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Multiomic analysis of mice epilepsy models suggest that miR-21a expression modulates mRNA and protein levels related to seizure deterioration

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Summary

Epilepsy is now recognized as the second most common neurological disease in China. To determine the genetic cause of epileptic encephalopathy, we performed a multiomics study using mouse models of controls, anticonvulsant mice treated with five drugs and epileptic mice. Based on genome-wide profiling analysis, we discovered four genes in the epileptic mouse group with differentially-expressed mRNA. After isobaric tags for relative and absolute quantification (iTRAQ) validation, only one gene, *SNCA*, remained, which was associated with apoptotic response of neuronal cells, and regulation of dopamine release and transport. We also identified three miRNAs targeting *SNCA*, out of which mmu-miR-21a-3p demonstrated a seven-fold change in expression between control and epileptic mice.

1. Introduction

After headache, epilepsy is now the second most common neurological disease in China. However, the precise molecular mechanisms underlying seizure disorders have not been identified. Genetic factors play a small role in causing epilepsy. Other factors include brain injury, stroke and brain tumors as well as drug and alcohol misuse (Berkovic *et al.*, 2006). In this study, we examine epilepsy caused by maximum electroshock (MES) in mouse models.

Several genes involved in the pathological process of epilepsy have been identified in molecular studies. Recently, genome-wide gene expression profiling studies have become popular. However, few have focused on the mechanisms of epilepsy. By direct sequencing with next-generation sequencing (NGS), tens of thousands, or even millions, of tags can be analyzed at the same time, generating precise and quantitative gene expression profiles. RNA-seq provides the most accurate transcription profiles used for further investigation at the cellular level. MicroRNA plays an important role in the regulation of gene expression, cell cycle, timing and other aspects of organism development by binding to the target (mRNA) and then inhibiting gene expression. MicroRNAs and mRNAs, whose expression levels are relevant in biological research, can be reliably and accurately quantified on a genome-wide scale with the help of NGS technologies (Zhou *et al.*, 2010).

Mouse models of epilepsy provide a framework for understanding critical features of the brain that regulate excitability, and will continue to serve as the basis for epilepsy research (Baraban, 2007). In this study, we used the MES test to induce epilepsy in mice. The MES test, developed by Toman and his collaborators more than 60 years ago (Castel-Branco *et al.*, 2009) is probably the best validated preclinical test to accurately predict the effect of drugs against generalized seizures. We selected five antiepileptic drugs that have been widely used in the clinical environment: Efexor, FT, RF, J601 and Q808 (Table 1). The former three drugs were manufactured by Sigma, the latter two were synthesized in the laboratory by ourselves. We studied the transcriptome of drug treated and epileptic (induced by MES) mice to confirm the existence of molecular mechanisms in the normal/epileptic mouse group.

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Table 1. *Sample information.*

Group	Symptom
Control	Normal
MES	Epilepsy
Efexor50	Returned to normal
FT-1	Returned to normal
J601-1	Returned to normal
Q808-1	Returned to normal
RF-1	Returned to normal

In this study, we performed a synthetic RNA-seq analysis profile of mRNAs by initially detecting expression levels in control, drug treated and epileptic (induced by MES) mouse brains using the Illumina HiSeq2000 platform. Furthermore, we selected miRNAs of the differentially-expressed target genes and analysed the proteomic data using isobaric tags for relative and absolute quantification (iTRAQ) to validate the genes. We also used the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6·7 to annotate these genes and pinpoint key signalling and metabolic pathways that were frequently abnormal in epileptic mice.

2. Materials and methods

(i) *Animals*

The experiments were performed on adult (18–22 g) KunMing male mice. Mice were individually housed in a quiet room with a 12-h light/dark cycle, at 22 ± 2 °C and 60 ± 5% humidity. All experiments were performed at the same time during the light period. The procedures used in this study were conducted in line with the Guidelines for the Care and Use of Laboratory Animals as adapted by the National Institute of Health, and were also approved by the local ethics committee (Wan, 2014; ethics committee number: 10/2013). All efforts were made to minimize the suffering of animals and to reduce the number of animals used. According to the results of the preliminary experiment, this study had seven treatment groups: control, MES, Efexor50, FT-1, J601-1, Q808-1 and RF-1. Every group included three mice. All animals were fed normal mouse food. Before being sacrificed, control group mice were injected with saline. All control group mice displayed general behaviour while mice in the MES group were given the MES test to induce epilepsy. We carried out the MES test in this study according to the standard description in the Antiepileptic Drug Development Program (ADD) of the National Institutes of Health (USA). We used an electric stimulation generator (JTC-1, ChengDu, China) to produce electroconvulsions. Seizures were induced with a 60-Hz alternating current of 50 mA intensity. The current was applied with ear-clip electrodes for 0·2 s. The criterion of the seizure activity was tonic hindlimb extension. The following groups were based on the MES group but were injected with drugs as follows: 10 mg/kg of Q808 for the Q808-1 group, 15 mg/kg RF for the RF-1 group, 50 mg/kg Efexor for the Efexor50 group, 18 mg/kg J601 for the J601 group and 35 mg/kg FT for the FT group. After receiving these drugs, the mice experiencing epilepsy caused by MES returned to normal.

(ii) *Brain collection, RNA isolation and small RNA library construction*

The hippocampus is the most epileptogenic region of the brain (Wiebe, 2000). Total RNA was extracted from the hippocampus of mice brains using TRIZOL (Invitrogen). The final RNA concentration and purity were measured using an Agilent 2100 Bioanalyzer. RNA samples were stored at -80 °C and then were sequenced with an Illumina HiSeq2000. After sequencing, dirty raw reads were filtered to obtain cleans reads and criteria were as follows: removed reads with sequence adaptors; reads with more than 2% 'N' bases; and low-quality reads (more than 50% quality assurance $\leqslant$15 bases). In the end we obtained no less than 6 million clean reads for each sample.

In order to construct the small RNA library and to enable sequencing, 18–30 nt RNA was obtained using PAGE and the purified small RNA from different samples was used for sequencing with an Illumina HiSeq2000. Fifty nt sequence tags from HiSeq sequencing were obtained, then adaptors and low-quality tags were removed to retain high-quality sequences. In the end we obtained no less than 8 million reads of sRNA.

A standard bioinformatics analysis was carried out, including annotation of the high-quality sequences into categories and using sequences unable to be annotated to predict novel miRNA editing. The small RNA reads were mapped to the mouse pre-miRNA and matured-miRNA of miRBase 19 in order to annotate the miRNA categories with the Blast tool. The genomic positions of known and novel miRNAs were identified from the miRBase 19 database.

(iii) *Statistical analysis of differential mRNA expression*

Due to the arrival of NGS, sequence-based expression analysis has become an increasingly prevalent trend. Expression profiling has logically become the next step after sequencing a genome to determine what is actually happening at any point in time. Referring to 'the significance of digital gene expression profiles'

Table 2. *Sample expression information.*

Sample	mRNA expression (TPM)
Control	14 814
MES	14 719
Efexor50	14 779
FT-1	14 528
J601-1	14 477
Q808-1	14 468
RF-1	14 338

Table 3. *Sample differential expression information.*

Sample pair	mRNA differential expression
Control/MES	5730
MES/Efexor50	4782
MES/FT-1	6726
MES/J601-1	7078
MES/Q808-1	8480
MES/RF-1	9701

(Audic & Claverie, 1997), we have developed a rigorous algorithm known as log2-Ratio (Ratio = the expression level of sample 2/the expression level of sample 1) to identify differentially expressed genes between two samples. The differentially-expressed statistical model used is Poisson distribution, and false discovery rate (FDR) was used to determine the *p*-value threshold. We selected differentially-expressed genes with *p*-values <0·05 and FDRs <0·01.

(iv) *Identification of miRNAs in differentially-expressed genes*

The predicted mRNA targets of differentially-expressed miRNAs were extracted from Targetscan software (Allen *et al.*, 2005; Schwab *et al.*, 2005).

3. Results

(i) *Sample information*

The sample information is shown in Table 1. After the MES test, mice showed epileptic symptoms, while those then exposed to Efexor50, FT-1, J601-1, Q808-1 or RF-1 returned to normal.

Our analysis contained seven groups including a control group, epilepsy group and five groups of anticonvulsant-treated mice. Every group contained three mice (Table 1).

(ii) *Overview of RNA-seq data generated in mouse brains*

Expression information of the seven samples is shown in Table 2. This table shows the total gene number of mRNA expression in the seven groups.

The gene numbers for differential expression of the seven samples are shown in Table 3. There were 5000–10 000 genes differentially expressed in the six normal/epilepsy pairs (Table 3 and Supplementary Table S1).

The expression levels of 14 814, 14 779, 14 528, 14 477, 14 468, 14 338 and 14 719 (transcript per million [TPM]) protein-coding genes in the control, Efexor50, FT-1, J601-1, Q808-1, RF-1 and MES were detected in one sample. A total of 5730, 4782, 6726, 7078, 8480 and 9701 differentially-expressed mRNAs were identified ($p < 0{\cdot}05$, FDR $\leqslant 0{\cdot}0001$) in epilepsy samples compared to controls, Efexor50, FT-1, J601-1, Q808-1 and RF-1. Additionally, 5575, 5301 and 4575 (TPM) miRNAs were detected in one sample in our pipeline analysis. The known miRNA expressions were compared between two samples to determine the differentially-expressed miRNA (Supplementary Table S2). Additionally, 43 and 56 known miRNAs were identified as significantly differentially-expressed genes in the MES/control and MES/Efexor50 pairs. We defined the significantly differentially-expressed genes as fold changes (log2-Ratio) > 1 and *p*-value <0·1. Figure 1 shows the differential expression of miRNA in MES/control and MES/Efexor50 pairs.

(iii) *Deregulated expression of mRNAs and miRNAs in mouse brains*

We selected the differentially-expressed mRNA according to fold changes (log2-Ratio >0·5) in mRNA expression between control/anticonvulsant-treated mice and epilepsy mice. There were 3339, 2769, 3181, 3134, 3575 and 4078 differentially-expressed mRNAs in control/MES, MES/Efexor50, MES/FT-1, MES/J601-1, MES/Q808-1 and MES/RF-1 pairs that had changed more than 0·5-fold. We then selected the same trend of differentially-expressed genes that existed in both the control/epilepsy group and anticonvulsant-treated mice/epilepsy group (Fig. 2 *a–e*). We also selected genes with the same trends in all groups (Fig. 2 *f*). After filtration, we had four genes remaining, including *SNCA* (synuclein alpha), which has been reported in Parkinson's disease (Beyer, 2006), *DLGAP4* (discs, large homolog-associated protein 4) whose family member *DLGAP2* has been reported in epilepsy (Ranta *et al.*, 2000), *CORO1B* (coronin, actin binding protein, 1B) and *AHSP* (erythroid associated factor).

We also selected the inverse expression miRNA of *SNCA* in control/MES and MES/Efexor50 pairs. There were three significantly differentially-expressed *SNCA* miRNA targets, of which mmu-miR-21a-3p was upregulated (seven-fold change) in epilepsy mice (Fig. 3).

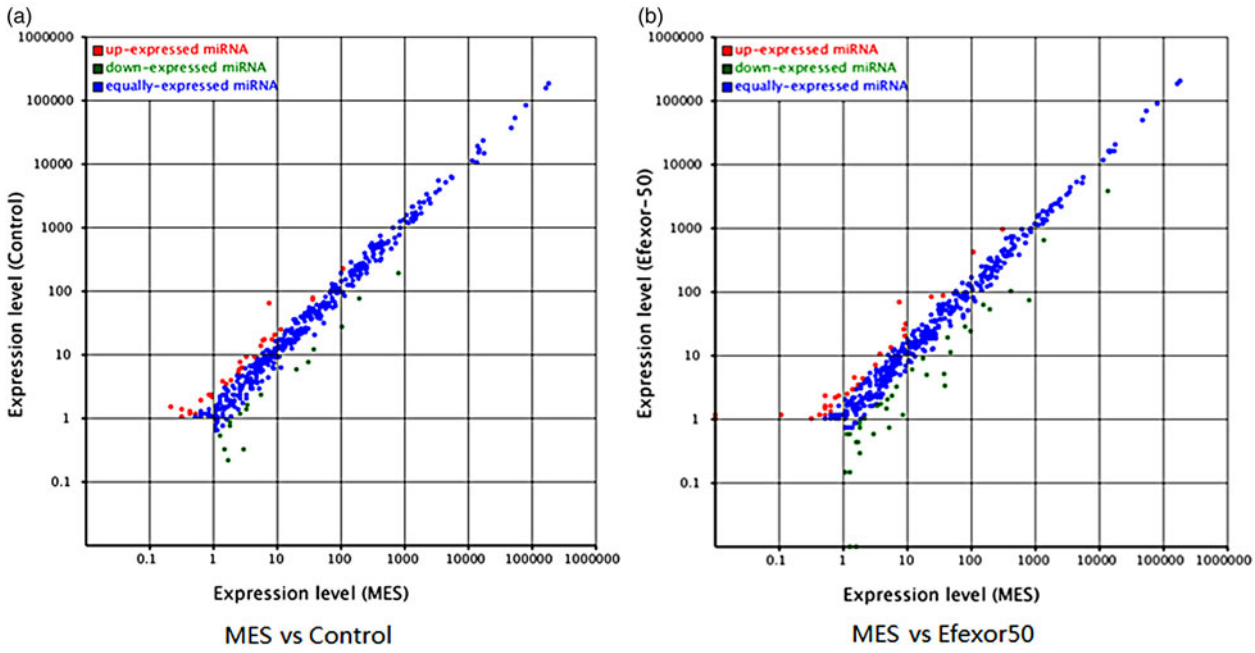

Fig. 1. Scatter plots showing (*a*) MES vs. control; (*b*) MES vs. Efexor50. Each point in the figure represents a miRNA. The x-axis and y-axis show expression levels of miRNA in two samples. Red points are miRNAs with log2-ratio > 2, blue points are miRNAs with 0·5 < log2-ratio ⩽ 2, green points are miRNAs with log2-ratio ⩽ 0·5.

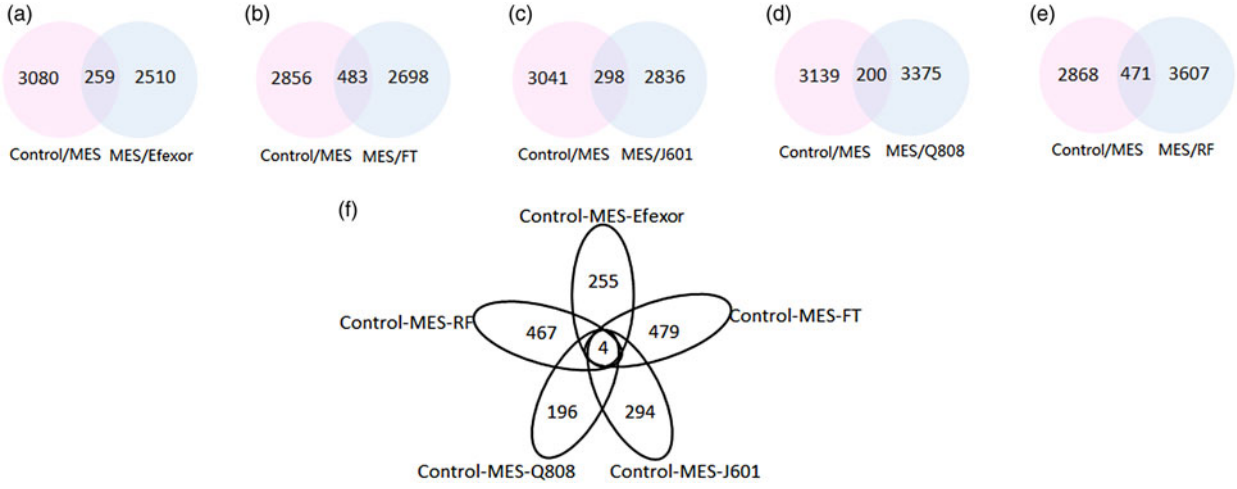

Fig. 2. Venn diagrams of the number of differentially-expressed mRNAs in control/MES and the five drug/MES groups are shown in (*a*), (*b*), (*c*), (*d*) and (*e*). (*f*) describes the genes present in all five groups.

(iv) *Pathway analysis of differentially-expressed genes in mice*

To gain a better understanding of the biological function of the above-mentioned genetic alterations in epileptic mice, we mapped differentially-expressed genes from our study to known pathways using DAVID v6·7 (https://david.ncifcrf.gov). The results of KEGG pathway comparison to differentially-expressed genes are listed in Table 4. We also annotated the *SNCA* gene with DAVID, and the results of KEGG pathway and GO term comparison with *SNCA* are listed in Table 5. After annotation one pathway remained which was involved in neuroactive ligand–receptor interaction found to be correlated with neuronal dysplasia.

(v) *Validation of the RNA-seq analysis using proteome information*

We sequenced the proteome of the control, Efexor50 and MES groups to validate the above-mentioned result, and the protein information is shown in Table 6. We used iTRAQ to quantify tandem mass spectrometry to determine the proteins from different mice. The total number of identified proteins from all

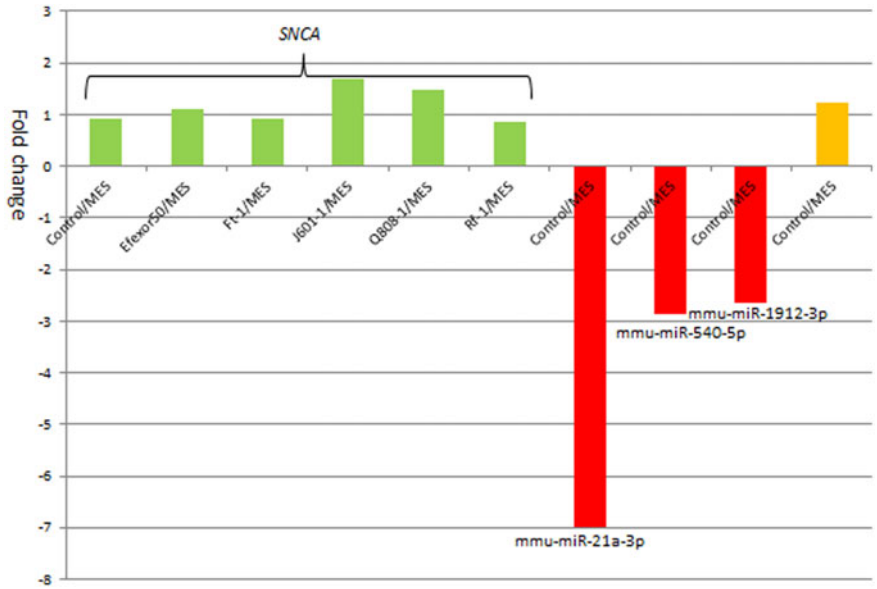

Fig. 3. mRNA expression (log2-Ratio, green) of *SNCA*, the inverse miRNA (log2-Ratio, red) it targeted and the protein (ratio, orange) it expressed. Downregulation of *SNCA* is evident in epilepsy with upregulation of miRNA.

Table 4. *KEGG pathway analysis of differentially-expressed mRNA in controls and MES.*

Term	Count	*p*-value	Genes	List total
mmu04080: Neuroactive ligand–receptor interaction	10	0·002414	*SSTR4, GABRR2, PTH2R, DRD2, S1PR4, F2, DRD4, HTR1D, PTGFR, HTR2A*	66

Table 5. *Gene annotation of SNCA.*

Source	Category
KEGG pathway	Function: involved in regulation of dopamine release and transport. Correlated with Parkinson's disease
GOTERM BP FAT	Regulation of dopamine secretion, regulation of amine transport

samples was 2977. The number of differentially-expressed proteins in control/MES was 174 and MES/Efexor50 was 307 (Supplementary Table S3). We extracted four differentially-expressed genes in control/MES and MES/Efexor50 pairs, of which only *SNCA* had the same protein expression trend as mRNA. The ratio of *SNCA* protein expression in control/MES was 1·24 and Efexor50/MES was 1·372. The log2-Ratio of *SNCA* mRNA expression in control/MES was 0·927 and Efexor50/MES was 1·117. The mRNA and *SNCA* protein in the MES group was underexpressed, but highly expressed in the control and Efexor50 groups without epilepsy. It was validated that *SNCA* changed after the MES test and after drugs were injected.

Table 6. *Sample protein information.*

Sample ID	Concentration (μg/μl)	Volume (μl)	Total protein (μg)
Control	2·57	350	899·5
MES	2·3	350	805
Efexor50	2·33	350	815·5

Table 7. *Statistics of differentially-expressed proteins from three samples.*

Pair	Downregulated protein	Upregulated protein	Total expressed protein
Control/MES	74	100	174
MES/Efexor50	183	124	307

Table 6 shows the concentration and volume of protein collected from control, epilepsy and anticonvulsant-treated mouse brains. From this information we can calculate the total protein for each sample: total protein (μg) = concentration (μg/μl) × volume (μl).

The numbers of differentially-expressed proteins in the three samples are shown in Table 7. We defined differentially-expressed proteins as ratio >1·2 and

Table 8. *Selected enrichment of KEGG pathway for proteins differentially-expressed in controls and MES.*

Term	Count	*p*-value	Genes	List total
mmu05012: Parkinson's disease	16	2·15E-07	NM_010887, NM_133666, NM_007505, NM_016774, NM_025899, NM_145518, NM_001136085, NM_025407, NM_023202, NM_020569, NM_011695, NM_007450, NM_025641, NM_025628, NM_025358, NM_011670	130
mmu05010: Alzheimer's disease	13	7·39E-04	NM_010887, NM_133666, NM_016774, NM_007505, NM_025899, NM_145518, NM_025407, NM_009722, NM_023202, NM_025641, NM_025628, NM_025358, NM_009696	130

p-value < 0·05. Selected enrichment of the KEGG pathway for proteins differentially-expressed in controls and MES is shown in Table 8. There were two significant pathways seen: Parkinson's disease and Alzheimer's disease.

4. Discussion

We studied comprehensive mRNA and miRNA profiles from control, drug treated and epileptic mouse brains. Comparing expression changes between control, epileptic and anticonvulsant-treated mice identified four differentially-expressed mRNAs, of which only one was validated by proteomic data. According to the mRNA and protein study, *SNCA* is underexpressed in mice with epilepsy. We extracted miRNA from *SNCA*, and mmu-miR-21a-3p, which targeted *SNCA*, caused a significant change in expression (seven-fold increase) in mice with epilepsy.

SNCA is a member of the synuclein family, which is abundantly expressed in the mouse brain. A previous study reported that *SNCA* controls p53 expression and transcriptional activity to inhibit caspase 3 activation, triggering apoptotic responses in neuronal cells (Jowaed *et al.*, 2010). In epileptic mice, *SNCA* was underexpressed, which led to an apoptotic response in neuronal cells, which may lead to epileptic neuropathology. This is not unique, as defects in *SNCA* have seen to be involved with the pathogenesis of Parkinson's disease (Jowaed *et al.*, 2010). Epilepsy is also a disease related to abnormal brain function. These two diseases may share some common molecular progress with similar symptoms, such as neural disabilities.

Epileptic seizures are the result of excessive and abnormal cortical nerve cell activity in the brain and *SNCA* is involved in the regulation of dopamine release and transport (Munoz *et al.*, 2015), which plays a significant role in nerve cell activity.

Besides *SNCA*, three other genes (*DLGAP4, AHSP* and *CORO1B*) were selected by RNA-seq. Despite not being validated by proteomics data, they were all present in the normal/epilepsy groups and may play a role in molecular progress of epilepsy.

DLGAP4 encodes a membrane-associated guanylate kinase found at the postsynaptic density in neuronal cells (Takeuchi *et al.*, 1997). The encoded protein may play a role in synapse organization and neuronal signalling. Alternatively spliced transcript variants encoding multiple isoforms have been found for this gene and *DLGAP2* has previously been associated with epilepsy (Ranta *et al.*, 2000).

AHSP acts as a chaperone protein that hinders harmful alpha-hemoglobin aggregation during normal erythroid cell development, and that protects free alpha-hemoglobin from precipitation (Kihm *et al.*, 2002). *AHSP* is induced by *GATA-1* during erythroid maturation. It belongs to the *AHSP* family and forms a heterodimer with free alpha-hemoglobin (Kihm *et al.*, 2002). *AHSP* does not bind beta-hemoglobin nor alpha (2) beta (2) hemoglobin A (Cai *et al.*, 2005). It has tissue specificity and is expressed in the spleen, bone marrow and blood, with the highest levels seen in bone marrow (Cai *et al.*, 2005).

CORO1B helps to regulate leading edge dynamics and cell motility in fibroblasts, and is involved in cytokinesis and signal transduction (Cai *et al.*, 2005). *CORO1B*, which regulates the Arp2/3 complex and cell motility in fibroblasts, has been seen to be phosphorylated on Ser-2 (Cai *et al.*, 2008). It belongs to the WD repeat coronin family (Cai *et al.*, 2008) and contains five WD repeats and interacts with Arp2/3 complex components, including ACTR2, ARPC1B and ARPC2.

The most prominent KEGG pathway associated with differentially-expressed mRNA is, as we speculated, the neuroactive ligand–receptor interaction. Additionally, the enrichment of the KEGG pathway suggests involvement in Parkinson's disease and Alzheimer's disease. As we know, Parkinson's disease, Alzheimer's disease and epilepsy are all the result of nervous system abnormalities.

5. Conclusion

RNA-seq is a strong and credible tool that accurately quantifies expression levels of mRNAs and helps identify distinct genes between target samples. The four

genes discovered by RNA-seq are important as they were present in all normal/epilepsy groups. The most significant deregulated gene was *SNCA*, which was underexpressed in epileptic mouse brain cells and may have an important relationship with the progression of epilepsy because of its direct relationship with synapses. Also, mmu-miR-21a-3p, which targeted *SNCA*, caused a significant seven-fold increase in expression in mice with epilepsy, which suggests that it might be involved in causing epilepsy in mice. Further research on the relationship between epilepsy and these four genes is required.

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Declaration of interest

None.

Supplementary material

The online supplementary material can be found available at http://dx.doi.org/10.1017/S0016672315000245

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