Acta virologica 58: 120 – 127, 2014 doi:10.4149/av_2014_02_120

Alterations in microRNA expression profile in rabies virus-infected mouse neurons

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Received September 3, 2013; accepted April 23, 2014

Summary. – Rabies virus (RABV) is known to cause a fatal infection in many mammalian species, yet its pathogenesis remains poorly understood. This study was performed to analyze the microRNA (miRNA) expression profiles in RABV-infected primary neurons of mice. A total of 53 miRNAs were found to be differentially expressed in RABV-infected samples compared with mock samples in a time-dependent manner. Among them, the expression of ten miRNAs was validated by real-time RT-PCR. Potential target genes of differentially expressed miRNAs were predicted by TargetScan. Further bioinformatics analysis indicated that these predicted targets were overrepresented in neuronal function-related Gene Ontology (GO) terms and biological pathways. The results of this study suggest that RABV may cause neuronal dysfunction by regulating cellular miRNA expression.

Keywords: rabies virus; microRNA; expression; microarray; neurons; mouse

Introduction

RABV, (the genus *Lyssavirus,* the family *Rhabdoviridae*) is a highly neurotropic virus that spreads along neural pathways and invades the CNS, where it causes an acute infection (Schnell *et al.*, 2010). Although significant advances have been made in rabies prevention and control, rabies remains a major threat to public health (Hemachudha *et al.*, 2002). Despite the catastrophic clinical outcome of rabies encephalomyelitis, the fact that inflammatory changes and degenerative neuronal changes may be minimal, or even absent, under natural conditions indicates that neuronal dysfunction, rather than neuronal death, is likely responsible for the clinical disease and fatal outcome of rabies under natural conditions (Jogai *et al.*, 2000; Fu *et al.*, 2005). Although there are several hypotheses under investigation at the present time, the fundamental basis for neuronal dysfunction in rabies has not yet been demonstrated.

miRNAs, an extensive class of non-coding RNA molecules, negatively regulate gene expression and play essential roles in many critical cellular processes such as development, differentiation, proliferation, and hematopoiesis (Ambros, 2004; Bartel, 2004). As is well known, viral infection represents a great change in the host cellular environment that generally results in dramatic changes in cellular gene expression. Similarly, because most miRNAs are also transcribed by RNA polymerase II, using many of the same cellular transcription factors, the viral infection would also affect their patterns of expression. It is expected that viruses may gain an advantage by reshaping the cellular miRNA composition to directly or indirectly affect viral replication and pathogenesis (Cullen, 2010). For example, a cellular miRNA required for virus replication is miR-122, which is important to the complete life cycle of hepatitis C virus (Jopling *et al.*, 2005); human T-cell lymphotropic virus type 1 modulates

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Abbreviations: CNS = central nervous systems; DAVID = Database for Annotation, Visualization, and Integrated Discovery; FDR = false discovery rate; GO = Gene Ontology; KEGG = Kyoto Encyclopedia of Genes and Genomes; miRNA = microRNA; p.i. = post infection; RABV = rabies virus

the expression of a number of cellular miRNAs in order to control T-cell differentiation (Bellon *et al.*, 2009); human cytomegalovirus selectively manipulates the expression of miR-100 and miR-101 to facilitate its own replication (Wang *et al.*, 2008); Epstein-Barr virus LMP1 induces miR-29b, which results in miR-29b-mediated downregulation of T-cell leukemia gene 1, a protein with roles in cell survival and proliferation (Anastasiadou *et al.*, 2010); miR-29c is involved in A549 cell apoptosis induced by influenza virus infection through repression of antiapoptotic factor BCL2L2 (Guan *et al.*, 2012); CCL8/MCP-2 is a target for miR-146a in HIV-1-infected microglia, as overexpression of miR-146a prevents HIV-induced secretion of MCP-2 chemokine (Rom *et al.*, 2010) .

Given the above mentioned information, this study was aimed to identify potential dysregulated miRNAs in RABV-infected neurons. We carried out a miRNA expression profile of primary neurons of mice infected with mock and RABV, and performed real-time RT-PCR validation of several differentially expressed miRNAs. Functional enrichment of the predicted miRNA target genes presented neuronal function-related GO terms and intracellular signaling pathways, which would be very helpful in understanding neuronal dysfunction in rabies.

Materials and Methods

Virus, cells and animals. The MRV strain C (GenBank Acc. No. DQ875050.1) of street RABV, a canine RABV variant isolated from the Henan province of China, was grown in mouse N2a neuroblastoma cells. Primocultures of neurons were derived from the cortex of embryonic day 16.5 (E 16.5) C57BL/6J mice. The cortexes were incubated with 0.25% trypsin, and 50 µl of 10 mg/ml DNase I stock was added for 30 sec to break down the DNA and avoid tissue clumping. The dissociated neurons were initially plated in DMEM with 10% FBS (GIBCO) at the plating density of 1×10^5 cells per cm^2. After 2 hr, the plating medium was carefully replaced with equilibrated neurobasal media containing B27 supplement (GIBCO) and 2 mmol/l L-glutamine (Invitrogen). The cells grew for up to 1 week without any further change of medium. The cells were infected with RABV at 10 $TCID_{50}$ per well in 6 well-plates. C57BL/6J mice had access to food and water *ad libitum* and were challenged with RABV by intracranial inoculation at a dose of 10 LD_{50} per 30 µl. All experiments with live virus challenge were carried out in Biosafety Level 2 facilities.

miRNA microarray. Total RNA was extracted from primocultures of neurons infected with RABV at 0, 48, 96, and 144 hr post infection (p.i.) using TRIZOL reagent (Invitrogen). The RNA samples were submitted to LC Sciences (Houston, TX) for quality control, processing and miRNA expression analysis in accordance with their specifications. All samples met quality control standards. Samples were hybridized to individual mouse miRNA microarray (LC Sciences), which were current with Sanger miRBase version 16.0. Data were analyzed by first subtracting the background and then normalizing the signal using a LOWESS method.

Real-time RT-PCR. For analysis of mature miRNAs, TaqMan miRNA assays (Applied Biosystems) were used. Total RNA was reverse transcribed using a miRNA-specific primer and Multiscribe reverse transcriptase. Real-time PCR was performed with a miRNA-specific probe according to the manufacturer's instructions. All reactions were run in triplicate on an Applied Biosystems 7500 Real-Time PCR System. Expression of the miRNA of interest was normalized to snRNA RNU6B and relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method. Data were expressed as mean ± standard deviation (SD) from at least three separate experiments.

Prediction of target genes of miRNAs. TargetScan (*http://www.targetscan.org/*) is a web resource that predicts biological targets of miRNAs by searching for the presence of 8mer and 7mer sites that match the seed region of each miRNA (Lewis *et al.*, 2005). We used TargetScan (Release 6.2) to predict target genes of miRNAs.

GO enrichment and biological pathway analyses of predicted target genes. All of the predicted genes were annotated with their GO information. Biological pathways were defined by Kyoto Encyclopedia of Genes and Genomes (KEGG). The GO enrichment and biological pathway analysis were performed using the web-based tool, Database for Annotation, Visualization, and Integrated Discovery (DAVID, *http://david.abcc.ncifcrf.gov/*) (Huang da *et al.*, 2009). This software uses enrichment or overrepresentation analysis to determine if gene sets are statistically overrepresented in the identified gene clusters or biological pathways more than a random chance as determined by the modified Fisher's exact *P*-value. *P*-values were adjusted using the method of Benjamini and Hochberg to control the false discovery rate (FDR) (Hochberg *et al.*, 1990).

Results

Effect of RABV infection on miRNA profile in cultured mouse neurons

To get an overview of host neuronal miRNA expression in response to RABV infection, we analyzed primocultures of neurons infected with RABV at 0, 48, 96, and 144 hr p.i. miRNAs whose relative expression levels showed a fold change ≥2 and $P \leq 0.01$ were considered significantly up-regulated, and those with fold change ≤-2 and $P \leq 0.01$ were considered significantly down-regulated. A total of 53 miRNAs were found to be differentially expressed in a time-dependent manner after infection. As shown in Fig. 1a, two distinct trends were presented, in which one trend was continuously upregulated with 19 miRNAs, while the other was continuously downregulated with 34 miRNAs.

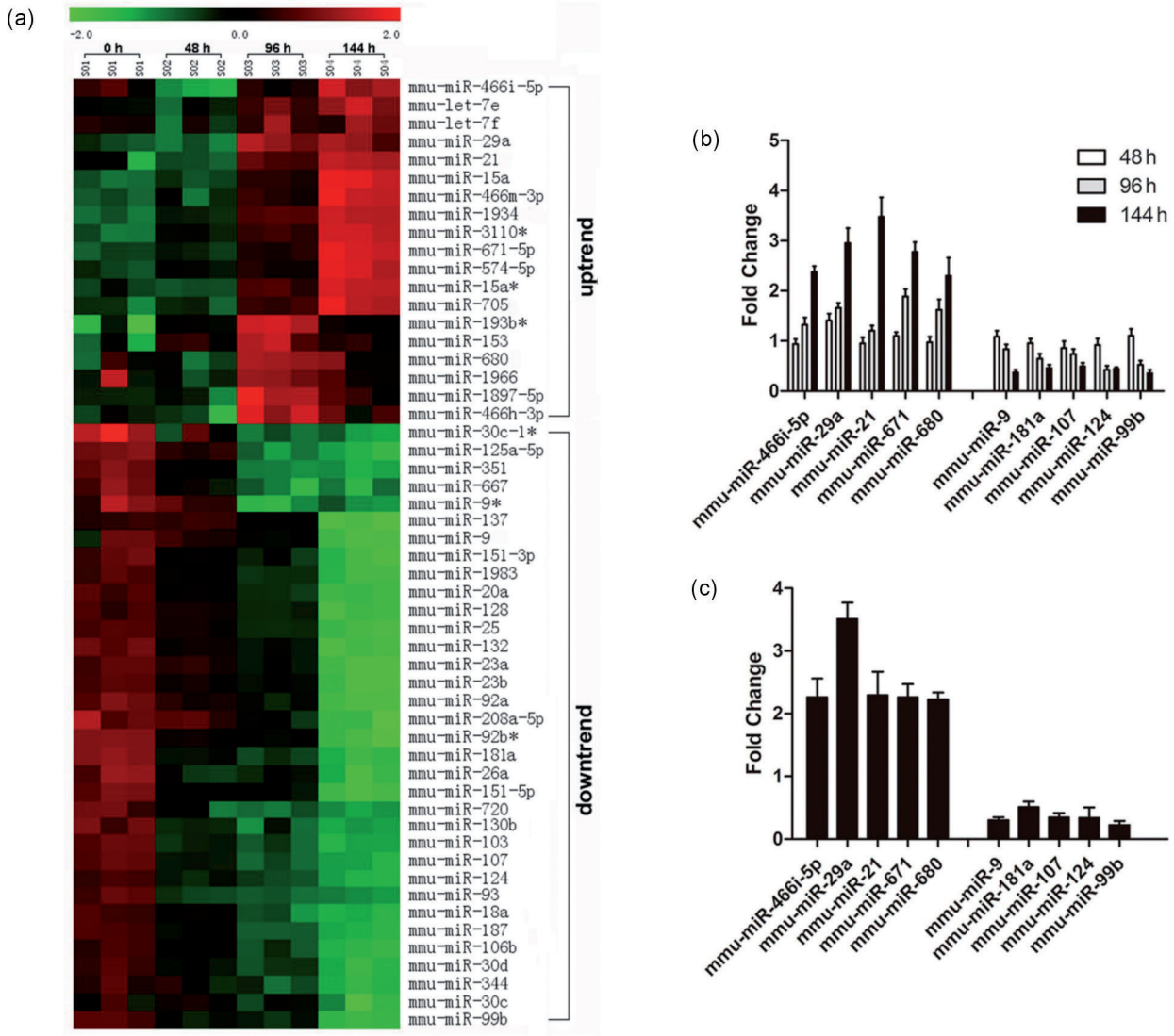

Fig. 1
Effect of RABV infection on miRNA profile in mouse neurons
(a) Two-way hierarchical cluster map of significantly up- or downregulated miRNAs in cultured neurons. (b) Selected differentially expressed miRNAs in cultured neurons as assayed by real-time RT-PCR. (c) Selected differentially expressed miRNAs in hippocampal neurons as assayed by real-time RT-PCR. Bars are represented from three independent experiments.

To verify the data from microarray analysis, we used real-time RT-PCR to detect 10 selected differentially expressed miRNAs, including miR-466i-5p, miR-29a, miR-21, miR-671, miR-680, miR-let-7e, miR-15a, miR-574-5p, miR-705, miR-153, miR-125a-5p, miR-107, miR-124, miR-181a, miR-9, miR-99b, miR-20a, miR-23b, miR-720, on RNA samples from primocultures of neurons (Fig. 1b). Furthermore, these selected miRNAs were also validated on hippocampi of mice challenged with RABV at moribund stage compared to mock infection (Fig. 1c). Although differences were observed between neuronal culture and hippocampal tissue, the results showed the same expression patterns of differentially expressed miRNAs. These results demonstrate that host neuronal miRNAs could be dysregulated by RABV infection.

Target prediction and GO enrichment analysis of target genes of differentially expressed miRNAs

To predict possible target genes for differentially expressed miRNAs, we searched the TargetScan mouse database. TargetScan successfully predicted target genes of 48 differentially expressed miRNAs. A total of 6,172

Table 1. Significant neuronal function-related GO annotations for the upregulated miRNAs

GO term	Count[a]	%[b]	*P*-value[c]	FDR[d]
Biological process				
GO:0035295~tube development	116	2.993548	1.77E-17	3.32E-14
GO:0030182~neuron differentiation	156	4.025806	2.15E-17	4.02E-14
GO:0031175~neuron projection development	90	2.322581	8.83E-12	1.65E-08
GO:0007169~transmembrane receptor protein tyrosine kinase signaling pathway	80	2.064516	8.25E-11	1.54E-07
GO:0048812~neuron projection morphogenesis	75	1.935484	9.78E-11	1.83E-07
GO:0007264~small GTPase mediated signal transduction	99	2.554839	1.10E-10	2.05E-07
GO:0007409~axonogenesis	69	1.780645	8.35E-10	1.56E-06
GO:0016192~vesicle-mediated transport	152	3.922581	1.40E-09	2.63E-06
GO:0046903~secretion	83	2.141935	1.27E-08	2.38E-05
GO:0030001~metal ion transport	142	3.664516	1.51E-08	2.82E-05
GO:0007268~synaptic transmission	69	1.780645	5.92E-08	1.11E-04
GO:0007411~axon guidance	45	1.16129	6.03E-08	1.13E-04
GO:0046907~intracellular transport	135	3.483871	1.86E-07	3.47E-04
GO:0010941~regulation of cell death	168	4.335484	2.02E-07	3.78E-04
GO:0050808~synapse organization	27	0.696774	5.81E-07	0.001085
GO:0006836~neurotransmitter transport	37	0.954839	8.80E-07	0.001644
GO:0050767~regulation of neurogenesis	51	1.316129	4.28E-06	0.007995
GO:0007269~neurotransmitter secretion	21	0.541935	4.61E-06	0.008617
GO:0030029~actin filament-based process	62	1.6	1.15E-05	0.02152
GO:0001764~neuron migration	31	0.8	2.29E-05	0.042745
Cellular component				
GO:0045202~synapse	133	3.432258	2.77E-20	4.09E-17
GO:0043005~neuron projection	104	2.683871	1.09E-16	1.67E-13
GO:0030425~dendrite	52	1.341935	1.05E-10	1.55E-07
GO:0030424~axon	46	1.187097	5.33E-08	7.87E-05
GO:0031410~cytoplasmic vesicle	148	3.819355	6.97E-08	1.03E-04
GO:0034703~cation channel complex	42	1.083871	8.70E-08	1.29E-04
GO:0031982~vesicle	150	3.870968	9.77E-08	1.44E-04
GO:0005856~cytoskeleton	283	7.303226	3.68E-07	5.44E-04
GO:0045211~postsynaptic membrane	48	1.23871	1.84E-06	0.002715
GO:0005912~adherens junction	42	1.083871	2.84E-06	0.004196
GO:0034702~ion channel complex	56	1.445161	3.15E-06	0.004646
GO:0019717~synaptosome	29	0.748387	1.40E-05	0.020692
GO:0014069~postsynaptic density	24	0.619355	2.48E-05	0.036536
GO:0015630~microtubule cytoskeleton	123	3.174194	2.90E-05	0.042794

[a]Numbers of target genes involved in the specific GO term. [b]Percentages represent involved genes/total genes annotated with GO information. [c]Enrichment *P*-value with modified Fisher's exact test. [d]FDR stands for the false discovery rate using Benjamini & Hochberg procedure.

genes were predicted as target genes for up-regulated miRNAs, while 14,780 genes were predicted for down-regulated miRNAs. To better understand the potential role of miRNA in the pathogenesis of RABV, we submitted the predicted target gene lists to DAVID database for performing functional annotation. As shown in table 1 and 2, the neuronal function-related GO biological processes and cellular components were significantly over-represented (P <0.01 and FDR <0.05), respectively. Furthermore, the target genes were also enriched into biological pathways related to neuronal function (P <0.01 and FDR <0.05) (Table 3). These results suggest that miRNAs with significant change are potentially involved in regulation of target genes related to neuronal dysfunction in response to RABV infection.

Discussion

As the perturbation of gene regulation networks based on miRNA activities may result in abnormal brain function (Im *et al.*, 2012), it is expected that the highly neurotropic

Table 2. Significant neuronal function-related GO annotations for the downregulated miRNAs

GO term	Count[a]	%[b]	*P*-value[c]	FDR[d]
Biological process				
GO:0035295~tube development	159	2.890384	1.02E-25	1.93E-22
GO:0030182~neuron differentiation	209	3.799309	9.92E-23	1.88E-19
GO:0048666~neuron development	161	2.926741	1.49E-20	2.82E-17
GO:0031175~neuron projection development	129	2.345028	4.28E-20	8.10E-17
GO:0048812~neuron projection morphogenesis	108	1.963279	1.42E-18	2.69E-15
GO:0016192~vesicle-mediated transport	221	4.017451	2.63E-17	4.97E-14
GO:0007409~axonogenesis	100	1.817851	2.96E-17	5.59E-14
GO:0007264~small GTPase mediated signal transduction	137	2.490456	1.19E-15	2.31E-12
GO:0007411~axon guidance	66	1.199782	2.49E-14	4.70E-11
GO:0030029~actin filament-based process	95	1.726959	1.13E-11	2.14E-08
GO:0030036~actin cytoskeleton organization	87	1.581531	4.37E-10	8.26E-07
GO:0030001~metal ion transport	190	3.453917	4.46E-10	8.44E-07
GO:0030900~forebrain development	84	1.526995	1.60E-08	3.03E-05
GO:0007423~sensory organ development	117	2.126886	3.22E-08	6.10E-05
GO:0006811~ion transport	274	4.980913	4.19E-08	7.92E-05
GO:0035023~regulation of Rho protein signal transduction	50	0.908926	6.50E-08	1.23E-04
GO:0007268~synaptic transmission	86	1.563352	1.09E-07	2.06E-04
GO:0007010~cytoskeleton organization	140	2.544992	1.12E-07	2.12E-04
GO:0043087~regulation of GTPase activity	51	0.927104	5.58E-07	0.001055
GO:0019226~transmission of nerve impulse	101	1.83603	8.82E-07	0.001668
GO:0000165~MAPKKK cascade	58	1.054354	2.14E-06	0.004047
GO:0030902~hindbrain development	39	0.708962	2.24E-06	0.004235
GO:0001764~neuron migration	40	0.727141	3.02E-06	0.005709
GO:0050808~synapse organization	31	0.563534	3.04E-06	0.005744
GO:0006836~neurotransmitter transport	43	0.781676	9.74E-06	0.018427
GO:0012501~programmed cell death	180	3.272132	2.26E-05	0.042725
Cellular component				
GO:0045202~synapse	180	3.272132	1.36E-28	2.03E-25
GO:0043005~neuron projection	136	2.472278	6.63E-21	9.89E-18
GO:0031982~vesicle	218	3.962916	9.88E-14	1.48E-10
GO:0005856~cytoskeleton	403	7.325941	1.09E-11	1.62E-08
GO:0014069~postsynaptic density	38	0.690783	1.66E-11	2.48E-08
GO:0030425~dendrite	64	1.163425	2.22E-11	3.31E-08
GO:0019717~synaptosome	42	0.763498	2.48E-09	3.70E-06
GO:0030424~axon	58	1.054354	7.96E-09	1.19E-05
GO:0045211~postsynaptic membrane	63	1.145246	9.06E-08	1.35E-04
GO:0015629~actin cytoskeleton	91	1.654245	1.35E-07	2.01E-04
GO:0034702~ion channel complex	71	1.290674	2.17E-06	0.003238
GO:0034703~cation channel complex	48	0.872569	2.62E-06	0.003913
GO:0030027~lamellipodium	35	0.636248	5.57E-06	0.008319
GO:0012506~vesicle membrane	47	0.85439	1.40E-05	0.02084
GO:0043197~dendritic spine	19	0.345392	1.98E-05	0.029622
GO:0042641~actomyosin	19	0.345392	1.98E-05	0.029622
GO:0005905~coated pit	21	0.381749	2.83E-05	0.042257

RABV would also affect neuronal miRNA expression. In previous studies, miRNA expression profiles of mouse brains infected with street or fixed RABV were described. Functional analysis showed that differentially expressed miRNAs were mainly involved in many immune-related signaling pathways, such as the Jak-STAT signaling pathway, cytokine-cytokine receptor interactions, and Fc gamma R-mediated phagocytosis (Zhao *et al.*, 2012a,b). Because neurons are outnumbered by the various non-neuronal supportive cellular elements in the brain, such as glial cells, cells of the blood vessels and cells of the choroid plexus, miRNA expression patterns from whole-brain may be somewhat difficult to unravel miRNA dysregulation in neuron induced by RABV.

Table 3. Significant neuronal function-related biological pathways

KEGG Pathway	Count[a]	%[b]	*P*-value[c]	FDR[d]
For upregulated miRNAs				
mmu04360:Axon guidance	64	1.651613	4.56E-14	5.62E-11
mmu04310:Wnt signaling pathway	65	1.677419	1.70E-11	2.10E-08
mmu04810:Regulation of actin cytoskeleton	80	2.064516	1.33E-09	1.63E-06
mmu04722:Neurotrophin signaling pathway	52	1.341935	7.85E-08	9.67E-05
mmu04150:mTOR signaling pathway	26	0.670968	5.52E-06	0.006796
mmu04120:Ubiquitin mediated proteolysis	48	1.23871	1.59E-05	0.019562
For downregulated miRNAs				
mmu04360:Axon guidance	86	1.563352	5.15E-20	6.41E-17
mmu04010:MAPK signaling pathway	139	2.526813	5.68E-19	7.07E-16
mmu04722:Neurotrophin signaling pathway	76	1.381567	1.07E-13	1.33E-10
mmu04810:Regulation of actin cytoskeleton	110	1.999636	1.19E-13	1.48E-10
mmu04310:Wnt signaling pathway	80	1.454281	9.79E-12	1.22E-08
mmu04150:mTOR signaling pathway	34	0.618069	1.43E-07	1.77E-04
mmu04350:TGF-beta signaling pathway	47	0.85439	2.50E-07	3.12E-04
mmu04720:Long-term potentiation	38	0.690783	3.79E-06	0.004715

In order to understand how cellular miRNAs play roles in neuronal dysfunction caused by RABV infection, we performed miRNA microarray assay in RABV-infected primocultures of mouse neurons. In our study, we have demonstrated that a number of miRNAs were significantly differentially expressed during RABV infection in a time-dependent manner. Some miRNAs have been well known for their important roles in neuronal development, function and pathology. MiR-9 exerts diverse cell-autonomous effects on the proliferation, migration and differentiation of neural progenitor cells by modulating different mRNA targets (Yuva-Aydemir *et al.*, 2011). Dysregulation of miR-9 in postmitotic neurons is also implicated in brain function and neurodegenerative diseases (Lukiw, 2007; Packer *et al.*, 2008). As one of the most highly conserved neuronal-specific miRNAs, miR-124 is involved in diversity in dendrite morphology, larval locomotion, synaptic release at the neuromuscular junction and neuronal fate determination (Visvanathan *et al.*, 2007; Sun *et al.*, 2012). Another brain-specific miRNA, miR-132, is linked to multiple functions, including neuronal cell development and synaptic plasticity (Lambert *et al.*, 2010; Tognini *et al.,* 2012). Precise control of SNAP-25 by miR-153 is necessary not only for presynaptic vesicle release, but also for protein secretion, motor neuron patterning, and outgrowth (Wei *et al.*, 2013). MiR-181a is a key regulator of mammalian AMPA-type glutamate receptors with potential implications for the regulation of drug-induced synaptic plasticity (Saba *et al.*, 2012). Targeting of Arpc3 by miR-29a/b fine-tunes structural plasticity by regulating actin network branching in mature and developing spines (Lippi *et al.*, 2011).

Previous observations suggest that neuronal dysfunction, rather than neuronal death, is likely responsible for the clinical disease and fatal outcome in rabies under natural conditions, such as electrophysiological alterations, effects on ion channels and neurotransmission (Gourmelon *et al.*, 1991; Iwata *et al.*, 2000; Fu *et al.*, 2005). To better understand the potential role of these differentially expressed miRNAs in RABV-induced neuronal dysfunction, we predicted target genes and performed bioinformatics analysis. Neuronal function-related GO biological processes were overrepresented, such as vesicle-mediated transport, synaptic transmission, axon guidance, synapse organization and neurotransmitter transport. The predicted target genes were also enriched into specific cellular components of neuron, such as synapse, axon, dendrite, synaptosome. Neuronal function depends on proper pathway activity. By pathway enrichment analysis, we found that predicted target genes of differentially expressed miRNAs upon rabies virus infection could be enriched into neuronal function-related pathway, such as axon guidance, neurotrophin signaling pathway, regulation of actin cytoskeleton, mTOR signaling pathway, long-term potentiation. These pathways regulate many aspects of neuron, including neuronal survival, differentiation, development, axon growth and navigation, dendritic arborization, synapse formation and synaptic plasticity (Reichardt, 2006; Swiech *et al.*, 2008; Park *et al.*, 2012). These results suggest that RABV causes an imbalance in host gene expression and intracellular signaling pathway of neuron potentially by modulating host miRNA expression, which may lead to malfunction of neuron or favor viral life cycle.

In conclusion, our study unveiled the RABV-induced dysregulation of miRNA in primary neurons of mice, whose expression patterns and bioinformatics analysis provide a useful clue for understanding of the neuronal dysfunction caused by RABV infection. Further investigation is needed to clarify the roles of identified miRNA in the pathogenesis of RABV.

Acknowledgements. This work was supported by grants from National Natural Science Foundation of China (grant No. 31172337) and Key Laboratory of Jilin Province for zoonosis prevention and control – Province of State Key Laboratory cultivation base construction the grant (grant No. 2012ZPC).

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