in female. Data also infer that glucoprivic patterns of phospho-p38 protein expression are governed primarily by GLUT2-independent mechanisms.

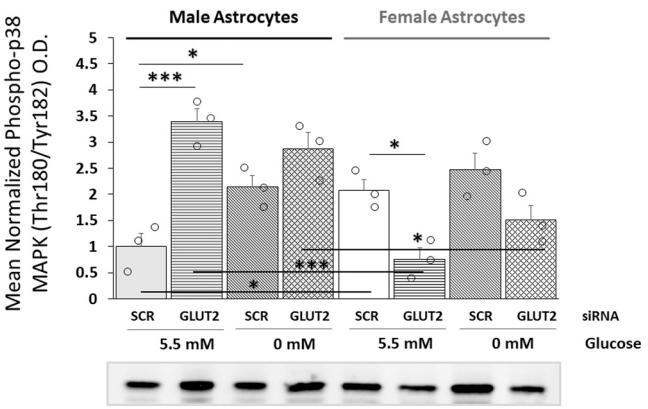

Fig. 4. Patterns of Phospho-p38 Protein Expression in Glucose-Supplied versus -Deprived Primary Hypothalamic Astrocyte Cell Cultures. Data depict mean normalized phospho-p38 protein O.D. measures ± S.E.M. for male (*at left*, gray bars) and female (at right, white bars) astrocytes from SCR siRNA/5.5 mM (gray (male) or white (female) solid bars); GLUT2 siRNA/5.5 mM (gray (male) or white (female) horizontal-striped bars); SCR siRNA/0 mM (gray (male) or white (female) diagonal-striped bars); GLUT2 siRNA/0 mM (gray (male) or white (female) cross-hatched bars) treatment groups. Open circles depict individual independent data points. Mean normalized protein O.D. data were analyzed by three-way ANOVA and Student-Newman-Keuls *post-hoc* test, using GraphPad Prism (Volume 8) software. Statistical differences between discrete pairs of treatment groups are denoted as follows: *p < 0.05; **p < 0.01; ***p < 0.001.

Discussion

Current studies addressed the premise that hypothalamic astrocytes manufacture one or more of the four principal p38 protein variants, and that the unique plasma membrane glucose transporter/sensor GLUT2 may control all or a subset of expressed p38 isoform profiles. Outcomes document detectable levels of p38α, p38β, p38γ, and p38δ proteins in astrocyte cultures of each sex; only the latter p38 variant exhibited a sex difference in expression profiles. Results also provide unique evidence that glucose imbalance causes sex-contingent adjustments in p38 isoform proteins, as p38δ was the only variant affected in the male, whereas the other three p38 proteins were glucoprivic-sensitive in the female. GLUT2 regulation of baseline p38 isoform expression deviates between sexes, as GLUT2 inhibits these proteins in male, yet has no effect on (p38α, p38β, p38γ) or stimulates (p38δ) these proteins in female. Glucose status evidently controls GLUT2 regulation of p38 isoform protein expression, particularly in female astrocytes, which display a shift from undetectable or stimulatory GLUT2 control to a suppressive impact in the presence versus absence of glucose. Further effort is required to characterize upstream molecular mechanisms that link GLUT2 to the p38 MAPK cascade, and to establish how glucose-dependent GLUT2 regulation of distinct p38 protein expression profiles is achieved in each sex. Ongoing research seeks to determine if and how p38 variant responses to glucose imbalance may shape sex-specific hypothalamic astrocyte adaptation to this metabolic stress.

Astrocyte-neuron metabolic coupling assures provision of the oxidizable energy fuel L-lactate for support of nerve cell mitochondrial oxidative phosphorylation. Astrocyte-derived lactate signal volume governs hypothalamic counterregulatory neurotransmission (Mahmood et al., 2019; Bheemanapally et al., 2021; Roy et al., 2022). The distinctive locations in the brain where astrocyte GLUT2 shapes neural responses to neuro-glucopenia are not known. Present evidence that the cell stress-activated p38 MAPK module is subject to GLUT2 control bolsters the need to investigate, within a neural system context, if and

how GLUT2-dependent astrocyte p38 MAPK isoform protein expression and net p38 phosphorylation state may affect hypothalamic counter-regulatory neurochemical and systemic glycemic profiles. It should be noted that future research incorporating *in vivo* experimental strategies will be crucial to substantiate the physiological relevance of hypothalamic astrocyte p38 protein variant sensitivity to GLUT2. GLUT2 glucose monitoring requires an intact cytoplasmic loop domain (Guillemain et al., 2000), yet it remains unclear how that distinctive GLUT2 component regulates p38 MAPK enzyme protein expression and activity. Activation of the p38 cascade involves the upstream small Rho GTPases Rac and Cdc42 (Cuenda and Rousseau, 2007); a pivotal question that emerges herein concerns their respective roles in GLUT2 regulation of p38 phosphorylation. There is also a need to identify the distinct upstream MAPKKK and MAPKK enzymes that undergo activation in hypothalamic astrocytes in reaction to GLUT2 sensory cues.

Present findings illustrate the capacity of GLUT2 to affect expression profiles of p38α, p38β, p38γ, and p38δ proteins in hypothalamic astrocytes, with the qualification that this control is sex-dimorphic. It would be informative to learn if GLUT2 glucose sensing cues affect transcriptional and/or translational aspects of p38 isoform protein manufacture. Data here show that GLUT2 uniformly suppressed baseline expression of all four p38 variants in male astrocytes, yet in the other sex, these protein profiles were either refractory to (p38α, p38β, p38γ) or stimulated by (p38δ) input from this sensor. Evidence that GLUT2 protein expression is equivalent in male versus female astrocyte cultures (Pasula et al., 2022) infers that net glucose mass conveyed to the astrocyte interior by this distinctive transporter is likely comparable between the two sexes. As siRNA knockdown decreased GLUT2 protein expression to a similar extent in male and female astrocytes (Pasula et al., 2022), a reasonable supposition here is that downstream GLUT2-sensitive targets that govern p38 variant protein profiles exhibit sex-contingent responses to this sensory signal. It is intriguing to speculate whether GLUT2-sensitive elements are a common target for multiple metabolic signals, i.e. derived from glucokinase and/or 5'-AMP-activated protein kinase, and may thus respond to down-regulated GLUT2-associated glucose uptake within a sex-specific context of overall cellular glucose accumulation and/or energy status. GLUT2 gene knockdown is presumed to diminish the volume of GLUT2-transported glucose into cultured cells; it is not clear if this decrement mimics *in vivo* physiological reductions in glucose uptake associated with systemic glucose dyshomeostasis, namely hypoglycemia. GLUT2 siRNA-mediated up-regulation of p38 MAPK protein profiles in male raises the prospect that increasing concentrations of GLUT2-transported glucose may impose a proportionate inhibitory tone on these proteins. The comparative inefficacy of this treatment paradigm to affect p38α, -β, and -γ protein expression in the female suggests that associated reductions in glucose uptake may be insufficient to affect these protein profiles, yet is able to influence p38δ. It would be insightful to learn if graded adjustments in GLUT2-transported glucose volume impose concentration-dependent effects on one or more p38 MAPK family proteins.

Present data disclose sex-dimorphic effects of glucose starvation on hypothalamic astrocyte p38 variant protein expression, as glucose-deprived male cultures exhibited enhanced p38δ levels, whereas female astrocytes showed augmented p38α and p38γ protein content. The latter stimulatory responses in the female contrast p38α and p38γ refractoriness to GLUT2 gene silencing. It is likely that additional decrements in glucose transport volume beyond that achieved by this genetic manipulation may be required to affect these specific p38 MAPK proteins in this sex. Sex-specific p38 MAPK protein responses to glucose starvation are evidently mediated by as-yet-uncharacterized GLUT2-independent mechanisms, as GLUT2 siRNA pretreatment exacerbated glucoprivic up-regulation of each profile. The latter observation infers that GLUT2 signaling may function to curb p38 MAPK stimulatory responses to glucose imbalance. As GLUT2 transport function is presumably minimal in glucose-deprived astrocytes, GLUT2 gene knockdown efficacy in the absence of glucose may reflect, in part, a reduction in number of individual sensor cues. GLUT2 siRNA may regulate glucoprivic-insensitive p38 isoform proteins (p38α and -γ proteins in male; p38β and -δ proteins in female) by a similar mechanism.

Project outcomes show that GLUT2 imposes divergent regulatory effects, i.e. inhibitory in male versus stimulatory in female, on net p38 phosphorylation in primary hypothalamic astrocyte cultures. Thus, in male, this sensor simultaneously suppresses p38 MAPK protein expression and activation. Outcomes described here do not disclose information on baseline phosphorylation status of individual p38 isoform proteins, and, moreover, do not reveal how GLUT2 siRNA may affect isoform activation. Antibody-based analytical tools for investigation of GLUT2 regulation of phosphorylation of individual p38 variants do not currently exist. Yet, there is promise from recent advances in mass spectrometry (MS) instrumentation and software that permit targeted protein quantification (Ebhardt et al., 2015; Bakalarski and Kirkpatrick, 2016; Wang et al., 2016; Smith and Martins-de-Souza, 2021). Indeed, multiplexed measurement of specific constituent peptides, including variants and modified forms, by triple quadrupole or quadrupole-ion trap multiple-reaction-monitoring after proteolytic digestion is one of the most rapidly growing applications of MS in protein analysis. Moreover, MS provides the most specific, unambiguous means of site-specific detection of post-translation modifications (Liebler and Zimmerman, 2013). Insight on physiological regulation of p38 variant protein activation in each sex will be forthcoming upon development of appropriate new quantitative methodologies. Interestingly, glucose starvation augmented net p38 protein phosphorylation in male, but not female astrocytes. Again, identification of discrete MAPK isoforms that exhibit altered activation status in response to this metabolic stress awaits the advent of methodological advances. Glucoprivic-associated loss of GLUT2 gene silencing effects on p38 phosphorylation infers, on one hand, that cellular glucose status may determine whether this sensor governs phosphorylation. This supposition aligns with the notion, discussed above, that multiple metabolic stimuli converge upon the p38 MAPK module. Alternatively, decrements in screened glucose due to media starvation may be of sufficient magnitude in each sex to abolish GLUT2 regulation of phosphorylation.

Novel evidence described here for sex-specific GLUT2 regulation of hypothalamic astrocyte p38 isoform protein profiles and net p38 protein phosphorylation bolsters the need for ongoing work to delineate the distinctive mechanisms that control these MAPK cascade responses to plasma membrane glucose sensor input in male versus female astrocytes. Since baseline GLUT2 protein levels are equivalent in the two sexes, and exhibit similar reductions due to siRNA knockdown in each sex (Pasula et al., 2022), it is possible that divergent, sex-dimorphic p38 variant protein responses to down-regulated GLUT2 expression may reflect, in part, inherent differences in sensitivity established during sexual differentiation of the brain. An alternative prospect is these sex-specific responses reflect, in part, actions by media estradiol. The current experimental design involved reintroduction of estradiol to steroid-starved primary astrocyte cultures of each sex, e.g. astrocytes previously incubated in charcoal-stripped media for the reason that the presence of this hormone in brain tissue is ubiquitous, and our studies show that hypothalamic tissue estradiol concentrations in vivo are equivalent in intact male versus ovariectomized female rats treated by replacement therapy to reinstate plasma estradiol levels within the physiological range (Bheemanapally et al., 2020). We reported that the estradiol dose used here, i.e. 10 nM, has a positive effect of equal magnitude on estrogen receptor-alpha and glycogen metabolic enzyme protein expression in glucose-supplied astrocytes of each sex (Ibrahim et al., 2020a). Nevertheless, we cannot discount the possibility here that this specific dosage may impose differential modulatory effects on p38 protein variant mRNA production and/or translation patterns elicited by GLUT2 down-regulation. The prospect that estradiol may affect net p38 phosphorylation by controlling cross-talk between this MAPK cascade and other signal transduction pathways is also plausible. The crucial question that will require further investigation involves identification of

specific estrogen receptor(s) that may mediate sex-specific protein responses described here if those are characterized as estradiol-dependent.

While it is tempting to speculate that extra-hypothalamic astrocyte populations that engage in GLUT2 glucose monitoring may exhibit sex-dimorphic signal transduction pathway responses, including p38 isoform protein expression profiles, when glucose supply deviates from the normal range, it is reasonable to presume that regional astrocyte populations are likely heterogeneous with respect to utilization of glucose screening data, regulatory mechanisms that function downstream of glucose sensors, estradiol sensitivity, and estrogen modulation of signal transduction pathways that transduce glucose uptake information. Undoubtedly, further research is justified to resolve this important issue.

In summary, current research provides novel evidence that hypothalamic astrocytes express the four principal p38 variants proteins, and that glucose deficiency elicits sex-specific changes in cell p38 isoform protein content. GLUT2 regulation of baseline p38 isoform expression deviates between sexes, as GLUT2 inhibits these proteins in male, yet has no effect on (p38α, p38β, p38γ) or stimulates (p38δ) these proteins in female. Glucose status evidently affects GLUT2 regulation of p38 isoform protein expression, particularly in female astrocytes, which display a shift from undetectable or stimulatory GLUT2 control to a suppressive impact in the presence versus absence of glucose. Further effort is required to characterize upstream molecular mechanisms that link GLUT2 to the p38 MAPK cascade, and to establish how glucose-dependent GLUT2 regulation of distinct p38 protein expression profiles is achieved in each sex. Ongoing research seeks to determine if and how p38 variant responses to glucose imbalance may shape sex-specific hypothalamic astrocyte adaptation to this metabolic stress. Additional effort is also warranted to address the possibility that p38 isoform gene expression and/or signal transduction mechanisms that establish p38 phosphorylation state may be intrinsically sex-dimorphic due to early programming, or alternatively, may occur as a result of programmed differences in estrogen receptor variant expression, receptivity to estradiol, and/or down-stream processing of receptor stimulation.

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Statement of ethics

Studies performed here were approved by the University of Louisiana Monroe Institutional Animal Care and Use Committee, reference no. 19AUG-KPB-01, in accordance with the National Institutes of Health (NIH) Guide for Care and Use of Laboratory Animals, 8th Edition.

CRediT authorship contribution statement

Karen P Briski: Conceptualization, Funding acquisition, Project administration, Writing – original draft, Writing – review & editing. **Madhu Babu Pasula:** Conceptualization, Data curation, Formal analysis, Investigation, Validation, Visualization, Writing – original draft, Writing – review & editing. **Paul W. Sylvester:** Resources, Writing – review & editing.

Declaration of Competing Interest

The authors have no conflicts interest to declare.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ibneur.2024.05.008.

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