Bisphenol A Induces *Agrp* Gene Expression in Hypothalamic Neurons through a Mechanism Involving ATF3

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Keywords

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Abstract

Background: Bisphenol A (BPA) is a ubiquitous endocrine disrupting chemical and obesogen. Although limited evidence exists of the effects of BPA on hypothalamic agouti-related peptide (AgRP) levels, the mechanisms underlying these effects remain unknown. Given that AgRP is a potent orexigenic neuropeptide, determining the mechanism by which BPA increases AgRP is critical to preventing the progression to metabolic disease. **Methods:** Using quantitative reverse transcriptase polymerase chain reaction, we investigated the response of *Agrp*-expressing mouse hypothalamic cell lines to BPA treatment. The percentage of total BPA entering hypothalamic cells in culture was quantified using an enzyme-linked immunosorbent assay. In order to identify the mechanism underlying BPA-mediated changes in *Agrp*, siRNA knockdown of transcription factors, FOXO1, CHOP, ATF3, ATF4, ATF6, and small-molecule inhibitors of endoplasmic reticulum stress, JNK or MEK/ERK were used. **Results:** BPA increased mRNA levels of *Agrp* in six hypothalamic

cell lines (mHypoA-59, mHypoE-41, mHypoA-2/12, mHypoE-46, mHypoE-44, and mHypoE-42). Interestingly, only 18% of the total BPA in the culture medium entered the cells after 24 h, suggesting that the exposure concentration is much lower than the treatment concentration. BPA increased *pre-Agrp* mRNA levels, indicating increased *Agrp* transcription. Knockdown of the transcription factor ATF3 prevented BPA-mediated increase in *Agrp*, *pre-Agrp*, and in part *Npy* mRNA levels. However, chemical chaperone, sodium phenylbutyrate, JNK inhibitor, SP600125, or the MEK/ERK inhibitor PD0352901 did not block BPA-induced *Agrp* upregulation. **Conclusion:** Overall, these results indicate that hypothalamic *Agrp* is susceptible to dysregulation by BPA and implicate ATF3 as a common mediator of the orexigenic effects of BPA in hypothalamic neurons.

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Introduction

Obesity is a pressing health concern as it leads to complications, including cardiovascular disease, diabetes, and some cancers. Although calorie-dense diets and a sedentary lifestyle are major reasons for the recent rise in obe-

sity, the contribution of environmental endocrine disrupting chemicals, termed “obesogens,” has recently gained attention [1, 2]. Bisphenol A (BPA), a chemical widely used in polycarbonate plastics and receipt paper, has been correlated to increased body mass index and diabetes [3–6], increases body weight in rodent models [7], and has recently been classified as a metabolism-disrupting chemical [1]. BPA is detected in the urine of 9 in 10 people [4] and accumulates in tissues, including the brain [8].

Although the obesogenic effects of BPA have been primarily described as targeting fat cells, leading to adipogenesis, lipogenesis, and ultimately weight gain [7, 9, 10], its effects on the hypothalamic control of energy homeostasis have been largely neglected. The hypothalamus houses orexigenic neuropeptide Y (NPY)/agouti-related peptide (AgRP) neurons and anorexigenic pro-opiomelanocortin (POMC) neurons that integrate peripheral indicators of energy status to maintain energy homeostasis [11]. Exogenous chemicals that affect these neurons can override this intricate control of energy balance, leading to dysregulation. In fact, maternal exposure to BPA increased protein levels of AgRP and decreased POMC in neural progenitor cells of newborn mice [12]. MacKay et al. [13] also described that in male offspring, perinatal exposure to BPA led to decreased POMC fiber innervation and increased *Npy* and *Agrp* mRNA levels when combined with a high-fat diet. The molecular mechanisms underlying these changes remained unknown, and these paradigms do not address the direct effects of BPA on neurons in the mature hypothalamus. The chronic activation of orexigenic NPY and AgRP will ultimately lead to obesity, as demonstrated with intracerebroventricular administration of the neuropeptides [11]. Notably, a single dose of AgRP was shown to increase food intake in a rodent model for 7 days, highlighting its potency [14].

We have recently described an increase in *Npy*, *Agrp*, and *Pomc* mRNA expression with BPA exposure in mouse hypothalamic neurons in vitro, suggesting an overall dysregulation of the hypothalamic melanocortin system beginning at the transcriptional level [15, 16]. Mechanistically, the increase in *Npy* mRNA levels was mediated by the circadian transcription factor BMAL1 [15], while the transient upregulation of *Pomc* at 4 h was mediated by neuroinflammation and the transcription factor PPAR γ [16]. However, blocking these pathways did not block the BPA-mediated upregulation in *Agrp* mRNA, suggesting that another distinct pathway is involved in mediating the changes in *Agrp*. Given that AgRP is a potent, orexigenic neuropeptide, determining the

mechanism by which BPA increases its transcription is critical to blocking the orexigenic effects of BPA.

As an orexigenic peptide, *Agrp* is suppressed by signals of high energy, including insulin [17], leptin [18], high glucose [19], and amino acids [20]. These signals ultimately regulate *Agrp* through a set of transcription factors. The understanding of the transcriptional regulation of mouse *Agrp* is limited, in part due to its large regulatory region that lies up to 43 kb upstream of its transcriptional start site [21]. Nevertheless, FOXO1 is a well-known activator of *Agrp* transcription, while STAT3 directly represses *Agrp* transcription [18]. We therefore hypothesized that BPA increases *Agrp* mRNA levels by activating FOXO1 or repressing STAT3. Many other transcription factors are likely involved in the promoter regulation of *Agrp*. Besides circadian dysregulation and neuroinflammation, BPA has been described to induce endoplasmic reticulum (ER) stress [22]. ER stress occurs as a result of elevated protein misfolding and also in states of nutrient deprivation [23] and obesity [24], both of which are accompanied by changes in *Agrp*. A number of transcription factors are involved in ER stress, such as CHOP, ATF3, ATF4, and ATF6 [23], that may be involved in *Agrp* regulation by direct binding to the *Agrp* regulatory region or through interaction with other *Agrp*-associated transcription factors. For example, ATF3 has been previously shown to dimerize with FOXO1 on the *Agrp* promoter, leading to increased transcription [25]. Therefore, we also hypothesized that BPA increases *Agrp* mRNA levels by altering ER stress-regulated transcription factors.

Herein, we describe that BPA does indeed lead to transcriptional upregulation of *Agrp* mRNA in both adult and embryonic-derived mouse hypothalamic cell lines, with concurrent upregulation in the expression of *Foxo1*, *Stat3*, *Chop*, *Atf3*, *Atf4*, and *Atf6*. We further identify that the increase in *Agrp* is dependent on the transcription factor ATF3, yet independent of FOXO1 activation and ER stress. We also describe that ATF3 is, in part, involved in BPA-mediated *Npy* upregulation. Overall, these results illustrate a FOXO1-independent action of ATF3 on *Agrp* and *Npy* and highlight a common mechanism to mitigate the orexigenic effects of BPA in the hypothalamus.

Methods

Cell Culture and Reagents

Hypothalamic neurons from adult or embryonic mice were immortalized as previously described to generate several *Agrp*-expressing clonal cell lines [26, 27]: mHypoA-59, mHypoE-41,

mHypoA-2/12, mHypoE-46, mHypoE-44, and mHypoE-42. Neurons were maintained in Dulbecco's modified Eagle's medium (DMEM; cat. D5796; MilliporeSigma, Oakville, ON, Canada) containing 4,500 mg/L glucose, supplemented with 2% fetal bovine serum (FBS; Gibco, Burlington, ON, Canada) and 1% penicillin-streptomycin (PS; Gibco) at 37 °C with 5% CO₂. Cells were split into 60-mm tissue culture plates approximately 24 h prior to treatment and grown to 75–85% confluency. Growth medium was replaced with treatment medium (as described below) on the day of treatment.

BPA (MilliporeSigma) was dissolved in 100% ethanol to a concentration 200 mM, then diluted 1:1 in sterile H₂O to generate a 100 mM stock solution. Vehicle (50% ethanol + 50% H₂O) or 100 mM BPA was then diluted 1:1,000 in phenol-red free DMEM (Hyclone Laboratories Inc., Whitby, ON, Canada) containing 4,500 mg/L glucose, supplemented with 1% charcoal-dextran stripped FBS (CS-FBS; Gemini Bio Products, Burlington, ON, Canada) and 1% PS, giving a final concentration of 0.05% ethanol or 100 μM BPA in 0.05% ethanol. For lower doses of BPA, 200 mM BPA was further diluted in 100% ethanol to 100, 50, or 20 mM BPA, then diluted 1:1 in H₂O, followed by 1:1,000 in medium to obtain final concentrations of 50, 25, and 10 μM BPA.

Primary culture experiments were conducted as previously described [15]. Briefly, cells from the hypothalami of 8-week-old male CD-1 mice (Charles River Laboratories, Senneville, QC, Canada) were dispersed by trituration and cultured in supplemented neurobasal A medium (ThermoFisher Scientific, Burlington, ON, Canada) for 7–9 days as described. Cells were treated with 0.05% ethanol or 100 μM BPA for 8 h in phenol-red free DMEM with 1% CS-FBS and 1% PS prior to collection for RNA isolation.

For inhibitor pretreatment experiments, pretreatments were added to the cells in 2.5 mL medium. BPA or ethanol was then added to the cells in 0.5 mL medium to achieve final concentrations of 100 μM BPA or 0.05% ethanol in 3 mL volume. Stock solutions of actinomycin D (ActD; 10 mg/mL), SP600125 (50 mM), and PD0352901 (10 mM) were prepared by dissolving in 100% dimethyl sulfoxide, followed by a 1:1,000 dilution in medium to obtain final concentrations of 10 μg/mL ActD, 50 μM SP600125, and 10 μM PD0352901. Sodium phenylbutyrate (PBA) was dissolved in sterile H₂O to a concentration of 500 mM, followed by a 1:100 or 1:50 dilution into medium to obtain final concentrations of 5 and 10 mM PBA. ActD (cat. A1410-5MG) and PBA (cat. SML0309-100MG) were purchased from MilliporeSigma, and SP600125 (cat. 1496/10) and PD0352901 (cat. 4192/10) were purchased from Tocris Bioscience (Cedarlane, Burlington, ON, Canada).

5-aminoimidazole-4-carboxamide ribonucleotide (AICAR; cat. 2840/50) was purchased from Tocris Bioscience, dissolved in H₂O to 50 mM, and diluted 1:100 in medium for a final concentration of 500 μM. Cells were pretreated with 500 μM AICAR or H₂O for 1 h, followed by 0.05% ethanol treatment for 8 h.

BPA Content Enzyme-Linked Immunosorbent Assay

Cells were grown to 75–80% confluency in 60-mm tissue culture plates 24 h prior to treatment with 0.05% ethanol, 10 μM BPA, or 100 μM BPA for 4 or 24 h in phenol-red free DMEM, supplemented with 1% CS-FBS and 1% PS. After treatment, 500 μL of medium from each plate was collected, immediately dried down using a vacuum concentrator, and stored at –20 °C. Cells were lysed with 500 μL/plate 1× cell lysis buffer (Cell Signaling Technology Inc., New England Biolabs, Whitby, ON, Canada) diluted in phosphate-buffered saline (PBS). BPA was then collected from the

cells using an ethyl acetate extraction method. Briefly, 250 μL of cell lysate was acidified with 1 μL of acetic acid to obtain a pH of 3–4. Then, 250 μL of ethyl acetate was added to each sample, vortexed, and centrifuged at 12,000 g for 3 min to separate organic and aqueous layers. The top organic layer was collected and the extraction was repeated twice using 250 μL ethyl acetate each time. A total of 700 μL organic layer was obtained after three extractions, which was immediately dried using compressed air and stored at –20 °C. The Estrogen BPA Environmental ELISA Kit (cat. ab175820) was purchased from Abcam Inc. (Toronto, ON, Canada) and the assay to measure BPA content was performed as per manufacturer's instructions. A 1:50 or 1:100 dilution of each sample in sample dilution buffer was performed prior to loading samples into assay wells. Percent BPA content was calculated using the following formula: % BPA content = [(content in cell lysate) / (content in cell lysate + content in medium)] × 100.

Quantitative Reverse Transcriptase Polymerase Chain Reaction

Total RNA was isolated with the PureLink RNA isolation kit (ThermoFisher Scientific) according to the manufacturer's instructions with genomic DNA removal using an on-column DNase step (PureLink DNase set, ThermoFisher Scientific). RNA was quantified on the NanoDrop 2000 (ThermoFisher Scientific) and 500–1,000 ng of cDNA was synthesized using the Applied Biosystems High Capacity cDNA Reverse Transcription kit (ThermoFisher Scientific). For the quantitative reverse transcriptase polymerase chain reaction (qRT-PCR), 12.5 ng of cDNA was amplified with gene-specific primers (Table 1) and the Platinum SYBR Green qPCR SuperMix-UDG with ROX using the Applied Biosystems Prism 7900HT machine as described previously [15]. Data were analyzed with the standard curve method or with the $\Delta\Delta C_T$ method and normalized to the reference gene, 60S ribosomal protein L7 (*Rpl7*).

Western Blotting

Cells were grown to 80–90% confluency in DMEM supplemented with 2% FBS and 1% PS. On the day of treatment, cells were treated with 100 μM BPA or 0.05% ethanol for 2, 4, or 8 h in phenol-red free DMEM supplemented with 1% CS-FBS and 1% PS. For short-term signaling experiments, cells were serum-starved in 2.5 mL of phenol-red free DMEM for 1 h prior to treatment with 100 μM BPA or 0.05% ethanol for 5, 15, or 30 min. For AMPK signaling, on the day of treatment, 2.5 mL of DMEM modified phenol-red free medium with 1% CS-FBS and 1% PS was added to the cells 1 h prior to treatment with 100 μM BPA or 0.05% ethanol for 8 h. Protein was harvested using 1× cell lysis buffer (Cell Signaling Technology Inc.) containing 1 mM phenylmethylsulfonyl fluoride, 1% protease inhibitor cocktail, and 1% phosphatase inhibitor cocktail 2 (MilliporeSigma) and quantified using the BCA protein assay kit (ThermoFisher Scientific). A total of 15–25 μg of total protein was separated on 10 or 12% SDS-polyacrylamide gels and transferred onto PVDF membranes (Bio-Rad). Membranes were blocked in 5% milk dissolved in Tris-buffered saline with Tween 20 (TBS-T) for 1 h, prior to incubation with primary antibody overnight at 4 °C. The primary antibodies CHOP (L63F7; cat. 2895), ATF3 (D2Y5W; cat. 33593), ATF4 (D4B8; cat. 11815), ATF6 (D4Z8V; cat. 65880), α -tubulin (cat. 2144), pJNK (Thr183/Tyr185, 81E11; cat. 4668), total JNK (cat. 9252), pERK1/2 (Thr202/Tyr204; cat. 9101), total ERK (137F5; cat. 4695), pAMPK α (Thr172;

Table 1. qRT-PCR primers

Gene name	Primer sequence (5' → 3')	Amplicon size
qRT-PCR primers		
<i>Agrp</i>	F: CGG AGG TGC TAG ATC CAC AGA R: AGG ACT CGT GCA GCC TTA CAC	69
<i>Pre-Agrp</i>	F: TGA CCT CAG TCC ACT GCC A R: AGG ACT CGT GCA GCC TTA CAC	139
<i>Rpl7</i>	F: TCG CAG AGT TGA AGG TGA AG R: GCC TGT ACT CCT TGT GAT AGT G	114
<i>Foxo1</i>	F: CAA AGT ACA CAT ACG GCC R: GAG AGT CAG AAG TCA ACA	147
<i>Stat3</i>	F: GCC ACG TTG GTG TTT CAT AAT C R: TTC GAA GGT TGT GCT GAT AGA G	97
<i>Chop</i>	F: TAT GAG GAT CTG CAG GAG R: CAG GGT CAA GAG TAG TGA AG	109
<i>Atf3</i>	F: CTC CTG GGT CAC TGG TAT TTG R: CCG ATG GCA GAG GTG TTT AT	98
<i>Atf4</i>	F: GGA GCA AAA CAA GAC AGC R: TTG CCT TAC GGA CCT CTT CT	179
<i>Atf6</i>	F: CAG ATG GTG ACA ACC AGA AAG A R: CAT GGA GGT GGA GGC ATA TAA AG	112
<i>Npy</i>	F: CAG AAA ACG CCC CCA GAA R: AAA AGT CGG GAG AAC AAG TTT CAT T	77
<i>Bmal1</i>	F: GGG AGG CCC ACA GTC AGA TT R: GTA CCA AAG CCA ATT CAT CAA	78
ChIP primers		
<i>FOXO1 site 1</i>	F: GCA GCC ATT AAC ACT AAT GAA GC R: TAC ACC TAT TTC CAT CCC CAC G	143
<i>FOXO1 site 2</i>	F: GTG CCC TTG ACA AAG TTC CTG GAA R: GCA GAA CCT AGG GAT GGG TCA TGC	152
<i>ATF:CRE site</i>	F: ATG GCA ACT GCC CCA AAC R: AAG TAG TCT CCA CCC GGT T	132
<i>CEBP site</i>	F: TTT GAG GGA AAG GGG ATT GG R: GTA AGA GCT AAT CCT AGG CGG T	185
<i>ATF3 on ATF3 promoter</i>	F: CCA GTT CTC CTG GAA GCT A R: CGT TGC ATC ACC CCT TTT AA	110
ChIP, chromatin immunoprecipitation; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction.		

cat. 2532S), and total AMPK α (cat. 2535S) were purchased from Cell Signaling Technology Inc. and were diluted 1:1,000 in 5% milk in TBS-T (for CHOP, JNK, ERK, AMPK) or in 5% bovine serum albumin in TBS-T. Membranes were washed and incubated with secondary HRP-linked antimouse (for CHOP) or antirabbit antibody (1:7,500 in 5% milk in TBS-T; Cell Signaling Technology

Inc.) for 1 h, washed, and imaged using the Signal Fire ECL Reagent (Cell Signaling Technology Inc.) on the Kodak Image Station 2000R. The Restore PLUS Western blot stripping buffer (Thermo-fisher Scientific) was used according to the manufacturer's instructions before probing the same blot for subsequent proteins. Protein density was quantified using ImageJ.

Table 2. siRNA duplex sequences

Gene target	siRNA duplex sequence (5' → 3')	Target region
<i>Foxo1</i>	mm.Ri.Foxo1.13.3 CUU CUG GAU AAU CUC AAC CUU CUC T AGA GAA GGU UGA GAU UAU CCA GAA GGU	exon 2
<i>Chop</i>	mm.Ri.Ddit3.13.3 AAC AGA GGU CAC ACG CAC AUC CCA A UUG GGA UGU GCG UGU GAC CUC UGU UGG	exon 3/4
<i>Atf3</i>	mm.Ri.Atf3.13.3 CUG GAG UCA GUU ACC GUC AAC A UGU UGA CGG UAA CUG ACU CCA GCG	exon 2
<i>Atf4</i>	mm.Ri.Atf4.13.1 AAG ACU GAG AAA UUG GAU AAG C GCU UCU UAU CCA AUU UCU CAG UCU UCA	exon 3/4
<i>Atf6</i>	mm.Ri.Atf6.13.2 GAC CAA AAA UGU CAA UUG UAU UAC C GGU AAU ACA AUU GAC AUU UUU GGU CUU	exon 15

siRNA Knockdown

Cells were grown to 75–80% confluency in 100-mm tissue culture plates for transfection. siRNA duplexes (Table 2) and negative control (cat. 51-01-14-03) were purchased from Integrated DNA Technologies Inc. (Coralville, IA, USA). A total of 25 nM of siRNA or negative control and 25 μ L/plate of Dharmafect 3 Transfection Reagent (Dharmacon; Cedarlane) were complexed for 20 min at room temperature in serum- and antibiotic-free DMEM (cat. D5796; MilliporeSigma). The complexed reagents were diluted in 2% FBS containing antibiotic-free DMEM. Cells were incubated with 5 mL/plate of transfection medium for 24 h. The cells were then washed with PBS and treated with 100 μ M BPA or 0.05% ethanol for 8 h in antibiotic-free phenol-red free DMEM containing 1% CS-FBS. Cells were lysed and RNA and protein were collected from the same plate using the mirVANA PARIS kit (ThermoFisher Scientific) to measure changes in mRNA and protein levels as described above. For the *Foxo1* siRNA experiments, siRNA knockdown was validated with qRT-PCR as an appropriate antibody was not available.

Promoter Analysis and Chromatin Immunoprecipitation

The mouse *Agrp* regulatory region was obtained, and the transcriptional start site was inferred from previously published papers on the *Agrp* promoter [18]. ATF:CRE (TGACGTCA) and CEBP (TG(T/A)TGCAAT) sites were manually identified [28].

mHypoA-59 cells were grown to 80–85% confluency in 100-mm tissue culture plates for treatment. Cells were treated with 100 μ M BPA or 0.05% ethanol for 1, 4, or 8 h. An additional plate of cells was treated with 0.05% ethanol (vehicle) per experimental replicate for immunoprecipitation (IP) with the negative control (normal rabbit IgG) antibody. Cells were harvested in PBS containing 1 $\times$ protease inhibitor cocktail, and chromatin IP (ChIP) was performed using the SimpleChIP Enzymatic Chromatin IP kit with magnetic beads (Cell Signaling Technology Inc.) according to the manufacturer's instructions. Two micro-

grams of ATF3 antibody (D2Y5W, cat. 33593, Cell Signaling Technology Inc.) or normal rabbit IgG antibody (provided with the kit) were used for the IP. DNA was purified using columns provided with the SimpleChIP kit or the PureLink PCR Purification Kit (ThermoFisher Scientific). Binding of ATF3 to the *Agrp* promoter region was analyzed using qRT-PCR with cycling conditions as described previously [15]. Each sample was run in triplicate using 3.5 μ L of purified DNA in 10 μ L reactions with primers specific for the *Agrp* promoter or the *Atf3* promoter (Table 1) and the Platinum SYBR Green qPCR SuperMix-UDG with ROX. The mean cycle of threshold (CT) of each IP sample and its respective 2% input sample were used to calculate the percent of DNA pulled down by the antibody relative to input: % of input = $2\% \times 2^{(CT\ 2\% \text{ input sample} - CT\ IP\ sample)}$. Relative binding was calculated by taking the average of the percent inputs of vehicle and treated groups per experimental replicate and dividing each value by the respective average.

Statistical Analysis

Data were analyzed for statistical significance using GraphPad Prism 6.0 (GraphPad Prism Software Inc., San Diego, CA, USA). Student *t* test, one-way ANOVA, or two-way ANOVA, followed by the Bonferroni or Tukey multiple comparison test, were performed as appropriate and are indicated in the figure legends. Differences were considered statistically significant when *p* was <0.05. Data are presented as mean $\pm$ SEM, with statistical significance denoted as **p* < 0.05, ***p* < 0.01, ****p* < 0.001, and *****p* < 0.0001 when comparing vehicle- versus BPA-treated groups, as #*p* < 0.05, ##*p* < 0.01, ###*p* < 0.001, and *****p* < 0.0001 when comparing the effect of inhibitor pretreatments or siRNA knockdowns within vehicle- or BPA-treated groups, and as +*p* < 0.05, ++*p* < 0.01, +++*p* < 0.001, and ++++*p* < 0.0001 when comparing the interaction between two factors. *n* values refer to independent experimental replicates.

Fig. 1. BPA increases *Agrp* mRNA expression in hypothalamic cell lines. **a** mHypoA-59 cells treated with 10 or 100 μ M BPA or vehicle (0.05% ethanol) for 4 h ($n = 3$). **b** mHypoE-41 cells treated with 10, 25, 50, or 100 μ M BPA or vehicle for 4 h ($n = 4$). **c** mHypoA-59 cells treated with 10 or 100 μ M BPA or vehicle for 4 or 24 h, followed by collection of both cells and media to determine BPA content ($n = 3$ –4). **d** Primary culture derived from the hypothalamus of male CD-1 mice treated with 100 μ M BPA or vehicle for 8 h ($n = 4$). **e** Six *Agrp*-expressing cell lines treated with 100 μ M BPA or vehicle for 2, 4, 8, 16, or 24 h ($n = 3$ –6). Time-matched vehicles are repre-

sented by the dotted line at $y = 1$ and asterisks indicate significant difference compared with the time-matched vehicle. *Agrp* gene expression was analyzed using qRT-PCR. Data are expressed as mean $\pm$ SEM, and statistical significance was determined using Student *t* test (**d**), one-way ANOVA (**a**, **b**), or two-way ANOVA (**c**, **e**), followed by Bonferroni post hoc test (**a**, **b**, **e**) or Tukey multiple comparison test (**c**). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. BPA, bisphenol A; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction.

Results

BPA Increases *Agrp* mRNA Levels in Several Hypothalamic Cell Lines

To characterize the effects of BPA and establish the optimal treatment dose, adult-derived mHypoA-59 cells were treated with 10 or 100 μ M BPA for 4 h; 100 μ M BPA increased *Agrp* expression in mHypoA-59 cells (Fig. 1a),

while 10 μ M BPA did not alter *Agrp* expression in the embryonic-derived mHypoE-41 cells; however, 25, 50, and 100 μ M BPA increased *Agrp* mRNA in this cell line (Fig. 1b). These results suggested that BPA does act as an orexigen in both adult- and embryonic-derived hypothalamic neurons, albeit at a higher concentration than what is reported in humans [29, 30].

For this reason, we aimed to determine the amount of BPA that was able to enter the neurons in culture. BPA may not readily dissolve in aqueous culture medium and enter cells due to its lipophilic nature [31]. To determine the percentage of BPA in the medium that enters hypothalamic neurons, an enzyme-linked immunosorbent assay was performed to determine the concentration of BPA within the cells and in the culture medium 4 and 24 h after treatment. At 4 h, 2 and 10% of the total measured BPA was found within the cells when treated with 10 and 100 μM BPA, respectively, or an equivalent concentration of 200 nM and 10 μM . At 24 h, 14% with the 10- μM treatment and 18% with the 100- μM treatment of the total BPA content was found within the cells (1.4 and 18 μM ; Fig. 1c). Thus, only a fraction of the BPA that is dissolved into the culture medium actually enters the hypothalamic cell, indicating a lower exposure concentration than the apparent treatment concentration. Furthermore, with lower treatment concentration, there is a lower percentage of entry into the cells. Accordingly, when mHypoA-59 cells were treated with 10 and 100 μM BPA (Fig. 1a), only the higher apparent concentration of 100 μM was able to increase *Agrp* expression. We therefore chose to use 100 μM BPA in our subsequent experiments to ensure adequate and consistent entry of BPA into hypothalamic cells in culture.

Treatment with 100 μM BPA for 8 h in hypothalamic primary culture derived from 8-week-old male mice (Fig. 1d) and in six hypothalamic cell lines from 2 to 24 h (Fig. 1e) revealed consistent upregulation of *Agrp* across cell models, again illustrating the orexigenic nature of BPA across an array of AgRP neurons. In embryonic-derived cell lines (mHypoE-), the upregulation occurred from as early as 2 h until 8 or 16 h, whereas in adult cell lines (mHypoA-), *Agrp* was significantly upregulated at 8 h alone (Fig. 1e).

Fig. 2. BPA increases *pre-Agrp* mRNA levels and associated transcription factors. **a, b** mHypoA-59 (**a**) or mHypoE-41 cells (**b**) pre-treated with transcriptional inhibitor 10 $\mu\text{g}/\text{mL}$ ActD or 0.1% DMSO for 1 h, followed by treatment with 100 μM BPA or vehicle (0.05% ethanol) for 8 h ($n = 4$). **c** Schematic of the locations of primers used to amplify mature *Agrp* and *pre-Agrp* using qRT-PCR. **d–f** mHypoA-59 or mHypoE-41 cells treated with 100 μM BPA or vehicle for 2, 4, or 8 h (same samples as in Fig. 1e; $n = 3–5$). Time-matched vehicles are represented by the dotted line at $y = 1$ and asterisks indicate significant difference compared with the time-matched vehicle. Gene expression was analyzed using qRT-PCR. **g, h** mHypoA-59 cells treated with 100 μM BPA or vehicle for 2, 4, or 8 h for protein quantification ($n = 3–4$ for CHOP, $n =$

BPA Increases Transcription of *Agrp* mRNA and Associated Transcription Factors

The mechanism underlying BPA-induced *Agrp* expression may be a result of transcription factor binding and transcriptional activation at the promotor level, or a result of increased stability of the transcript if there is no de novo transcription occurring. To test this, the transcriptional inhibitor ActD was used prior to treatment with BPA. ActD decreased basal and BPA-induced levels of *Agrp* at 8 h in the mHypoA-59 and mHypoE-41 cell lines, indicative of the inhibition of de novo *Agrp* transcription and subsequent mRNA degradation. Furthermore, in the presence of ActD, BPA did not increase *Agrp* mRNA levels compared to vehicle, suggesting that transcription is required for the BPA-induced upregulation of *Agrp* (Fig. 2a, b). To determine whether *Agrp* itself is regulated at the transcriptional level, changes in a region containing an intronic portion of *Agrp* (*pre-Agrp*) were measured (Fig. 2c). The expression of *pre-Agrp* mRNA was upregulated by BPA at 2 h in the mHypoA-59 cells (Fig. 2d) and at 2 and 8 h in the mHypoE-41 cells (Fig. 2e), suggesting de novo transcription of *Agrp*.

Given that BPA increases transcription of *Agrp*, we hypothesized that the mechanism of BPA action depends on one or more transcription factors that regulate *Agrp* at the promoter level.

We took two approaches to answer this question. First, we investigated transcription factors that are known to regulate *Agrp*: *Foxo1*, and *Stat3* [18]. Second, we had previously found that BPA-induced pathways, including circadian dysregulation and neuroinflammation, did not mediate its effects on *Agrp*. ER stress is often activated by BPA [22] and is tightly linked to hypothalamic dysregulation seen in obesity [24]. Furthermore, ER stress activates a myriad of transcription factors that may directly activate *Agrp* transcription or interact with *Agrp*-activating transcription factors [23]. As such, we first determined

2 for ATF3). Protein levels were analyzed using Western blotting and representative blots from one experimental replicate are shown. No bands for CHOP or ATF3 appeared in vehicle-treated samples, thus only BPA-treated samples were quantified by densitometry. Data are expressed as mean $\pm$ SEM (except for **h**), and statistical significance was determined using two-way ANOVA followed by Tukey multiple comparison test (**a, b**) or Bonferroni post hoc test (**d–f**) or one-way ANOVA followed by Bonferroni post hoc test (**g**). Vehicle versus BPA: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; DMSO versus ActD: ## $p < 0.01$, ### $p < 0.001$, **** $p < 0.0001$; interaction: ++ $p < 0.01$. ActD, actinomycin D; BPA, bisphenol A; DMSO, dimethyl sulfoxide; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction.

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whether these transcription factors are altered with BPA treatment. In both the mHypoA-59 and mHypoE-41 cells, *Foxo1* and *Stat3* as well as the stress-responsive transcription factors *Chop*, *Atf3*, *Atf4*, and *Atf6* were increased with 100 μ M BPA treatment from 2 to 8 h (Fig. 2f). *Atf3* and *Chop* had the greatest fold change of approximately 10-fold (both cell lines) and 8- or 5-fold (mHypoA-59 and mHypoE-41, respectively) at 8 h (Fig. 2f). The protein levels of both CHOP and ATF3 were also increased, indicating their potential to bind to and alter transcription at the promoter level (Fig. 2g, h). As *Stat3* levels were only modestly increased, rather than decreased as would have been expected from previous studies [18], we did not follow up on the potential role of this transcription factor in BPA-mediated *Agrp* upregulation.

Knockdown of ATF3 Blocks the BPA-Induced Increase in Agrp and pre-Agrp mRNA

To determine whether the BPA-mediated increase in *Agrp* was dependent on any of the above-mentioned transcription factors, siRNA-mediated knockdown was performed (Fig. 3a), and the transcription and translation of each transcription factor was significantly decreased with siRNA exposure. Knockdown of ATF3 in the mHypoA-59 cells increased basal expression of *Agrp*, but blocked an increase in *Agrp* with BPA (Fig. 3b), suggesting that ATF3 is necessary for the BPA-mediated orexi-genic effect. Interestingly, knockdown of FOXO1, CHOP, ATF4, and ATF6 did not prevent the upregulation of *Agrp* by BPA (Fig. 3b). ATF3 knockdown also prevented BPA-mediated *Agrp* induction in the embryonic-derived mHypoE-41 cell line (Fig. 3c, d). Similar results were seen with *pre-Agrp* mRNA (Fig. 3e), suggesting that ATF3 is required for BPA-induced de novo transcription of *Agrp*.

BPA Does Not Increase ATF3 Binding to Agrp Regulatory Elements

ATF3 is a transcription factor that can either bind ATF:CRE elements or dimerize with other transcription

factors, including FOXO1, CEBP β , p53, and CREB among several others [28]. The ATF3:FOXO1 dimer has been previously validated to bind to and increase transcription of *Agrp* in response to low glucose conditions [25]. Alternatively, ATF:CRE, AP1, and CEBP sites were found to be the most common ATF3 binding sites in the entire genome [28]. Several AP1 binding sites are present in the *Agrp* regulatory region. We identified a potential ATF:CRE (TGACGTCA) and a potential CEBP (TGTT-GCAAT) site in the distal regulatory region of *Agrp* (Fig. 4a).

ChIP experiments revealed that increased ATF3 binding to the previously validated FOXO1 binding sites on *Agrp* did not occur with 100 μ M BPA treatment for 1 or 4 h (Fig. 4b, c). Relative binding of ATF3 was greater than IgG at the ATF:CRE site at 1 h, suggesting a basal degree of ATF3 binding to this region. However, BPA did not increase ATF3 binding at the ATF:CRE site or the CEBP site in the *Agrp* distal regulatory region (Fig. 4b). Thus, an alternative binding site within the large 43-kb *Agrp* promoter is likely involved. ATF3 is known to bind to its own promoter, which was thus used as a positive control for the antibody with previously validated primers [32]. With 4 or 8 h of BPA treatment, ATF3 binding to the ATF3 promoter was increased (Fig. 4c).

BPA Induces ER Stress, JNK, ERK, and AMPK, but Does Not Require ER Stress, JNK, or ERK to Increase Agrp mRNA

The factors upstream of ATF3, leading to its induction and ultimately the increase in *Agrp*, remain unknown. Treatment with ActD suggests that transcription of (a) gene(s) is required for the increase in *Atf3* (Fig. 5a). MAP kinases and ER stress are well-characterized regulators of ATF3, with ATF4 being the transcription factor directly upstream of ATF3 in the ER stress pathway [33, 34]. Interestingly, in the mHypoA-59 cells, although ATF4 knockdown decreased the basal levels of ATF3, it did not prevent the BPA-mediated increase in

Fig. 3. ATF3 knockdown prevents BPA-mediated increase in *Agrp* and *pre-Agrp* mRNA. **a, b, e** mHypoA-59 cells transfected with siRNA duplexes targeting *Foxo1*, *Chop*, *Atf3*, *Atf4*, or *Atf6* or negative control (NC) for 24 h, followed by treatment with 100 μ M BPA or vehicle (0.05% ethanol) for 8 h ($n = 3-5$). **c-e** mHypoE-41 cells transfected with a siRNA duplex targeting *Atf3* or NC for 24 h, followed by treatment with 100 μ M BPA or vehicle for 8 h ($n = 6$). siRNA knockdown of the various transcription factors (**a**) or ATF3 (**c**) was confirmed using qRT-PCR and Western blotting prior to analyzing changes in *Agrp* (**b, d**) or *pre-Agrp*

(**e**) mRNA levels using qRT-PCR. Representative blots from one experimental replicate of the siRNA knockdown validation are shown below the qRT-PCR validation (**a, c**). Data are expressed as mean $\pm$ SEM, and statistical significance was determined using two-way ANOVA followed by the Tukey multiple comparison test (**a-e**). Vehicle versus BPA: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; NC versus siRNA: # $p < 0.05$, ## $p < 0.01$, ### $p < 0.0001$; interaction: + $p < 0.05$, ++ $p < 0.01$, +++ $p < 0.001$, ++++ $p < 0.0001$. BPA, bisphenol A; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction.

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ATF3 (Fig. 5b), suggesting that akin to *Agrp* (Fig. 3b), BPA acts independently of ATF4 to upregulate *Atf3*. We next investigated whether common upstream regulators of ATF3 may mediate the BPA-induced changes in *Agrp*. As BPA induces ER stress (Fig. 2f), we used the generalized chemical chaperone PBA to mitigate ER stress.

However, PBA did not significantly abolish the BPA-mediated upregulation of *Agrp* (Fig. 5c), suggesting that this effect is independent of ER stress. The MAP kinases JNK and ERK were also induced with BPA (Fig. 5d, e, g), but inhibition of each using SP600125 (JNK) or PD0352901 (ERK) did not block the increase in *Agrp*

Fig. 4. BPA does not increase ATF3 binding to specific *Agrp* regulatory regions. **a** Schematic of transcription factor binding sites in the *Agrp* regulatory region. Circles and sequences represent potential ATF3 binding sites. Boxes represent the sites encompassed by each primer pair used for ChIP (**b**, **c**). **b**, **c** mHypoA-59 cells treated with 100 μ M BPA or vehicle (0.05% ethanol) for 1 h ($n = 3$ –5; **b**) or 4 h ($n = 3$; **c**), followed by ChIP with an ATF3 antibody or IgG. Samples

were treated for 4 or 8 h to determine binding of ATF3 to the ATF3 promoter. The level of ATF3 or IgG binding to each region was assayed using qRT-PCR. Data are expressed as mean $\pm$ SEM, and statistical significance at each site was determined using one-way ANOVA followed by the Bonferroni post hoc test (**b**, **c**). * $p < 0.05$. BPA, bisphenol A; ChIP, chromatin immunoprecipitation; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction.

Fig. 5. BPA induces ER stress, JNK, ERK, and AMPK, but does not require ER stress, JNK, or ERK to increase *Agrp* mRNA. **a** mHypoA-59 or mHypoE-41 cells pretreated with transcriptional inhibitor 10 μ M ActD or 0.1% DMSO for 1 h, followed by treatment with 100 μ M BPA or vehicle (0.05% ethanol) for 8 h ($n = 4$). **b** mHypoA-59 cells transfected with a siRNA duplex targeting *Atf4* or negative control (NC) for 24 h, followed by treatment with 100 μ M BPA or vehicle for 8 h ($n = 3$). **c** mHypoA-59 or mHypoE-41 cells pretreated with H₂O, 5 mM PBA, or 10 mM PBA for 1 h prior to treatment with 100 μ M BPA or vehicle for 8 h. **d**, **e** mHypoA-59 (**d**) and mHypoE-41 cells (**e**) serum starved for 1 h and treated with vehicle, 10 μ M BPA, or 100 μ M BPA for 15 or 30 min. **f**, **h** mHypoA-59 or mHypoE-41 cells pretreated with 0.1% DMSO or 50 μ M SP600125 (SP; JNK inhibitor) (**f**) or 0.1% DMSO or 10 μ M PD0352901 (PD; MEK/ERK inhibitor) (**h**) for 1 h, followed by treatment with 100 μ M BPA or vehicle for 8 h. **g** mHypoA-

59 cells serum starved for 1 h and treated with 100 μ M BPA or vehicle for 5 min. **i** mHypoA-59 cells treated with 100 μ M BPA or vehicle for 8 h. **j** mHypoA-59 cells treated with 500 μ M AICAR (AMPK activator) for 9 h. Gene expression was analyzed using qRT-PCR. Protein levels were analyzed using Western blotting and representative blots from one experimental replicate are shown. Data are expressed as mean $\pm$ SEM, and statistical significance was determined using two-way ANOVA followed by Tukey multiple comparison test (**a**–**c**, **f**, **h**) or by Bonferroni post hoc test (**d**, **e**) or using the Student *t* test (**g**, **i**, **j**). Vehicle versus BPA/H₂O versus AICAR: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; NC versus siRNA/H₂O/DMSO versus inhibitor: # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, **** $p < 0.0001$; interaction: **** $p < 0.0001$. ActD, actinomycin D; AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; BPA, bisphenol A; DMSO, dimethyl sulfoxide; ER, endoplasmic reticulum; ns, not significant.

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with BPA (Fig. 5f, h). Finally, the energy sensor AMPK can upregulate ATF3 [35]. Remarkably, BPA increased AMPK phosphorylation at 8 h in the mHypoA-59 cells (Fig. 5i), and AMPK activation using AICAR increased *Agrp* expression, pointing to a potential molecular link between BPA, ATF3, and *Agrp*.

ATF3 Is Also Involved in the BPA-Mediated Increase in *Npy* Expression

Given that ATF3 plays a crucial role in regulating BPA-induced *Agrp* expression, we questioned whether it affects the expression of the other major orexigenic neuropeptide in the arcuate nucleus of the hypothalamus, *Npy*. We have

Fig. 6. ATF3 knockdown attenuates the BPA-mediated increase in *Npy* mRNA expression. mHypoA-59 cells (**a**) ($n = 3$) or mHypoE-41 cells (**a, b**) ($n = 6$ for *Npy*, $n = 3$ for *Bmal1*) transfected with a siRNA duplex targeting *Atf3* or negative control (NC) for 24 h, followed by treatment with 100 μ M BPA or vehicle for 8 h. *Npy* (**a**) and *Bmal1* (**b**) mRNA expression was measured using qRT-PCR. Data are expressed as mean $\pm$ SEM, and statistical significance was determined using two-way ANOVA followed by Tukey multiple comparison test (**a, b**). Vehicle versus BPA: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; NC versus siRNA: # $p < 0.05$, ## $p < 0.01$; interaction: + $p < 0.05$. BPA, bisphenol A; ns, not significant; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction.

previously shown that BPA-induced *Npy* upregulation, but not *Agrp* or *Pomc*, requires the circadian regulator and transcription factor BMAL1 [15]. Knockdown of ATF3 decreased the basal levels of *Npy* in both vehicle- and BPA-treated groups in the mHypoA-59 cells (Fig. 6a). In the mHypoE-41 cells, the BPA-induced upregulation of *Npy* was prevented with ATF3 knockdown (Fig. 6a). Prevention of the *Npy* increase by BPA occurred with a concurrent decrease in *Bmal1* expression when mHypoE-41 cells were treated with BPA in the presence of ATF3

siRNA (Fig. 6b). These results suggest that ATF3, at least in part, also plays a role in the BPA-mediated increase in *Npy* expression in hypothalamic cells.

Discussion

The obesogenic effects of BPA have been described with respect to peripheral metabolism [2, 7, 36]; however, how BPA affects the hypothalamic neurons that control whole-body energy homeostasis is far less studied. In fact, BPA has been shown to accumulate in the human hypothalamus [8], highlighting the importance of studying the hypothalamic effects of BPA. Here, we add to the current pool of evidence that BPA affects the hypothalamic NPY/AgRP neurons by describing that BPA increases de novo transcription of the orexigenic neuropeptide *Agrp* in hypothalamic neurons through an ATF3-dependent mechanism. We also demonstrate that BPA can alter *Agrp* expression in not only embryonic-derived but also adult-derived neurons using similar mechanisms, underscoring the importance of studying the effects of BPA in adulthood. We further describe that ATF3 is also an important regulator of the BPA-mediated induction of *Npy*, pointing to a common mediator of appetite-increasing effects of BPA in the hypothalamus.

Perinatal exposure to BPA in rodents increases body weight [13], leads to pancreatic dysfunction [37], increases *Agrp* mRNA levels when combined with a high-fat diet [13], and upregulates AgRP protein levels in neural progenitor cells in vivo and in vitro [12]. In line with this evidence, BPA increased *Agrp* mRNA expression in four embryonic-derived cell lines. However, BPA increased *Agrp* expression in adult-derived cell lines and importantly adult-derived hypothalamic primary culture, suggesting that adult neurons are also susceptible to BPA-mediated dysregulation of the neuropeptides controlling energy homeostasis, albeit perhaps to a lesser degree, as both the magnitude and the temporal response of increased *Agrp* were limited in the adult-derived cells. Pubertal or adult exposure to BPA has also been shown to have detrimental consequences in rodent models. For instance, 5-week-old rodents exposed to dietary BPA had increased body weights [7]. Adult-rodent exposure to BPA also led to abnormal insulin production in the pancreas [38], insulin resistance in the muscle [39], and triglyceride accumulation in the liver [40]. Furthermore, urine levels of BPA are correlated with body mass index in children [41] as well as adults [42], suggesting that acute adulthood exposure to BPA is also harmful. Never-

theless, it is important to note that the timing of exposure during development or in adulthood plays a significant role in the effects of BPA; for example, perinatal exposure starting only at embryonic day 18 and subsequent postnatal exposure up to postnatal day 7 altered neither body weight nor *Npy* or *Agrp* expression in the hypothalamus of adult female rats [43].

BPA concentrations were initially thought to be in the low nM range in human urine samples (~5 nM) [30]. However, new measurement techniques [29] and the fact that BPA accumulates in tissues [44] have indicated that BPA concentrations may be higher than initially thought. Gerona et al. [29] demonstrated that older detection methods largely underestimated the levels of BPA metabolites in human urine samples, and reported mean concentrations to be approximately 230 nM, with BPA as high as 3 μ M detected in human urine. Our treatment concentration of 100 μ M was much higher than the reported human concentrations. The relevance of the concentration of BPA and other hormones such as estrogen used in in vitro studies is a primary question. As an example, circulating concentrations of 17 β -estradiol are in the picomolar range [45], while in vitro experiments often require 10–100 nM (1,000-fold greater) to detect a significant effect on function [46]. This is in part due to the lipophilic nature of these compounds, which would be appropriate for circulation and fat storage, but the inherent aqueous environment of cell culture experiments may not be conducive to easy access to the neuron. We therefore quantified how much BPA entered the neurons, with the assumption that BPA predominantly affects cells by entering through the plasma membrane rather than via cell surface receptor activation [47]. One exception to this assumption is the membrane-bound estrogen receptor GPER1. Although primarily characterized as residing on the ER membrane [48–50], GPER1 also localizes to the plasma membrane [51], and BPA has been shown to act through this receptor [52]. However, in both mHypoA-59 and mHypoE-41 cells, a GPER1 antagonist, G15, did not block the BPA-mediated induction of *Agrp* (data not shown), ruling out the involvement of both plasma-membrane and ER-membrane bound GPER1. Besides GPER1, the majority of mechanisms through which BPA affects cells have been intracellular in nature [47]. Based on our quantification, only a fraction of BPA entered the hypothalamic neurons in culture (10 μ M at 4 h, 18 μ M at 24 h), suggesting that the exposure concentration was much less than the treatment concentration. Furthermore, the percentage decreased with lower concentrations of BPA, alluding to

the need to use higher concentrations to ensure adequate cellular entry.

ATF3 is a stress-responsive transcription factor of the ATF/CREB family and has been directly linked to the transcriptional upregulation of *Agrp* in response to low glucose [25]. This effect occurs through binding of ATF3 to FOXO1 and then binding of both to FOXO1-responsive elements on the *Agrp* promoter [25]. Here, we describe a FOXO1-independent effect of ATF3 since knockdown of FOXO1 did not block the increase in *Agrp* and ATF3 did not show increased binding to the FOXO1 sites in the *Agrp* promoter. This FOXO1-independent effect could either occur due to ATF3 homodimer binding, ATF3 dimerizing with another bZIP transcription factor, followed by promoter activation, or through ATF3 altering the expression and/or binding of another transcription factor [28]. The latter may be true for *Npy*. We have previously linked the transcription factor BMAL1 to BPA-induced upregulation of *Npy* and shown that BPA induces increased BMAL1 binding at the *Npy* promoter [15]. The mechanism by which BPA increases BMAL1 expression and binding was not elucidated. We show here that when ATF3 is knocked down, BPA leads to a downregulation of *Bmal1* expression in the mHypoE-41 cell line. Early growth response 1 is a primary target of ATF3 [53] and is required for the daily rhythm of *Bmal1* in the suprachiasmatic nucleus of mice [54]. Thus, although direct promoter binding of ATF3 cannot be ruled out, it is possible that ATF3 may, in part, mediate BMAL1 binding to the *Npy* promoter with BPA exposure.

In terms of *Agrp*, either or both forms of regulation may be involved. In a study investigating the consensus sequences enriched for ATF3 binding across the human genome, Zhao et al. [28] described that 81% of ATF3 binding sites contained the ATF:CRE, AP1, or CEBP:ATF consensus sequences. In fact, over half of these binding sites were in distal or enhancer regions far from gene transcriptional start sites [28]. In our study, we determined whether there was increased binding of ATF3 to two putative ATF3 binding sites in the distal regulatory region of *Agrp* with BPA treatment. Although increased binding with BPA did not occur at either site, there was basal binding at the ATF:CRE site, as compared with IgG in the mHypoA-59 cells. Given that ATF3 knockdown increased *Agrp* mRNA levels in vehicle-treated mHypoA-59 cells, it is plausible that ATF3 binds and represses *Agrp* under unstimulated conditions. Similarly, although *Agrp* expression itself was not studied, knocking out ATF3 in LepRb neurons led to increased body weight in female mice [55], suggesting that ATF3 may contribute to maintaining basal energy homeostasis.

With low glucose [25] or with BPA exposure, the changing transcription factor pool may result in different factors that then interact with ATF3 and change its transcriptional activity. Interestingly, Zhao et al. [28] found that many genes whose expression was regulated by a stress event, such as DNA damage, were prebound by ATF3, and other transcription factors can then be recruited to these sites. Thus, the idea that prebound ATF3 (promoter bookmarking/priming) at this site may recruit and bind to alternative transcription factors to increase transcription cannot be ruled out for *Agrp*. Nevertheless, depending on the binding site, stress-induced elevation of ATF3 can lead to decreased binding of ATF3 to the DNA, and binding of ATF3 activates and represses an approximately equal number of genes, demonstrating the complexity in ATF3-induced regulation [28]. Upon ATF3 knockdown, basal mRNA levels of *Agrp* and *Npy* were significantly altered in the mHypoA-59 cells but remained unchanged in the mHypoE-41 cells. This may be due to the presence of specific transcription factors in the adult-derived cell line that interact with ATF3 to control basal levels of *Agrp* and *Npy* expression.

Curiously, *Stat3* expression was increased with BPA treatment in our experiment and BPA has been shown to induce STAT3 phosphorylation in adipocytes and macrophages [56, 57]. Both *Stat3* overexpression and phosphorylation would be expected to suppress *Agrp* expression [18, 58], suggesting that in these hypothalamic neurons, BPA increases *Agrp* independently of STAT3 or that the effects of other transcription factors override the effect of STAT3. In fact, although *Stat3* overexpression directly increases *Agrp* transcription in in vitro reporter assays [18] and *Stat3* knockdown is correlated with an increase in *Agrp*, STAT3 may not play a significant role in direct *Agrp* transcription in vivo as initially thought [59]. The *Stat3* induction in hypothalamic neurons in response to BPA may occur secondary to inflammatory cytokine signaling [16], which routinely activates STAT3 [56, 57] and occurs independently to *Agrp* transcription.

Other transcription factors that directly upregulate *Agrp* that we did not investigate here include the glucocorticoid receptor, brain-specific homeobox (BSX), and Krüppel-like factor 4 (KLF4). BPA is known to bind to and activate several nuclear receptors, including the glucocorticoid receptor [60]. However, the glucocorticoid receptor antagonist RU486 did not block the BPA-mediated increase in *Agrp* in the mHypoA-59 cells (data not shown). BSX is a transcription factor, highly expressed in the arcuate nucleus of the hypothalamus, and binds to the *Agrp* promoter adjacent to the FOXO1 binding sites [61, 62]. It has also been shown to interact with both FOXO1

and CREB to upregulate *Agrp* transcription [62]. KLF4 is a zinc finger transcription factor that binds to CACCC element in the *Agrp* promoter [63]. Interestingly, it has been described to activate transcription of *Atf3* in human colorectal cancer cells [64]. Whether BSX and KLF4 are altered with BPA in hypothalamic cells and whether they independently and/or with ATF3 contribute to BPA-mediated *Agrp* warrants further investigation.

As a stress-responsive transcription factor, ATF3 is activated by a myriad of upstream pathways and kinases. For instance, ER stress induces ATF3, along with other classic ER stress-related factors, such as ATF4 and ATF6 [23]. Belonging to the same family, these have similar consensus sequences for promoter binding. However, the DNA binding profile and gene regulation profile of ATF3 is distinct from ATF4 and ATF6, largely due to its ability to interact with a variety of other transcription factors [28]. This is corroborated by our findings that although BPA induced ER stress, ATF3, ATF4, and ATF6, the BPA-mediated increase in *Agrp* is only dependent on ATF3. The MAP kinases JNK and ERK were also activated with BPA treatment and are known upstream regulators of ATF3 [34]. However, in our study, the BPA-mediated upregulation of *Agrp* or *Atf3* (data not shown) was not blocked by JNK and ERK pathway inhibition. Interestingly, there was a nonsignificant trend in the mHypoA-59 cells, where the magnitude of upregulation in *Agrp* was decreased in the presence of the JNK inhibitor and the chemical chaperone PBA, whereas this did not occur in the mHypoE-41 cells. Again, this may allude to the greater vulnerability of the *Agrp* system in embryonic neurons to BPA treatment as blocking these pathways may, in part, alleviate some of the BPA-induced alterations in the mHypoA-59 neurons.

Examining the mechanisms by which nutrient signals regulate *Agrp* may give clues as to how BPA may be regulating *Agrp*. Amino acid deprivation increases *Agrp* and ATF3 [65]. Amino acid supplementation decreases levels of *Agrp* in hypothalamic neurons via mTOR activation [20]. mTOR is active in states of high energy, while AMPK activation in the hypothalamus occurs during states of low energy, such as fasting. In fact, constitutively active AMPK has been shown to increase *Agrp* [66], and ATF3 is a downstream target of AMPK [35]. We therefore evaluated whether BPA leads to AMPK activation and found that BPA increases AMPK phosphorylation and thus activation in mHypoA-59 cells. Furthermore, activation of AMPK using AICAR led to an increase in *Agrp* mRNA expression. These results suggest that BPA may mimic a state of low energy in hypothalamic neurons, leading to the induction of AMPK, ATF3, and ultimately orexigenic

Agrp mRNA expression. Future experiments using inhibitors of AMPK activity are required to confirm the relationship between AMPK, ATF3, and *Agrp* as AMPK can also target other factors, including BSX [67] and KLF4 [68], which can lead to the increase in *Agrp*.

In addition to increasing food intake, AgRP neurons also act as the main center of actions of the satiety factors insulin and leptin and maintain whole-body glucose homeostasis by suppressing gluconeogenesis [63]. Given that insulin and leptin both act on AgRP neurons to mediate satiety by directly decreasing *Agrp* transcription, the fact that BPA leads to de novo *Agrp* transcription may override the effects of such satiety factors. In fact, insulin and leptin resistance have both been described with BPA exposure [69, 70].

In conclusion, we describe for the first time that BPA increases de novo transcription of *Agrp* through an ATF3-dependent mechanism in hypothalamic neurons. ATF3 is also, at least in part, involved in the BPA-mediated increase in *Npy*, suggesting a common, targetable mediator to alleviate the orexigenic effects of BPA in the hypothalamus.

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Statement of Ethics

All animal procedures were conducted in accordance with the regulations of the Canadian Council on Animal Care and approved by the University of Toronto Animal Care Committee.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

N. Loganathan and E.K. McIlwraith designed and performed the experiments and analyzed the data. N. Loganathan wrote the first draft of the manuscript. D.D. Belsham conceived the study, obtained funding, designed the study, and interpreted the data. All authors edited the manuscript and read and approved the final manuscript.

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