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Up-regulation of *POMC* and *CART* mRNAs by intermittent hypoxia via GATA transcription factors in human neuronal cells

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ABSTRACT

Sleep apnea syndrome (SAS) is characterized by intermittent hypoxia (IH) during sleep. SAS and obesity are strongly related to each other. Here, we investigated the effect of IH on the expression of major appetite regulatory genes in human neuronal cells. We exposed NB-1, SH-SY5Y, and SK-N-SH human neuronal cells to IH (64 cycles of 5 min hypoxia and 10 min normoxia), normoxia, or sustained hypoxia for 24 h and measured the mRNA levels of *proopiomelanocortin* (*POMC*), *cocaine- and amphetamine-regulated transcript* (*CART*), *galanin*, *galanin-like peptide*, *ghrelin*, *pyroglutamylated RFamide peptide*, *agouti-related peptide*, *neuropeptide Y*, and *melanocortin 4 receptor* by real-time RT-PCR. IH significantly increased the mRNA levels of *POMC* and *CART* in all the neuronal cells. Deletion analysis revealed that the −705 to −686 promoter region of *POMC* and the −950 to −929 region of *CART* were essential for the IH-induced promoter activity. As possible GATA factor binding sequences were found in the two regions, we performed real-time RT-PCR to determine which *GATA* family members were expressed and found that *GATA2* and *GATA3* mRNAs were predominantly expressed. Therefore, we introduced siRNAs against *GATA2* and *GATA3* into NB-1 cells and found that *GATA2* and *GATA3* siRNAs abolished the IH-induced up-regulation of both *POMC* and *CART* mRNAs. These results indicate that IH stress up-regulates the mRNA levels of anorexigenic peptides, POMC and CART, in human neuronal cells via GATA2 and GATA3. IH can have an anorexigenic effect on SAS patients through the transcriptional activation of *POMC* and *CART* in the central nervous system.

1. Introduction

Sleep apnea syndrome (SAS) is a common disorder characterized by repetitive episodes of oxygen desaturation during sleep, the development of daytime sleepiness, and the deterioration of quality of life (Dempsey et al., 2010). SAS is caused by the obstruction of the upper airway, and moderate-to-severe cases of SAS affect 10–17% of men and 3–9% of women aged between 30 and 70 years (Peppard et al., 2013). During sleep, the repeated upper airway obstruction in SAS patients can cause serious recurrent apnea, and it causes these patients to be exposed to alternating low oxygen pressure and normal oxygen pressure, that is, intermittent hypoxia (IH) (Ota et al., 2015).

Hypothalamus is a key brain area that controls energy homeostasis. Particularly, the hypothalamic arcuate nucleus (ARC) is considered one of the best-characterized areas of the brain involved in the regulation of feeding behavior through the close coordination among the multiple neuronal populations (Gautron et al., 2015). The ARC contains two main neuronal populations with opposite effects on the feeding behavior, namely, the orexigenic neuropeptide Y/agouti-related peptide (NPY/AGRP)-expressing neurons and the anorexigenic proopiomelanocortin/cocaine- and amphetamine-regulated transcript (POMC/CART)-expressing neurons, both of which constitute the central

Abbreviations: AGRP, agouti-related peptide; ARC, hypothalamic arcuate nucleus; BMI, body mass index; CART, cocaine- and amphetamine-regulated transcript; CNS, central nervous system; FCS, fetal calf serum; GAL, galanin; GALP, galanin-like peptide; GATA, GATA-binding factor; GHRL, ghrelin; IH, intermittent hypoxia; MC4R, melanocortin 4 receptor; NPY, neuropeptide Y; OSA, obstructive sleep apnea; POMC, proopiomelanocortin; QRFP, pyroglutamylated RFamide peptide; RT-PCR, reverse transcription-polymerase chain reaction; ROS, reactive oxygen species; SAS, sleep apnea syndrome; SH, sustained hypoxia; siRNA, small interfering RNA

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melanocortin system with downstream target neurons expressing the melanocortin 3 receptor (MC3R) and melanocortin 4 receptor (MC4R) (Schneeberger et al., 2014). NPY/AGRP neurons are inhibited by leptin, insulin, and an enteric hormone Peptide YY_{3-36}, and they are stimulated by ghrelin (GHRL), an orexigenic hormone released from gastric mucosa (Flier, 2004). In addition to these appetite regulatory players, galanin (GAL) is an orexigenic neuropeptide expressed by majority of the noradrenergic neurons in many tissues throughout the body, including the hypothalamus (Robinson and Brewer, 2008). Pyroglutamylated RFamide peptide (QRFP) is also an orexigenic neuropeptide produced in cells of the paraventricular and ventromedial nuclei of the hypothalamus in humans (Bruzzone et al., 2006). Galanin-like peptide (GALP) is a neuropeptide responsible for energy homeostasis discovered in the porcine hypothalamus. *GALP* mRNA was also detected in the human brain, and it has both species- and time-dependent effects on feeding and body weight in rodents (Lawrence and Fraley, 2011).

In the present study, using human neuronal cells and an *in vitro* IH system, which is a controlled gas delivery system that regulates the flow of nitrogen and oxygen to generate IH, we investigated the effect of IH, a hallmark of SAS, on the expression(s) of major appetite regulatory neuropeptide and receptor genes such as *POMC*, *CART*, *GAL, GALP*, *GHRL*, *QRFP*, *AGRP*, *NPY*, and *MC4R*. We also explored the gene regulatory mechanism in human neuronal cells under the influence of IH.

2. Materials and methods

2.1. Cell culture

We used NB-1, SH-SY5Y and SK-N-SH human neuroblastoma cells. NB-1 cells were maintained in RPMI1640 medium (nacalai tesque, Kyoto, Japan) containing 10% FCS, 100 units/mL penicillin G (Wako), and 100 μg/mL streptomycin (Wako) (Itoh et al., 1983; Nakazawa et al., 2005). SH-SY5Y and SK-N-SH cells were obtained from RIKEN BioResource Center (Tsukuba, Japan) and were maintained in DMEM medium (Wako Pure Chemical Industries, Ltd., Osaka, Japan) with 10% fetal calf serum (FCS), 100 units/mL penicillin G (Wako), and 100 μg/mL streptomycin (Wako). The cells were cultured at 37 °C under 5% CO_2.

We exposed the NB-1, SH-SY5Y, and SK-N-SH cells to normoxia (21% O_2, 5% CO_2, and balance N_2), sustained hypoxia (SH: 1% O_2, 5% CO_2, and balance N_2), or intermittent hypoxia (IH: 64 cycles of 5 min SH and 10 min normoxia), mimicking the SAS environment in 24-well plate (2×10^5 cells/mL, 0.5 mL/dish) for 24 h using a custom-designed, computer-controlled incubation chamber attached to an external O_2–CO_2–N_2 computer-driven controller (O_2 programmable control, 9200EX, Wakenyaku Co., Ltd., Kyoto, Japan) as described (Ota et al., 2012, 2015). This condition is almost similar to that described in patients with a severe degree of SAS: patients with severe SAS are repeatedly exposed to severe hypoxemia followed by mild hypoxemia or normoxic condition, that is, IH. As previously reported, the magnitude of IH expressed by peripheral oxygen saturation fluctuated between 75%–98% and 50%–80% in SAS patients (Ota et al., 2015): these values were nearly equal to the medium condition in the present study (Ota et al., 2013).

2.2. Real-time reverse transcription-polymerase chain reaction (RT-PCR)

After the normoxia, SH, or IH treatment, total RNA was isolated from NB-1, SH-SY5Y, and SK-N-SH cells with an RNeasy Protect Cell Mini Kit (Qiagen, Hilden, Germany), as described previously (Ota et al., 2012, 2013; Yoshimoto et al., 2013). The isolated RNA was reverse-transcribed to the cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) for real-time PCR, as described previously (Ota et al., 2012; Fujimura et al., 2015; Tsujinaka et al., 2015; Tohma et al., 2017; Tsuchida et al., 2017; Tsujinaka et al., 2017; Uchiyama et al., 2017). The mRNA levels of *POMC*, *CART*, *GAL*, *GALP*, *GHRL*, *QRFP*, *AGRP*, *NPY*, and *MC4R* were measured by real-time RT-PCR. To examine which members of the GATA family of transcription factors were expressed in human neuronal cells, using the total RNA isolated from control NB-1 cells, the mRNA levels of *GATA1*, *2*, *3*, *4*, *5*, and *6* were also measured by real-time RT-PCR. All the primers used for real-time RT-PCR were synthesized by Nihon Gene Research Laboratories, Inc. (NGRL; Sendai, Japan) and described in Table 1. Real-time PCR was performed by the KAPA SYBR® Fast qPCR Master Mix (Kapa Biosystems, Boston, MA) and the Thermal Cycler Dice Real Time System (Takara, Kusatsu, Japan), as described previously (Ota et al., 2012; Yoshimoto et al., 2013). PCR was performed with an initial step of 3 min at 95 °C followed by 40 cycles of 3 s at 95 °C and 20 s at 60 °C for *POMC*, *CART*, *GAL*, *GALP*, *GHRL*, *QRFP*, *AGRP*, *NPY*, and *MC4R*, and was conducted with an initial step of 3 min at 95 °C followed by 45 cycles of 3 s at 95 °C, 5 s at 55 °C, and 20 s at 72 °C for *GATA1*, *2*, *3*, *4*, *5*, and *6*. Target cDNAs were cloned into pBluescript SK(−) plasmid (Stratagene, La Jolla, CA), and sequential 10-fold dilutions from 10^2 to 10^7 copies/μL were prepared. The serial dilutions were run to verify the specificity and to test the sensitivity of the SYBR Green-based real-time RT-PCR. The respective mRNA levels were normalized by those of the *β-actin* mRNA as an internal standard.

Table 1
PCR primers for real-time RT-PCR.

Target mRNA	Primer sequence (Position)
POMC	5′-TGGAAGTGCGTGGCTGGT-3′ (NM_000937: 278-295)
	5′-TGCACTCCAGCAGGTTGCT-3′ (NM_006343: 334-352)
CART	5′-CCCGAGCCCTGGACATCTA-3′ (NM_004291: 235-253)
	5′-CGCTTCGATCAGCTCCTTCT-3′ (NM_004291: 280-299)
GAL	5′-CAGGAAGCTTTGACAGGTCC-3′ (BC030241: 397-416)
	5′-ACACAGACAAACATGCCCA-3′ (BC030241: 557-575)
GALP	5′-CGAGGAGGCTGGACCCTCAA-3′ (NM_033106: 173-192)
	5′-CAGGTCTAGGATCTCAAG-3′ (NM_033106: 269-286)
GHRL	5′-GGCAGAGGATGAACTGGAAG-3′ (NM_016362: 250-269)
	5′-TCCCAGAGGATGTCCTGAAG-3′ (NM_016362: 353-372)
QRFP	5′-GCATGCTGGCTGCAGATTCC-3′ (NM_198180: 240-259)
	5′-TGAGCTCCTCAGCCAGGTTC-3′ (NM_198180: 339-358)
AGRP	5′-TGCAGAACAGGCAGAAGAGGAT-3′ (NM_001138: 465-486)
	5′-GCAGGACTCATGCAGCCTTAC-3′ (NM_001138: 562-582)
NPY	5′-TCGGCGCTGCGACACTA-3′ (NM_000905: 236-252)
	5′-TGTCTCTGGGCTGGATCGTT-3′ (NM_000905: 285-304)
MC4R	5′-CCAACCCGCTTAACTGTCAT-3′ (NW_004078095: 500-519)
	5′-GTAGCTCCTTGCTTGCATCC-3′ (NW_004078095: 592-611)
GATA1	5′-CCAAGCTTCGTGGAACTCTC-3′ (NM_002049: 654-663)
	5′-AGGCGTTGCATAGGTAGTGG-3′ (NM_002049: 754-773)
GATA2	5′-GCTGCACAATGTTAACAGGC-3′ (NM_00145661: 1569-1588)
	5′-TCTCCTGCATGCACTTTGAC-3′ (NM_00145661: 1686-1705)
GATA3	5′-TTAACATCGACGGTCAAGGC-3′ (NM_001002295: 709-728)
	5′-GGTAGGGATCCATGAAGCAG-3′ (NM_001002295: 822-841)
GATA4	5′-CTCTCCCAGGAACATCAAACC-3′ (NM_008092: 2603-2623)
	5′-GTGTGAAGGGGTGAAAAGG-3′ (NM_008092: 2710-2728)
GATA5	5′-TCGCCAGCACTGACAGCTCAG-3′ (NM_080473: 1012-1032)
	5′-TGGTCTGTTCCAGGCTGTTCC-3′ (NM_080473: 1281-1301)
GATA6	5′-CCACTCGTGTCTGCTTTTGTGC-3′ (NM_005257: 2118-2139)
	5′-CCCTTCCCTTCCATCTTCTCTCAC-3′ (NM_005257: 2233-2256)
β-actin	5′-GCGAGAAGATGACCCAGA-3′ (NM_001101: 420-437)
	5′-CAGAGGCGTACAGGGATA-3′ (NM_001101: 492-509)

2.3. Construction of reporter plasmids and luciferase reporter assay

Reporter plasmids were constructed by inserting progressively deleted fragments of the *POMC* promoter (−1862 to +1, −742 to +1, −705 to +1, −685 to +1, −681 to +1, −675 to +1, −666 to +1, and −593 to +1) and the *CART* promoter (−1551 to +30, −1081 to +30, −950 to +30, −928 to +30, and −906 to +30) upstream of a firefly luciferase reporter gene in the pGL4.17[*luc2*/Neo] vector (Promega, Madison, WI). NB-1 cells were seeded in a 24-well plate at 1×10^5 cells per well and were transfected with reporter plasmids by

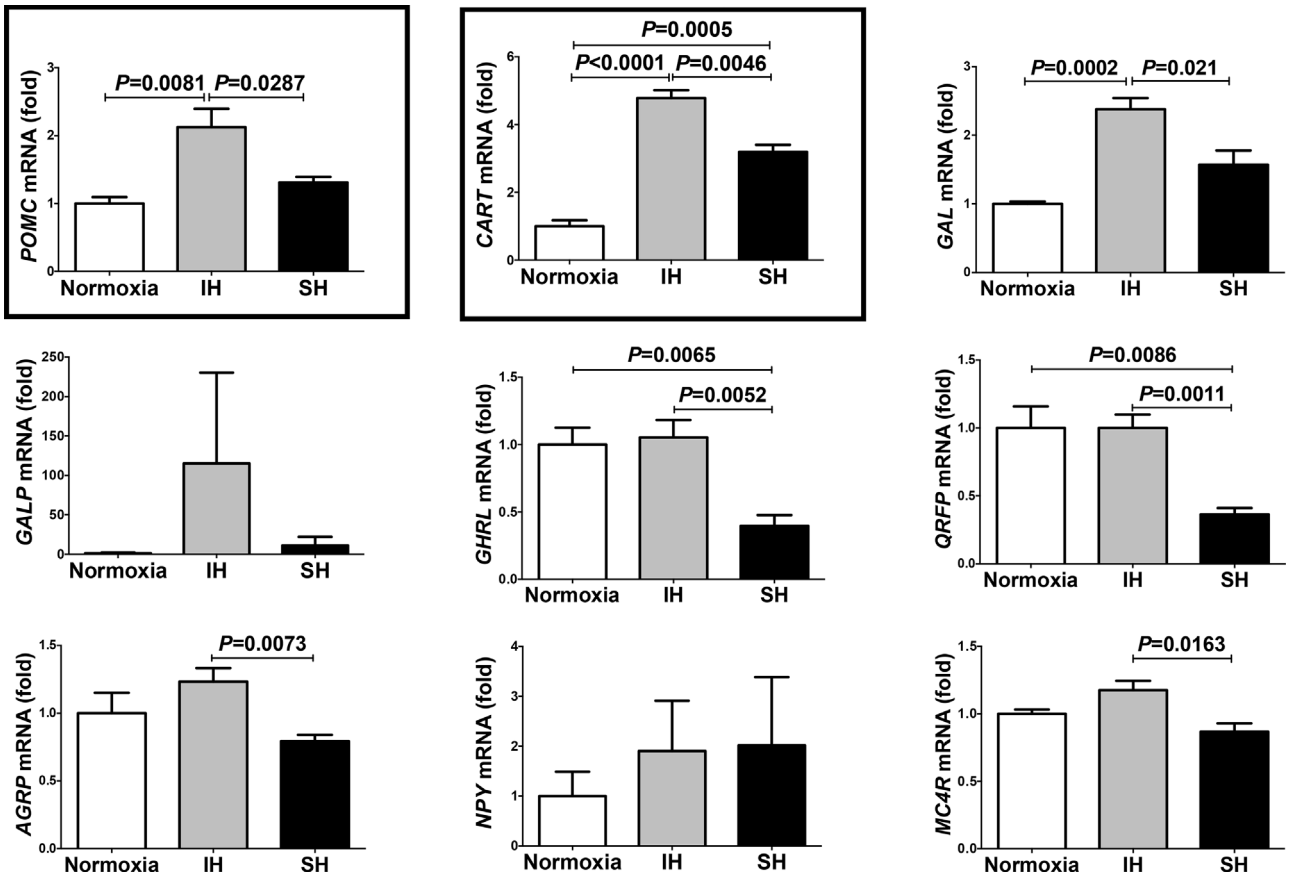

Fig. 1. The mRNA levels of *POMC*, *CART*, *GAL*, *GALP*, *GHRL*, *QRFP*, *AGRP*, *NPY*, and *MC4R* in human neuronal cells treated with normoxia, IH, or SH. **A:** The mRNA levels in NB-1 cells. **B:** The mRNA levels in SH-SY5Y cells. **C:** The mRNA levels in SK-N-SH cells. The mRNA levels were measured by real-time RT-PCR and normalized by *β-actin* as an internal standard. Data are expressed as the mean ± SE of the samples (n = 4). Statistical analyses were performed using Student's *t*-test. IH significantly increased the mRNA levels of *POMC* and *CART* in all the neuronal cells (NB-1, SH-SY5Y, and SK-N-SH).

Lipofectamine®3000 (Life Technologies), as described previously (Fujimura et al., 2015; Tsujinaka et al., 2015; Tsuchida et al., 2017; Tsujinaka et al., 2017; Uchiyama et al., 2017). After the treatment with IH or normoxia for 24 h, the cells were harvested and cell extracts were prepared in Extraction Buffer (0.1 M potassium phosphate, pH 7.8/0.2% Triton X-100; Life Technologies). To monitor transfection efficiency, the pCMV-SPORT-βgal plasmid (Life Technologies) was co-transfected in all experiments at a 1:10 dilution. Luciferase activity was measured by using the PicaGene Luciferase assay system (Toyo-ink, Tokyo, Japan) and normalized by the β-galactosidase activity, as previously described (Nakazawa et al., 2005; Ota et al., 2012).

2.4. RNA interference (RNAi)

Small interfering RNAs (siRNAs) directed against human *GATA2* and *GATA3* mRNAs were synthesized by NGRL. The sense sequences of siRNA for human *GATA2* and *GATA3* were 5′-GGCUCGUUCCUGUUC AGAAtt-3′ and 5′-AAGAAAGAGUGCCUCAAGUAC-3′, respectively. The Silencer Select® scrambled siRNA was purchased from Ambion® and used as the control. NB-1 cells were transfected with 5 pmol each of siRNA in a 24-well culture dish (4×10^5 cells/mL) using the Lipofectamine® RNAiMAX Transfection Reagent (Life technologies), as previously described (Ota et al., 2013; Fujimura et al., 2015; Tsujinaka et al., 2015; Tohma et al., 2017; Tsuchida et al., 2017; Tsujinaka et al., 2017).

2.5. Measurement of viable cell numbers by tetrazoliu salt cleavage

NB-1 cells (2×10^4cells/0.1 mL in 96-well plate) were incubated at 37 °C over night and the medium was replaced with RPMI1640 + 10% FCS just before normoxia/IH exposure. After a 24-h treatment of normoxia or IH, the viable cell numbers were determined by a Cell Counting kit-8 (Dojindo Laboratories, Mashiki-machi, Japan) according to the manufacturer's instructions. Briefly, WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(40nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt) solution was added to cells in 96-well plates, and the cells were incubated at 37 °C for 30 min–4 h. The optical density of each well was read at 450 nm (reference wave length at 650 nm) using a Sunrise™ microplate reader (Tecan, Mannedorf, Switzerland), as described (Ota et al., 2013; Tsujinaka et al., 2015; Tohma et al., 2017; Uchiyama et al., 2017).

2.6. Data analysis

All the values were expressed as the mean ± SE. The data obtained were analyzed by Student's *t*-test using the GraphPad Prism6 software (GraphPad Software, La Jolla, CA). A P value of < 0.05 was considered statistically significant.

3. Results

3.1. IH significantly up-regulates the mRNA levels of POMC and CART in human neuronal cells

We exposed human neuronal cells (NB-1, SH-SY5Y, and SK-N-SH) to normoxia, SH, or IH for 24 h. After the treatment, we measured the mRNA levels of *POMC*, *CART*, *GAL*, *GALP*, *GHRL*, *QRFP*, *AGRP*, *NPY*, and *MC4R* by real-time RT-PCR. As shown in Fig. 1, IH significantly

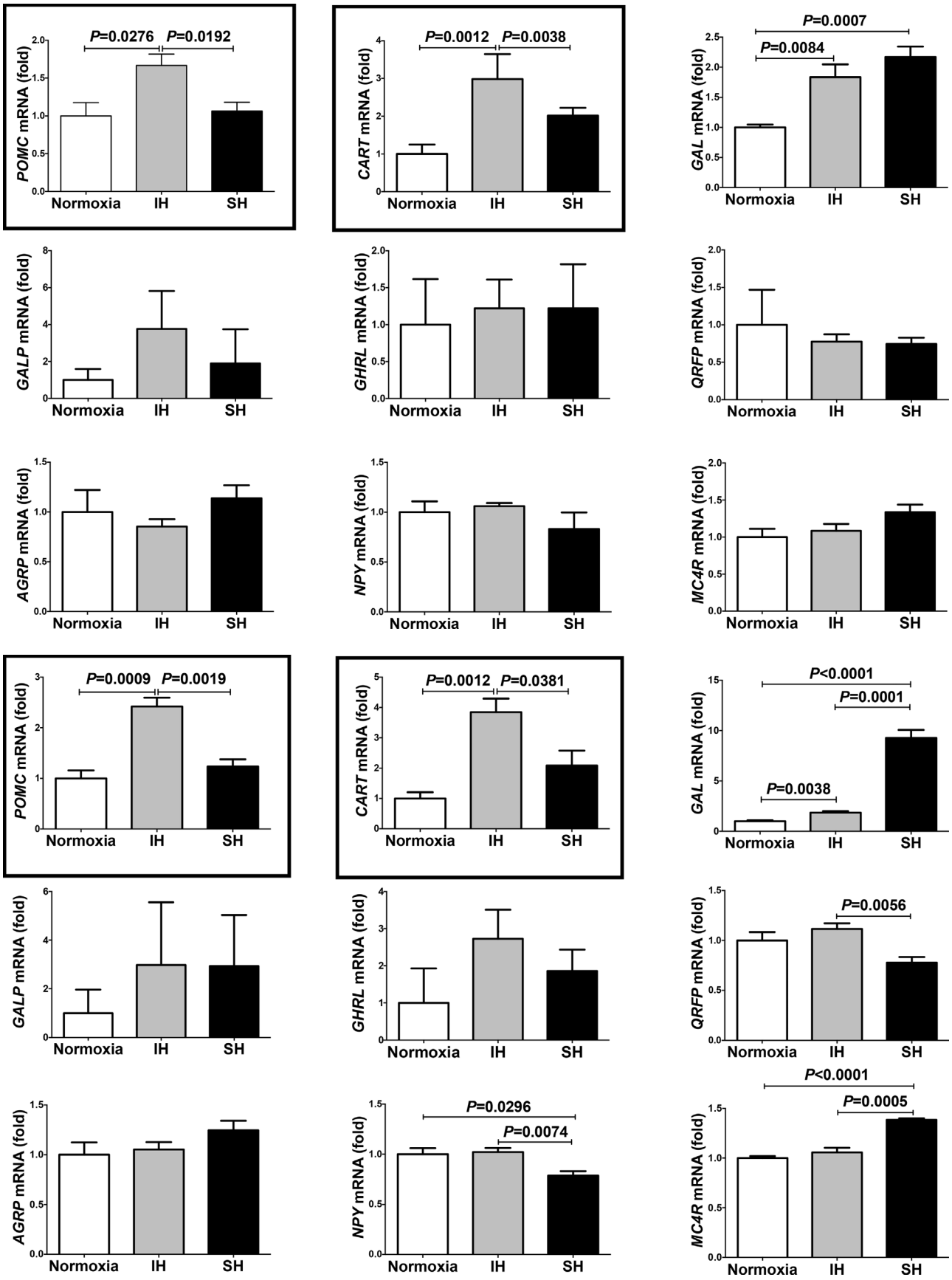

Fig. 1. (*continued*)

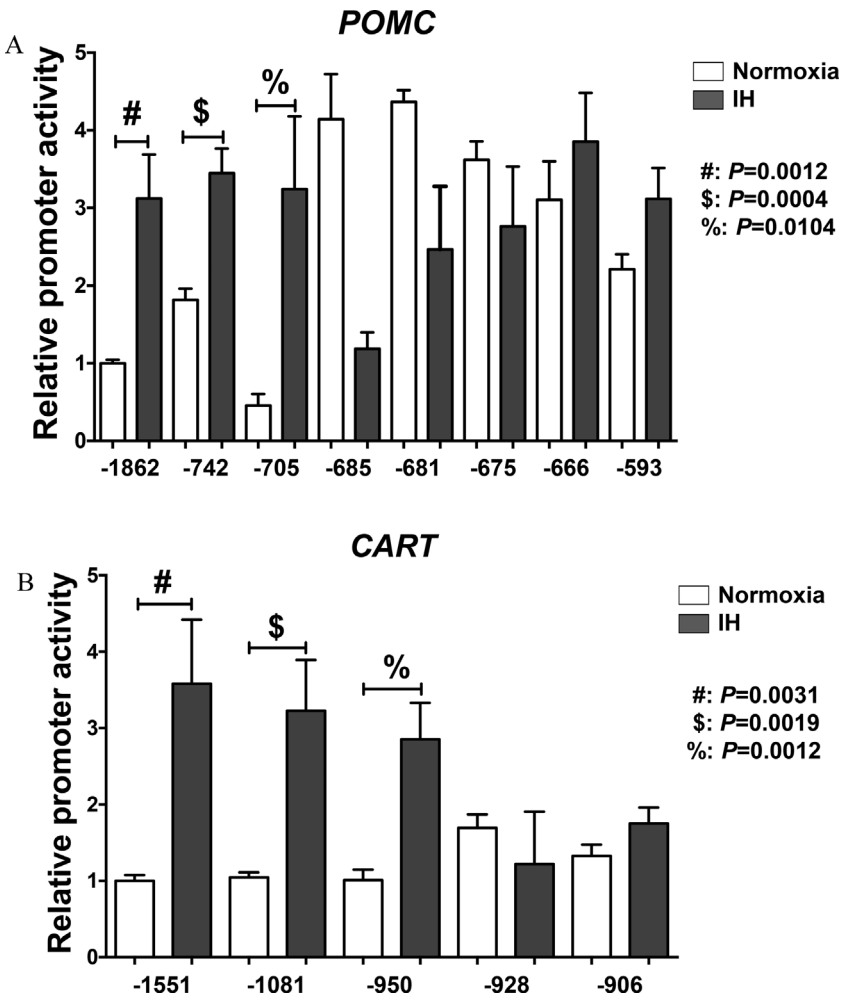

Fig. 2. Deletion analysis of *POMC* and *CART* promoters. A: Localization of the essential region for IH-induced *POMC* promoter activities. B: Localization of the essential region for IH-induced *CART* promoter activities. NB-1 cells were transfected with constructs containing various lengths of promoter fragments upstream of a firefly luciferase reporter gene in the pGL4.17 vector. After the treatment with normoxia or IH, the luciferase activity was measured. The diagram represents relative luciferase activities to the normoxia group of (A) "−1862" or (B) "−1551". All data are expressed as the means ± SE for each group (n = 4). The statistical analyses were performed using Student's *t*-test.

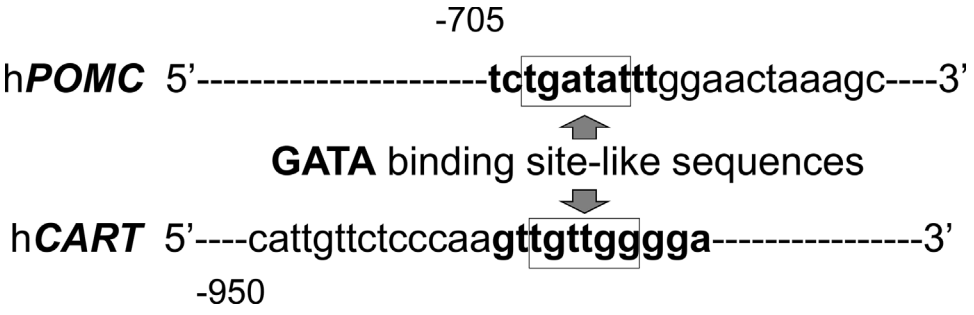

Fig. 3. Comparison of the −705 to −686 region of the *POMC* promoter and the −950 to −929 region of the *CART* promoter with the GATA transcription factors binding sequence. The possible GATA binding sequences are bolded, and the binding core sequences are boxed.

increased the mRNA levels of *POMC* and *CART* in all the neuronal cells (NB-1 (Fig. 1A), SH-SY5Y (Fig. 1B), and SK-N-SH (Fig. 1C)), whereas IH-specific increases were not observed in *GAL*, *GALP*, *GHRL*, *QRFP*, *AGRP*, *NPY*, and *MC4R*.

3.2. Localization of regions essential for the IH-induced POMC and CART promoter activities

To investigate the mechanism by which the mRNA levels of *POMC* and *CART* were up-regulated by IH, we prepared the reporter plasmids by inserting various lengths of *POMC* and *CART* promoter fragments upstream of a firefly luciferase reporter gene in the pGL4.17 vector, transfected them into NB-1 cells, and measured the luciferase activity after IH treatment. As shown in Fig. 2A, the deletion down to position −705 of the *POMC* promoter region resulted in the IH-induced up-regulation of the reporter gene expression, but an additional deletion to nucleotide −685 attenuated the IH-induced promoter activity. Similarly, the deletion down to position −950 of the *CART* promoter caused the IH-induced up-regulation of the reporter gene expression, but an additional deletion to nucleotide −928 attenuated the IH-induced promoter activity (Fig. 2B). These results indicate that the IH-induced up-regulation of *POMC* and *CART* mRNAs is caused by the transcriptional activation of the *POMC* and *CART* genes and that the −705 to −686 promoter region of the *POMC* gene and the −950 to −929 region of the *CART* gene are essential for the IH-induced promoter activity.

3.3. Both GATA2 and GATA3 are key factors for the IH-induced up-regulation of POMC and CART mRNA expressions

To further study the mechanism of the up-regulation of *POMC* and *CART* mRNAs by IH, we conducted a computer-aided search for

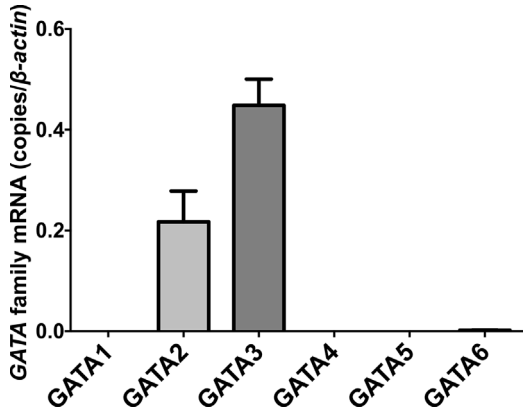

Fig. 4. The mRNA expression profile of the GATA family in human neuronal cells. The mRNA expressions of *GATA1*, *2*, *3*, *4*, *5* and *6* were shown by real-time RT-PCR from the mRNA isolated from control NB-1 cells and normalized by *β-actin* as an internal standard. Data are expressed as the means ± SE for each group (n = 4).

sequences similar to known *cis*-acting elements in the −705 to −686 promoter region of the *POMC* gene and the −950 to −929 region of the *CART* gene using the TFBIND program (http://tfbind.hgc.jp). The result showed that both the −705 to −686 promoter region of the *POMC* gene and the −950 to −929 region of the *CART* gene contained possible GATA transcription factor binding sequences (Fig. 3). As the GATA family has six members (Lentjes et al., 2016), we examined the expression of each GATA family member in NB-1 cells to determine which member(s) could be involved in the IH-induced *POMC/CART* expression in neural cells. Real-time RT-PCR revealed that the *GATA2* and *GATA3* mRNAs were mainly expressed in NB-1 cells, but the other GATA family member mRNAs (*GATA1*, *4*, *5*, and *6*) were scarcely detected (Fig. 4).

To investigate whether GATA2 and/or GATA3 were essential for the IH-induced up-regulation of *POMC* and *CART* mRNAs, we introduced siRNAs against human *GATA2* and *GATA3* mRNAs into NB-1 cells and analyzed the IH-induced *POMC* and *CART* mRNA expressions by real-time RT-PCR. Knockdown of GATA2 induced drastic up-regulation of *POMC* and *CART* mRNAs in normoxia and knockdown of GATA3 induced up-regulation of *POMC* and *CART* mRNAs in normoxia (data not shown). As a result, both human *GATA2* and *GATA3* siRNAs abolished the IH-induced up-regulation of *POMC* and *CART* mRNAs (Fig. 5A and B), thus indicating that both GATA2 and GATA3 are key factors for the IH-induced up-regulation of *POMC* and *CART* mRNA expressions.

4. Discussion

Obesity is recognized as a challenging healthcare problem, and a high prevalence exists in its association with the metabolic syndrome, the commonly used term for the cluster of obesity, insulin resistance, hypertension and dyslipidemia around the world (Tasali and Ip, 2008). Accumulating evidence indicates that obesity and SAS are strongly related to each other (Romero-Corral et al., 2010). Obesity can cause SAS due to the anatomical reason that airway narrowing induced by an excess of fat tissue around the neck can predispose an individual to airway obstruction (Young et al., 2005). However, a prospective non-randomized controlled study revealed that BMI was significantly lower in SAS Far-East Asian men than that in SAS white men when controlled for sex, age, and disease severity and that the mean BMI of the Far-East Asian men with SAS was below the norms for men in the United States (Li et al., 2000). Therefore, the mechanism by which SAS affects

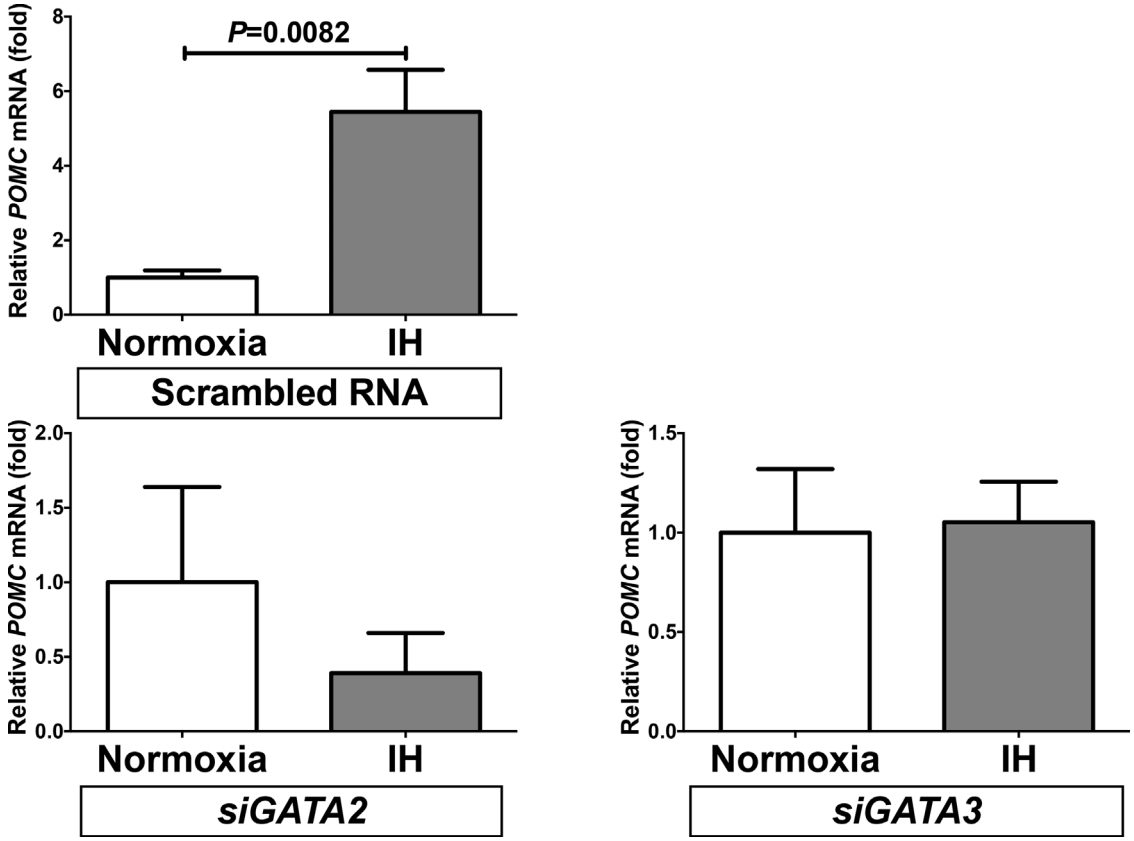

Fig. 5. Inhibition of the IH-induced up-regulation of the *POMC* and *CART* mRNAs by *GATA2* and *GATA3* siRNAs transfection into NB-1 cells. **A:** After the introduction of *GATA2* and *GATA3* siRNAs, NB-1 cells were treated with normoxia or IH for 24 h. The mRNA expression of *POMC* was measured by real-time RT-PCR and normalized by *β-actin* as an internal standard. **B:** The mRNA expression of *CART* was measured by real-time RT-PCR and normalized by *β-actin* as an internal standard. Data are expressed as the means ± SE for each group (n = 4). The statistical analyses were performed using Student's *t*-test.

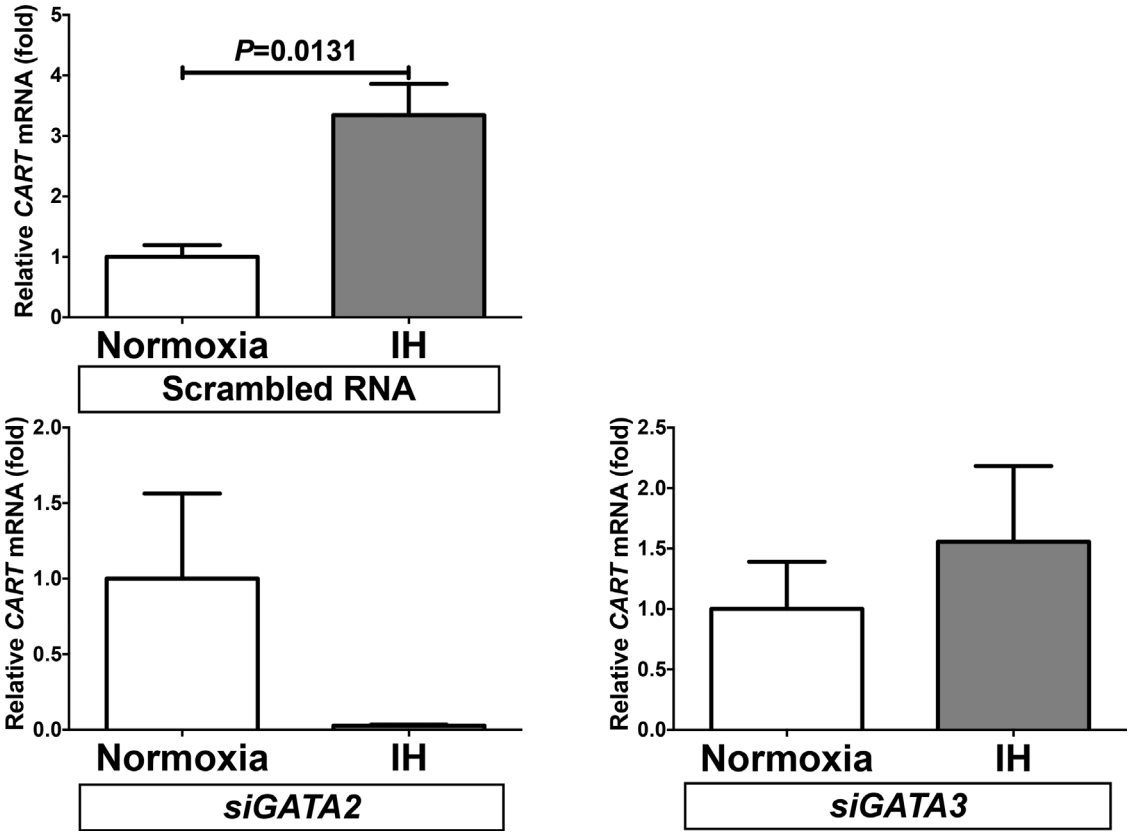

Fig. 5. (*continued*)

patients' body weight remains unclear. Although the etiology of overweight and obesity is complex and energy balance is regulated by many neurobiological and physiological mechanisms, weight gain is generally supposed to result from excessive food intake leading to an imbalance between calorie intake and energy expenditure. The effect of IH on the regulation of appetite and feeding behavior in SAS patients has been obscure, however, no reports have examined the changes in the expression of appetite regulatory genes under the influence of IH.

In the present study, we attempted to untangle the "chicken-or-egg" question of the SAS-obesity paradox by examining the expression of major appetite regulatory neuropeptide and receptor genes in IH-treated human neuronal cells. We focused on major appetite regulatory genes and analyzed their changes in the mRNA expression by real-time RT-PCR using three different human neuronal cell lines and an *in vitro* IH system. Judged by WST-8 assay, IH exposure did not affect cellular viability of human NB-1 neuronal cells (Fig. S1; $P = 0.242$ [n = 12]). Interestingly, significant increases by IH were observed only in the *POMC* and *CART* genes, both of which encode anorexigenic peptide hormones. The subsequent promoter assays indicated that the IH-induced up-regulation of *POMC* and *CART* mRNAs was caused by the transcriptional activation of the *POMC* and *CART* genes. In addition, RNA interference experiments revealed that the knockdown of *GATA2/GATA3* did not affect cellular viability prior to IH (Fig. S2; $P = 0.768$ [Control vs Scrambled], $P = 0.0987$ [Control vs *siGATA2*], $P = 0.0921$ [Control vs *siGATA3*], $P = 0.0756$ [Scrambled vs *siGATA2*], $P = 0.0708$ [Scrambled vs *siGATA3*], and $P = 0.677$ [*siGATA2* vs *siGATA3*], n = 12) and that the transcriptional activation of *POMC* and *CART* by IH required both GATA2 and GATA3. Contrary to our expectation, the present results suggest the possibility that the cyclic alternation of hypoxia-reoxygenation, or IH, inhibits appetite and food intake by increasing mRNAs for *POMC* and *CART* at the transcriptional level in the central nervous system (CNS). Based on the present result, IH seems to suppress food intake rather than increase appetite in SAS patients, thus suggesting that IH itself is not likely to make SAS patients eat to excess and subsequently obese.

Furthermore, we demonstrated that both GATA2 and GATA3 are essential for the IH-induced up-regulation of *POMC* and *CART* mRNA expressions. It is remarkable that the gene expression of *POMC* and *CART*, which express in an identical neuron, is up-regulated by IH through common transcription factors, GATA2 and GATA3. As a computer-aided search revealed that the −705 to −686 region of the *POMC* promoter and the −950 to −929 region of the *CART* promoter, both of which are essential for the IH-induced *POMC* and *CART* transcription, include possible GATA transcription factor family binding sequences, we focused on GATA transcription factors as important players in the IH-induced up-regulation of *POMC* and *CART* mRNAs. The GATA transcription factors consist of six members, GATA1-6, and the expression of GATA2, 3, 4, and 6 was reported in the CNS during vertebrate development (Lentjes et al., 2016). We demonstrated that the expression of *GATA2* and *GATA3* mRNA was predominant in human neuronal cells (Fig. 4) in the present study. Therefore, we conducted RNA interference against *GATA2* and *GATA3* mRNAs and found that GATA2 and GATA3 are indispensable for the IH-induced up-regulation of *POMC* and *CART* expression. Related with the significance of GATA factor(s) in IH-condition, Park et al. (2007) reported the involvement of Gata4 in IH-induced up-regulation of Bcl-2 and Bcl-x_L in mouse myocardial cells although the mechanism how IH activates Gata4 to induce Bcl-2 and Bcl-x_L has been elusive.

In conclusion, IH stress up-regulates the mRNA levels of *POMC* and *CART*, which are anorexigenic, in human neuronal cells via GATA2 and GATA3 acting on the −705 to −686 region of the *POMC* promoter and the −950 to −929 region of the *CART* promoter. The cyclic changes in hypoxia-reoxygenation may have anorexigenic effects on SAS patients through the transcriptional activation of *POMC* and *CART* in the CNS.

Disclosures

All authors state that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.biocel.2017.12.012.

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