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Changes in neuropeptides related to food intake in the rat arcuate nucleus after chronic immobilization stress and the effect of comfortable music exposure

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Key words: Hypothalamic arcuate nucleus, Neuropeptides, Food intake, Body weight, Chronic immobilization stress, Music exposure

Abstract: Stress is an inevitable interference factor that seriously affects health. Listening to music is an economical, non-invasive, and highly accepted tool for easing stress. However, physiological studies investigating the ability of music to reduce stress in daily life are limited. We established rat models of chronic immobilization stress (CIS) to observe changes in the hypothalamic arcuate nucleus (ARC) neurons involved in the regulation of food intake and the effect of comfortable classical music exposure. Twenty-one days of stress resulted in decreased food intake and delayed body weight gain; up-regulation of leptin receptor (Ob-R), cocaine- and amphetamine-regulated transcript (CART), proopiomelanocortin (POMC), and alpha-melanocyte-stimulating hormone (α-MSH) expression; and down-regulation of neuropeptide Y (NPY) and agouti-related protein (AgRP) expression in the ARC. Thus, peripheral leptin entered the ARC under chronic stress conditions, bound to Ob-R, and affected downstream nerve pathways related to appetite, such as the NPY/AgRP and CART/POMC pathways. Gentle classical music played at 65 dB reversed the abnormal expression of Ob-R and NPY induced by chronic stress. Thus, listening to comfortable music improves changes in ARC neurons related to the regulation of food intake in CIS rats, and these results provide a reference for basic research regarding how music therapy alleviates stress and stress-related health issues.

Introduction

Chronic stress has negative effects on individual health. The primary objectives of current research efforts are to search for convenient, effective, and economical stress intervention methods.

Music was applied as a method to enhance mental and physical well-being in ancient China. "Huangdi Neijing", which is the earliest existing Chinese medical book, states that music is related to the body's physiology and pathology and alleviates pathological processes to aid rehabilitation.

Growing evidence from multidisciplinary fields has demonstrated that music activates physiological pathways to modulate physical responses (DeNora, 2013; Fredericks *et al.*, 2012; Phumdoung and Good, 2003; Ueda *et al.*, 2013). Listening to music is associated with higher food intake in the natural environment, and can stimulate the appetite of demented patients and chemotherapy patients (Bilgic and Acaroglu, 2017; Ragneskog *et al.*, 1996; Stroebele and de Castro, 2006). Music also reduces stress and stress-related health issues (Pereira and Barbosa, 2013). Music therapy is an economical, non-invasive, and highly accepted intervention that has been proposed for the management of stress and stress-related health issues (Thoma *et al.*, 2013).

As in human studies, several animal experiments have demonstrated that appropriate music can improve negative emotions, relieve pain (Gao *et al.*, 2016), enhance learning, memory and cognitive abilities (Lee *et al.*, 2016; Xing *et al.*, 2016), and modulate brain development and neuroplasticity (Angelucci *et al.*, 2007a; Angelucci *et al.*, 2007b; Kim *et al.*, 2013; Sheikhi and Saboory, 2015; Sihvonen *et al.*, 2017). However, the physiological studies of music are limited, and the robust experimental evidence that explains the major effects of music is lacking (Angelucci *et al.*, 2007a).

The arcuate nucleus (ARC) is a key hypothalamic nucleus involved in the regulation of food intake, body weight, and energy balance. Neuropeptide Y (NPY)/agouti-related peptide

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(AgRP)- and proopiomelanocortin (POMC)/cocaine- and amphetamine-regulated transcript (CART)-expressing neurons in the ARC are the two major neuronal populations involved in appetite regulation (Abdalla, 2017; Loh *et al*., 2015; Morton *et al*., 2006). These inspired our investigation of changes of neuropeptides related to food intake and body weight in the hypothalamic ARC of rats undergoing chronic immobilization stress (CIS), and observation of the effects of classical music exposure intervention, and examination of the central mechanisms underlying the effects of music on energy balance.

Materials and Methods

Animals

Eight-week-old male Sprague-Dawley (SD) rats weighing 210 ± 20 g were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd., Beijing, China. The animals were housed under controlled conditions at 21 ± 1°C temperature, 40% to 60% humidity, and under 12 h of light (from 07:00 to 19:00) and 12 h of darkness (from 19:00 to 07:00). Purified water and rodent food were provided ad libitum. After adaptation for 1 week, the animals were randomly divided into three groups (a control group, a stress group, and a music group) with 24 rats per group. Each group had 8 cages with 3 rats per cage. The rats in the normal control group were routinely fed for 21 days. The rats in the stress and music groups were exposed to a continuous immobilization stressor for 3 h daily for 21 days. The rats in the music group were exposed to classical Chinese music.

All animals in the study were bred and treated in strict accordance with "The Regulations for the Administration of Affairs Concerning Experimental Animals" promulgated by decree No. 2 of the State Scientific and Technological Commission of China. The Committee on the Ethics of Animal Experiments of the Hebei University of Chinese Medicine approved the experimental procedures. We reduced the number of experimental animals used and minimized animal suffering as much as possible.

Chronic immobilization stress

We adopted the CIS method (CIS 3 h daily for 21 days) to replicate rat models based on specific procedures that have been previously described (Chen *et al*., 2008; Wang *et al*., 2013; Wang *et al*., 2012). Briefly, rats were bound to a binding rack with the chest and abdomen fixed with two adjustable soft belts. The head and limbs were free and comfortable. Body weight and food intake were recorded before stress (0th) and stress induction on the 7th, 14th, and 21st days.

Music exposure

The rats in the music group were exposed to comfortable classical music at 65 dB for 3 h daily for 21 consecutive days. The music included "Three Variations of Plum Flowers" played on flute and xiao (a Chinese vertical bamboo flute), "The Homebound Fishermen" played on zheng (a 21-or 25-stringed plucked instrument in some ways similar to the zither), and "Hu Jia Shi Ba Pai" played on guqin (a seven-stringed plucked instrument in some ways similar to the zither).

Sample preparation

Samples were prepared as previously reported (Wang *et al*., 2012). The rats in each group were anesthetized and then decapitated. The ARC tissues were removed and placed into sterile RNase-free Eppendorf tubes for total RNA (n = 6) and protein (n = 3) preparation. The other six rats per group were anesthetized and fixed via cardiac perfusion. Then, the entire brain was removed and fixed in a 4% paraformaldehyde solution, dehydrated in 20% and 30% sucrose solutions and sectioned at approximately 25-μm thickness for immunofluorescence staining.

Protein isolation and western blotting analysis (WB analysis)

The ARC tissues were homogenized in RIPA lysis buffer (Pierce Biotechnology Inc., Rockford, IL, USA). The lysates were reacted in an ice bath for 30 min and centrifuged at 8000 rpm for 10 min at 4°C. The supernatant was extracted, PMSF (Amresco LLC, Solon, OH, USA) was added to achieve a final concentration of 1% PMSF, and the samples were stored at −20°C. The protein concentrations were quantified by measuring the absorbance at 595 nm using a spectrophotometer (Hitachi UV-330, Japan).

Proteins (30 μg per sample) were separated on SDS-polyacrylamide gels and transferred to polyvinylidene difluoride (0.45 μm) membranes (EMD Millipore, Inc., Billerica, MA, USA). The membranes were blocked for 1 h in 5% non-fat milk and incubated at 4°C overnight with the following primary antibodies: rabbit polyclonal anti-β-actin (1:300, Abcam, Inc., Cambridge, UK), anti-AgRP (1:200, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-Ob-R (1:300, Invitrogen Corporation, Carlsbad, CA, USA), anti-POMC (1:300, Signalway Antibody LLC, College Park, MD, USA), rabbit anti-CART monoclonal antibody (mAb) (1:300, Cell Signaling Technologies, Danvers, MA, USA), and anti-NPY (1:300, Cell Signaling Technologies, Danvers, MA, USA). The membranes were washed and incubated at 4°C for 1 h with a horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibody (1:5000, Beijing Zhongshan Biological Technology Co., Ltd., Beijing, China). The target bands were detected using an enhanced chemiluminescence detection system (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) according to the manufacturer's instructions. β-actin was used as the loading control. Images were acquired using a Gel Imaging System (UVP, LLC., Upland, CA, USA). The optical density of each band was measured using Image-Pro Plus 6.0, and the results were expressed as the $IOD_{sample/\beta\text{-}actin}$.

RNA isolation, cDNA synthesis, and quantitative real-time PCR analysis (qRT-PCR)

ARC tissues (approximately 50 μg/sample) were homogenized, and total RNA was extracted using TRIzol (Invitrogen Corporation, Carlsbad, CA, USA). An M-MLV reverse transcriptase kit (Promega Corporation, Madison, WI, USA) was used to convert the total RNA (1 μg/sample) into cDNA.

The PCR reaction system was prepared, and qRT-PCR reactions were performed using a SYBR Green I fluorescent quantitative PCR kit (Fermentas Inc., Burlington, ON, Canada) and an ABI 7300 Real-Time PCR System according to the manufacturer's instructions using standard conditions and the following primers: β-actin, forward

5'-GGTCATCACCATTGGCAA-3' and reverse 5'-GAGTTGAAGGTAGTTTCGTGGA-3', NM_001101.3, 105 bp; NPY, forward 5'-CGTGTGTTTGGGCATTCT-3' and reverse 5'-CAGTGTCTCAGGGCTGGAT-3', XM_003749745.2, 165 bp; AgRP, forward 5'-CTGCCGCTTCTTCAATACC-3' and reverse 5'-CTTTGCCCAACATCCGTT-3', NM_033650.1, 196 bp; Ob-R, forward 5'-GCCAAAGTCAACTACGCTCTT-3' and reverse 5'-CTTCCATACGCAAACCCA-3', NM_012596.1, 133 bp; POMC, forward 5'-TGCTTCAGACCTCCATAGACG-3' and reverse: 5'-AGGGCTG-TTCATCTCCGTT-3', NM_139326.2, 159 bp; CART, forward 5'-GACATCTACTCTGCCGTGGA-3' and reverse 5'-CGGAATGCGTTTACTCTTGA-3', NM_017110.1, 135 bp.

β-Actin was included as the internal reference. The first sample of the control group was set to a value of 1 to calculate the Ct values of the genes in each sample. The formula RQ = $2^{-\Delta\Delta Ct}$ was used to calculate the relative quantitative Ct values (RQs) for the statistical analysis.

Double-labeling immunofluorescence

Double-labeling immunofluorescence was performed as previously described (Wang *et al*., 2012; Wang *et al*., 2013). First, the sections were washed, blocked, and incubated with primary rabbit anti-NPY (1:500, EMD Millipore, Inc., Billerica, MA, USA), rabbit anti-CART (1:50, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), or rabbit anti-α-MSH (1:100, Phoenix Pharmaceuticals, Inc., Belmont, CA, USA) for two nights at 4°C. Then, the samples were incubated with secondary Alexa Fluor 594-conjugated donkey anti-rabbit IgG antibody (1:200, Thermo Fisher Scientific Inc., Waltham, MA, USA) in the dark for 4 h at room temperature. Second, the sections were washed, blocked, and incubated with a goat anti-Ob-R primary antibody (1:50, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for two nights at 4°C and Alexa Fluor 488-conjugated anti-goat IgG (1:200, Thermo Fisher Scientific Inc., Waltham, MA, USA) in the dark for 4 h at room temperature. Finally, the sections were washed and mounted with Vector H-1500 (Vector Laboratories, Inc., Burlingame, CA, USA).

A Leica TCS SP8 confocal laser-scanning microscope was used for imaging. Image-Pro Plus 6.0 was used to analyze the number, area, and integral optical density (IOD) of neurons positive for Ob-R, NPY, CART, and α-MSH. The co-localization of NPY and Ob-R, CART and Ob-R, or α-MSH and Ob-R were assessed using Pearson's correlation coefficient.

Statistical analysis

The data are expressed as the mean ± standard error of the mean (SEM). The body weights and food intake were analyzed using a one-way analysis of variance with a general linear model in SPSS 23.0. Multivariate analysis of variance with the least significant difference (LSD) test was used to assess differences between groups at each time point (0, 7, 14, and 21 days). One-way analysis of variance was used to assess the other data. The LSD test was used to compare two groups. $p < 0.05$ was considered a significant difference.

Results

Changes in body weight and food intake in the three groups

No differences in body weight or food intake were observed before CIS. The body weights and food intake of the stress and music group rats were significantly lower than those of the control rats on the 7th, 14th, and 21st days ($p < 0.01$ or $p < 0.05$). The body weights and food intake of the music rats were higher than those of the stressed rats on the 14th and 21st days, but this difference was not significant (Tabs. 1 and 2, Figs. 1A and 1B).

Protein and mRNA expression of neuropeptides in the ARC

The Western blotting results were consistent with the quantitative real-time PCR results. CIS affected the levels of neuropeptides related to food intake in the ARC. CIS reduced the NPY and AgRP mRNA and protein levels and increased the Ob-R, CART, and POMC mRNA and protein levels compared to those of the control rats ($p < 0.05$ or

TABLE 1

Changes in body weight in the three groups (n = 24, g, $\bar{x}$ ± SEM)

Group	Day 0	Day 7	Day 14	Day 21
Control group	264.813 ± 1.501	300.688 ± 1.807	333.938 ± 2.797	358.625 ± 2.650
Stress group	265.111 ± 1.649	282.167 ± 1.969※※	303.722 ± 2.803※※	324.222 ± 2.969※※
Music group	268.867 ± 2.727	284.067 ± 3.031※※	309.333 ± 3.538※※	331.800 ± 3.845※※

※$p < 0.05$, ※※$p < 0.01$ *vs*. the control group.

TABLE 2

Changes in food intake in the three groups (n = 8, g, $\bar{x}$ ± SEM)

Group	Day 0	Day 7	Day 14	Day 21
Control group	21.465 ± 0.305	22.485 ± 0.463	22.705 ± 0.343	22.658 ± 0.203
Stress group	20.422 ± 0.497	19.780 ± 0.494※※	20.172 ± 0.492※※	20.500 ± 0.875※
Music group	20.575 ± 0.164	19.880 ± 0.392※※	20.783 ± 0.309※※	20.933 ± 0.267※

※$p < 0.05$, ※※$p < 0.01$ *vs*. the control group.

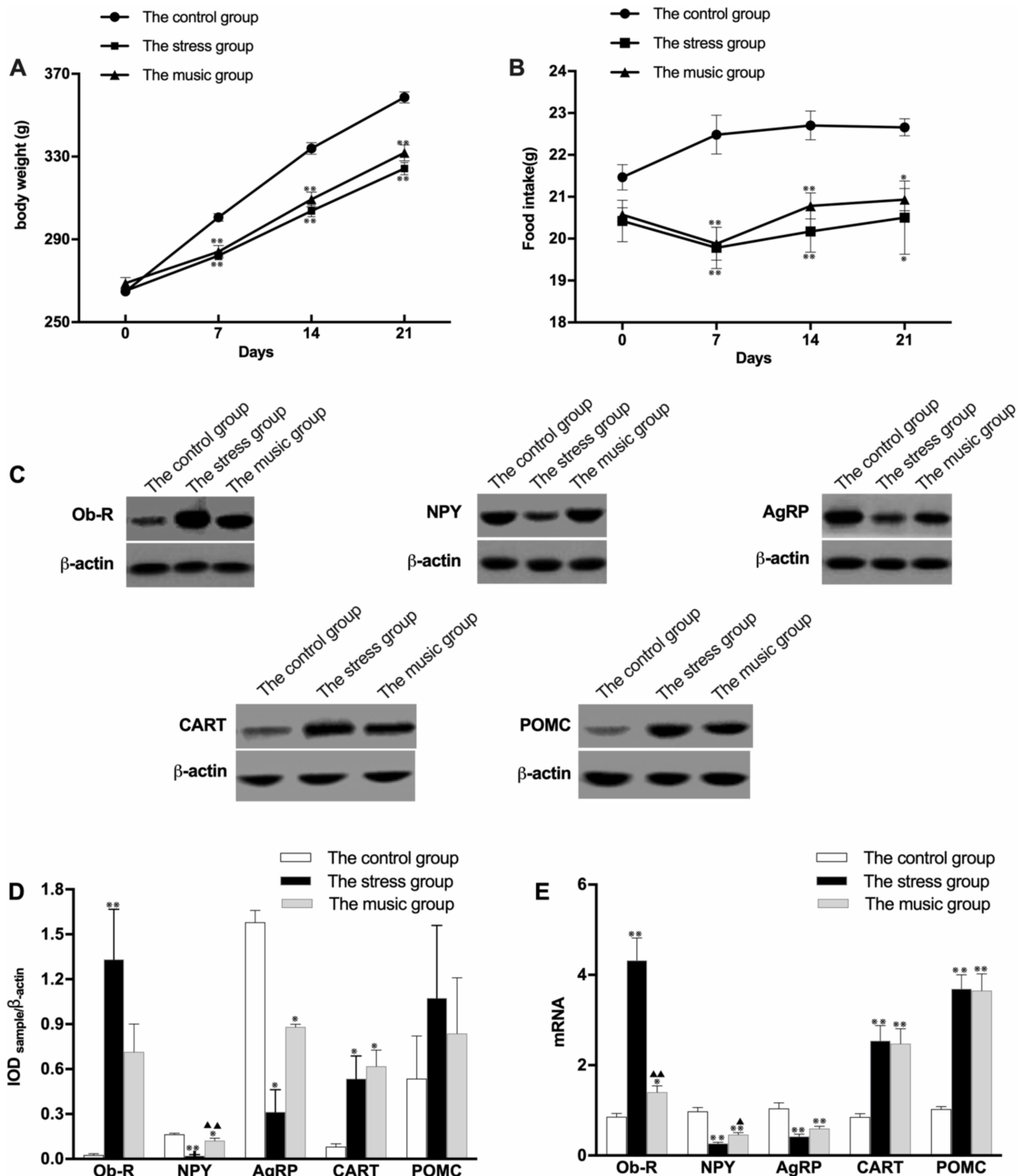

FIGURE 1. Changes in body weight and food intake in the three groups and neuropeptide expression in the ARC measured using qRT-PCR and Western blotting analyses. (A) Body weight and (B) food intake of the rats on the 7th, 14th, and 21st days in the three groups. (C and D) Western blotting analyses of Ob-R, NPY, AgRP, CART, and POMC levels in the three groups. β-Actin was used as the loading control. The $IOD_{sample/\beta\text{-}actin}$ value was used for the statistical analysis. (E) qRT-PCR analyses of Ob-R, NPY, AgRP, CART, and POMC mRNA levels in the three groups. β-Actin was used as the internal reference. Each point or bar represents the mean ± SEM. ※$p < 0.05$, ※※$p < 0.01$ *vs.* the control group; ▲$p < 0.05$, ▲▲$p < 0.01$ *vs.* the stress group.

$p < 0.01$). The Ob-R, NPY, AgRP, CART, and POMC mRNA and protein levels were altered in the ARCs of rats exposed to CIS in combination with music compared to those of the rats exposed to CIS alone, with a significant decrease in Ob-R levels and a significant increase in NPY levels ($p < 0.05$ or $p < 0.01$, respectively) (Tabs. 3 and 4, Figs. 1C–1E). Music thus affected neuropeptides related to appetite in the ARC.

Double-labeling immunofluorescence of ARC neuropeptides related to food intake

NPY-, CART-, and α-MSH-positive ARC neurons were labeled with red fluorescence, and Ob-R-positive ARC neurons were labeled with green fluorescence. Double-stained neurons for Ob-R and NPY, Ob-R and CART, or Ob-R and α-MSH were labeled in yellow (Figs. 2A–2C).

TABLE 3

Neuropeptide expression in the ARC measured by Western blotting analyses ($\bar{x}$ ± SEM)

Group	Ob-R IOD	NPY IOD	AgRP IOD	CART IOD	POMC IOD
Control group	0.026 ± 0.009	0.164 ± 0.007	1.579 ± 0.080	0.082 ± 0.019	0.536 ± 0.286
Stress group	1.333 ± 0.335※※	0.021 ± 0.008※※	0.314 ± 0.148※	0.536 ± 0.153※	1.075 ± 0.483
Music group	0.714 ± 0.186	0.122 ± 0.016※▲▲	0.882 ± 0.018※	0.619 ± 0.108※	0.837 ± 0.373

※$p < 0.05$, ※※$p < 0.01$ *vs.* the control group; ▲$p < 0.05$, ▲▲$p < 0.01$ *vs.* the stress group.

TABLE 4

Neuropeptide expression in the ARC measured by qRT-PCR analyses ($\bar{x}$ ± SEM)

Group	Ob-R mRNA	NPY mRNA	AgRP mRNA	CART mRNA	POMC mRNA
Control group	0.855 ± 0.078	0.979 ± 0.083	1.041 ± 0.125	0.855 ± 0.075	1.029 ± 0.054
Stress group	4.320 ± 0.499※※	0.268 ± 0.024※※	0.424 ± 0.051※※	2.544 ± 0.333※※	3.692 ± 0.309※※
Music group	1.402 ± 0.142※▲▲	0.462 ± 0.047※※▲	0.596 ± 0.050※※	2.480 ± 0.328※※	3.651 ± 0.371※※

※$p < 0.05$, ※※$p < 0.01$ *vs.* the control group; ▲$p < 0.05$, ▲▲$p < 0.01$ *vs.* the stress group.

The results showed that the number, area, and IOD of the Ob-R-, CART-, and α-MSH-positive neurons in the ARC were higher in the stressed rats and the music rats than in the control rats, while NPY-positive neurons were decreased ($p < 0.01$ or $p < 0.05$). The number of Ob-R-positive neurons was significantly lower ($p < 0.01$) in the music rats than in the stressed rats, but the area of the NPY-positive neurons increased ($p < 0.01$). The expressions of CART- and α-MSH-positive neurons were reduced in the music group, but no significant difference was detected compared to the stress group (Tabs. 5–8, Figs. 2D–2G).

Immunofluorescence staining of Ob-R and NPY double-labeling was significantly lower in the stressed rats than in the control rats, while Ob-R and CART double-labeling and Ob-R and α-MSH double-labeling were significantly higher ($p < 0.01$ and $p < 0.05$). In addition, double-labeling immunofluorescence of Ob-R and CART was significantly lower in the music rats than in the stressed rats ($p < 0.01$), while changes of Ob-R and NPY double-labeling and Ob-R and α-MSH double-labeling were no statistic difference (Tab. 9, Fig. 2H).

Discussion

In this study, the food intake and body weight of CIS rats were lower than those of control CIS-free rats, which was consistent with our previous experimental results (Wang *et al.*, 2012; Wang *et al.*, 2013) and the results from other studies (Elbassuoni, 2014; Liu *et al.*, 2014; Monteiro *et al.*, 1989; Valles *et al.*, 2000).

In ARC, NPY/AgRP expressing neurons promote appetite, and POMC/CART expressing neurons inhibit appetite (Abdalla, 2017; Schwartz *et al.*, 2000). Alpha-melanocyte-stimulating hormone (α-MSH), produced by POMC, act via the interaction with its receptors in inhibiting appetite, decreasing body weight gain, and promoting energy consumption (Cone, 2006; Lu, 2001). The functional isoform of the leptin receptor (Ob-Rb) is widely expressed within hypothalamic nuclei (Elmquist *et al.*, 1998; Fei *et al.*, 1997), particularly in the ARC, lateral hypothalamic area (LHA), ventromedial hypothalamic nucleus (VMN), and dorsomedial hypothalamic nucleus (DMN) (Bagnasco *et al.*, 2002). NPY/AgRP- and CART/POMC-positive neurons in the ARC express Ob-Rb (Baskin *et al.*, 1999; Cheung *et al.*, 1997; Elmquist *et al.*, 1998). Ob-Rb is the main functional receptor that is mediated by leptin. Leptin is a hormone secreted by adipose cells. Circulating leptin crosses and binds to Ob-Rb in the ARC to inhibit NPY/AgRP expression and activate CART/POMC expression, which alters food intake and energy balance (Faouzi *et al.*, 2007).

We established rat models of CIS. The results showed that Ob-R expression was increased in CIS conditions. Peripheral leptin entered the ARC, bound to Ob-R and affected downstream nerve pathways, as shown in our previous results (Wang *et al.*, 2012; Wang *et al.*, 2013). We utilized immunofluorescence, qRT-PCR, and WB analyses to observe changes in ARC neurons involved in the regulation of appetite. Our comprehensive results demonstrated that NPY and AgRP expressions were down-regulated and that CART and POMC expressions were up-regulated in the ARC after observing reduced food intake and delayed body weight gain in CIS rats.

Music is an advanced experience of human mental activity. Many studies have suggested that listening to music may reduce stress in daily life (Linnemann *et al.*, 2015). Some experiments have demonstrated that music also affects animal physiology, especially by reducing stress responses (Alworth and Buerkle, 2013). The present study used classical Chinese music pieces, including “Three Variations of Plum Flowers”, played on flute and xiao, “The Homebound Fishermen” played on zheng, and “Hu Jia Shi Ba Pai” played on the guqin. The tone color of these pieces

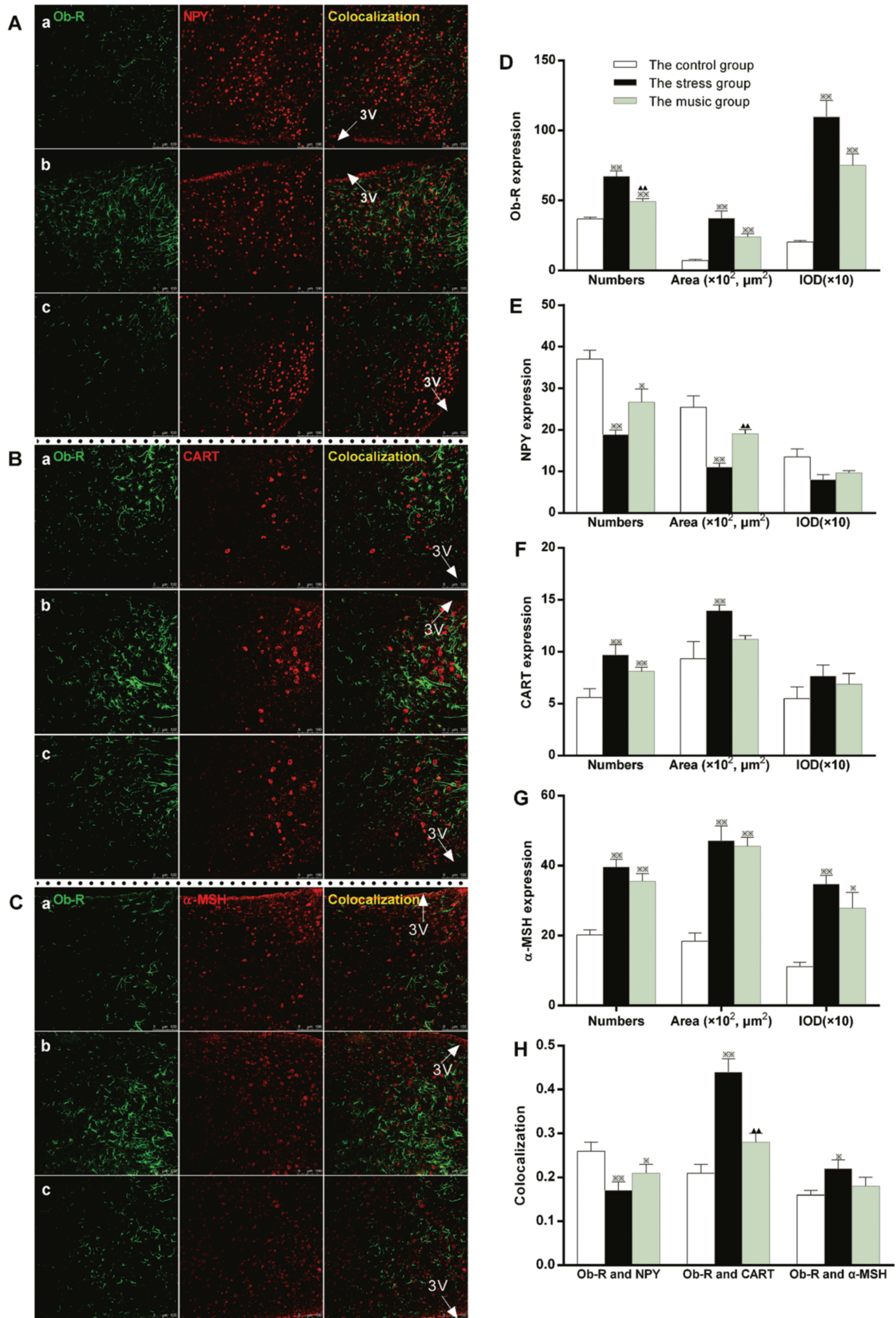

FIGURE 2. Immunofluorescence of Ob-R, NPY, CART, and α-MSH in the ARC. (A–C) Confocal images of the ARC of the three groups assessed for Ob-R (green), NPY (red), CART (red), α-MSH (red), and co-localization (yellow) are shown. (a) The control group. (b) The stress group. (c) The music group. Numbers, areas, IODs, and co-localization coefficients (H) of Ob-R (D), NPY (E), CART (F), and α-MSH (G)-positive neurons were measured using Image-Pro Plus 6.0. Each bar represents the mean ± SEM. ※$p < 0.05$, ※※$p < 0.01$ *vs.* the control group; ▲$p < 0.05$, ▲▲$p < 0.01$ *vs.* the stress group. 3V indicates the third ventricle. Scale bar = 100 μm. Original magnification 200×.

TABLE 5

Ob-R-positive neurons expression in the ARC in three groups ($\bar{x}$ ± SEM)

Group	Numbers	Areas (×10^2, μm^2)	IOD (×10)
Control group	36.80 ± 1.30	7.17 ± 0.75	20.34 ± 1.02
Stress group	67.20 ± 3.80※※	37.27 ± 5.25※※	109.77 ± 11.70※※
Music group	49.40 ± 1.82※※▲▲	24.05 ± 2.11※※	75.07 ± 8.28※※

※$p < 0.05$, ※※$p < 0.01$ *vs.* the control group; ▲$p < 0.05$, ▲▲$p < 0.01$ *vs.* the stress group.

TABLE 6

NPY-positive neurons in the ARC in the three groups ($\bar{x}$ ± SEM)

Group	Numbers	Areas (×10^2, μm^2)	IOD (×10)
Control group	37.08 ± 2.14	25.44 ± 2.73	13.46 ± 1.98
Stress group	18.83 ± 1.09※※	11.03 ± 0.93※※	7.97 ± 1.24
Music group	26.67 ± 3.16※	19.06 ± 1.06▲▲	9.64 ± 0.56

※$p < 0.05$, ※※$p < 0.01$ *vs.* the control group; ▲$p < 0.05$, ▲▲$p < 0.01$ *vs.* the stress group.

TABLE 7

CART-positive neurons in the ARC in the three groups ($\bar{x}$ ± SEM)

Group	Numbers	Areas (×10^2, μm^2)	IOD (×10)
Control group	5.6 ± 0.85	9.33 ± 1.64	5.47 ± 1.12
Stress group	9.7 ± 0.97※※	13.94 ± 0.56※※	7.64 ± 1.08
Music group	8.1 ± 0.42※※	11.20 ± 0.35	6.88 ± 1.02

※$p < 0.05$, ※※$p < 0.01$ *vs.* the control group.

TABLE 8

α-MSH-positive neurons in the ARC in the three groups ($\bar{x}$ ± SEM)

Group	Numbers	Areas (×10^2, μm^2)	IOD (×10)
Control group	20.2 ± 1.43	18.41 ± 2.36	11.10 ± 1.25
Stress group	39.6 ± 2.24※※	47.12 ± 4.27※※	34.69 ± 2.55※※
Music group	35.6 ± 2.15※※	45.58 ± 2.52※※	27.89 ± 4.46※

※$p < 0.05$, ※※$p < 0.01$ *vs.* the control group.

TABLE 9

Co-localized expression in the ARC ($\bar{x}$ ± SEM)

Group	NPY and ob-R	CART and ob-R	MSH and ob-R
Control group	0.26 ± 0.02	0.21 ± 0.02	0.16 ± 0.01
Stress group	0.17 ± 0.02※※	0.44 ± 0.03※※	0.22 ± 0.02※
Music group	0.21 ± 0.02※	0.28 ± 0.02▲▲	0.18 ± 0.02

※$p < 0.05$, ※※$p < 0.01$ *vs.* the control group; ▲$p < 0.05$, ▲▲$p < 0.01$ *vs.* the stress group.

is light and soft and induces comfortable and gentle feelings that may alleviate depression and anxiety and promote spontaneous regulation of physiological functions (Hou and Chen, 2009; Zhang and Tian, 2011).

Although music exposure had no obvious effect on the body weight or food intake of the stressed rats in this study, comfortable classical music played at 65 dB did exhibit a regulatory effect, particularly on Ob-R and NPY expressions

in the ARC of stressed rats. Supportively, Cristina Russo has shown in the study that the music interventions can not only increase the rats' body weights but also up-regulated the secretion of NPY in rat hypothalamus (Russo *et al.*, 2017).

In conclusion, these results reveal that listening to comfortable music contributes to regulating changes in central neurons of Ob-R and NPY in the ARC of CIS rats that are related to appetite and provides some experimental evidence that music therapy alleviates stress and stress-related health issues.

Authors Contributions

RZ and SW conceived and designed the study. HW, FF, and CF performed the experiments. HW, FF, RZ, and SW wrote the paper. RZ, SW, and CF reviewed and edited the manuscript. All authors read and approved the manuscript.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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