miR-212 mediates counter-regulation on CRH expression and HPA axis activity in male mice

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Abstract

The mechanisms of hypothalamic–pituitary–adrenal (HPA) axis regulation have been studied persistently but still are not elucidated. Considering the emerging roles of microRNA in stress response, we conducted a microRNA microarray in mice hypothalamus to identify the potential role of microRNAs in regulating the HPA axis. In total, 41 microRNAs changed during heat stress in which we found that miR-212 contains a binding sequence with corticotropin-releasing hormone (*Crh*) 3′UTR according to a sequence analysis. We observed that miR-212 expression in the hypothalamus was escalated by repeated heat and restraint stress. By overexpression or inhibition of miR-212 and the dual-luciferase reporter assay, we proved that miR-212 could bind with *Crh* 3′UTR to regulate its expression in mice hypothalamus primary cells and in the hippocampus neuron cell line HT-22. In addition, we injected miR-212 agomir or antagomir in mice hypothalamus to overexpress or inhibit miR-212, which leads to alterations of CRH expression and HPA axis activity *in vivo*. Furthermore, miR-212 and CRH were both transcribed by the cAMP response element-binding protein (CREB). Overexpression and inhibition of miR-212 affect CREB-dependent CRH expression. Taken together, our results suggest an inhibitory role of miR-212 on the HPA axis, which acts in a counter-regulatory manner.

Introduction

The hypothalamic–pituitary–adrenal (HPA) axis plays a pivotal role in prompting management, adaptation and recovery from stress (Sapolsky *et al*. 2000, de Kloet *et al*. 2005, McEwen 2007). It is well documented, however, that overactivity of the HPA axis is accompanied by impaired physical functions, including growth, metabolism, circulation, reproduction, inflammation and immunity (Charmandari *et al*. 2005, Chrousos & Kino 2007, Nicolaides *et al*. 2015). The mechanisms of the HPA axis regulation continue to intrigue researchers (McEwen 1998, 2008, Glaser & Kiecolt-Glaser 2005, Reynolds 2013). As the starting point of the HPA axis, corticotropin-releasing hormone (CRH) is a key element in managing HPA axis activity, and numerous researchers have studied its regulation extensively (Kovacs 2013). The dominant way by which CRH is regulated is through the negative feedback of glucocorticoids (GC) (Herman *et al*. 2016). Other factors also seem to be involved, however, as

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treatment with GC in adrenalectomized rats does not prevent an increase in stress-induced CRH hnRNA (Kageyama & Suda 2009). In addition, a decline in CRH after stress exposure is independent of circulating GC because adrenalectomized rats, which lack GC, display an identical pattern (Shepard *et al*. 2005). Aside from GC, many negative regulating factors of CRH have been identified so far, including GABAergic neurons (Gunn *et al*. 2015), endocannabinoid signaling (Hill & Tasker 2012) and inflammatory cytokines (Felger & Lotrich 2013). The network of transcription factors and co-regulators of regulation of CRH expression, however, remain to be explored (Kovacs 2013).

Since their discovery, microRNAs have been found to be involved in various biological processes and usually function as negative regulators through post-transcription regulation by binding target mRNA to inhibit mRNA translation or facilitate mRNA degradation (Zeng *et al*. 2002, Bartel 2004). Recently, it has been indicated that microRNAs participate in stress response (Leung & Sharp 2010, Hollins & Cairns 2016). A study reported that adult male rats subjected to both acute and chronic restraint stresses show altered expressions of numerous miRNAs in the brain (Meerson *et al*. 2010). More specifically, Rinaldi *et al*. found that expressions of let-7a, miR-9 and miR-26a/b increase in the frontal cortex in acute-restraint stress mice (Rinaldi *et al*. 2010). Furthermore, several studies have reported that alterations of microRNA expression, induced by stress, indirectly affect regulation of HPA axis activity. An *in vitro* study demonstrates that miR-34c reduces the responsiveness of cells to CRH by regulating corticotrophin-releasing hormone receptor 1 (CRHR1) (Haramati *et al*. 2011). Additionally, miR-449a contributes to CRHR1 downregulation induced by GC in the pituitary during stress (Nemoto *et al*. 2013). Another proof is disclosed by the finding that the GC receptor is regulated by miR-18 and miR-124 in the brain (Vreugdenhil *et al*. 2009). Nevertheless, direct connections between microRNAs and regulation of the HPA axis are absent. Whether the CRH expression is regulated by microRNAs in the hypothalamus remains unknown.

To this end, our study investigates the potential role of microRNAs in regulating CRH and HPA axis activity. By using a microRNA microarray in the hypothalamus and conducting subsequent validation experiments, we prove that miR-212 can bind with *Crh* 3′UTR to regulate its expression in a counter-regulatory manner *in vitro* and can further affect HPA axis activity *in vivo*. On the basis of our findings, we propose a new perspective of HPA axis regulation, which may be helpful for developing gene therapy interventions for patients with HPA axis overactivity diseases.

Materials and methods

Animals

We purchased male C57BL/6 (8 weeks old) mice from Slac Laboratory Animal (Shanghai, China) and maintained at $22\pm2°C$, 12 h light/12 h dark cycle (07:00 light on/19:00 light off) with food and water freely available. We conducted all animal experiments in accordance with the 'Guide for the Care and Use of Laboratory Animals', and with the approval of the Second Military Medical University Institutional Animal Care and Use Committees.

Repeated stress process

After being acclimated for 1 week, 40 mice weighing approximately 22 ± 2 g were divided randomly into five groups ($n=8$) for repeated heat stress, including day 0 (without exposure), day 1 (exposed 1 time), day 3 (exposed 3 times), day 7 (exposed 7 times) and day 14 (exposed 14 times). We also randomly divided another 40 mice in the same batch into five groups for repeated restraint stress with an identical set of groups. Details of the stress procedures are as follows: (1) Heat stimulus: mice were placed in an artificial climate cabin with a temperature of 40°C and relative humidity of 60% for 1 h; (2) immobilization: mice were placed in a 50-mL centrifuge tube (28 mm diameter × 105 mm long) with a punching hole for 1 h. All experiments were conducted between 09:00 h and 10:00 h every day. We decapitated the mice at the end of exposure, and then we immediately collected the blood and hypothalamus. We centrifuged the blood at 3000 ***g*** for 20 min to obtain serum for adrenocorticotropic hormone (ACTH) and corticosterone (CORT) detection. We used the hypothalamus to extract RNA and protein for real-time polymerase chain reaction (PCR) and Western blot.

MicroRNA microarray and data analysis

We obtained the hypothalamus from another 15 decapitated mice at day 0, day 1 and day 7 (five mice in each group). We abstracted a total of 15 RNA samples by using Trizol reagent (Catalog #15596-026, Invitrogen), which we processed in an Affymetrix GeneChip miRNA3.0 (Catalog #902017, Invitrogen) to profile the patterns of microRNA

expression. The random-variance model (RVM, which is commonly used for comparison of more than two groups) *F*-test was applied to filter the differentially expressed microRNAs for the three groups because the RVM *F*-test can raise degrees of freedom effectively in small samples. After conducting analyses to determine significance and the false discovery rate, we selected the differentially expressed microRNAs according to the *P* value threshold (Wright & Simon 2003, Clarke *et al.* 2008).

Injection of miR-212 agomir and antagomir in mice hypothalamus

We injected microRNAs agomir and antagomir to the local site for functional research (Hou *et al.* 2011). We divided the 45 mice weighing between 26 g and 28 g into five groups (day 0, 1, 3, 7 and 14), and each group had three subgroups (*n*=3), which we injected with 2 µL negative control, miR-212 agomir and miR-212 antagomir (Genepharma, Shanghai, China) at a concentration of 1 OD/µL (~2.5 nmol/µL) in the paraventricular nucleus PVN, respectively. We completed the injection by mouse brain stereotaxic apparatus. The injection coordinates were 0.82 mm caudal and 0.1 mm lateral from Bregma and 4.75 mm ventral from the surface of the skull at Bregma, according to Paxinos & Franklin (2003).

Determination of serum hormone concentrations

We measured serum ACTH and CORT by radioimmunoassay kits (Catalog #D14DJB and Catalog # D10PJB, North Institute of Biological Technology Co. Beijing, China) following the manual protocol.

Mice hypothalamic primary cells and HT-22 cells

We purchased primary cells of mice hypothalamus and HT-22 cells, a kind of mice hippocampus neuron cell line, with identification reports from Zhongqiaoxinzhou Biotech Inc. (Shanghai, China) and cultured the cells in the primary neurons' culture system (Catalog #PriMed-iCell-005, iCell, Shanghai, China) with precoated poly-L-lysine or in Dulbecco's Modified Eagle Medium (Catalog #SH30022.01B, HyClone; GE Healthcare) supplemented with 10% fetal bovine serum (Catalog #10099-141, Gibco Laboratories) and 1% antibiotic solution (Catalog #15140-122, Gibco Laboratories) and incubated the cells in a humidified 5% carbon dioxide atmosphere at 37°C.

Transfection

For hypothalamic primary cell transfection, we used miR-212 agomir and antagomir (Genepharma) at a concentration of 50 nM and 100 nM, respectively. For HT-22 transfection, we used miR-212 mimics and inhibitors (Genepharma) at a concentration of 50 nM and 100 nM, respectively. We transfected plasmids for cAMP response element-binding protein (CREB; Genepharma) at a concentration of 3 µg/mL. We performed all transfections by using micropoly-transfecter cell reagent (Catalog #MT115, Micropoly, NanTong, China), and extracted RNA and protein 48 h after the transfection for downstream experiments.

Dual-Luciferase reporter assay

We grew HT-22 cells on 96-well plates to approximately 50% confluence and co-transfected plasmids of GP-miRGLO (Genepharma) containing either the wild-type or mutated *Crh* 3′UTR with miR-212 mimics or negative control. Forty-eight hours after transfection, the cells underwent lysis for luciferase activity with a Dual-Luciferase Reporter Assay System (Catalog #E1910, Promega). We normalized firefly luciferase activity to Renilla luciferase activity for each cell culture.

Total RNA and protein extracted from mice hypothalamus and cells

Because of the small size of mice hypothalamus, we used All-In-One DNA/RNA/Protein Mini-preps Kit (Catalog #B618003, Sangon Biotech, Shanghai, China) to extract RNA and protein simultaneously. In general, after tissue homogenization and lysis, we centrifuged the liquid in an RNA purification column to obtain RNA and added the flow-through with protein precipitation solution to precipitate protein. In cells, we extracted RNA by Trizol reagent (Catalog #15596-026, Invitrogen) and extracted protein using a whole-protein extraction kit (Catalog #KGP250, Keygene, Nanjing, China).

Real-time quantitative PCR analysis

We reverse-transcribed 1 µg RNA of each sample to cDNA using a Reverse-Transcription Reagent Kit (Catalog #RR036A & #RR037A, Takara Bio). We performed real-time quantitative PCR amplification with the SYBR Green Kit (Catalog #QPK-201, Toyobo Bio Inc., Osaka,

Japan) using the StepOnePlus (Applied Biosystems). We normalized the mRNA expression of the target gene to 18s and normalized miRNA to U6. We used the following primers: 18s-forward, GTAACCCGTTGAACCCCATT; 18s-reverse, CCATCCAATCGGTAGTAGCG; *Crh*-forward, TCTCACCTTCCACCTTCTGC; *Crh*-reverse, AAGCGCAACATTTCATTTCC; *Creb*-forward, TGCCAACTCCAATTACCAA and *Creb*-reverse, ACCCCATCGGTACCATTGTT. We purchased the primer set for U6 and miR-212 from Genepharma.

Western blot

We separated 30 μg denatured proteins by electrophoresis in SDS-page gel and transferred the proteins to the polyvinylidene difluoride membrane (Catalog #BSP0161, PALL Life Science, Port Washington, NY, USA) using the Bio-Rad system. We purchased the following antibodies from Abcam: anti-CRH antibody (Catalog #ab184238), anti-CREB antibody (Catalog #ab32515), anti-CREB (Phospho-S133, Catalog #ab32096) and anti-β-actin (Catalog #D110001, Sangon Biotech, Shanghai, China). We normalized grayscales of target protein, measured by GeneSnap from SynGene software package, to β-actin.

Statistics

We represented the data as mean±S.E.M. The statistical difference between the two groups was assessed by an independent *T*-test. We performed one-way analysis of variance, followed by a Bonferroni test as the post-test, to analyze differences among the three or more groups. Differences were considered significant at the 95% confidence level ($P<0.05$): *$P<0.05$, **$P<0.01$, and ***$P<0.001$; #$P<0.05$, ##$P<0.01$, and ###$P<0.001$; &$P<0.05$, &&$P<0.01$, and &&&$P<0.001$; and $$P<0.05$, $$$P<0.01$, and $$$$P<0.001$.

Results

Hypothalamic miR-212 expression was escalated in mice exposed to repeated stress

To observe changes of hypothalamic microRNAs expression in mice exposed to repeated stress, we used Affymetrix GeneChip miRNA3.0 to investigate the expression pattern of microRNAs in the hypothalamus during heat stress as described in the Materials and methods section. Among all the microRNAs detected, we identified 41 microRNAs with significant changes

and a cluster analysis was exhibited in Fig. 1A. Through sequence-matching analysis of these 41 microRNAs, we identified 1136 target genes in three microRNA databases: Targetscan, miRdb and miRanda. The most relevant gene ontology and pathway pertaining to these genes were the DNA-dependent regulation of transcription and the PI3K-Akt signaling pathway, respectively. We did find the direct link between the microRNA and the HPA axis in which miR-212-5p could bind with *Crh* 3′UTR at position 284–290 (Fig. 2E) according to Targetscan. The expression of miR-212 increased gradually at day 1 and day 7 compared with day 0 in the microarray.

To elucidate changes of miR-212 expression and HPA axis activity, we conducted two repeated stress processes on mice and added two groups: day 3 and day 14. Results showed that miR-212 expression escalated by the amount of time exposed to stress, whereas *Crh* expression was enhanced in the mice that were exposed to the stimulus at day 1 and climaxed at around day 3. Subsequently, the *Crh* expression showed a significant decrease at day 7 and day 14 in both heat and restraint stress models (Fig. 1B and C) as well as protein level (Fig. 1E and F). In addition to the CRH expression in the hypothalamus, the serum content of ACTH and CORT exhibited identical alterations (Fig. 1D and G) in both models, which indicated that the inhibitory mechanisms of HPA axis produced effects at day 7 and day 14.

Because of the inverse relationship between a gradual increase in miR-212 and a subsequent decrease in HPA axis activity in mice subjected to repeated stress, and more meaningfully, considering potential binding site for miR-212 exists in *Crh* 3′UTR, we hypothesized that miR-212 may bind with *Crh* and could be involved in regulating its expression and HPA axis activity during repeated stress.

miR-212-regulated CRH expression in primary cells of mice hypothalamus and hippocampus cell HT-22

To explore whether miR-212 had a role in regulating the CRH expression, we first observed changes of *Crh* expression in primary hypothalamic cells after overexpression or inhibition of miR-212. Hypothalamic primary cells extracted from mice were identified by immunofluorescence using the neuron specific enolase antibody. The purity of these primary cells was greater than 95% according to the analysis of immunofluorescence (Fig. 2A). As shown in Fig. 2B, overexpression of miR-212 by miR-212 agomir transfection decreased
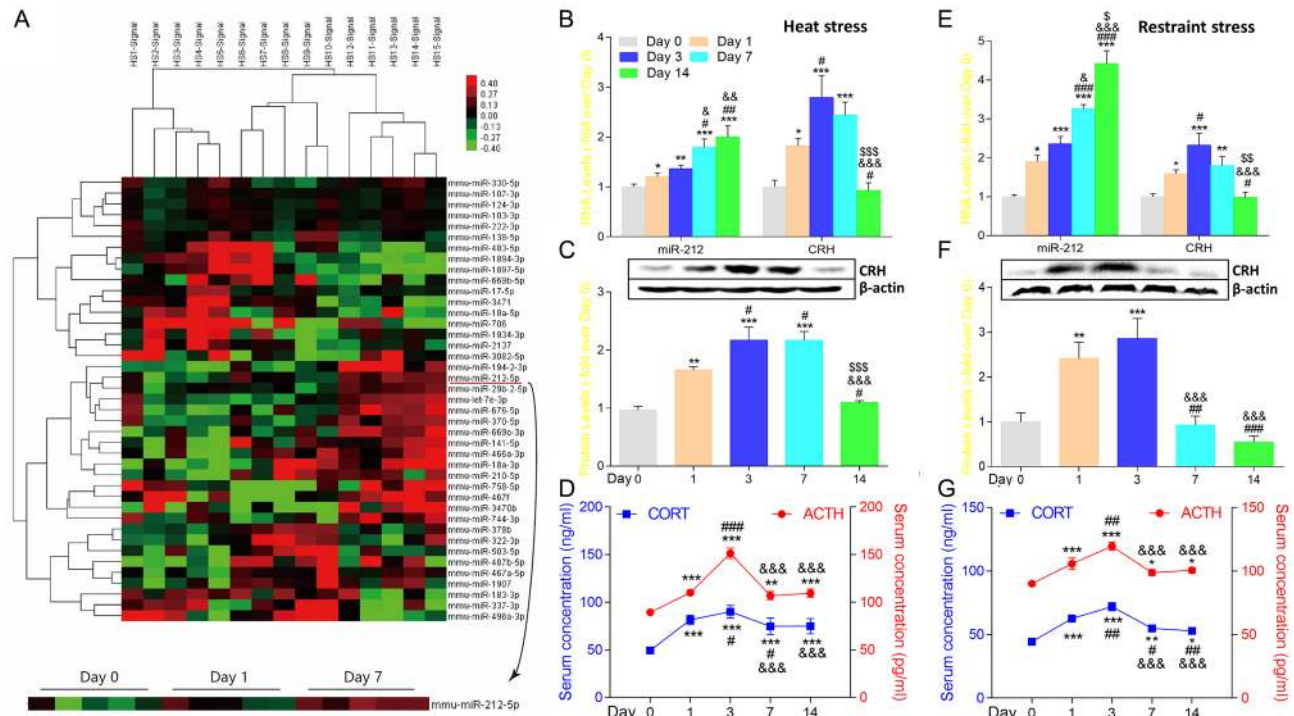
Figure 1

Hypothalamic miR-212 expression was escalated in mice exposed to repeated stress. (A) Cluster analysis of 41 significantly changed microRNAs in the hypothalamus of heat-exposed mice ($n=5$ in groups of day 0, 1 and 7). (B) miR-212 and CRH mRNA expressions ($n=8$) in a 14-day heat stress process. (C) CRH protein expression in a 14-day heat stress. Grayscale of CRH protein bands normalized to β-actin. (D) Serum contents ($n=8$) of ACTH and CORT in a 14-day heat stress process. (E) miR-212 and CRH mRNA expressions ($n=8$) in a 14-day restraint stress process. (F) CRH protein expression in a 14-day restraint stress process. (G) Serum contents ($n=8$) of ACTH and CORT in a 14-day restraint stress process. Data were presented as mean ± s.e.m. *$P<0.05$, **$P<0.01$, and ***$P<0.001$ (vs group of day 0); #$P<0.05$, ##$P<0.01$, and ###$P<0.001$ (vs group of day 1); &$P<0.05$, &&$P<0.01$, and &&&$P<0.001$ (vs group of day 3); and $P<0.05$, $$P<0.01$, and $$$P<0.001$ (vs group of day 7). A full color version of this figure is available at http://dx.doi.org/10.1530/JME-17-0124.

mRNA expression of *Crh*, whereas inhibition of miR-212 by miR-212 antagomir transfection increased mRNA expression of *Crh*.

We used the cell line HT-22, a kind of hippocampus cell that also expresses CRH, to conduct a dual-luciferase reporter assay to further identify the binding activity of miR-212 and *Crh* 3′UTR. At first, we proved that transfection with miR-212 mimics could decrease mRNA and the protein expression of CRH and miR-212 inhibitors could increase mRNA and protein expression of CRH (Fig. 2C and D). Next, we constructed GP-miRGLO plasmids that contained either a wild-type or mutated binding site in *Crh* 3′UTR for miR-212 (Fig. 2E). The result of the dual-luciferase reporter assay showed that co-transfection with wild-type plasmids and miR-212 mimics could significantly reduce the relative luciferase activity (firefly luminescence and Renilla luminescence); however, when the binding site was mutated, the effect of the miR-212 mimics disappeared (Fig. 2F). Taken together, our results showed that

miR-212 could bind directly to *Crh* 3′UTR and could regulate the CRH expression *in vitro*.

A counter-regulatory pathway mediated by miR-212 to inhibit CRH expression *in vitro*

It has been reported that CREB-responsive elements in both miR-212 and CRH promoters are required for pCREB-dependent transcription of miR-212 in primary cortical neurons and THP-1 monocytes (Remenyi *et al.* 2010, Nahid *et al.* 2013) and CRH in response to stress (Seasholtz *et al.* 1991, Kovacs & Sawchenko 1996, Kovacs 2013). This finding inspired our curiosity to determine the underlying mechanism since we observed that miR-212 and CRH displayed different expression patterns in the hypothalamus after stress (Fig. 1). To explore this, we first observed the expression of CREB in the hypothalamus. Results showed that mRNA expression (Fig. 3A), protein expression and phosphorylation level of CREB in both heat stress (Fig. 3B) and restraint stress (Fig. 3C) had the

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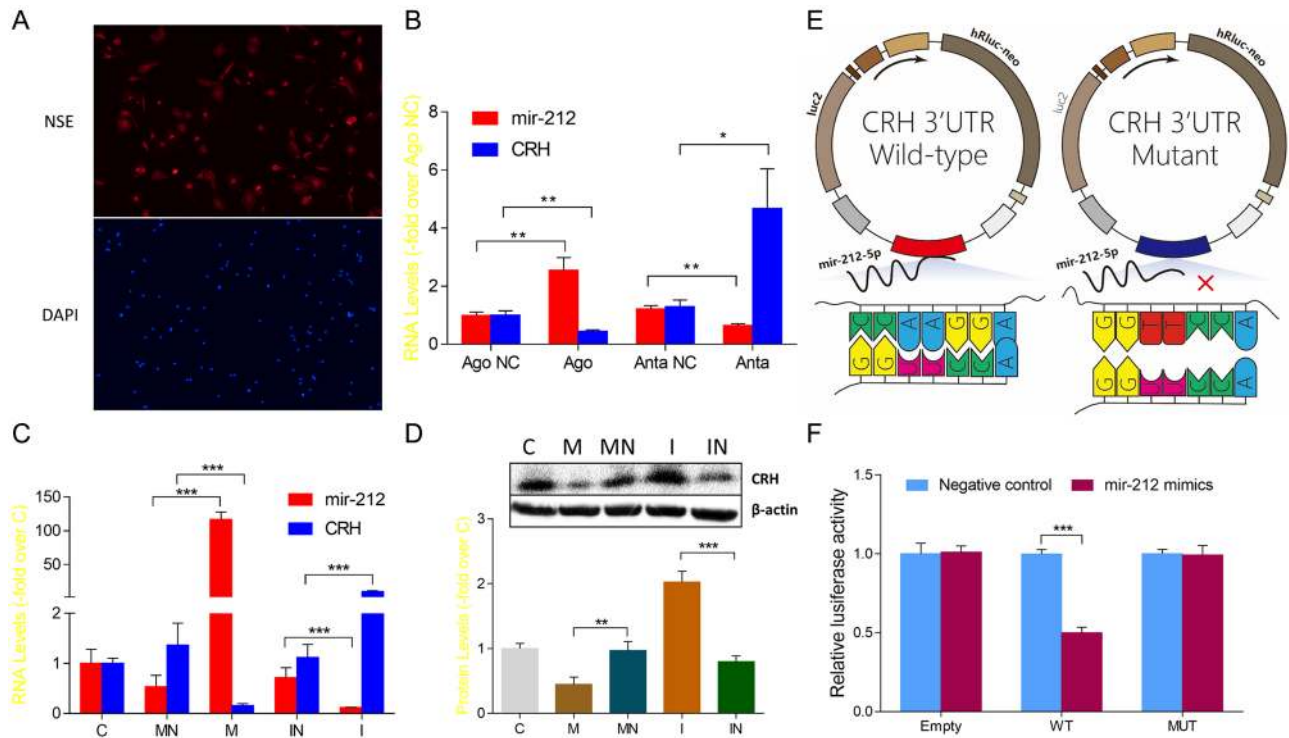
Figure 2

miR-212-regulated CRH expression via direct binding in hypothalamic primary cells and HT-22 cells. (A) Identification of primary hypothalamic cells by immunofluorescence using NSE antibody. The purity of these primary cells was more than 95%. (B) Transfecting hypothalamic primary cells with miR-212 agomir led to a decrease in the CRH mRNA expression, whereas miR-212 antagomir led to an increase in the CRH mRNA expression. Ago NC, negative control of miR-212 agomir; Ago, miR-212 agomir; Anta NC, negative control of miR-212 antagomir; Anta, miR-212 antagomir. (C) Transfecting miR-212 mimics to HT-22 cells led to a decrease in CRH mRNA, whereas miR-212 inhibitors led to an increase in CRH mRNA. C, blank control; MN, miR-212 mimics negative control; M, miR-212 mimics; IN, miR-212 inhibitors negative control; I, miR-212 inhibitors. (D) Effects of miR-212 mimics and inhibitors transfection on the protein expression of CRH were consistent with the mRNA expression. (E) Binding sequences of wild-type and mutant CRH 3′UTR with miR-212-5p in GP-miRGLO plasmids. (F) Relative luciferase activity of co-transfection of (1) empty plasmids (empty) and miR-212 mimics or negative control; (2) wild-type CRH 3′UTR plasmids (WT) and miR-212 mimics or negative control and (3) mutant CRH 3′UTR plasmids (MUT) and miR-212 mimics or negative control. miR-212 mimics led to a decrease in relative luciferase activity of wild-type plasmid, but this effect disappeared as a result of a mutated binding sequence. Data were presented as mean ± s.e.m. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. A full color version of this figure is available at http://dx.doi.org/10.1530/JME-17-0124.

same trend as miR-212. The CRH expression, however, increased at first and subsequently decreased under the condition that CREB persistently increased during repeated stress process.

To observe the role of miR-212 in CREB-dependent CRH expression, we used HT-22 cells for transfection and set 5 groups: blank control (C), negative control of CREB plasmids (NC), CREB overexpression plasmids (CR), co-transfection of CREB plasmids and miR-212 mimics (CRM), and co-transfection of CREB plasmids and miR-212 inhibitors (CRI). Transfection with CREB plasmids resulted in an increase in CREB, miR-212 and CRH (Fig. 3D and E; the group of CR), which meant that CREB was a transcriptional activator of miR-212 and CRH gene expressions, which is in accordance with other research works (Remenyi *et al.* 2010, Kovacs 2013, Nahid *et al.* 2013). Co-transfection of CREB plasmids

and miR-212 mimics reduced the increase in the CRH expression, whereas miR-212 inhibitors amplified the role of CREB overexpression on the CRH expression compared with the group that had only CREB overexpression (Fig. 3D and E; the group of CRM and CRI). Thus, we thought that miR-212 acted in a counter-regulatory way to prevent the overexpression of CRH when CREB was activated during stress.

miR-212-regulated CRH expression and further affected HPA axis activity *in vivo*

To observe the effects of miR-212 on CRH expression and HPA axis activity *in vivo*, we overexpressed or inhibited miR-212 in the hypothalamus through injection of miR-212 agomir or antagomir. At first, we observed that injection of miR-212 agomir could significantly increase
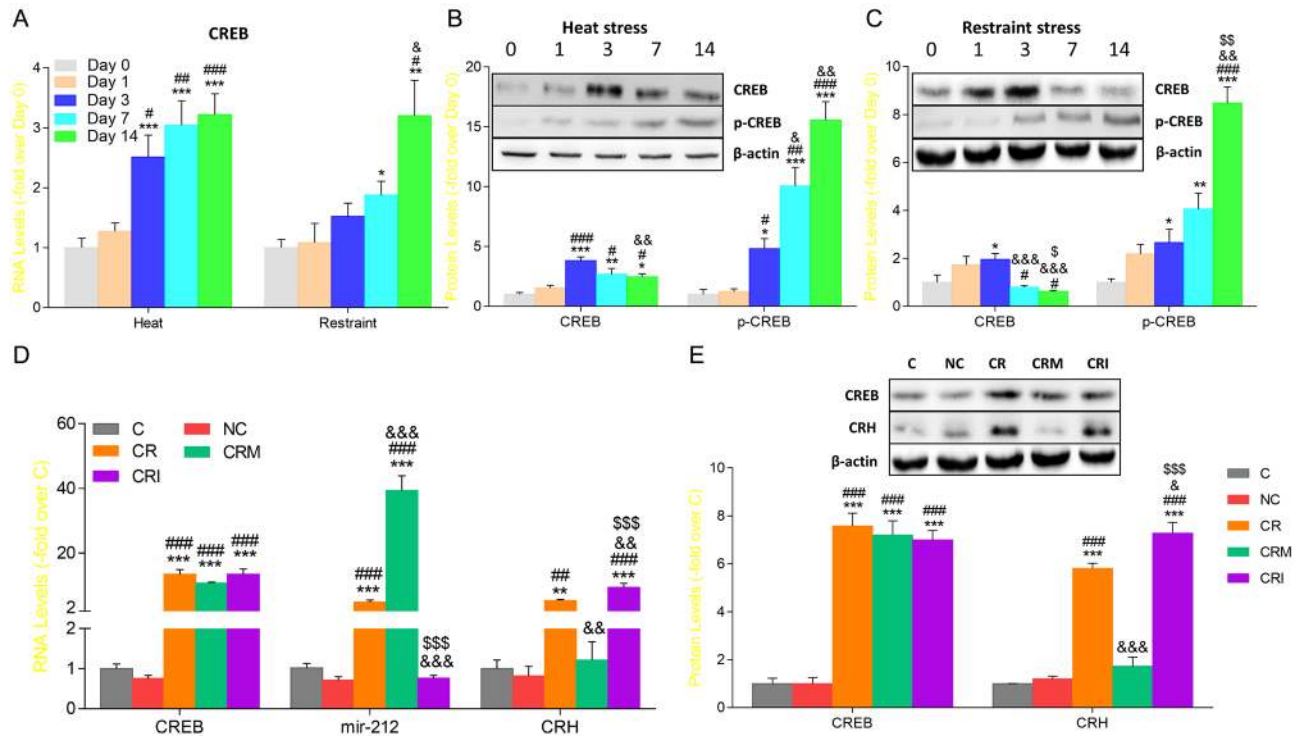
Figure 3

CREB-mediated CRH expression was regulated by miR-212. (A) Effects of heat–restraint stress on the expression of CREB mRNA ($n=8$) in a 14-day repeated stress process. (B) Protein expression of CREB and p-CREB in the heat stress process ($n=3$). (C) Protein expression of CREB and p-CREB in the restraint stress process ($n=3$). Data were presented as mean±s.e.m. *$P<0.05$, **$P<0.01$, and ***$P<0.001$ (vs group of day 0); #$P<0.05$, ##$P<0.01$, and ###$P<0.001$ (vs group of day 1); &$P<0.05$, &&$P<0.01$, and &&&$P<0.001$ (vs group of day 3); and $$P<0.05$, $$$P<0.01$, and $$$$P<0.001$ (vs group of day 7). (D) In HT-22 cells, overexpression of CREB (CR) led to an increase in miR-212 and CRH mRNA compared with group of control (C) or negative control (NC), co-transfection of CREB plasmids and miR-212 mimics (CRM) inhibited the increase in CRH mRNA, whereas co-transfection of CREB plasmids and miR-212 inhibitors (CRI) amplified the increase in CRH mRNA compared with the group of CR. (E) Changes of CRH and CREB protein resulted from co-transfection of CREB plasmids and miR-212 mimics or miR-212 inhibitors in HT-22 cells. Data were presented as mean±s.e.m. *$P<0.05$, **$P<0.01$, and ***$P<0.001$ (vs group of C); #$P<0.05$, ##$P<0.01$, and ###$P<0.001$ (vs group of NC); &$P<0.05$, &&$P<0.01$, and &&&$P<0.001$ (vs group of CR); $$P<0.05$, $$$P<0.01$, and $$$$P<0.001$ (vs group of CRM). A full color version of this figure is available at http://dx.doi.org/10.1530/JME-17-0124.
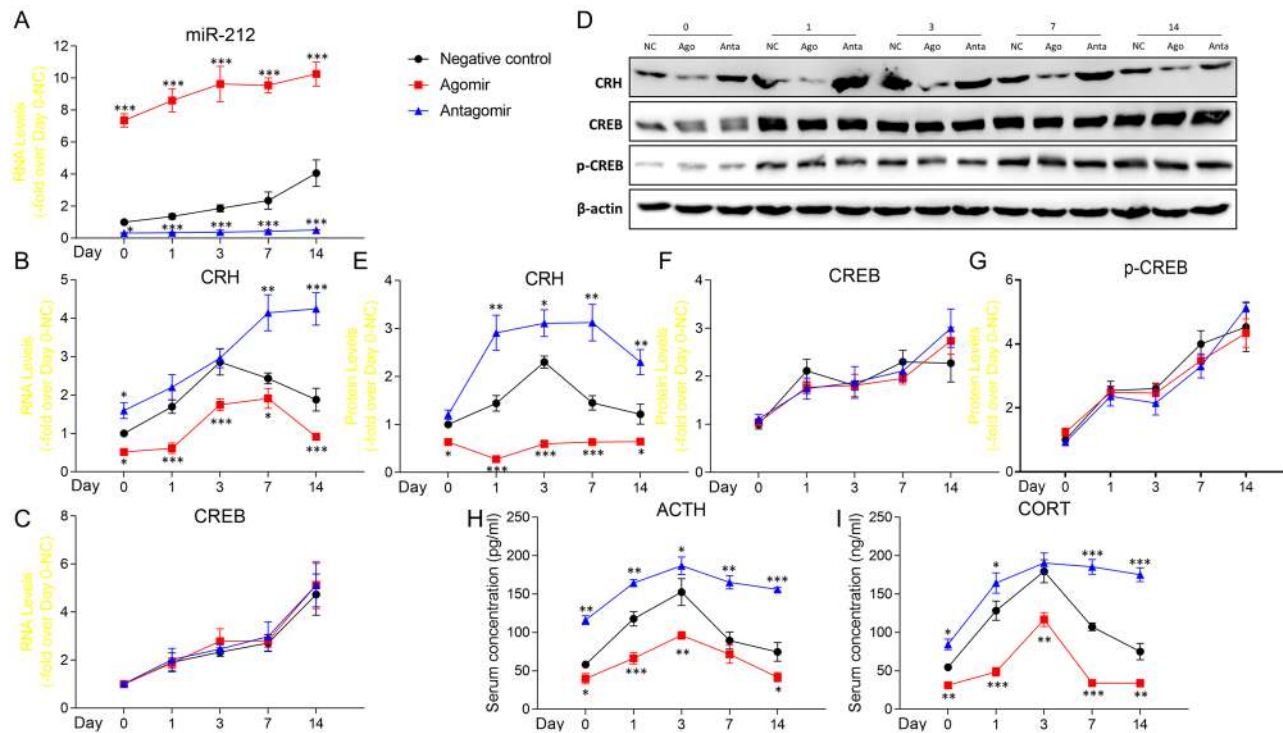
the miR-212 expression in hypothalamus. Meanwhile, CRH expression and HPA axis activity decreased after one-time heat stimulus (data not shown). After that, we conducted a 14-day heat stress process on mice by injecting a negative control, miR-212 agomir or miR-212 antagomir into the hypothalamus.

During repeated stress, mice injected with miR-212 agomir or antagomir showed a significant increase or decrease in miR-212 in the hypothalamus (Fig. 4A). Consistent with the results of experiments *in vitro*, overexpression of miR-212 decreased the CRH expression and inhibition of miR-212 increased the CRH expression at both mRNA and protein levels (Fig. 4B, D and E). CREB and pCREB levels increased gradually during repeated stress, independent of the injection (Fig. 4C, D, F and G). A more meaningful result was that injection of miR-212 agomir led to a decreased serum content of ACTH and CORT compared with injection of the negative control

(Fig. 4H), whereas injection of miR-212 antagomir augmented the stress response with an increased serum content of ACTH and CORT (Fig. 4I). Taken together, these results demonstrate that miR-212 could modulate HPA axis activity by regulating the CRH expression *in vivo*.

Discussion

Much is known about CRH regulation to date, and many aspects of the inhibitory mechanisms of CRH have been elucidated, including the following two main aspects: (1) negative feedback, which includes GC negative feedback (Di *et al*. 2003, Evanson *et al*. 2010), ACTH negative feedback (Silverman & Sternberg 2012) and negative feedback of other hormone or cytokine (Uchoa *et al*. 2014); and (2) counter-regulation, including an inhibitory neuron projecting to the PVN (Evanson & Herman 2015), an inhibitory factor inducible cyclic AMP

Figure 4

Effects of overexpression or inhibition of miR-212 on CRH expression and HPA activity in mice exposed to the heat stress process. (A) miR-212 expression in mice hypothalamus. Injection of miR-212 agomir significantly increased the miR-212 expression in the hypothalamus, whereas injection of miR-212 antagomir had an inhibitory effect. (B) Effects of miR-212 agomir and antagomir on the mRNA expression of CRH during the 14-day heat stress process. In mice injected with negative control, level of CRH mRNA increased at beginning and returned to baseline at the end. Compared to group of the negative control, the miR-212 agomir injection group showed a significant decrease in the CRH mRNA expression. At the same time, the miR-212 antagomir injection group showed an increase in the CRH mRNA expression. (D and E) The protein expression of CRH was in accord with the mRNA expression. (C) The CREB mRNA expression was escalated during the stress process independent of the miR-212 injection, as well as the protein expression of CREB and p-CREB (D, F and G). (H and I) Effects of miR-212 agomir and antagomir injection on serum contents of ACTH and CORT. Like CRH expression in the hypothalamus, serum contents of ACTH and CORT were also regulated by miR-212. The results show that miR-212 had a role in regulating HPA axis activity by modulating hypothalamic CRH expression. Data were presented as mean±S.E.M. *$P<0.05$, **$P<0.01$, and ***$P<0.001$ (vs group of NC at each time-point respectively). A full color version of this figure is available at http://dx.doi.org/10.1530/JME-17-0124.

early repressor (Shepard *et al*. 2005), and the signaling of endocannabinoid (Hill & Tasker 2012). The complicated and precise networks of CRH regulators, however, are still not elucidated. Thus, it is meaningful to determine these networks to fully understand the regulation of HPA axis activity (Kovacs 2013, Gold 2015).

As verified by other research works, the HPA axis is activated when subjected to stressors and partly alleviated in long-term stress (Dallman 1993, Nyhuis *et al*. 2010*a,b*, Zimmerman *et al*. 2015). This study also shows that the HPA axis activity is partially returned to normal level when subjected to stressors for 14 days. By using a microRNA microarray analysis in the hypothalamus of mice subjected to heat stress, we found that miR-212, which is necessary for the proper development, maturation and function of neurons (Wanet *et al*. 2012), is capable of regulating CRH expression and HPA axis activity both *in vitro* and *in vivo*. As a concern, it was reported that dexamethasone treatment

has no effect on miR-212 expression in rat adrenal glands (Hu *et al*. 2013) and level of pCREB and CREB in hypothalamic 4B cells (Evans *et al*. 2013). In our studies, the trend of corticosterone, which peaks at day 3, and that of miR-212, which rises continuously in 14 days, also suggested that the expression of miR212 is independent of the stress-induced adrenal steroid. In addition, our results show that treatment of CREB-overexpressed cells with miR-212 inhibitors resulted in higher levels of CRH expression compared with the CREB-overexpressed group, whereas the lower level of CRH expression was exhibited in the group treated with miR-212 mimics. These results suggest that miR-212 acts as a counter-regulatory factor in regulating CRH expression and HPA axis activity. Another explanation for the alleviation of HPA axis activity is habituation, which is a whole body response to rebuilding homeostasis during unlethal repeated stress. Our results showed that miR-212, which escalated during repeated stress, may be a part of

habituation processes since miR-212 could regulate HPA axis activity and level of serum corticosterone, which is important in adaptation and recovery from stress (Sapolsky *et al.* 2000, de Kloet *et al.* 2005, McEwen 2007). More important, because of the simultaneously synthesis of CRH and miR-212, we thought that regulation of miR-212 on CRH expression and HPA axis activity was prompt and efficient. Therefore, we proposed that once exposed to stress, a switch is turned on to activate the HPA axis but at the same time, a fuse (which miR-212 seems to be) is triggered to prevent overactivation of HPA axis (Fig. 5). Future studies will continue to explore the relationships among miR-212, HPA axis and habituation.

Currently, increasing numbers of people are threatened by excess HPA axis activity, especially those suffering from chronic physiological or psychological stress. Epidemiological investigations show that high levels of endogenous GCs, or treatment with exogenous GCs, are associated with adverse metabolic profile, increased cardiovascular disease and altered mood and cognitive decline (Reynolds 2013). Additionally, a major depressive disorder is characterized by an increased release of GCs and hyperactivity of the HPA axis (Keller *et al.* 2017). Other meaningful reports concluded that a decrease in miR-212 was linked with postpartum psychosis (Weigelt *et al.* 2013), mesial temporal lobe epilepsy (Haenisch *et al.* 2015) and Alzheimer's disease (Pichler *et al.* 2017). More significantly, dysregulation of miR-212 was associated with schizophrenia and bipolar disorders (Kim *et al.* 2010). Because our study indicates that inhibition of miR-212 leads to an increase in CRH expression and overactivity of the HPA axis both *in vitro* and *in vivo*, loss of miR-212 may be one of the reasons resulting in overactivity of the HPA axis and therefore may induce these mental disorders. The relationships among loss of miR-212, overactivity of the HPA axis and relative mental diseases deserve further investigation.

Another concern is that interventions for people who suffer from an overreactive HPA axis are lacking (Reiche *et al.* 2004, Wingenfeld & Wolf 2011, Doom & Gunnar 2013, Walker *et al.* 2016, Roelofs & Pasman 2017). As a kind of gene-based therapy, many researchers are intrigued by microRNA therapy, and currently, these therapies are undergoing testing in clinical trials (Kasinski & Slack 2011, Yin *et al.* 2014, Fernandez-Pineiro *et al.* 2017, Ji *et al.* 2017). Therefore, microRNA intervention may be a promising method to help people who suffer from an overreactive HPA axis as our study shows that overexpression of miR-212 could significantly reduce CRH expression and HPA axis activity *in vitro* and *in vivo*.

In conclusion, our study illustrates that microRNA participates directly in the regulation of the HPA axis
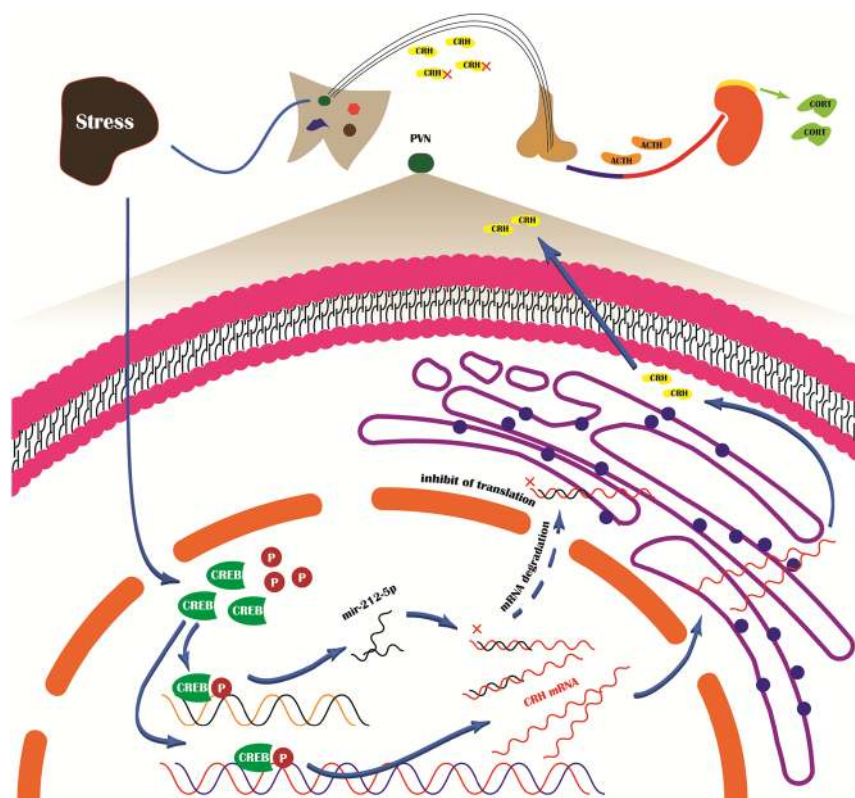

Figure 5
A counter-regulatory pathway is mediated by miR-212 to prevent overactivity of the HPA axis by regulating the CRH expression. A full color version of this figure is available at http://dx.doi.org/10.1530/JME-17-0124.

and suggests that miR-212 may be the link between overactivity of the HPA axis and relative diseases. Preventive or therapeutic measures based on miR-212 will be helpful for those people who suffer from the adverse effects of an overactive HPA axis.

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