Comparison of miRNA expression profiles in pituitary–adrenal axis between Beagle and Chinese Field dogs after chronic stress exposure

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MicoRNAs (miRNAs), usually as gene regulators, participate in various biological processes, of which stress response is included. Hypothalamus–pituitary–adrenal axis (HPA axis) is an important pathway in regulating stress response. Although the mechanism that HPA axis regulates stress response has been basically revealed, the knowledge that miRNAs regulate stress response within HPA axis, still remains poor. The object of this study was to investigate the miRNAs in pituitary and adrenal cortex that regulated chronic stress response with high-throughput sequencing. The pituitary and adrenal cortex of Beagle and Chinese Field Dog (CFD) from a stress exposure group [including Beagle pituitary 1 (BP1), CFD pituitary 1 (CFDP1), Beagle adrenal cortex 1 (BAC1), CFD adrenal cortex 1 (CFDAC1)] and a control group [including Beagle pituitary 2 (BP2), CFD pituitary 2 (CFDP2), Beagle adrenal cortex 2 (BAC2), CFD adrenal cortex 2 (CFDAC2)], were selected for miRNA-seq comparisons. Comparisons, that were made in pituitary (including BP1 vs. BP2, CFDP1 vs. CFDP2, BP1 vs. CFDP1 and BP2 vs. CFDP2) and adrenal cortex (including BAC1 vs. BAC2, CFDAC1 vs. CFDAC2, BAC1 vs. CFDAC1 and BAC2 vs. CFDAC2), showed that a total of 33 and 22 common differentially expressed miRNAs (DE-miRNAs) (Fold change >2 & *P*-value <0.001), that shared in at least two pituitary comparisons and at least two adrenal cortex comparisons, were detected separately. These identified DE-miRNAs were predicted for target genes, thus resulting in 2436 and 1363 target genes in pituitary and adrenal cortex, respectively. Further, 78 and 6 differentially expressed genes (DEGs) (Fold change >2 & *P*-value <0.05) from those target genes in pituitary and adrenal cortex were obtained separately, in combination with our previous corresponding transcriptome study. Meanwhile, in line with that miRNAs usually negatively regulated their target genes, we finally identified two DE-miRNAs possibly targeting 8 DEGs in pituitary. Of that, cfa-miR-30a possibly functionally targeted *SLC1A2*, *GRIA2*, *GRIN2A*, and *SORCS3*, thereby affecting the neuronal excitability, the synaptic plasticity and the neuronal activity in chronic stress. Our results shed light on the miRNA expression profiles in dog pituitary and dog adrenal cortex with and without stress exposure, and provide a new insight into miR-30a with its feasible roles in regulating chronic stress in pituitary .

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Short title: the dog miRNA expression profile

Abstract

MicoRNAs (miRNAs), usually as gene regulators, participate in various biological processes, of which stress response is included. Hypothalamus–pituitary–adrenal axis (HPA axis) is an important pathway in regulating stress response. Although the mechanism that HPA axis regulates stress response has been basically revealed, the knowledge that miRNAs regulate stress response within HPA axis, still remains poor. The object of this study was to investigate the miRNAs in pituitary and adrenal cortex that regulated chronic stress response with high-throughput sequencing. The pituitary and adrenal cortex of Beagle and Chinese Field Dog (CFD) from a stress exposure group [including Beagle pituitary 1 (BP1), CFD pituitary 1 (CFDP1), Beagle adrenal cortex 1 (BAC1), CFD adrenal cortex 1 (CFDAC1)] and a control group [including Beagle pituitary 2 (BP2), CFD pituitary 2 (CFDP2), Beagle adrenal cortex 2 (BAC2), CFD adrenal cortex 2 (CFDAC2)], were selected for miRNA-seq comparisons. Comparisons, that were made in pituitary (including BP1 vs. BP2, CFDP1 vs. CFDP2, BP1 vs. CFDP1 and BP2 vs. CFDP2) and adrenal cortex (including BAC1 vs. BAC2, CFDAC1 vs. CFDAC2, BAC1 vs. CFDAC1 and BAC2 vs. CFDAC2), showed that a total of 33 and 22 common differentially expressed miRNAs (DE-miRNAs) (Fold change >2 & *P*-value <0.001), that shared in at least two pituitary comparisons and at least two adrenal cortex comparisons, were detected separately. These identified DE-miRNAs were predicted for target genes, thus resulting in 2436 and 1363 target genes in pituitary and adrenal cortex, respectively. Further, 78 and 6 differentially expressed genes (DEGs) (Fold change >2 & *P*-value <0.05) from those target genes in pituitary and adrenal cortex were obtained separately, in combination with our previous corresponding transcriptome study. Meanwhile, in line with that miRNAs usually negatively regulated their target genes, we finally identified two DE-miRNAs possibly targeting 8 DEGs in pituitary. Of that, cfa-miR-30a possibly functionally targeted *SLC1A2*, *GRIA2*, *GRIN2A*, and *SORCS3*, thereby affecting the neuronal excitability, the synaptic plasticity and the neuronal activity in chronic stress. Our results shed light on the miRNA expression profiles in dog pituitary and dog adrenal cortex with and without stress exposure, and provide a new insight into miR-30a with its feasible roles in regulating chronic stress in pituitary.

Keywords dog, chronic stress exposure, HPA axis, miRNA-seq, cfa-miR-30a

Introduction

MiRNAs pertain to a class of non-coding endogenous RNAs with the lengths ranging from 18nt to 25nt. They have been much explored as gene expression regulators that involve many biological processes, including differentiation, proliferation, development and apoptosis of cells, hormone secretions, virus diseases and cancers (Stefani & Slark. 2008; Cordes et al. 2010; Taft et al. 2010). MiRNAs are also important in neuroendocrine system and functionally associated with in postembryonic development, axon guidance, synaptic plasticity and astrocyte activity (Bartel 2004; Krichevsky et al. 2006; Mor et al. 2011). Previous studies showed miR-18a could down-regulate the expression of glucocorticoid receptor in vitro (Uchida et al. 2008; Vreugdenhil et al. 2009). And in paraventricular nucleus, the miR-18a expression levels of the excessively stress-induced rats were higher than that in the natural rats. In addition, with the neuron treated by glucocorticoid (Kawashima et al. 2010), the miRNA-132 in neuron showed a lower expression level compared with that in negative control. Experiments in rats indicated that, compared with non-stress groups, the expression levels of some miRNAs in prefrontal cortex changed a bit within the acute stress exposure groups, but significantly up-regulated within the chronic stress exposure groups (Rinaldi et al. 2010). Collectively, the existing knowledge indicates that the miRNAs play important roles in neuroendocrine system and are closely associated with the stress response.

Hypothalamus–pituitary–adrenal axis (HPA axis) is an important part of the neuroendocrine system. Stress response, including acute and chronic ones, is regulated by the activity of the HPA axis in part (Frodl & O'Keane 2013; Griffiths & Hunter 2014). HPA axis is involved in maintaining the homeostasis of the body, with its regulations in digestion, immune system, mood, sex behavior, energy storage and consumption (Bodera et al. 2014; Kennedy et al. 2014; Gelman et al. 2015). Currently, the mechanism of HPA axis in regulating stress response has been basically revealed. However, especially in dog, the knowledge of the miRNAs associated with stress response in HPA axis is still devoid of a systemic revelation. In addition, to our knowledge, the systemic study of miRNAs in dog tissues still remains poor compared with that in other frequently-used medical animals, just with a study concerning dog trachea and dog lung reported (Zhao et al. 2014). In 2008, 357 candidate miRNAs in the Canis familiaris genome were identified with a comparative analysis of the whole genome in silico. Of them, 300 miRNAs were homological with characterized human miRNAs (Zhou et al. 2008). Virtually, miRNA is

characterized by its high homology among species, thus the miRNA study targeting HPA axis in dog can further its corresponding understanding of that in human or other animals as well.

High-throughput sequencing has been a powerful method to investigate the expression profiles of miRNAs, and has been widely utilized in various organisms (Zhao et al. 2014; Zhan et al. 2014; Li et al. 2015; Wongwarangkana et al. 2015). In this study, we aimed to investigate the miRNAs involving chronic stress response in dog pituitary and dog adrenal cortex (pituitary-adrenal axis). To achieve it, the pituitary and adrenal cortex tissues of Beagles and Chinese Field Dogs (CFD) that were treated with chronic stress exposure and non-treatment, were separately performed with miRNA-seq. Then, in pituitary and adrenal cortex, miRNA profiles were compared between the chronic stress exposure groups and the control groups within and between breeds to identify the miRNAs associated with chronic stress response in the pituitary–adrenal axis.

To better pinpoint the pivotal miRNAs of regulating chronic stress in pituitary and adrenal cortex, we combined the miRNA-seq of this study with our previous transcriptome study (Luo et al. 2015. In press). Importantly, the animals used, stress exposure treatment, sampling and differential expression analysis strategy in this study were all the same with our previous transcriptome study (Luo et al. 2015. In press). In our transcriptome study, glucocorticoid levels of pre-and post-stress exposure in each day were detected, and hippocampal sections of stress exposure groups and control groups in both breeds were conducted, both giving an evidence that our stress exposure was potent and valid. Besides, our previous transcriptome study identified a total of 511 and 171 differentially expressed genes in pituitary and adrenal cortex, respectively (Table S1).

The two dog breeds used here, including Beagle and CFD, own a distinct character in stress tolerance. Beagle is of good manageability, good environmental adaptability and good stress tolerance, while CFD is of excitability. We herein utilize two breeds that are distinct in stress tolerance but not just a single breed to avoid drawing a sweeping conclusion and pinpoint the miRNAs concerning chronic stress response more accurately.

2. Material and Method

2.1 Ethics Statements

In this experiment, all animals used were approved by the Animal Care Committee of Jinan University (Guangzhou, People's Republic of China) with approval number 20131018001, and strictly implemented in line with the experimental basic principles.

2.2 *Chronic stress treatment*

Chronic stress treatment was given briefly as follows: 6 unrelated purebred male Chinese Field Dogs (CFD) and 6 unrelated purebred male Beagles, similar in health, weight, and other aspects were selected randomly. From each of these two breeds, three dogs were selected for stress exposure via intermittent electrical stimulation and restraint stress, and the other 3 dogs of each breed, used as normal control, were not exposed to the stress. Each morning for 10 days, dogs were restrained and electrically stimulated with a stable current of 10 mA for 6 s followed by a 6-s interval without stimulation, lasting for 20 min every day. Meanwhile, before and after each 20-min stress exposure session, 4 ml of blood was collected and isolated to examine cortisol level with a cortisol radiation immunoassay kit used.

2.3 *Samples and RNA preparation*

All 12 dogs were killed by air embolism on the 11^{th} day, along with all parts of the adrenal cortex and pituitary tissues collected and fast frozen in liquid nitrogen until for miRNA-seq. Further, Trizol reagent (Invitrogen, USA) was used to isolate the RNAs of all the adrenal cortex and pituitary tissues collected above. The quantity and quality of RNA were examined by Agarose gel electrophoresis, NanoDrop 2000 (Thermo, USA), and Agilent 2100 (Agilent, USA). The RNA collected from each group, including 3 dogs of each, were pooled with equal mass respectively, thus obtaining 8 RNA pooled samples, including CFDP1 (CFD pituitary with stress exposure), CFDP2 (CFD pituitary with non-disposal), CFDAC1 (CFD adrenal cortex with stress exposure), CFDAC2 (CFD adrenal cortex with non-disposal), BP1 (Beagle pituitary with stress exposure), BP2 (Beagle pituitary with non-disposal), BAC1 (Beagle adrenal cortex with stress exposure), and BAC2 (Beagle adrenal cortex with non-disposal). In addition, tissues from the hippocampus were collected to perform slice analysis (Nissl staining), aiming to evaluate the influences of stress exposure on hippocampal region cells.

2.4 *small RNA library construction and sequencing*

A total of 1 μg RNA was collected from each RNA pooled sample above. Then, specific 3' and 5' adaptors were added to the RNA ends of each group (Truseq TM Small RNA sample prep Kit, Illumina). After that, the RNA with adaptors were reverse transcribed into its 1st cDNA, with random primers added. PCR was performed to amplify the cDNA with 11-12 cycles run and then resulted in 8 cDNA libraries, including CFDP1, CFDP2, CFDAC1, CFDAC2, BP1, BP2, BAC1 and BAC2. After purified further by polyacrylamide gel electrophoresis (PAGE), each cDNA library was performed by 1*50 bp high-sequencing on Hiseq-2000 (Majorbio Inc, Shanghai, China).

2.5 *Sequence data analysis*

The raw reads obtained by high-sequencing were further performed with quality control to result in clean reads. In brief, the quality control was that of trimming low-quality reads (ambiguous N and length < 18 nt), 3′ adapters, 5′ adapters and poly (A) sequences. Thereby, the lengths of 18-32 bp reads, as the clean ones, were obtained and calculated for their length distributions. Meanwhile, the identical reads were collapsed to obtain the unique reads for the statistics of the small RNA's species and abundance. Moreover, the clean reads were mapped to Rfam database (ftp://sanger.ac.uk/pub/databases/Rfam/) and GenBank noncoding RNA database (http://blast.ncbi.nlm.nih.gov/) to annotate the miscellaneous RNAs, thus filtering out the rRNA, scRNA, snoRNA, snRNA, tRNA and other non-coding RNA. The remaining sRNAs obtained above, including known miRNAs and unknown miRNAs, were then mapped to the reference genome to analyze their distributions on genome and calculate their expressive quantity. In addition, the remaining sRNAs were mapped to the Canis Canine miRNA data of miRBase 20.0 to identify the known miRNAs. Meanwhile, the novel miRNAs were predicted using miRDeep2 (http://www.mdcberlin.de/en/research/research_teams/systems_biology_of_gene_regulatory_elements/projects/miRDeep/index.html) from unannotated sRNAs (Bonnet et al. 2004; Langmead et al. 2009; Friedländer et al. 2012).

2.6 *Analysis of differentially expressed miRNAs*

To explore the differentially expressed miRNAs (DE-miRNAs) in pituitary and adrenal, on the basis of miRNA expression profiles, eight comparisons were made, including BP1_vs_BP2,

CFDP1_vs_CFDP2, BP1_vs_CFDP1 and BP2_vs_CFDP2 in pituitary; BAC1_vs_BAC2, CFDAC1_vs_CFDAC2, BAC1_vs_CFDAC1 and BAC2_vs_CFDAC2 in adrenal cortex. Herein, software DEGseq (http://www.bioconductor.org/packages/release/bioc/html/DEGseq.html) (Wang et al. 2010) was used to screen out the DE-miRNAs (fold change>2 and corrected *p-value*≤0.001). Furthermore, the common miRNAs that differentially expressed in at least two comparisons from pituitary and adrenal cortex were selected for the following target prediction, respectively.

2.7 *Target gene prediction of DE-miRNA and its GO and KEGG analysis*

For the target gene prediction, we herein utilized two different software, including Targetscan and miRDB target prediction (http://www.targetscan.org/vert_61/, http://mirdb.org/miRDB/index.html). Importantly, it's just the target genes that were both predicted by the two software above, were selected for further analysis. Then, the common target genes were selected to perform GO and KEGG pathway analysis, with KOBAS 2.0 Functional Annotation Tool (http://kobas.cbi.pku.edu.cn/program.inputForm.do?program=Annotate) employed (Corrected *p-value*<0.05).

2.8 *Conjoint analysis of DE-miRNAs and DEGs*

To better identify the important miRNAs in regulating chronic stress response, the DEGs identified in our previous transcriptome study in pituitary and adrenal cortex were compared with the target genes of DE-miRNAs in pituitary and adrenal cortex separately, thereby obtaining the genes that were both DEGs and target genes in pituitary and adrenal cortex, respectively (as a matter of convenience, the genes that were both DEGs and target genes were named "DE-target genes"). Then IPA (http://www.ingenuity.com/) was utilized to analyze the DE-target genes from pituitary and adrenal cortex separately, thus constructing the DE-target genes network in pituitary and adrenal cortex, respectively. Further, according to a negative correlation between miRNAs and their target genes in expression pattern, the DE-miRNAs and their corresponding DE-target genes that against this pattern, were then removed, thus resulting in the final DE-miRNA and its corresponding DE-target gene. Subsequently, the final DE-miRNAs above were integrated into the gene network above, thus constructing the final interaction network between DE-miRNAs and DEGs.

195 2.9 *Validation of DE-miRNAs by qPCR*

196 To elucidate the validity of the miRNA-seq data, DE-miRNAs, including miR-30a, miR-124,
197 and miR-222, were further detected by qPCR. RNA samples used here were the same with that
198 for miRNA-seq. In each group, 1 μg of pooled RNA was reverse transcribed using ReverTra Ace
199 qPCR RT Kit 101 (TOYOBO, Japan), along with bulge-loop RT primers added. The bulge-loop
200 RT primers and primers for qPCR were both synthesized by RIBOBIO (Guangzhou, China).
201 QPCR was performed on the Bio-Rad S1000 with Bestar SYBR Green RT-PCR Master Mix
202 (DBI Bioscience, Germany). Besides, *U6*, as the reference gene, was used to normalize the
203 miRNA expression on the basis of $2^{-\Delta\Delta CT}$ method (Schmittgen & Livak 2008). The detailed
204 information of the primers and the annealing temperatures was presented in Table S2.

205

206 **3. Results**

207 3.1 *High-sequencing of small RNA*

208 In this study, eight libraries including BAC2, BAC1, CFDAC2, CFDAC1, BP2, BP1, CFDP2
209 and CFDP1 were constructed for miRNA-seq. As a result, a number of raw reads ranging from
210 15,926,611 to 81,028,035 were obtained from these 8 groups (Table 1). After eliminating reads
211 of low quality (ambiguous N and length < 18 nt), 3′ adapters, 5′ adapters and poly (A) sequences,
212 a number of clean reads ranging from 11,900,933 to 74,445,014 were obtained from the 8 groups
213 (Table 1). Further, the clean reads from each group were mapped to Rfam database (11.0,
214 http://Rfam.sanger.ac.uk/) and GenBank noncoding RNA database (http://blast.ncbi.nlm.nih.gov/)
215 to annotate miscellaneous RNAs. And the detailed annotations of small RNA in each library
216 were presented in Figure S1.

217 The statistics of size distributions showed that the majority of sRNAs were 21–23 nt in length
218 across 8 samples, along with a significantly down-regulated profile in post-stress group
219 compared with that in pre-stress (Figure 1A and 1B). Besides, when mapped to the reference
220 genome of Canis Canine with the remaining sRNA reads (including known miRNAs and
221 unknown miRNAs), Chromosome 1, 3, 5, 20 and 31 were found to be mapped most abundantly
222 (ration>10%) in dog (Figure 2). Meanwhile, a bigger number of small RNA reads were showed

in pituitary samples (especially in CFD pituitary samples) compared with that in adrenal cortex samples.

3.2 *Identification of known miRNAs*

When mapped to the Canis Canine data of miRBase 20.0 by the remaining sRNA reads, a total of 276 out of 289 known miRNAs (Table S3) were identified across eight samples, while the 13 known miRNAs, including cfa-let-7j, cfa-miR-207, cfa-miR-302b, cfa-miR-302c, cfa-miR-302d, cfa-miR-367, cfa-miR-578, cfa-miR-589, cfa-miR-631, cfa-miR-665, cfa-miR-718, cfa-miR-759 and cfa-miR-872 were not found to be expressed in all samples. Of the 276 identified known miRNAs, BAC2, BAC1, CFDAC2, CFDAC1, BP2, BP1, CFDP2 and CFDP1 contributed to 263, 257, 264, 263, 269, 265, 272 and 267 miRNAs, respectively.

Besides, the importance attached to the abundance of the miRNA expression in these two breeds was conducive to identify the miRNAs of high activity in dog pituitary and dog adrenal cortex. In adrenal cortex samples, cfa-miR-99a was the most abundant known miRNA, followed with cfa-miR-30 family (including cfa-miR-30a, and cfa-miR-30d), cfa-miR-26 family (including cfa-miR-26a and cfa-miR-26b) and cfa-miR-7 family (including cfa-miR-7 and cfa-miR-7g) (The top 20 known miRNAs of abundance in adrenal cortex were listed in Table 2). Meanwhile, cfa-miR-7 family (including cfa-miR-7 and cfa-miR-7g), cfa-miR-99a, cfa-miR-30 family (including cfa-miR-30a and cfa-miR-30d), and cfa-miR-125 family (including cfa-miR-125a and cfa-miR-125b) were found to be the most abundant known miRNAs in pituitary samples (The top 20 known miRNAs of abundance in pituitary were listed in Table 3). In previous high-throughput sequencing studies of other species, various isoforms of the miRNAs were always detected (Li et al. 2011; Visser et al. 2014). In this study, we found almost all known cfa-miRNAs were of isoforms, with a larger number of isoforms possessed by the miRNAs of higher abundance. Moreover, most of the isoforms showing the highest expression level were identical to the canonical forms in miRBase 20.0, but for some other miRNAs, like the cfa-miR-217, the isoforms that showed the highest expression level were not the canonical ones at all (Figure S2), indicating the variability of miRNA expression pattern.

3.3 *Identification of novel miRNAs*

In this study, a total of 182 novel miRNAs were identified across eight samples (Table S4). Compared with the known miRNAs identified here, rather lower expression levels were showed

by the identified novel miRNAs. Of the 182 novel miRNAs, there were just 20 ones that were greater than 1000 reads in expression level (Table S5). MiR-8_37869 and miR- 24_20231 as the top two of the 20 novel miRNAs above, their predicted secondary structures were presented in Figure S3. Besides, of the 182 novel miRNAs, 97 ones shared a same seed sequence with some other known miRNAs of other species (especially mammals).

3.4 *Identification of differentially expressed known miRNAs*

In this study, a total of 29 (7 up, 22 down), 37 (16 up, 21 down), 30 (22 up, 8 down), 23 (16 up, 7 down), 21 (15 up, 6 down), 50 (11 up, 39 down), 28 (6 up, 22 down) and 40 (29 up, 11 down) DE-miRNAs (*P-value* < 0.001, $\log_2$(Fold_change) >1) were detected in the comparisons of BAC1_vs_BAC2, BAC1_vs_CFDAC1, BAC2_vs_CFDAC2, CFDAC1_vs_CFDAC2, BP1_vs_BP2, BP1_vs_CFDP1, BP2_vs_CFDP2 and CFDP1_vs_CFDP2 respectively (the DE-miRNAs identified from adrenal cortex were listed in Table S6, and that from pituitary were listed in Table S7). Furthermore, a total of 79 miRNAs expressing differentially in pituitary comparisons were obtained, with 45 common DE-miRNAs shared in at least 2 pituitary comparisons (common DE-miRNAs). Of the 45 common DE-miRNAs, cfa-miR-105a, cfa-miR-219-3p and cfa-miR-802 were the common DE-miRNAs of the four pituitary comparisons (Figure 3A). Meanwhile, of the 63 DE-miRNAs detected in adrenal cortex comparisons, there were 40 common DE-miRNAs, with the five common miRNAs of cfa-miR-196a, cfa-miR-216b, cfa-miR-300, cfa-miR-514 and cfa-miR-448 shared in all these four adrenal cortex comparisons (Figure 3B). In consideration of the functional impact exerted by miRNA expression level, we herein chose the DE-miRNAs that whose total reads within pituitary or adrenal cortex were greater than 1000 for subsequent analysis, thus resulting in a total of 33 and 22 common DE-miRNAs in pituitary and adrenal cortex separately (Table S8).

To validate the miRNA expression levels obtained by high-sequencing, 3 random DE-miRNAs, including miR-30a, miR-124 and miR-222 were selected to perform qPCR (Figure 4). The results showed miR-124, and miR-222 both were significantly up-regulated in CFDP1_vs_CFDP2, along with miR-30a in CFDP1_vs_CFDP2 and miR-124 in BAC1_vs_BAC2 both were significantly down-regulated. These observations were consistent with those obtained by high-sequencing, indicating the high-throughput sequencing data was reliable.

3.5 *miRNA target prediction, and GO and KEGG analysis*

The common DE-miRNAs, including 33 and 22 in pituitary and adrenal cortex were used for miRNA target prediction, with the methods of Targetscan and miRDB target prediction used. Consequently, 2436 and 1363 target genes were obtained from pituitary and adrenal cortex respectively, with the target genes predicted by both methods selected (Table S9). Then 2436 and 1363 target genes were performed with GO and KEGG analysis separately. Results showed that neither of the target genes from pituitary and adrenal cortex could be clustered into a certain GO term or KEGG pathway, due to the corrected p-value > 0.05.

3.6 *Interaction network of DE-miRNAs and DEGs*

In our previous transcriptome study (Luo et al. 2015. In press), a total of 511 and 171 DEGs were identified in pituitary and adrenal cortex, respectively (Table S1). The DEGs identified in our transcriptome study were separately compared with the target genes in pituitary and adrenal cortex (i.e., 2436 and 1363 respectively), thus resulting in 78 and 6 common genes that were both included in DEGs and target genes (DE-target genes), respectively. Furthermore, the 78 and 6 DE-target genes from pituitary and adrenal cortex were performed IPA separately, to construct the gene networks in pituitary and adrenal cortex respectively. Subsequently, the expression patterns of the 78 and 6 DE-target genes were compared with that of their corresponding miRNAs, without prejudice to the negative correlations between miRNAs and their target genes in both breeds. Consequently, 8 pairs of (DE-miRNA)-(DE-target gene) in pituitary were obtained, including cfa-miR-30a-*SLC1A2*, cfa-miR-30a-*GRIN2A*, cfa-miR-30a-*GRIA2*, cfa-miR-30a-*CAMTA1*, cfa-miR-30a-*SORCS3*, cfa-miR-30a-*ATP2B2*, cfa-miR-30a-*FAM49A* and cfa-miR-205-*CHN1* (Table 4). Furthermore, cfa-miR-30a and cfa-miR-205 were projected onto the above gene network in pituitary, thereby obtaining the final interaction network between DEGs and DE-miRNAs (Figure 5).

4. Discussion

Dog, especially Beagle, as a typical medical experimental animal has been utilized prevalently (Lu et al. 2015; Ji et al. 2015; Li et al. 2015). In this study, we took advantage of the good

manageability and good stress tolerance in Beagle, and the excitability in CFD, to explore the differentially expressed miRNAs in pituitary-adrenal axis under chronic stress exposure within and between breeds, with miRNA-seq. Our miRNA-seq study yielded 28,667,214, 15,926,611, 32,583,375, 32,665,593, 27,591,701, 18,359,529, 81,028,035, and 51,202,889 raw reads in BAC2, BAC1, CFDAC2, CFDAC1, BP2, BP1, CFDP2 and CFDP1, respectively. To our knowledge, this is the first time that the miRNA profiles of dog pituitary and dog adrenal cortex were presented. We found cfa-miR-30 family (including cfa-miR-30a and cfa-miR-30d), cfa-miR-7 family (including cfa-miR-7 and cfa-miR-7g) and cfa-miR-125 (including cfa-miR-125a and cfa-miR-125b) were the miRNAs of highest activity in dog pituitary. Besides, cfa-miR-99a, cfa-miR-30 family (including cfa-miR-30a and cfa-miR-30d), cfa-miR-26 family (including cfa-miR-26a and cfa-miR-26b) and cfa-miR-7 family (including cfa-miR-7 and cfa-miR-7g) were the miRNAs of highest activity in dog adrenal cortex. These observations in pituitary and adrenal cortex were distinct from those in dog lung and dog trachea, in which the cfa-miRNA-143 and the cfa-let-7 were the ones of highest activity (Zhao et al. 2014) respectively. Furthermore, when we compared the miRNA expression profiles of pre-and post- stress exposure groups with that of their corresponding gene expression profiles, especially in pituitary, a strikingly converse expression pattern was observed between the miRNA expression profiles and gene expression profiles, indirectly proving that the negatively regulated pattern between miRNAs and genes existed. Intriguingly, in non-disposal groups, within both breeds, the total miRNA expression levels of the pituitary were significantly higher than that of the adrenal cortex (The total reads of 21, 22, and 23 nt detected in CFD pituitary were all greater than 15 million, while all less than 8 million in CFD adrenal cortex, and this phenomenon was also found in Beagle but not so significantly compared with that in CFD). And this might be attributable to the wider roles played in biological functions by pituitary than that by adrenal cortex, as a larger miRNA reserve pool could irrigate more. Besides, in non-disposal groups, CFD pituitary exhibited a much higher miRNA expression level than that of Beagle pituitary. This might in part explain the different stress tolerance between Beagle and CFD: Because the expression pattern of the miRNA was negatively correlated with its target genes, and the genes involving chronic stress response in pituitary were mainly up-regulated, hence a larger miRNA reserve pool harbored by CFD pituitary indicated that the CFD had greater potential energy to up-regulate the genes involving stress response to a greater degree, thus expressing symptoms more severely in CFD.

Because the miRNAs function as gene regulators, it's of limited significance to just discuss the roles of miRNAs without concerning their target gene expressions. In this study, we combined the miRNA expression profiles with their corresponding transcriptome profiles to analyze the potential DE-(miRNAs-target genes). Consequently, cfa-miR-30a-*SLC1A2*, cfa-miR-30a-*GRIN2A*, cfa-miR-30a-*GRIA2*, cfa-miR-30a-*CAMTA1*, cfa-miR-30a-*SORCS3*, cfa-miR-30a-*ATP2B2*, cfa-miR-30a-*FAM49A* and cfa-miR-205-*CHN1* were identified as the candidate miRNAs and their corresponding target genes. We herein just identified two miRNAs and 8 corresponding target genes, and this could be accounted for our rigorous strategy of the statistical analysis and the control group set. In this study, miRNAs that were differentially expressed in at least two comparisons within pituitary or adrenal cortex groups were regarded as the candidate miRNAs for target prediction. Furthermore, the target genes were the predicted genes covered by both target prediction methods, and must be the DEGs as well. In addition, we herein didn't take the miRNA and its corresponding target genes into consideration if the expression pattern between the miRNA and its target genes was just negatively correlated in one breed but not in another. These measures all contributed to a smaller number of the candidate DE-miRNAs and their corresponding target genes, but enabling higher fidelity.

Cfa-miR-30a was identified as a pivotal miRNA concerning chronic stress response, with its potentially regulatory roles in *GRIN2A*, *GRIA2*, *SORCS3* and *SLC1A2* in this study. MiR-30a is a member of miR-30 family. So far, the researches about it mainly center on cancer. A large body of studies has reported its dysregulation in human was closely associated with breast cancer, lung cancer, thyroid cancer, gastric cancer and leukemia (Calin et al. 2004; Li et al. 2010; Cheng et al. 2012; Kumarswamy et al. 2012). In most cancer cases, miRNA-30a usually functions as a cancer inhibitor, with its roles in anti-proliferation, anti-migration and anti-infection. Importantly, for the 7 potential target genes of cfa-miR-30a, *GRIN2A*, *GRIA2*, and *SLC1A2* were identified as the pivotal genes that regulated chronic stress response in transcriptome study (Luo et al. 2015. In press). In our transcriptome study, *GRIN2A*, *GRIA2*, and *SLC1A2* were found to be enriched in one or more pathways of the GABAergic synapse, Neuroactive ligand-receptor interaction, Retrograde endocannabinoid signaling, Dopaminergic synapse and Long-term potentiation (Table 5). Importantly, the pathways above were all involved in nervous system, and were identified to be related to chronic stress (Luo et al. 2015). *SLC1A2* pertains to the excitatory amino acid transporter, and is mainly responsible for transporting the glutamate back to the

presynaptic terminal to maintain the glutamate reserve pool of it. Meanwhile, previous studies found it played a pivotal role in glutamate uptake activity and glutamate homeostasis (Haugeto et al. 1996; Tanaka et al. 1997; Otisand & Kavanaugh 2000; Matsugami et al. 2006; Kiryk et al. 2008; Bjørnsen et al. 2014). So under chronic stress exposure, cfa-miR-30a seemed to upregulate the *SLC1A2* expression to maintain the normal neuronal excitability of the body. *GRIN2A* is a member of NMDA (N-methyl-D-aspartate) receptors and *GRIA2* pertains to the AMPA receptors. These two types of receptors both were the glutamate receptors at the postsynaptic membrane, and were integral to neuronal excitability and synaptic plasticity (Dingledine et al. 1999; Li & Tsien 2009). Thereby, in chronic stress, cfa-miR-30a possibly regulated *GRIN2A* and *GRIA2* expression level, thus affecting the neuronal excitability and synaptic plasticity. Besides, *SORCS3*, as another identified target gene of the cfa-miR-30a, is a member of the receptor family containing a Vps10p-domain. It has been studied with its feasible roles in neuronal activity (Hermey et al. 2004; Hermey 2009). Taken together, we herein made a brief chart to summarize the roles of cfa-miR-30a in regulating chronic stress response (Figure 6).

Conclusions

In conclusion, we herein compared the miRNA expression profiles of the adrenal cortex and pituitary in Beagle and CFD with and without stress exposure. We detected 276 known miRNAs and 183 novel miRNAs in total across 8 samples, and of the 276 known miRNAs, a total of 33 and 22 miRNAs were found to be differentially expressed in pituitary and adrenal cortex respectively. In combination with the transcriptome profiles corresponding to the miRNA profiles of this study, we identified cfa-miR-30a as a pivotal candidate miRNA in regulating the chronic stress. Cfa-miR-30a in this study was found to possibly play roles in affecting neuronal excitability, synaptic plasticity and neuronal activity by targeting *SLC1A2*, *GRIA2*, *GRIN2A* and *SORCS3*. Our results shed light on the miRNA expression profiles in pituitary and adrenal cortex with and without chronic stress exposure, and provide a new insight into miR-30a with its feasible roles in regulating chronic stress in pituitary.

Competing interests

The authors have declared that no competing interests exist.

Authors' contributions

WL and MF carried out the molecular genetic studies, participated in the sequence alignment, statistical analysis, and drafted the manuscript. HPX reviewed drafts of the paper. MF and HJX contributed reagents/materials/analysis tools. QN and FJ conceived and designed the study. All authors read and approved the final manuscript.

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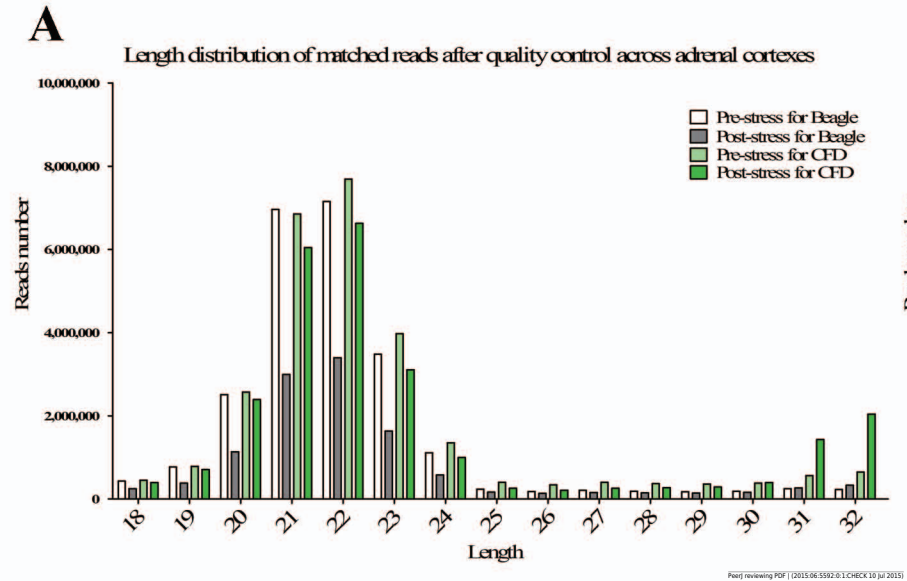
547

Figure 1(on next page)

Length distribution of matched reads after quality control in adrenal cortex (A) and pituitary (B)

The white, light black, light green, and green columns represent the samples of pre-stress for Beagle, post-stress for Beagle, pre-stress for CFD and post-stress for CFD, respectively.


A
Length distribution of matched reads after quality control across adrenal cortexes
Pre-stress for Beagle
Post-stress for Beagle
Pre-stress for CFD
Post-stress for CFD
Reads number
10,000,000
8,000,000
6,000,000
4,000,000
2,000,000
0
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
Length

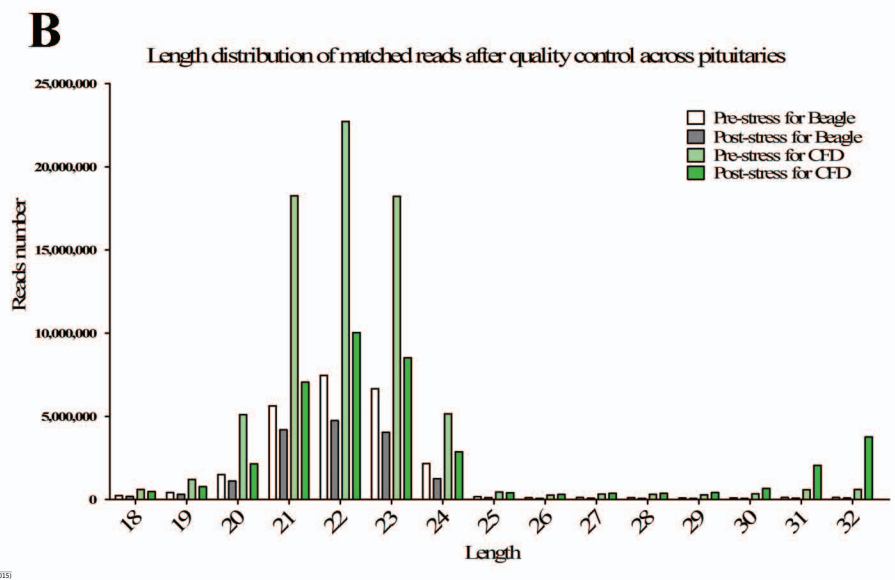

B
Length distribution of matched reads after quality control across pituitaries
Pre-stress for Beagle
Post-stress for Beagle
Pre-stress for CFD
Post-stress for CFD
Reads number
25,000,000
20,000,000
15,000,000
10,000,000
5,000,000
0
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
Length

Figure 2(on next page)

Distribution ration by miRNAs on genome. erif";m4K

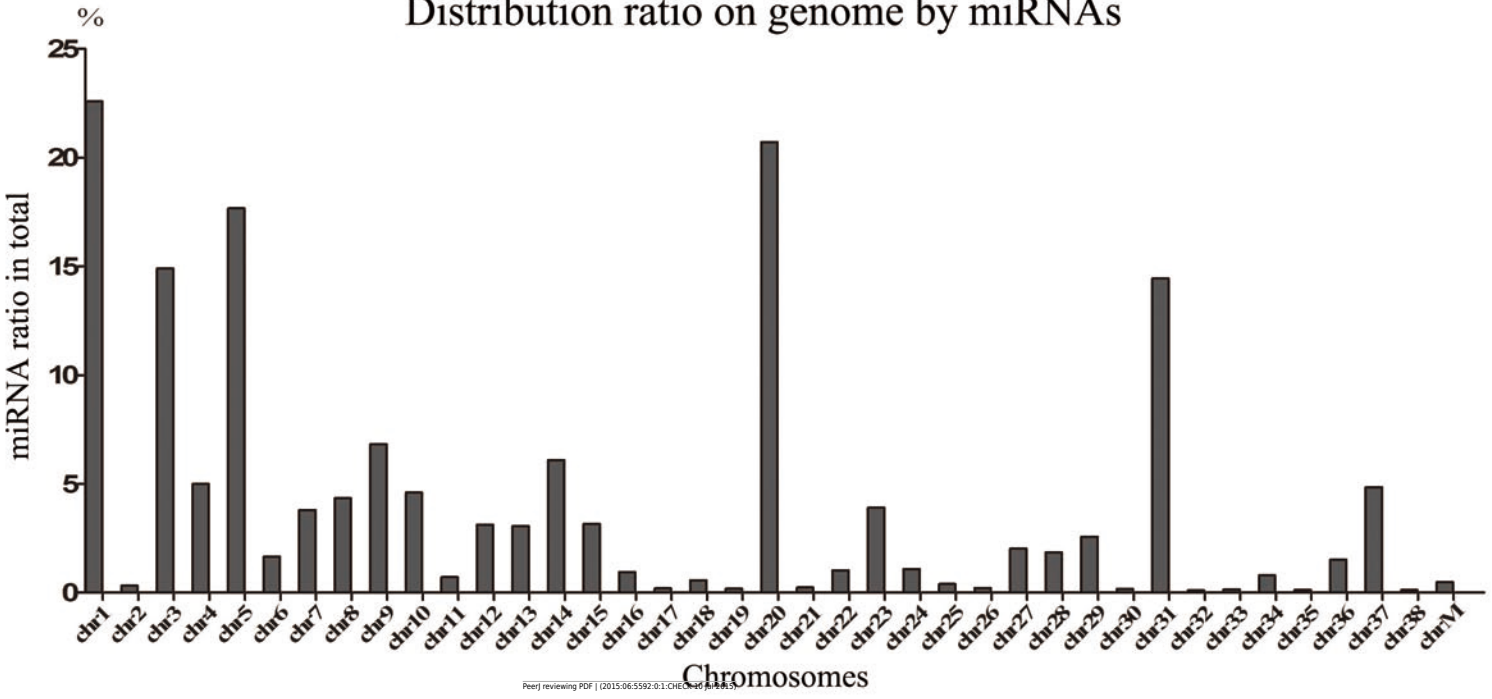

Distribution ratio on genome by miRNAs
%
25
20
15
10
5
0
miRNA ratio in total
chr1
chr2
chr3
chr4
chr5
chr6
chr7
chr8
chr9
chr10
chr11
chr12
chr13
chr14
chr15
chr16
chr17
chr18
chr19
chr20
chr21
chr22
chr23
chr24
chr25
chr26
chr27
chr28
chr29
chr30
chr31
chr32
chr33
chr34
chr35
chr36
chr37
chr38
chrM
Chromosomes

Figure 3(on next page)

Venn diagrams of differentially expressed miRNAs (DE-miRNAs) among comparisons of each tissue.

A- The DE-miRNAs distributions in pituitary, including BP1, BP2, CFDP1 and CFDP2. **B-** The DE-miRNAs distributions in adrenal cortex, including BAC1, BAC2, CFDAC1 and CFDAC2. The white number denotes the number of the common DE-miRNAs shared by at least two comparisons and are the ones selected for further analysis.

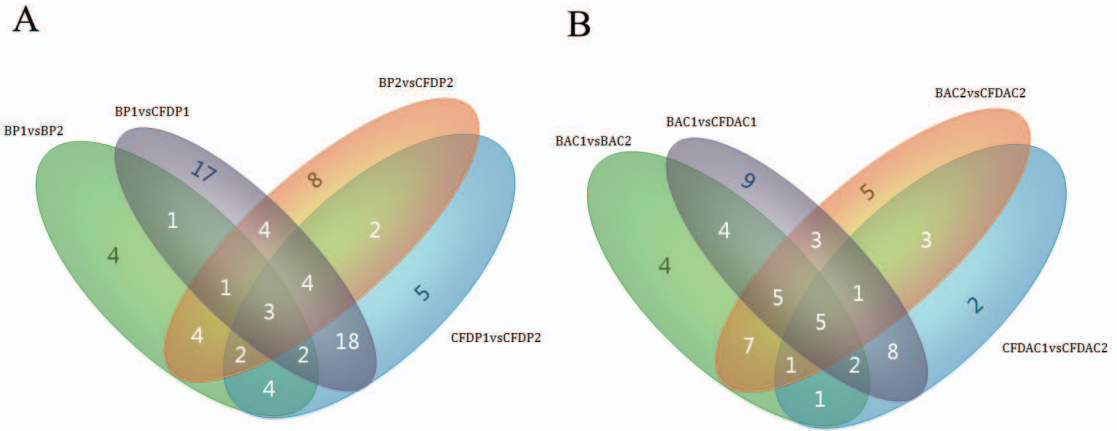

A
BP1vsBP2
BP1vsCFDP1
BP2vsCFDP2
CFDP1vsCFDP2
4
17
1
8
4
2
1
3
4
5
4
2
2
18
4
B
BAC1vsBAC2
BAC1vsCFDAC1
BAC2vsCFDAC2
CFDAC1vsCFDAC2
4
9
4
3
5
3
5
1
2
7
5
1
2
8
1

Figure 4(on next page)

Validations of miRNA-seq by qPCR.

U6 is the reference gene to normalize the expression level of cfa-miR-30a, cfa-miR-124, and cfa-miR-222.

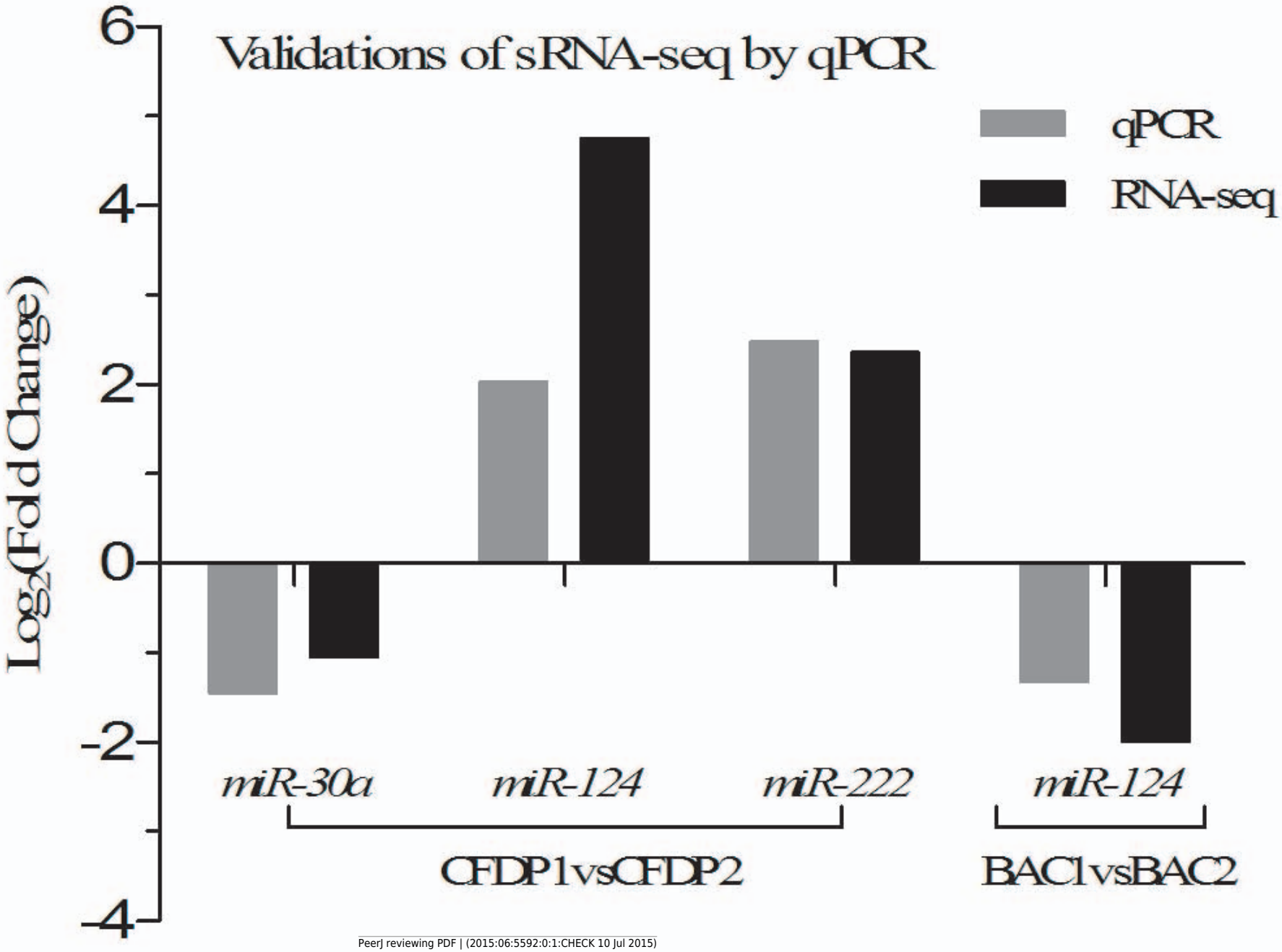

Validations of sRNA-seq by qPCR
qPCR
RNA-seq
Log2(Fold Change)
6
4
2
0
-2
-4
miR-30a
miR-124
miR-222
miR-124
CFDP1vsCFDP2
BAC1vsBAC2

Figure 5(on next page)

Interaction network integrated differentially expressed miRNAs (DE-miRNAs) and their differentially expressed target genes (DE-target genes) in pituitary.

78 target genes pertaining to differentially expressed genes are constructed by IPA, with the genes of direct relations selected for this network. Meanwhile, cfa-miR-30a and cfa-miR-205 are projected onto the gene network to reconstruct the final network of DE-miRNA and DE-target genes. The yellow circle denotes the DE-target gene and the pink rectangle represents the DE-miRNA. Dashed line denotes the relation between DE-miRNA and DE-target gene, while the solid line denotes that between DE-target gene and DE-target gene. Arrow indicates the effect direction between genes, and the ones without arrow indicate a reciprocal effect of genes. ? K&#65533;]%h-

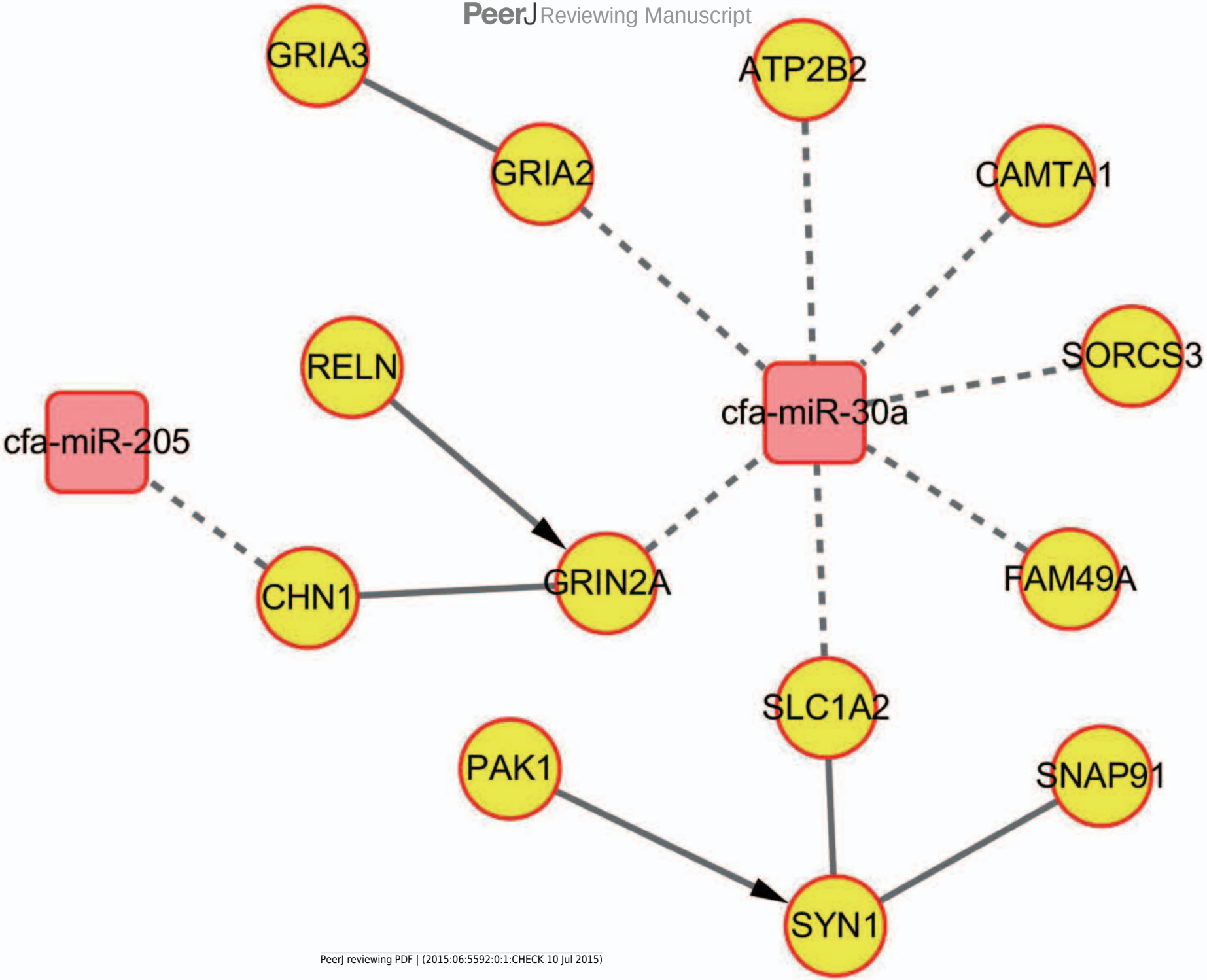

GRIA3
GRIA2
ATP2B2
CAMTA1
SORCS3
RELN
cfa-miR-30a
cfa-miR-205
CHN1
GRIN2A
FAM49A
SLC1A2
PAK1
SNAP91
SYN1

Figure 6(on next page)

Flowchart of cfa-miR-30a regulating the chronic stress.

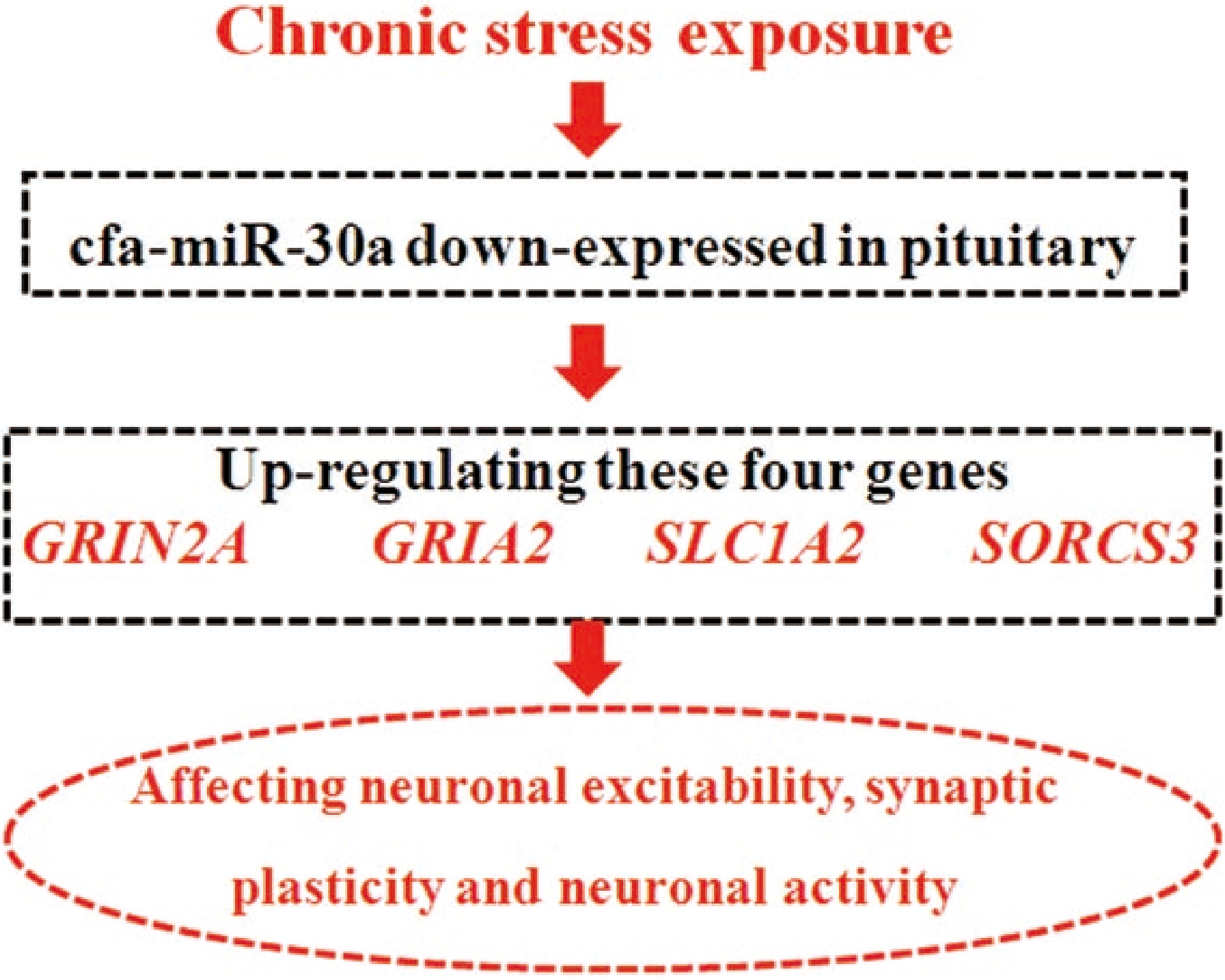

Chronic stress exposure
cfa-miR-30a down-expressed in pituitary
Up-regulating these four genes
GRIN2A
GRIA2
SLC1A2
SORCS3
Affecting neuronal excitability, synaptic plasticity and neuronal activity

Table 1(on next page)

Statistics of miRNA-seq across 8 samples in brief.

1 **Table 1. Statistics of miRNA-seq across 8 samples in brief.**

	BAC2	BAC1	CFDAC2	CFDAC1	BP2	BP1	CFDP2	CFDP1
Raw reads number	28,667,214	15,926,611	32,583,375	32,665,593	27,591,701	18,359,529	81,028,035	51,202,889
Clean reads number	24,078,122	11,900,933	27,143,726	25,448,102	24,957,693	16,409,592	74,445,014	40,249,802
Unique reads	975,309	693,607	1,508,439	1,118,474	646,032	477,154	1,644,704	1,491,566
Perfectly matched unique reads	211,901	178,920	84,239	257,486	175,320	140,709	426,965	423,546
Unique gene miRNA number	68,246	48,170	100,880	68,735	68,482	56,260	167,784	89,791
Total percent of miRNA in clean reads	82.08%	73.63%	74.32%	69.32%	88.97%	88.42%	88.27%	70.16%

2

Table 2(on next page)

The top 20 known miRNAs of abundance in adrenal cortex samples.

miRNAs ranked up to down with the total reads decreasing.

Table 2. The top 20 known miRNAs of abundance in adrenal cortex samples.

miRNA_name	Normalized reads				Total reads
	BAC1	BAC2	CFDAC1	CFDAC2	
cfa-miR-99a	23885090.2	23698720.8	19810873.5	23061916.4	90456600.8
cfa-miR-21	8270829.64	9166958.59	11362478.6	8917961.41	37718228.2
cfa-miR-10b	10830756.4	7734338.98	8593051.2	10484330.2	37642476.7
cfa-miR-26a	9373878.77	8741314.22	9605727.73	9394090.89	37115011.6
cfa-miR-143	9142690.81	9274211.39	10111291.1	7168041.93	35696235.2
cfa-miR-7	7976997.97	9396609.61	4858309	8864939.7	31096856.3
cfa-miR-374a	6606705.58	8459683.27	8402230.7	7377763.45	30846383
cfa-miR-202	7427822.25	6877350.91	7718714.55	7747423.14	29771310.9
cfa-miR-30a	5658608.42	4963782.4	5489755.45	4844690.05	20956836.3
cfa-miR-27b	5122166.51	5830649.29	5225156.81	4532650.73	20710623.3
cfa-miR-30d	4390377.34	3785721.96	5056338.25	5151399.78	18383837.3
cfa-miR-29a	3368500.34	3569168.82	3936458.01	3129052.45	14003179.6
cfa-miR-186	2984768.87	2481664.28	3402541	3581630.27	12450604.4
cfa-miR-125b	3009473.43	3206618.06	2674398.7	3404810.02	12295300.2
cfa-miR-145	2467809.23	2956808.32	3740812.11	2661095	11826524.7
cfa-let-7g	2758636.29	2813195.96	3306573.07	2869080.6	11747485.9
cfa-miR-26b	2535794.47	2246271.72	2753612.75	2877932.78	10413611.7
cfa-miR-22	2290489.62	2258852.45	2820006.03	2040488.21	9409836.31
cfa-miR-101	2700737.95	2525644.05	2090208.31	1740113.06	9056703.37

miRNAs ranked up to down with the total reads decreasing.

Table 3(on next page)

The top 20 known miRNAs of abundance in pituitary samples.

miRNAs ranked up to down with the total reads decreasing.

1 **Table 3. The top 20 known miRNAs of abundance in pituitary samples.**

miRNA_name	Normalized reads				Total reads
	BPC1	BPC2	TPC1	TPC2	
cfa-miR-7	42072356.23	52972181.04	48819574.79	33991920.3	177856032.4
cfa-miR-99a	25061757.21	21580416.66	19940044.75	27288015.58	93870234.2
cfa-miR-375	9585912.85	11114510.51	10726742.08	12287973.7	43715139.14
cfa-miR-26a	6396173.42	6323870.8	7900619.26	7717515.74	28338179.22
cfa-miR-125b	4403503.21	4879716.86	5966317.3	7841984.54	23091521.91
cfa-miR-374a	7711742.07	3956626.97	2786702.77	4550098	19005169.81
cfa-miR-30a	6285675.13	4436272.48	2553207.51	5289957.28	18565112.4
cfa-miR-335	4001569.02	4075890.5	4575892.58	4631148.6	17284500.7
cfa-miR-29a	4014629.12	4175757.79	4012135.75	3820759.64	16023282.3
cfa-miR-27b	3439612.01	3324948.83	3322590.19	4124008	14211159.03
cfa-miR-141	4626709.76	3372330.11	2315039.09	3512334.22	13826413.18
cfa-miR-30d	2632734.87	3220912.82	3332492.68	3599249.62	12785389.99
cfa-miR-148a	2807002.65	3022651.6	2048044.93	1881415.83	9759115.01
cfa-miR-96	2440241.53	1787999.7	1437373.37	2083438	7749052.6
cfa-let-7g	1902394.44	1849689.83	1938222.26	1773452.74	7463759.27
cfa-miR-411	1911780.14	1837131.67	1849683.16	1745117.95	7343712.92
cfa-miR-24	1717835.67	1667696.15	1868057.03	2044620.34	7298209.19
cfa-miR-181a	1600920.5	1297382.05	2144383.94	2085170.51	7127857
cfa-miR-125a	1108391.02	1323945.72	1729022.01	1935352.01	6096710.76
cfa-miR-183	1789539.74	1079464.57	910031.93	1686212.5	5465248.74

2 miRNAs ranked up to down with the total reads decreasing.

Table 4(on next page)

The potential differentially expressed miRNAs targeting the differentially expressed genes, without prejudice to a negative correlation between miRNA and its corresponding target genes in both breeds.

Each square categorizes the miRNA and its target genes.

Table 4. The potential differentially expressed miRNAs targeting the differentially expressed genes, without prejudice to a negative correlation between miRNA and its corresponding target genes in both breeds.

Genes \ Counts	BP1	BP2	CFDP1	CFDP2
cfa-miR-30a	6285675	4436273	2553208	5289957
SLC1A2	137.5	170.4	4665	98.91
GRIN2A	0.923	5.744	113.1	0
GRIA2	1035	1054	2754	767.1
CAMTA1	608.2	724.7	1941	749.3
SORCS3	1083	2084	1098	472.2
ATP2B2	158.7	180	1094	184.8
FAM49A	577.7	649.1	2884	633.6
cfa-miR-205	10730	22724	12765	55847
CHN1	581.4	409.7	2940	495.5

Each square categorized the miRNA and its target genes.

Table 5(on next page)

Pathways enriched by *GRIN2A*, *GRIA2* and *SLC1A2*, identified in CFDP1_vs_CFDP2 of transcriptome study.

The superscript number 1, 2, 3, 4, 5 denote the pathways enriched by the target gene, corresponding to GABAergic synapse, Neuroactive ligand-receptor interaction, Retrograde endocannabinoid signaling, Dopaminergic synapse and Long-term potentiation, respectively.

Table 5. Pathways enriched by *GRIN2A*, *GRIA2* and *SLC1A2*, identified in CFDP1_vs_CFDP2 of transcriptome study.

Gene	Description
***GRIN2A*[1,2,4,5]**	glutamate receptor, ionotropic, N-methyl D-aspartate 2A
***GRIA2*[1,2,3,4]**	glutamate receptor ionotropic AMPA 3
***SLC1A2*[1]**	Canis lupus familiaris solute carrier family 1 (glial high affinity glutamate transporter) member 2

The superscript number 1, 2, 3, 4, 5 denote the pathways enriched by the target gene, corresponding to GABAergic synapse, Neuroactive ligand-receptor interaction, Retrograde endocannabinoid signaling, Dopaminergic synapse and Long-term potentiation, respectively.