Glucose responsiveness in a novel adult-derived GnRH cell line, mHypoA–GnRH/GFP: Involvement of AMP-activated protein kinase

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Abstract

Glucose regulates energy homeostasis and reproductive function within the hypothalamus. The underlying mechanisms responsible for glucose regulation of GnRH gene transcription were investigated using a novel murine immortalized, adult-derived hypothalamic cell line, mHypoA–GnRH/GFP. Analysis of GnRH mRNA synthesis and secretion following agonist treatment demonstrated that the mHypoA–GnRH/GFP cell line is a representative model of in vivo GnRH neurons. c-fos mRNA levels, following glucose exposure, indicated that these neurons were responsive to low (0.5 mM) and high (5 mM) glucose, and high glucose stimulated GnRH mRNA transcription in a metabolism-dependent manner. Glucose inhibited AMPK activity, and was linked to the downstream stimulation of GnRH mRNA levels. The effect was confirmed with an AMPK antagonist, Compound C. Collectively, these findings demonstrate that glucose can directly regulate GnRH transcription, while implicating the AMPK pathway as an essential mediator of nutritional signaling in a novel GnRH neuronal cell model.

1. Introduction

Gonadotropin-releasing hormone (GnRH) neurons are a converging point of hormonal, neuropeptide, and nutrient signals to regulate the hypothalamic–pituitary–gonadal (HPG) axis, and are essential for reproduction. GnRH is transported to the gonadotropes to control the release of lutetizing hormone (LH) and follicle-stimulating hormone (FSH), and ultimately gonadal steroids, forming a feedback loop with the hypothalamus. Numerous studies suggest that apart from being regulated by gonadal hormone and steroid input, glucose may also play a role in directly regulating GnRH transcription, while implicating the AMPK pathway as an essential mediator of nutritional sensing, the effect of increased glucose concentration on GnRH transcription has not yet been studied in detail (Ohkura et al., 2004; Zabuli et al., 2009).

In the present study, we use a newly generated mHypoA–GnRH/GFP cell line, derived from primary culture of a 2-month old female GnRH-GFP transgenic mouse hypothalamus, immortalized and then fluorescence-activated cell (FAC)-sorted to generate a GnRH cell model consisting of a highly enriched complement of GnRH

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http://dx.doi.org/10.1016/j.mce.2013.06.035
neurons (Suter et al., 2000). We performed a series of experiments aimed at characterizing and investigating regulation of this novel adult-derived, non-clonal mHypoA-GnRH/GFP cell line in response to agonist and glucose treatment. Our results demonstrate the mHypoA-GnRH/GFP cell line is responsive to agonist treatments stimulating the NO, PKA and PKC pathways, indicative of in vivo GnRH neuronal properties. The mHypoA-GnRH/GFP cell line also responds to changes in extracellular glucose treatment in an AMPK dependant manner. We hypothesize that the mHypoA-GnRH/GFP cell line is a representative model of the in vivo complement of adult GnRH neurons, and can be used as a population of GnRH neurons to study direct effects of reproductive components on GnRH transcription and secretion.

2. Materials and methods

2.1. Fluorescence-activated cell sorting (FACS)

The GnRH-GFP transgenic mice generated by Dr. Suzanne Moenter (University of Michigan, Ann Arbor, MI) were obtained from The Jackson Laboratory, Bar Harbor, ME (Hogan et al., 1994; Suter et al., 2000). Mice were housed under standard vivarium procedures and were conducted in accordance with the regulations of the Council of Animal Care and approved by the University of Toronto Animal Care Committee. mHypoA-GnRH/GFP transgenic mouse hypothalami were dissected from 2-month old female mice individually, and placed in Hank’s balanced salt solution and maintained in phenol red-free DMEM, supplemented with 1% FBS, 1% P/S, 0.5 mM Glucose DMEM (Sigma–Aldrich, Oakville, Ontario, Canada). For glucose studies, cells were maintained in phenol red-free DMEM, supplemented with 1% FBS, 1% P/S and maintained in 5% CO2 at 37 °C as previously described (Belsham et al., 2004; Gingerich et al., 2009). Forskolin and TPA, purchased from Sigma were reconstituted in 100% EtOH and used at a final concentration of 10 μM or 20 μM and 100 nM, respectively (Wetsel et al., 1993a,b). Sodium nitroprusside (SNP) was purchased from Sigma, reconstituted in water and used at a final concentration of 20 μM. We utilized D-Glucose, and D-Deoxyglucose obtained from Sigma reconstituted in MilliQ water and treated at concentrations of 0.5, 2.5 and 5 mM (Dhillon et al., 2011). AMPK antagonist, 6-[(2-Piperidin-1-yl)ethoxy]phenyl]pyridin-4-ylpyrazolo[1,5-a]pyrimidine (Compound C) (Tocris Bioscience, Ellisville, MS) was reconstituted in dimethyl-sulfoxide DMSO and applied at a final concentration of 20 μM (Peairs et al., 2009).

2.2. Cell culture and reagents

mHypoA-GnRH/GFP neurons were cultured in monolayer, submersed in Dulbecco’s Modified Eagle Medium (DMEM) (Sigma–Aldrich, Oakville, Ontario, Canada) supplemented with 5% fetal bovine serum (FBS), and 1% penicillin–streptomycin (P/S) (Gibco, Burlington, Ontario, Canada). For glucose studies, cells were maintained in phenol red-free DMEM, supplemented with 1% FBS, 1% P/S and maintained in 5% CO2 at 37 °C as previously described (Belsham et al., 2004; Gingerich et al., 2009). Forskolin and TPA, purchased from Sigma were reconstituted in 100% EtOH and used at a final concentration of 10 μM or 20 μM and 100 nM, respectively (Wetsel et al., 1993a,b). Sodium nitroprusside (SNP) was purchased from Sigma, reconstituted in water and used at a final concentration of 20 μM. We utilized D-Glucose, and D-Deoxyglucose obtained from Sigma reconstituted in MilliQ water and treated at concentrations of 0.5, 2.5 and 5 mM (Dhillon et al., 2011). AMPK antagonist, 6-[(2-Piperidin-1-yl)ethoxy]phenyl]pyridin-4-ylpyrazolo[1,5-a]pyrimidine (Compound C) (Tocris Bioscience, Ellisville, MS) was reconstituted in dimethyl-sulfoxide DMSO and applied at a final concentration of 20 μM (Peairs et al., 2009).

2.3. RT-PCR and quantitative RT-PCR (qRT-PCR)

RNA was extracted using the guanidinium thiocyanate–phenol–chloroform method (Chomczynski and Sacchi, 2006). cDNA was synthesized using the Applied Biosystems High Capacity cDNA Reverse Transcriptase kit (Applied Biosystems, Streetsville, Ontario, Canada). Mango Taq polymerase was used for semi-quantitative PCR according to manufacturer’s protocols. OneStep PCR products were run on a 2% agarose, 0.5 μg/μL ethidium bromide gel and visualized under ultraviolet light using Kodak 1D Image Analysis Software 3.6 (Eastman Kodak Company, Rochester, NY). A Ct value above 35 was considered negative expression in the screening analysis (Fig. 1B). For quantitative RT-PCR experiments RNA was harvested at specified time-points using the guanidinium thiocyanate–phenol–chloroform method (Chomczynski and Sacchi, 2006). Following quantification the RNA was treated with Turbo DNase to eliminate genomic DNA from the samples. DNase treated RNA, 2 μg, was then reverse transcribed using a High Capacity cDNA Reverse Transcription Kit as described. Samples were run in triplicate on Applied Biosystems Prism 7000 Sequence Detection 384 well plates according to the following cycle protocol: 50 °C for 2 min, 95 °C for 10 min; 40 cycles of 95 °C for 15 s, 60 °C for 1 min; 95 °C for 15 s, 60 °C for 15 s, 95 °C for 15 s. qRT-PCR data were then quantified by the standard curve method and normalized to the housekeeping gene, histone 3a. The GnRH Ct values for the mHypoA-GnRH/GFP were around 23, whereas the GT1-7 were around 15, indicating a higher baseline level of GnRH transcription. The higher levels of GnRH in the GT1-7 cells are also in accordance with the ICC and EIA secretory results.

For the agonist studies, mHypoA-GnRH/GFP or GT1-7 cells were grown to confluence and placed in 5 mM glucose, serum-free, DMEM-free, medium 4 h prior to the initiation of the experiment. Treatments with forskolin [10 μM], or TPA [100 nm] or time matched control (EtOH) were added to the media over a 2 (c-fos experiments) or 16 h (GnRH experiment) time course and total RNA was isolated at indicated time points. For the glucose-sensing experiments, mHypoA-GnRH/GFP neurons were grown to confluence and starved in low glucose [0.5 mM], 1% FBS media overnight (12–16 h). Following starvation, cells were either rechallenged with 0.5, 2.5, 5 mM glucose or 2-DG, or the medium was not changed as a control (no-rechallenge). Cells were harvested for total RNA extraction at indicated time points following treatment. For the AMPK inhibition studies using Compound C, we followed a similar protocol, starving the cells overnight then treating with 20 μM Compound C or controls treated with DMSO and isolated total RNA at indicated timepoints. Primer sequences, annealing temperatures, and amplicon sizes are described in Supplementary Table 1.

2.4. Western blot analysis and Immunocytochemistry (ICC)

mHypoA-GnRH/GFP neurons were grown to 85–90% confluence, serum-starved in 1% FBS, 0.5 mM phenol-red free media, for 4–16 h and then treated with 100 nM TPA and 10 μM forskolin, or vehicle control (EtOH). Western blot analysis was performed exactly as previously described (Dhillon et al., 2011). Phospho-specific proteins were quantified using densitometry and normalized to total targeted protein levels. Primary antibodies can be found in Supplementary Table 2. For ICC, mHypoA-GnRH/GFP or GT1-7 neurons were plated into eight well chamber slides (5 mm sq. BD Biosciences) in DMEM, 5 mM glucose media until cells reached 80–90% confluence. At confluence they were fixed in 4% paraformaldehyde for 15 min at room temperature. Cells were then washed twice with room temperature PBS and permeated with 0.2% triton-X-100 in PBS (Sigma–Aldrich Inc.) for 20 min at room temperature. ICC was performed as previously described (Dhillon et al., 2011).

2.5. Enzyme immunoassay (EIA)

mHypoA-GnRH/GFP neurons were grown to 90–95% confluence and serum starved in 0% FBS, 1% penicillin–streptomycin P/S, 5 mM glucose DMEM for 4 h prior to incubation with 100 nM TPA, 20 μM forskolin, or vehicle (EtOH) alone for 1 h, at 37 °C. Cells were treated with 60 mM KCl for 15 min prior to isolation, to induce depolarization of the cells and serve as a positive control. For glucose-sensing experiments, mHypoA-GnRH/GFP neurons were grown to 90–95% confluence and serum/glucose starved overnight in 1% FBS, 1% P/S, 0.5 mM Glucose phenol-red free DMEM prior to rechallenge with 0.5 mM glucose, 2.5 mM glucose, 5 mM glucose or no
rechallenge control. For the no rechallenge, medium was not changed, in order to measure the effects that addition of fresh low [0.5 mM] glucose has on the cells. Cell supernatants were collected in triplicate and GnRH-like immunoreactivity was measured by EIA (Phoenix Pharmaceuticals, CA), according to manufacturers protocol and values were normalized to total protein.

2.6. Statistical analysis

Data are presented as the mean ± the standard error of the mean (SEM). Data were analyzed by one-way or two-way analysis of variance (ANOVA) followed by Bonferroni post hoc test using Graphpad Prism (Graphpad Software Inc., CA). Experiments were performed in three to five replicates. Statistical significance was indicated if a $p < 0.05$ was achieved.

3. Results

3.1. Characterization of mHypoA-GnRH/GFP neuronal cell model

To create an adult-derived immortalized murine hypothalamic GnRH cell line, we exploited the ability of CNTF to stimulate proliferation in adult primary hypothalamic cultures from a female

Fig. 1. mHypoA-GnRH/GFP neurons co-express GnRH and GFP peptides as well as markers associated with mature GnRH neurons and glucosensing. (A) The immortalized mHypoA-GnRH/GFP line expresses GFP and GnRH, as determined by ICC. The levels of GnRH expression are comparable to the GT1-7 cell line. To-PRO-3 is a nuclear stain, whereas the differential interference contrast (DIC) image is also shown. (B) RNA was isolated from mHypoA-GnRH/GFP cell line, as well as comparable controls GT1-7 and Gn3 as indicated on the images, and assessed for expression of relevant genes by RT-PCR. Listed in the table is the presence (+) or absence (−) of a number of markers, as assessed by a cut-off Ct of >35. Representative agarose gels are included for specific genes, as indicated. Abbr.: NSE, neuron-specific enolase; Nestin; T-Ag, T-antigen; OTX2, orthodenticle homeobox 2; GFAP, glial fibrillary acidic protein; GP 130, CNTF receptor; GnRHR, GnRH-receptor; ER, estrogen receptor; GPR 30, G-protein coupled receptor 30; AR, Androgen receptor; Tacr3, Neurokinin B Receptor; Tac2, Neurokinin B, GLUT1-5; Glucose Transporter 1-5, SGLT1; Sodium-Like Glucose Transporter1, Kir6.1, Kir6.2, Sur1, Sur2, KATP Channel Subunits.
transgenic GnRH/GFP mouse. mHypoA-GnRH/GFP cells were immortalized and FAC-sorted as previously described (Dhillon et al., 2011). The mHypoA-GnRH/GFP cell line was assessed for GnRH and GFP protein expression using specific antibodies and visualized using ICC to confirm cell phenotype (Fig. 1A). The expression profiles of our novel GnRH cell line, the mHypoA-GnRH/GFP was analyzed for the presence of neuronal markers, reproductive related receptors as well as glucose transporters and glycolytic enzymes through RT-PCR (Fig. 1B). Representative ICC and screening results are presented for comparison with the GnRH-expressing GT1-7 and/or GnV cell lines. Basal levels of GnRH are approximately 2-fold higher in the GT1-7 cells compared to the mHypoA-GnRH/GFP cells, although highly detectable levels are demonstrated in both lines. The screening results suggest the presence of neuronal glucose transporters and machinery for the study of glucose responsiveness.

3.2. Regulation of c-fos and GnRH mRNA levels by forskolin and TPA in the mHypoA-GnRH/GFP cell line

Previous studies of in vivo GnRH neuronal populations and the in vitro GT1-7 cell line indicate that stimulation of PKA and PKC signaling pathways leads to activation of GnRH neuronal cells (Wetsel et al., 1993a,b). We therefore, assessed activation of these pathways in the mHypoA-GnRH/GFP cell line using forskolin, an adenyl cyclase agonist that indirectly activates PKA, and TPA, an agonist that directly stimulates PKC. Changes in c-fos expression, an immediate early gene, are commonly used as an anatomical marker of neuronal activation (Hoffman et al., 1993; Kovacs, 2008); however, activation of the neuron does not always directly correlate with changes in downstream gene expression. Levels of c-fos mRNA were assessed following stimulation with forskolin [10 μM] and TPA [100 nM]. The mHypoA-GnRH/GFP was treated with forskolin, TPA or time-matched vehicle controls (EtOH). RNA was isolated at 30, 60, and 120 min following treatment and changes in c-fos mRNA were measured with qRT-PCR. The mHypo-A-GnRH/GFP had significant induction of c-fos mRNA levels following treatment with forskolin and TPA. Specifically, mHypoA-GnRH/GFP neurons demonstrated an increase in c-fos mRNA with a significant expression at 30 min following either 10 μM forskolin (forskolin: 2.61 ± 0.08 vs. control: 0.73 ± 0.08); and TPA treatment (TPA 4.76 ± 0.13 vs. control: 0.34 ± 0.04) (Fig. 2A and B). Treatment with forskolin and TPA was also associated with a concomitant increase in activity of phosphoproteins CREB and ERK1/2, respectively, suggesting PKA and PKC pathway activation (Supplementary Fig. 1). The mHypoA-GnRH/GFP was then treated with the same concentrations of forskolin and TPA over an extended time-course and GnRH mRNA levels were measured. We found that 10 μM forskolin had no effect on GnRH mRNA levels. Whereas, treatment of the mHypoA-GnRH/GFP cell line with 100 nM TPA caused an acute and significant repression in GnRH mRNA levels at 2 and 4 h (EtOH, 1.31 ± 0.06, TPA 0.89 ± 0.02). Prolonged TPA exposure revealed that mHypoA-GnRH/GFP cells had a biphasic response to PKC pathway activation, with induction at 16 h (EtOH 0.92 ± 0.09, TPA 1.75 ± 0.19). These findings were similar to those reported in the GT1-7 clonally derived GnRH cell line, as well as our own findings (Supplementary Fig. 2). Furthermore, these findings suggest that the mHypoA-GnRH/GFP cell line is a functional model of GnRH neurons, which will be useful in the elucidation of glucose effects on GnRH cellular activation and mRNA production.

3.3. Glucose-mediated regulation of gene expression in the mHypoA-GnRH/GFP cell line

To determine whether mHypoA-GnRH/GFP cells respond to glucose, changes in c-fos mRNA levels were assessed. c-fos has previously been used as a measurement of GnRH neuronal activity due to nutrient challenge (Berriman et al., 1992). Berriman et al. found that food deprivation decreases c-fos expression in GnRH neurons of the forebrain of Syrian hamsters. Prior to initiation of our experimental paradigm, cells were placed in phenol red-free,

![Fig. 2](image-url)
low glucose (0.5 mM) media for 12–16 h overnight to lower cellular ATP levels, allowing for more accurate measurements of changes in cellular activity induced by glucose metabolism. No significant cell death was observed in the starved condition. After addition of fresh media containing low (0.5 mM) or high (5 mM) glucose concentrations, c-fos activity was assessed by qRT-PCR. Addition of both 0.5 mM and 5 mM glucose media caused a rapid upregulation in c-fos mRNA levels, with the strongest effects seen at 30 min with high glucose media treatment (vehicle 0.54 ± 0.145, 0.5 mM glucose 2.09 ± 0.11, 5 mM glucose 3.0 ± 0.11) (Fig. 3A). These data indicate that the mHypoA-GnRH/GFP hypothalamic cell model is responsive to changes in extracellular glucose concentrations.

Although evidence suggests that glucose regulates GnRH secretion in vivo (He et al., 1999; Rodriguez et al., 1999), it is not yet known whether glucose directly regulates GnRH mRNA levels. mHypoA-GnRH/GFP neurons were exposed to 0 (no rechallenge), 0.5 or 5 mM glucose concentrations over an 8 h time-course following a glucose starve overnight. GnRH mRNA levels were analyzed using qRT-PCR. 5 mM glucose treatment resulted in a 3.6-fold increase in GnRH mRNA levels, while 0.5 mM glucose did not significantly stimulate GnRH mRNA levels. Results indicated that GnRH transcription was maximally stimulated 2 h after glucose challenge (no rechallenge, 0.6 ± 0.05, 5 mM glucose 2.16 ± 0.14) (Fig. 3B). Relative expression levels of cpt1c, a regulator of β-oxidation (Schreurs et al., 2010), were also analyzed; however, there was no significant difference in CPT1c mRNA levels at the time points examined. This finding suggests that the stimulatory effect of glucose was specific to the GnRH mRNA (Fig. 3C). These data suggest that GnRH transcription is stimulated, or GnRH mRNA stability is increased by an increase in extracellular glucose concentration.

To determine whether the stimulatory effect of glucose on GnRH transcription was mediated through glycolysis and subsequent ATP production, we utilized a non-metabolizable potent glucose mimetic, 2-DG, which, once internalized, does not yield a net generation of ATP. 2-DG actively blocks glycolysis of glucose and is commonly used to study metabolic activity with regard to cancer, and is in clinical trials as an anti-cancer treatment due to these properties (Pelicano et al., 2006; Singh et al., 2005). Following overnight starvation of the cells, media was replaced with 0.5, 2.5 or 5.0 mM glucose or 2-DG. Analysis of GnRH mRNA levels indicated significant induction of GnRH transcription in 2.5 mM and 5.0 mM glucose conditions, but no significant change with the 2-DG treatment condition at the concentrations examined (Fig. 3D). These data suggest that the stimulatory effect of glucose on GnRH transcription is dependent on glycolysis and ATP production.

3.4. Glucose alters the phosphorylation status of key AMPK signal transduction pathway second messengers

In an attempt to delineate the molecular mechanisms responsible for the stimulatory effect of glucose on GnRH transcription, we utilized Western blot analysis to characterize the phosphorylation status of cellular signaling molecules following treatment. As expected, we observed a significant decrease in the phosphorylation levels of AMPK (Fig. 4A), and acetyl CoA carboxylase (ACC) (Fig. 4B) following 5 mM glucose treatment. These data suggest that AMPK and its associated downstream signaling molecules are modulated by increased glucose concentrations, and suggest their involvement in the stimulatory effect of glucose to increase GnRH mRNA levels.

To determine if the induction of GnRH mRNA transcription is due to modulation of AMPK phosphorylation status, we treated our cells with the AMPK antagonist, Compound C, using a concentration of 20 μM (Peairs et al., 2009). The decrease in pAMPK by

![Fig. 3](https://example.com/fig3)

**Fig. 3.** High glucose (5 mM), but not 2-DG, treatment induces c-fos and GnRH mRNA synthesis mHypoA-GnRH/GFP cells were placed in low glucose (0.5 mM) medium for 12–16 h before treatment with 0.5 mM, 5 mM glucose, no-rechallenge or 2-DG. At indicated time points, RNA was extracted for qRT-PCR analysis. (A) c-fos mRNA levels were assessed, showing a significant upregulation at each time point with 0.5 and 5 mM glucose. (B and D) GnRH and (C) Cpt1c mRNA levels were analyzed following an 8 h time course. Glucose directly increases GnRH mRNA levels at each time point with 5 mM glucose treatment, however CPT1c levels were unchanged at all time points. mRNA was isolated and analyzed using qRT-PCR. Each column represents the mean ± SEM. Significance is indicated by ‘p < 0.05’, ‘p < 0.01’, and ‘p < 0.001’ as per two-way ANOVA with Bonferroni’s post hoc test. n = 3–5 independent experiments.

Compound C (Fig. 4D) indicates that the neurons have sufficient nutrients, thus the neurons respond similarly to the high glucose conditions (Fig. 4A). Thus even in low glucose, when AMPK is inhibited by Compound C, the levels of GnRH mRNA increase (Fig. 4C) as seen in the high glucose conditions (Fig. 3). Changes in GnRH mRNA transcription due to Compound C were accompanied by a decrease in AMPK phosphorylation status, while the vehicle (DMSO) had no effect on GnRH mRNA levels or AMPK phosphorylation status (Fig. 4D). Together, these findings confirm that AMPK is an important second messenger in the regulation of the mHypoA-GnRH/GFP neurons and GnRH transcription.
3.5. Relative GnRH secretion in mHypoA-GnRH/GFP following NO, PKA, PKC signal transduction pathway activation and glucose stimulation

In order to assess secretory capacity in the mHypoA-GnRH/GFP neurons, we exposed both mHypoA-GnRH/GFP cells to the depolarizing agent KCl, the potent NO agonist, sodium nitroprusside (SNP), as well as 10 μM forskolin and 100 nM TPA to stimulate secretion (Fig. 5A and B). SNP has been utilized as a stimulatory reagent in previous studies involving the GT1-7 cell line, demonstrating an increase in GnRH secretion (Mahachoklertwattana et al., 1994). We report that stimulation of the mHypoA-GnRH/GFP cells with 60 mM SNP and 100 μM SNP, lead to an approximately 1.5–2-fold increase in GnRH secretion (mHypoA-GnRH/GFP: vehicle 1.03 ± 0.05, KCl 1.56 ± 0.14, SNP 1.59 ± 0.07). However, while we report significant induction of GnRH secretion following 1-h TPA treatment (mHypoA-GnRH/GFP: EtOH, 1.03 ± 0.11, TPA 1.52 ± 0.07), forskolin had no significant effect on GnRH secretion. These findings suggest slight differences in the mHypoA-GnRH/GFP cell line in response to PKA, PKC and NO signaling pathway activation to what has been reported in the GT1-7, as well as our own analysis of GnRH secretion by the GT1-7 cell line (Supplementary Fig. 3). Basal levels of GnRH secretion are higher in the GT1-7 by approximately 2-fold over the mHypoA-GnRH/GFP neurons (4 ng/ml vs. 2 ng/ml), however the stimulated levels of secretion are comparable between the two lines.

Accumulating evidence suggests that glucose can act directly to regulate GnRH neuronal activity and secretion (Beall et al., 2012; Zhang et al., 2007). To confirm secretory responsiveness of the mHypoA-GnRH/GFP neurons with increasing glucose concentrations, cells were starved overnight in low glucose media followed by rechallenge of 0.5 mM glucose and 5 mM glucose, together with the depolarizing agent KCl as a positive control. We found that only 5 mM glucose treatment elicited an increase in GnRH secretion (no-rechallenge, 1.0 ± 0.05; 0.5 mM Glucose; 0.99 ± 0.03, 5 mM glucose 1.35 ± 0.13; KCl 1.28 ± 0.05) (Fig. 5C). Comparable to KCl, high glucose treatment resulted in an approximately 1.3-fold increase in GnRH secretion, indicating that the mHypoA-GnRH/GFP cells are depolarized by glucose. This conforms to what has been reported in other GnRH cell lines, where glucose withdrawal decreases firing rate in GT1-7 cells (Beall et al., 2012). These findings suggest that the mHypoA-GnRH/GFP neurons are stimulated by high glucose exposure, causing GnRH secretion, but do not elevate secretion in response to resting glucose concentrations.

4. Discussion

The availability of an adult-derived GnRH cell model representing the entire complement of GnRH neurons in the hypothalamus has not been possible until it was possible to use a new technology for inducing neurogenesis in adult neurons to produce the mHypoA-GnRH/GFP cell line (Belsham et al., 2009; Dhillon et al., 2011). To date, the GT1-7 cell line has been instrumental in defining the cellular and molecular pathways underlying the regulation of GnRH synthesis and secretion (Belsham and Lovejoy, 2005). Yet the GT1-7 cell line is representative of a single GnRH neuron with an unknown developmental origin, and may not respond similarly to all GnRH neurons, particularly those of fully differentiated adult neurons. Less research has been performed on the other available GnRH cell lines, including other clones of GT1, GN, GnV, and GRT.
A study by Sim et al. identified four membrane properties (Sim et al., 2001). These findings suggest immortalized, adult derived hypothalamic cell line, mHypoA- and nutrient levels. To address this issue, we have generated an immortalized GnRH/GFP neurons, FAC-sorted from primary culture taken from an adult transgenic GnRH-GFP mouse hypothalamus. This cell line will be useful in determining the direct effects of glucose on the complement of different subpopulations of GnRH neurons.

It is beneficial to have multiple cell lines to get a more global, generalized view of neuronal function. Depending upon the developmental origin of the line, there can be divergent gene expression patterns and functional differences or similarities that should be defined (Dhillon et al., 2012). For this reason, we surmised that creating a novel GnRH cell model from the GnRH-GFP transgenic mouse could bridge a gap in the available models and provide a platform for comparison to the embryonic lines widely used in the field. We therefore expect there to be some variation in the phenotype of our neuronal culture, which may consist of a heterogeneous mixture of the sub-populations of GnRH neurons found within the hypothalamus. Thus the responses may be different between this and the widely used GT1-7 line. Although our study is based on glucose sensing in the GnRH neuron and the transcriptional response to glucose, we thought it prudent to undergo some comparative studies before assessing glucose effects on GnRH transcription. Overall, the two lines express similar phenotypic markers, and express and secrete GnRH in an expected manner, albeit the basal levels of GnRH are 2-fold higher in the GT1-7 cells. This may reflect the integrations site of the immortalizing factor T-anti- gen or the difference in the developmental origin of the two lines (one from primary adult culture vs. a tumor). Nonetheless, there are highly detectable levels of GnRH transcript and peptide in both lines, making them suitable for molecular analysis. While it would be counter-productive and impractical to repeat all of the studies undertaken in the GT1-7 line over the past 20 years, we found that the mHypoA-GnRH/GFP line responds in a similar fashion to the GT1-7 cells with regards to some of the reported signal transduction pathways and secretory responses (Wetsel, 1995; Wetsel et al., 1993a,b). However, there were some temporal differences in responses, indicating that indeed the lines could reflect their origin and overall composition, as might be expected from a more heterogeneous group of neurons. This may be more reflective of the additive response of the entire complement of GnRH neurons in the hypothalamus. These comparative studies should be pursued in more detail. However, since the mHypoA-GnRH/GFP line proved to be a suitable model, we began with the glucose-sensing analysis.

The primary function of GnRH neurons is to integrate regulatory control over the HPG axis through their interaction with a multitude of hormones and neuropeptides. It is recognized that hypothalamic neurons directly sense extracellular nutrient concentrations, leading to modulation of fertility (Wade and Schneider, 1992). The role of glucose within the hypothalamus to regulate orexigenic and anorexigenic neuronal populations, thereby altering feeding behavior, is well defined (Borg et al., 1995; Murphy et al., 2009). However, recent evidence suggests that glucose may also regulate fertility by directly modulating GnRH neuronal activity (Zhang et al., 2007). Recently, AMPK has emerged as a vital second messenger, mediating metabolic hormone and nutrient effects in GnRH neurons (Cheng et al., 2011; Coytal-Castel et al., 2008; Wen et al., 2008). However, the cellular and molecular mechanisms underlying glucose action in GnRH neurons are still being elucidated. Using the mHypoA-GnRH/GFP cell line, we investigated the cellular events underlying regulation of GnRH mRNA levels following treatment with glucose. In this study, the AMPK signal transduction pathway was implicated in the effect of glucose directly regulating GnRH mRNA levels. Inhibition of AMPK with Compound C was also shown to increase GnRH mRNA, further supporting a role for AMPK in the control of the GnRH transcription.

Fig. 5. Depolarization, NO or PKC agonists and high glucose (5 mM) directly induced GnRH secretion in mHypoA-GnRH/GFP. Cell culture medium was assayed for GnRH-like immunoreactivity by enzyme immunoassay (EIA) following treatment with (A) 60 mM KCl and 100 μM SNP or control (H2O) or (B) 20 μM forskolin, 100 nM TPA or control (EtOH). (C) For glucose experiments, neurons were starved 12 h overnight in low glucose (0.5 mM) before treatment with 0.5 mM, 5 mM or no rechallenge control. Results are shown relative to control and are expressed as mean ± SEM, (n = 3–4 independent experiments each run in triplicate). *p < 0.05, **p < 0.01 vs. control, as per one-way ANOVA with Bonferroni’s post hoc test.

cell lines (Mellon et al., 1990; Radovick et al., 1991; Salvi et al., 2006; Wolfe et al., 2008). Recent evidence suggests that GnRH neurons may consist of subpopulations, given their differing morphology and dendrite complexity reported in vivo (Campbell et al., 2005, 2009; Herde et al., 2011). A study by Sim et al. identified four different populations of GnRH neurons, which had heterogeneous membrane properties (Sim et al., 2001). These findings suggest that GnRH neurons in vivo may represent multiple subpopulations, which display divergent morphological properties and may be functionally different. These findings highlight the need for a model, which is more representative of the entire complement of GnRH neurons, to better study the direct effects of changes in hormone and nutrient levels. To address this issue, we have generated an immortalized, adult derived hypothalamic cell line, mHypoA- GnRH/GFP that represents a highly enriched population of immortalized GnRH/GFP neurons, FAC-sorted from primary culture taken from an adult transgenic GnRH-GFP mouse hypothalamus. This cell line will be useful in determining the direct effects of glucose on the complement of different subpopulations of GnRH neurons.
Furthermore, it was determined that the mHypoA-GnRH/GFP cells secrete GnRH in response to glucose treatment.

Glucose utilizes a number of mechanisms to enter the brain. The blood–brain barrier is a major site of glucose entry into the central nervous system, where facilitated transport across endothelial cells results in brain glucose concentrations of approximately, 10–30% of that found in the periphery (de Vries et al., 2003; McNay and Gold, 1999; Silver and Erecinska, 1994). However, due to the presence of fenestrated capillaries in the arcuate nucleus, which allow for passive transport of glucose into the brain, glucose concentration that neurons are exposed to might vary depending on their anatomical position within the hypothalamus (Peruzzo et al., 2000; Wang et al., 2004). While studies of glucose responsiveness in cultured cells have used concentrations of glucose from 10 to 20 mM (Cai et al., 2007; Wang et al., 2004), it is very unlikely that GnRH neurons in the preoptic area would be exposed to such a high concentration. However, we chose a more physiologically relevant 0.5–5 mM change in glucose concentration for our experiments, which is in accordance with previous in vivo GnRH glucose-sensing studies (Roland and Moenter, 2011a,b,c; Zhang et al., 2007). The anatomical position of GnRH neurons within the preoptic area (POA) of the hypothalamus makes them especially vulnerable to peripheral nutrient changes due to their close proximity to the blood brain barrier within the third ventricle (Cottrell et al., 2006; Prevot et al., 2010; Silverman and Desnoyers, 1976). Recent evidence suggests that a proportion of GnRH neurons extend through the blood brain barrier, possibly exposing these populations to peripheral nutrient levels (Herde et al., 2011). How GnRH neurons respond to glucose is not completely clear, particularly if the effect is director at the level of transcription.

Activation of AMPK through treatment with the pharmacological agonist AICAR, an AMP mimetic, leads to suppression of GnRH neuronal activity in vivo and in vitro (Roland and Moenter, 2011a,b,c). Conversely, addition of Compound C, an AMPK antagonist, to cells pretreated with AMPK agonists, restores GnRH neuronal activity (Roland and Moenter, 2011a,b,c). These studies indicate a functional role for AMPK in the modulation of GnRH neuronal activity. Further evidence for a role of AMPK in regulating fertility comes from the study of an AMPK knock out knockout mouse model. These mice are subfertile but exhibit no apparent metabolic phenotype, suggesting that the alterations in fertility are not secondary to metabolic deregulation (Jorgensen et al., 2004). These findings highlight a role for AMPK in regulating GnRH cellular activity. However, until now it was unknown whether altered AMPK phosphorylation status leads to modulation of GnRH mRNA levels. We found that following treatment with glucose [5 mM] in mHypoA-GnRH/GFP cells, which have been starved overnight, AMPK phosphorylation status is significantly reduced. Supporting our hypothesis that inhibition of AMPK phosphorylation directly regulates GnRH transcription, we showed that treatment of mHypoA-GnRH/GFP neurons with Compound C causes an increase in GnRH mRNA levels similar to those seen in the high glucose [5 mM] conditions. These findings implicate AMPK as an important second messenger in the regulation of GnRH mRNA in mHypoA-GnRH/GFP neurons.

While we have elucidated a role for an AMPK-dependant mechanism in the regulation of GnRH mRNA, how glucose regulates GnRH secretion in the mHypoA-GnRH/GFP cell line is yet to be determined. Much of the information regarding the regulation of cellular activity and secretion of hypothalamic GE neuronal populations comes from the study of POMC-expressing neurons in the arcuate nucleus (Claret et al., 2007; Ibrahim et al., 2003; Porton et al., 2007). The mHypoA-GnRH/GFP cell line differs from these neurons based on expression of components associated with the pancreatic beta-cell glucose-signaling pathway, which is made up of the ATP-sensitive potassium channel (KATP) subunits, SUR1 and Kir6.2 and the glycolytic enzyme glucokinase (GCK) (Cai et al., 2007; Ibrahim et al., 2003). The KATP channel functions by sensing the increase in ATP/ADP ratio within the cell following glycolysis. Increased ATP levels cause closure of the channel leading to depolarization of the cell. The importance of the KATP channel in POMC-expressing neuronal glucose-sensing was demonstrated by Porton et al. when mice with selective Kir6.2 mutations were no longer able to respond to glucose when tested electrophysiologically. These effects were attributed to mild glucose intolerance within the whole mouse (Porton et al., 2007).

Recent evidence has suggested that a subpopulation of GnRH neurons in vivo may use a similar mechanism to sense changes to extracellular glucose levels. In fact, approximately 30% of GnRH neurons have been shown to express GCK. However, its role in mediating glucose-sensing in GnRH neurons is not yet known, as more than 30% of GnRH neurons have been shown to exhibit glucose-sensing properties (Roland and Moenter, 2011a,b,c). In accordance, we report that the mHypoA-GnRH/GFP cell line expresses GCK; however, the functionality and distribution of this enzyme throughout our neuronal cell model needs to be validated. Zhang et al. reported that single-cell RT-PCR of a number of GnRH neurons of a mHypoA-GnRH/GFP mouse expressed the (KATP) channel subunits SUR1 and Kir6.1 (Zhang et al., 2007). These findings were later confirmed in the clonally generated GT1-7 GnRH cell model, where functional (KATP) channels modulated firing of these cells in response to extracellular glucose (Beall et al., 2012). While expression of the (KATP) subunits has been confirmed in a proportion of GnRH neurons, whether this channel participates in GnRH glucosensing is controversial. In a recent study mHypoA-GnRH/GFP hypothalamic slices were bathed in low glucose media and treated with the KATP channel agonist, tolbutamide. Tolbutamide treatment only evoked firing in a subset of GnRH neurons, and failed to attenuate the inhibitory response, which low glucose concentrations exerted on the GnRH neurons. This finding suggests that although KATP channels are expressed in a proportion of GnRH neurons, this channel may not be the primary mechanism responsible for glucosensing (Zhang et al., 2007). An alternative mechanism that glucose may use to modulate GnRH neuronal activity is transport of glucose through the high affinity Na+-D-glucose co-transporter (SGLT1). This transporter is expressed in the brain (Poppe et al., 1997), and pharmacological inhibition with phlorizin has been shown to reduce activity of GE neurons in the hypothalamus (Yang et al., 1999). SGLT1 is also expressed in the mHypoA-GnRH/GFP neurons, and may mediate the stimulatory effect of glucose on GnRH secretion. These findings suggest that the mHypoA-GnRH/GFP cell line is a novel model, which will be effective in delineating potential mechanisms through which GnRH neurons respond to glucose challenge in a KATP-independent mechanism.

The mHypoA-GnRH/GFP cell line has properties that are advantageous over the existing clonally generated models available, and will allow for an in vitro analysis of the entire complement of GnRH neurons from the mHypoA-GnRH/GFP mouse hypothalamus. The studies performed highlight the need for further cellular and molecular investigation of GnRH neuronal models to more accurately approximate how in vivo GnRH populations respond to nutrient, hormone and drug exposure. Until these studies can be routinely performed in the whole brain, the mHypoA-GnRH/GFP cell line may be a valid representative model for these studies.

Acknowledgements

We acknowledge funding from the Canadian Institutes for Health Research (CIHR) and Canadian Diabetes Association (DDB); Canada Foundation for Innovation and Canada Research
Chairs Program (DBB). SAM and JM were supported by Banting and Best Diabetes Centre (BBDC) Studentships and JJ was supported by a BBDC Summer Studentship. Many thanks to Drs. Leigh Wellhaus-mer for critical reading of the manuscript, Sandeep Dhillon for initial screening assistance, and Margaret Koleter for technical assistance.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.mce.2013.06.035.

References


