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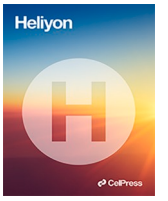

Research article

Temporal expression profiles of microRNAs associated with acute phase of brain ischemia in gerbil hippocampus

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ABSTRACT

Neuroprotective therapeutic potential for restoring dysregulated microRNA (miRNA) expression has previously been demonstrated in a gerbil cerebral infarction model. However, since temporal changes in miRNA expression profiles following stroke onset are unknown, miRNAs proving to be useful therapeutic targets have yet to be identified. We evaluated cognitive function, hippocampal neuronal cell death, and microarray-based miRNA expression profiles at 5, 9, 18, 36, and 72 h after 5-min whole brain ischemia in gerbils. A decline in cognitive function occurred in parallel with increased neuronal cell death 36–72 h after ischemia. The Jonckheere-Terpstra test was used to analyze miRNA expression trends 5–72 h after ischemia. The expression levels of 63 miRNAs were significantly upregulated, whereas 32 miRNAs were significantly downregulated, monotonically. Of the 32 monotonically downregulated miRNAs, 18 showed the largest decrease in expression 5–9 h after ischemia. A subset of these dysregulated miRNAs (miR-378a-5p, miR-204-5p, miR-34c-5p, miR-211-5p, miR-34b-3p, and miR-199b-3p) could be associated with brain ischemia and neuropsychiatric disorders.

1. Introduction

Cognitive dysfunction is one of the sequelae of ischemic stroke [1]. Many neuroprotective drugs have been developed for ischemic stroke [2]. However, the effectiveness of these drugs is inadequate. Although reperfusion therapy is effective in the treatment of cerebral infarction, reactive oxygen species generated after reperfusion could cause brain tissue damage [3], calling for more effective neuroprotective therapies.

Mongolian gerbils are widely used as a model animal for ischemic stroke. They have dysplasia of the circle of Willis [4]; bilateral occlusion of the carotid arteries could cause transient global cerebral ischemia including the hippocampus.

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MicroRNAs (miRNAs) are 21–25 nucleotides long, non-coding, and single-stranded RNAs, which suppress gene expression by binding to the target messenger RNAs [5]. MiRNAs hold an immense potential for serving as diagnostic biomarkers and therapeutic targets for various diseases [6]. In an animal model of cerebral infarction, we have previously shown that mild exercise prior to transient brain ischemia in gerbils had neuroprotective effects through the restoration of dysregulated miRNA expression [7]. Reportedly, the expression patterns of certain miRNAs undergo time-dependent alterations from 24 h to 6 months after cerebral ischemia in gerbils [8]. Thus, the restoration of dysregulated miRNA expression after ischemia could be a potential neuroprotective therapy. However, the temporal changes in miRNA expression profiles immediately after disease onset, which is the most effective time for treatment of ischemic stroke, have not yet been elucidated. Furthermore, miRNAs that should be restored as potential treatments have not been identified. Temporal changes in the expression of certain miRNAs have been reported in mice and gerbils during the early phase (from 3 h to 4 days) after cerebral ischemia [9]. However, as we previously reported in gerbils [7], the expression of a large number of miRNAs was dysregulated in cerebral ischemia and restored by prior exercise. Therefore, a comprehensive evaluation of temporal changes in miRNA expression profiles is required.

In this report, we aimed to elucidate temporal changes in miRNA expression in the hippocampus, one of the brain regions most vulnerable to ischemia, in a gerbil model of the acute phase of ischemic stroke (5–72 h after transient brain ischemia).

2. Materials and methods

2.1. Animals

Eight-week-old male Mongolian gerbils (Japan SLC, Inc., Shizuoka, Japan) with an average body weight of 61.4 ± 4.0 (standard deviation) g were used for this study. The experimental protocols were approved by the Animal Committee of Kagawa University Faculty of Medicine. The gerbils were randomly divided into ischemic and sham groups. The ischemic group was subdivided according to the post-ischemic recovery period: 5-, 9-, 18-, 36-, and 72-h groups. In each ischemic subgroup, four and three gerbils were used for miRNA evaluation and histological examination, respectively. In the sham group, three animals were used for histological evaluation 72 h after sham surgery.

2.2. Transient brain ischemia

Details of the procedure have been described in our previous study [7]. Briefly, gerbils were anesthetized by intraperitoneal injection of pentobarbital (30 mg/kg), followed by transient global ischemia, which was induced by 5-min occlusion of the bilateral common carotid arteries with microaneurysm clips. After surgery, they were allowed to recover for 5, 9, 18, 36, or 72 h. After short-term memory examination using the Y-maze spontaneous alternation test, gerbils were euthanized by intraperitoneal injection of pentobarbital (150 mg/kg). The brains were then removed and processed to evaluate the miRNA expression or histology in the hippocampi.

2.3. miRNA array analysis

3D-Gene miRNA oligo chips (version 21; Toray Industries, Inc., Tokyo, Japan), which could detect 1900 miRNAs, were used to assess miRNA expression in the hippocampus. The experimental procedures for miRNA extraction and array analysis have also been previously described [7]. miR-1153 was detected in all samples and thereby used as an internal control for quantile normalization of the signal intensities.

A heatmap was created to illustrate the time-dependent differential expression of significantly dysregulated miRNAs. The columns in the heatmap indicate the respective samples, while the rows indicate the miRNAs. The expression levels of the miRNAs were $\log_2$-transformed and assigned a color code. The center of the color code (white) is the median of all values used in the heatmap; blue and red colors indicate lower expression and higher expression, respectively. A common color code was used for all heatmaps presented in this report.

2.4. Pathological analysis

Paraffin-embedded brains were cut in the coronal plane into a thickness of 6 μm. Hematoxylin-eosin (HE)-stained sections containing dorsal hippocampal areas were evaluated. To evaluate the ischemia-induced morphological changes in neurons in the Cornet d'Ammon 1 (CA1) region of the hippocampus, pyramidal cells with normal nuclei in the CA1 region were counted. Thereafter, the density of viable neurons (cells/mm) was calculated. In this study, pathological evaluation was performed only in the CA1 region. According to our method, ischemia-induced neuronal death was observed only in the CA1 region [10,11], the area most susceptible to ischemia in the hippocampus.

2.5. Statistical analysis

The Jonckheere-Terpstra test was performed to screen for the miRNAs that exhibited a monotonic upward/downward trend in the response variable (signal intensity) at 5, 9, 18, 36, and 72 h after ischemia (significance level was set at $p < 0.01$). The Benjamini-Hochberg procedure was used to assess the expected false discovery rate (FDR) to account for multiple testing. Fold change (FC)

was defined as the ratio of the signal intensity at 5 h to that at 72 h miRNAs with a FC > 2-fold or less than 0.5-fold were selected. Statistical analyses were performed using R version 3.5.2 (https://www.R-project.org).

The significance of differences in alternation of the Y-maze spontaneous alternation test and number of viable neurons were assessed with one-way analysis of variance (ANOVA) followed by Tukey's and Dunnett's post-hoc tests. Statistical significance was set at $p < 0.05$, using EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan) [12], which is a graphical user interface for R.

3. Results

3.1. Ischemia-induced behavioral changes

The Y-maze alternation, measuring short-term memory, was significantly different between the groups ($p = 0.042$) compared using one-way ANOVA (Fig. 1). However, no significant difference was found between the sham group and any of the post ischemia recovery time point groups (e.g., $p = 0.091$ for sham group vs. 72 h time point, Dunnett's test). Contrastingly, when Tukey's test was applied, a significant difference (decrease) in Y-maze alternation was observed only between the 36 h and 72 h time points ($p = 0.024$).

3.2. Histological evaluation of ischemia-induced hippocampal neuronal death

HE-staining of the gerbil hippocampus is shown in Fig. 2 (A). Although tissue staining conditions were standardized, uneven color density among these photographs occurred after staining, which was difficult to avoid. However, we encountered no challenges in measuring the number of viable neurons. At the recovery time point 72 h post ischemia, most pyramidal neurons within the CA1 area were shrunken and darkly stained with minimal cytoplasm in contrast to earlier recovery time points (Fig. 2 (A)). No significant decrease in the number of viable neurons was observed at recovery time points 5–36 h post ischemia ($p > 0.05$). However, the number

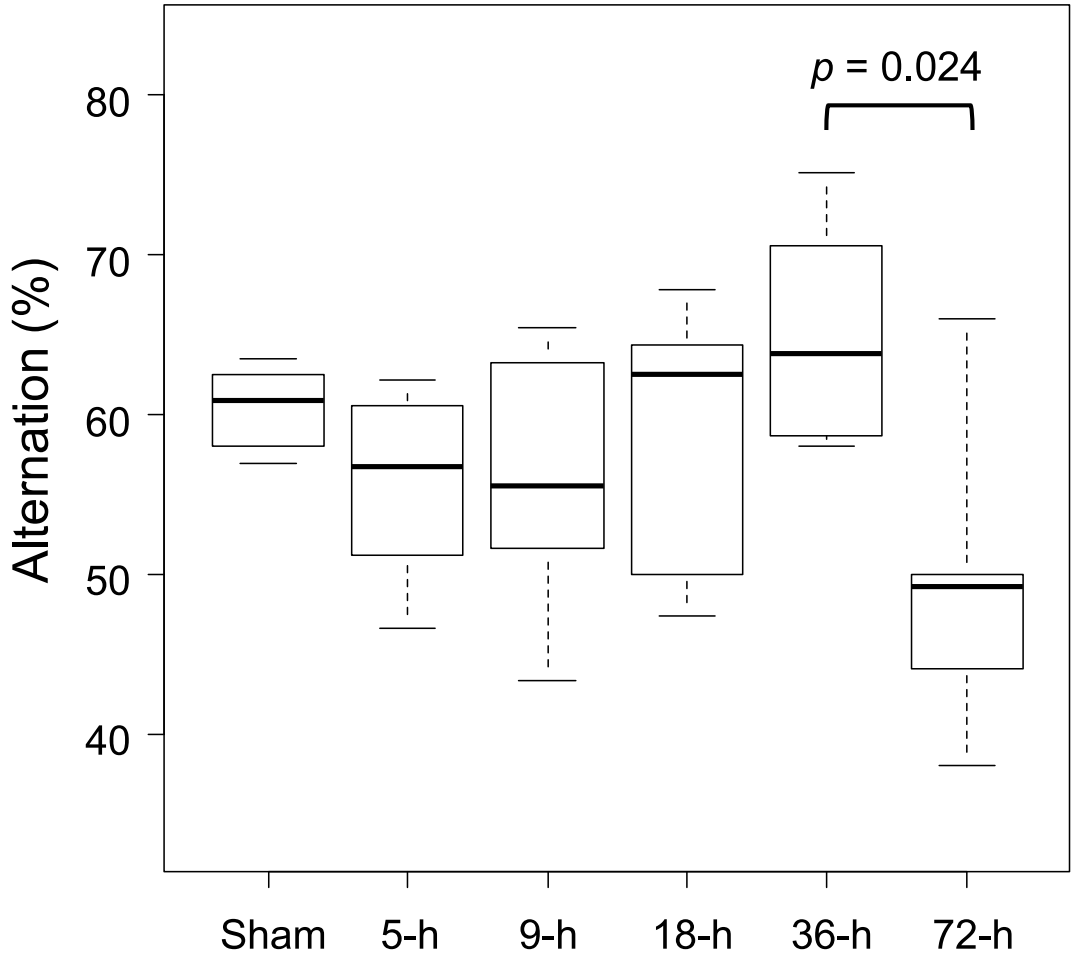

Fig. 1. Alternation behavior rates with the Y-maze spontaneous alternation test of the 5-h, 9-h, 18-h, 36-h, the 72-h groups, and the sham group, are shown (n = 7 per group). The significance of the difference between 36 and 72 h was determined by the Tukey test.

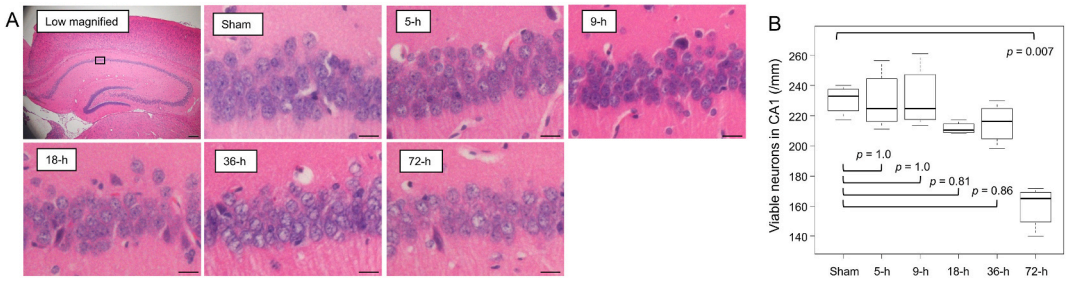

Fig. 2. (A) Hematoxylin-eosin staining of the gerbil hippocampus (upper leftmost panel; scale bar: 200 μm). Representative sections of the Cornet d'Ammon 1 (CA1) region, enclosed by a rectangle of the upper leftmost panel, (scale bar: 20 μm for all subsequent images) sham group (upper, second from the left panel), the 5-h group (upper, third from the left panel), the 9-h group (upper, rightmost panel), the 18-h group (lower left panel), the 36-h group (lower middle panel), and the 72-h group (lower right panel), are shown. (B) Quantitative data of viable neuros per millimeter of the hippocampal CA1 region of the 5-h, 9-h, 18-h, 36-h, the 72-h groups, and the sham group (n = 3 per group). The difference between sham and 72-h groups was significant (Dunnett test).

of viable neurons decreased significantly at 72 h post ischemia compared with the sham group ($p = 0.007$, Dunnett's test). A one-way ANOVA comparison of the number of viable neurons showed a significant difference between the groups ($p = 0.009$) (Fig. 2 (B)).

3.3. Identification of differentially expressed miRNAs in the hippocampus

miRNA expression levels in the hippocampi of gerbils were compared between each post-ischemia recovery time point groups. A total of 63 miRNAs were significantly upregulated, whereas 32 miRNAs were significantly downregulated, monotonically, from 5 to 72 h post transient ischemia (Fig. 3, Tables 1 and 2). The heatmap (Fig. 4 (A) and (B)) shows increasing and decreasing trends for dysregulated miRNAs. Approximately 59 of the 63 monotonically upregulated miRNAs showed the largest increase from 36 to 72 h after ischemia. In contrast, 18 of the 32 monotonically downregulated miRNAs showed the largest decrease from 5 to 9 h after ischemia.

Some monotonically upregulated miRNAs, such as miR-3473b, miR-669c-3p, miR-139-5p, miR-132-5p, miR-376b-3p, miR-378a-5p, and miR-138-5p have been reported to be associated with cerebral ischemia or neuropsychiatric disorders. In addition, some monotonically downregulated miRNAs, particularly those with the largest changes from 5 to 9 h after transient ischemia, such as miR-204-5p, miR-34c-5p, miR-34b-5p, miR-211-5p, miR-141-3p, miR-34b-3p, miR-199b-3p, and miR-199a-5p are known to be dysregulated in similar disease conditions. The relationship between the expression levels of these miRNAs and the recovery period after

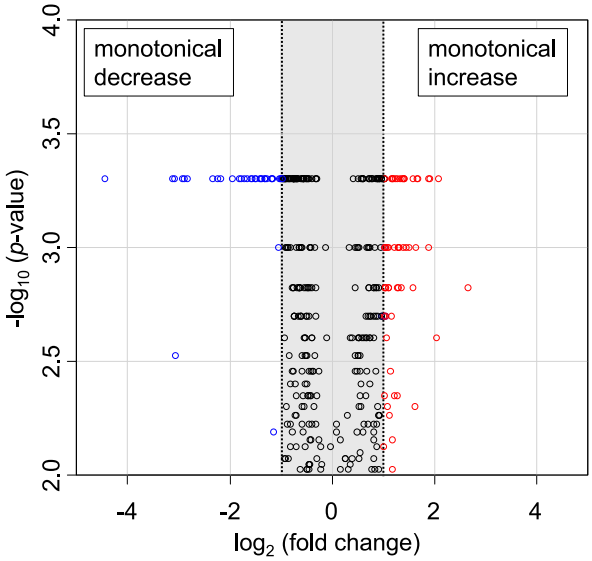

Fig. 3. Volcano plot of the microRNAs extracted from the hippocampi of gerbils after ischemia. The plot shows the relationship between the fold change and significance of the difference. The fold change was defined as the ratio of the expression level of the 72-h group to the expression level of the 5-h group. A Jonckheere-Terpstra test was performed to identify microRNAs that significantly increased or decreased, monotonically, with the passage of time after cerebral ischemia (the 5-h, 9-h, 18-h, 36-h, and the 72-h groups, n = 4 per group). The vertical lines indicate that microRNAs are either upregulated or downregulated above a fold change of 2 and 0.5, respectively. The blue color and red color indicate significantly monotonically decreased microRNAs and significantly monotonically increased microRNAs, respectively.

Table 1
Statistical results of significantly monotonically increased microRNAs from 5 to 72 h after transient brain ischemia. FDR: false discovery rate.

microRNA	Fold change	p-value	FDR
mmu-miR-6379	6.28	0.0015	0.01943
mmu-miR-3473a	4.24	0.0005	0.01281
mmu-miR-6541	4.12	0.0025	0.02464
mmu-miR-706	3.75	0.0005	0.01281
mmu-miR-6481	3.71	0.001	0.01624
mmu-miR-6946-5p	3.70	0.0005	0.01281
mmu-miR-3473b	3.19	0.0005	0.01281
mmu-miR-3473e	3.17	0.0005	0.01281
mmu-miR-5128	3.12	0.001	0.01624
mmu-miR-7000-5p	3.05	0.005	0.04147
mmu-miR-6981-5p	3.00	0.0005	0.01281
mmu-miR-6996-5p	2.98	0.0015	0.01943
mmu-miR-7217-3p	2.84	0.001	0.01624
mmu-miR-377-3p	2.71	0.001	0.01624
mmu-miR-466g	2.67	0.0005	0.01281
mmu-miR-6972-5p	2.64	0.0005	0.01281
mmu-miR-6989-5p	2.62	0.0005	0.01281
mmu-miR-5107-5p	2.61	0.001	0.01624
mmu-miR-6408	2.57	0.0005	0.01281
mmu-miR-7007-5p	2.55	0.0015	0.01943
mmu-miR-3060-3p	2.50	0.0005	0.01281
mmu-miR-669c-3p	2.49	0.001	0.01624
mmu-miR-466f-3p	2.46	0.0015	0.01943
mmu-miR-451b	2.46	0.001	0.01624
mmu-miR-139-5p	2.45	0.001	0.01624
mmu-miR-1967	2.43	0.0045	0.03872
mmu-miR-466i-3p	2.43	0.0005	0.01281
mmu-miR-7082-5p	2.40	0.0015	0.01943
mmu-miR-504-3p	2.35	0.0005	0.01281
mmu-miR-7216-5p	2.34	0.001	0.01624
mmu-miR-335-3p	2.33	0.0045	0.03872
mmu-miR-6973b-5p	2.28	0.0005	0.01281
mmu-miR-6385	2.27	0.0095	0.06443
mmu-miR-5113	2.25	0.0005	0.01281
mmu-miR-467d-3p	2.25	0.0005	0.01281
mmu-miR-6239	2.25	0.007	0.05207
mmu-miR-1907	2.24	0.002	0.02239
mmu-miR-3075-5p	2.22	0.0005	0.01281
mmu-miR-8090	2.20	0.0035	0.03178
mmu-miR-7050-5p	2.17	0.0055	0.04404
mmu-miR-467a-3p	2.16	0.001	0.01624
mmu-miR-132-5p	2.13	0.0015	0.01943
mmu-miR-877-5p	2.13	0.0015	0.01943
mmu-miR-7652-5p	2.12	0.001	0.01624
mmu-miR-376b-3p	2.12	0.005	0.04147
mmu-miR-6354	2.08	0.0025	0.02464
mmu-miR-6405	2.07	0.0015	0.01943
mmu-miR-6978-5p	2.06	0.001	0.01624
mmu-miR-6369	2.06	0.0015	0.01943
mmu-miR-7035-5p	2.05	0.001	0.01624
mmu-miR-7051-5p	2.05	0.002	0.02239
mmu-miR-8100	2.05	0.002	0.02239
mmu-miR-466m-3p	2.04	0.0015	0.01943
mmu-miR-7079-5p	2.04	0.0005	0.01281
mmu-miR-466i-5p	2.03	0.001	0.01624
mmu-miR-378a-5p	2.03	0.0005	0.01281
mmu-miR-669a-3p, mmu-miR-669o-3p	2.03	0.002	0.02239
mmu-miR-6987-5p	2.03	0.001	0.01624
mmu-miR-138-5p	2.02	0.0005	0.01281
mmu-miR-7681-3p	2.02	0.0005	0.01281
mmu-miR-6947-5p	2.02	0.0045	0.03872
mmu-miR-137-3p	2.02	0.002	0.02239
mmu-miR-5622-5p	2.01	0.0075	0.05508

Table 2
Statistical results of significantly monotonically decreased microRNAs from 5 to 72 h after transient brain ischemia. FDR: false discovery rate.

microRNA	Fold change	p-value	FDR
mmu-miR-204-5p	0.05	0.0005	0.00703
mmu-miR-34c-5p	0.11	0.0005	0.00703
mmu-miR-34b-5p	0.12	0.0005	0.00703
mmu-miR-211-5p	0.12	0.003	0.02488
mmu-miR-141-3p	0.13	0.0005	0.00703
mmu-miR-1298-5p	0.14	0.0005	0.00703
mmu-miR-34b-3p	0.14	0.0005	0.00703
mmu-miR-1298-3p	0.20	0.0005	0.00703
mmu-miR-1a-3p	0.21	0.0005	0.00703
mmu-miR-205-5p	0.22	0.0005	0.00703
mmu-miR-7008-3p	0.26	0.0005	0.00703
mmu-miR-6917-5p	0.29	0.0005	0.00703
mmu-miR-6918-5p	0.29	0.0005	0.00703
mmu-miR-199b-5p	0.31	0.0005	0.00703
mmu-miR-199a-3p, mmu-miR-199b-3p	0.31	0.0005	0.00703
mmu-miR-219a-2-3p	0.33	0.0005	0.00703
mmu-miR-16-2-3p	0.34	0.0005	0.00703
mmu-miR-182-3p	0.35	0.0005	0.00703
mmu-miR-702-3p	0.35	0.0005	0.00703
mmu-miR-669b-5p	0.37	0.0005	0.00703
mmu-miR-322-5p	0.38	0.0005	0.00703
mmu-miR-338-5p	0.38	0.0005	0.00703
mmu-miR-133b-3p	0.40	0.0005	0.00703
mmu-miR-152-3p	0.40	0.0005	0.00703
mmu-miR-5124a	0.41	0.0005	0.00703
mmu-miR-199a-5p	0.44	0.0005	0.00703
mmu-miR-34c-3p	0.44	0.0005	0.00703
mmu-miR-18a-5p	0.44	0.0005	0.00703
mmu-miR-466n-5p	0.45	0.0065	0.04307
mmu-miR-92b-3p	0.49	0.001	0.01165
mmu-miR-6933-3p	0.49	0.0005	0.00703
mmu-miR-6919-5p	0.50	0.0005	0.00703

ischemia is shown in Fig. 5(A)–(D).

4. Discussions

In this study, we identified gerbil hippocampus specific miRNAs that showed significant monotonic changes in their expression levels at various time points after exposing the animals to transient ischemia. A subset of these dysregulated miRNAs exhibited significantly reduced expression levels at 9 h post-ischemia compared with the 5 h recovery time point. However, in the case of monotonous upregulation, a significant difference in expression levels of upregulated miRNAs was observed only between the 36 h and 72 h groups. Contrastingly, the number of viable neurons in the CA1 region and cognitive function were preserved up to 36 h after transient ischemia. A significant difference in cognitive decline and increased hippocampal neuronal cell death was observed only between the 36 h and 72 h groups (Figs. 1 and 2). Our results indicated that downregulation of a subset of miRNAs could occur as early as 9 h post transient ischemia, long before observing any decrease in cell numbers or cognitive dysfunction, thus they could be used as baseline data for further assessment of the therapeutic efficacy of restoring miRNA dysregulation.

Our results showed that neuronal death in the CA1 region occurred several days after transient brain ischemia in gerbils; it is referred to as “delayed neuronal death” [10]. Since cognitive function is thought to decline at the same time phase as hippocampal neuronal cell death, it is reasonable that cognitive function also declines several days after a transient ischemic event. In this study, dysregulation of several miRNAs occurred as early as 9 h after ischemia, while no behavioral or histological changes were yet evident. Since miRNA is located upstream of protein synthesis, its expression may change rapidly when cells receive external stimuli. In experiments with rats, miRNA expression changed several hours after hepatotoxic drug administration, much earlier than the occurrence of hepatocellular necrosis or elevation in conventional biomarkers [13].

4.1. Monotonically downregulated miRNAs

4.1.1. MiR-204-5p

Some of these downregulated miRNAs have been reported to be associated with cerebral ischemia or neuropsychiatric disorders. MiR-204-5p was downregulated by cerebral ischemia and its overexpression protected against cerebral infarction [14]. Reduced expression levels of miR-204-5p in the blood of patients with stroke, the brain of middle cerebral artery occlusion (MCAO)-treated rats and oxygen-glucose deprivation (OGD)-treated neurons has been reported. Moreover, overexpression of miR-204-5p has been reported to reduce infarct size in a rat model. In rats, ischemic preconditioning upregulated miR-204-5p and decreased the cerebral infarct

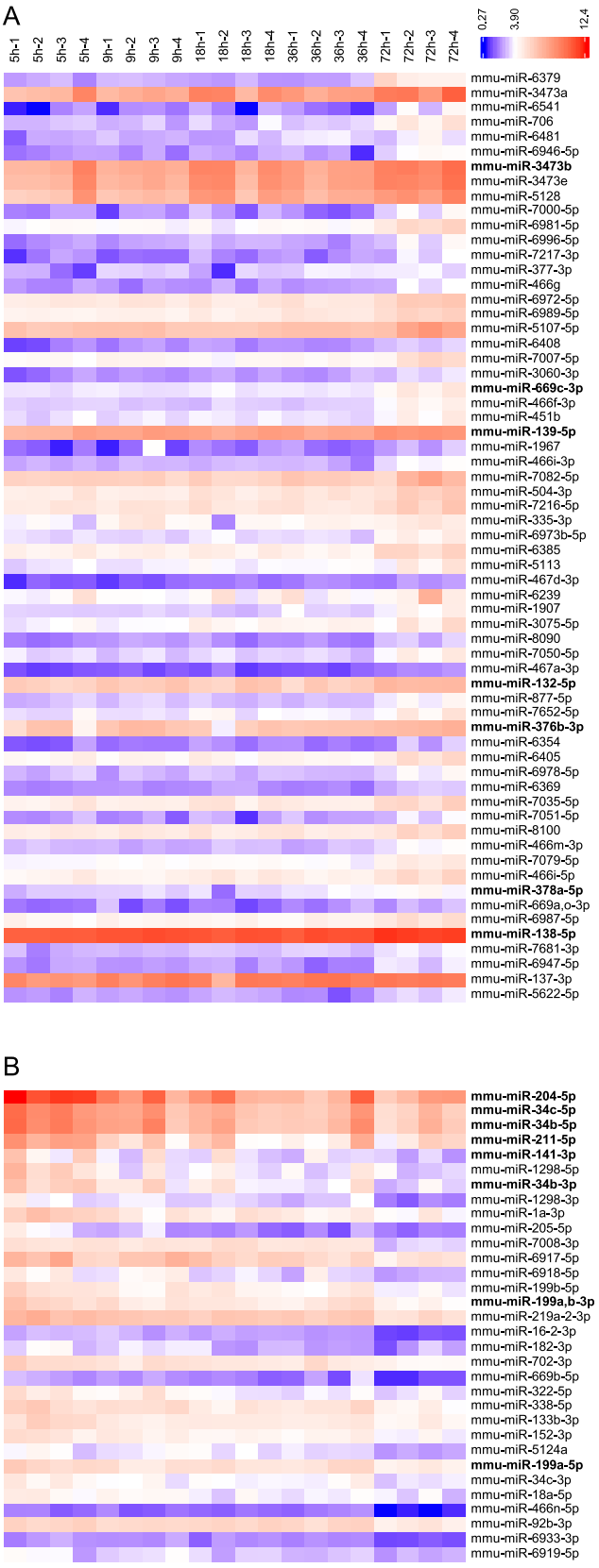

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Fig. 4. A heatmap illustrating (A) increasing and (B) decreasing trends of significantly dysregulated microRNAs. The columns and rows indicate samples (four gerbils for each post-ischemic recovery period: 5, 9, 18, 36, and 72 h) and microRNAs, respectively. The expression levels of the microRNAs were $\log_2$-transformed and assigned in a color code. White indicates median of all values used in the heatmap, blue indicates lower expression and red indicates higher expression.

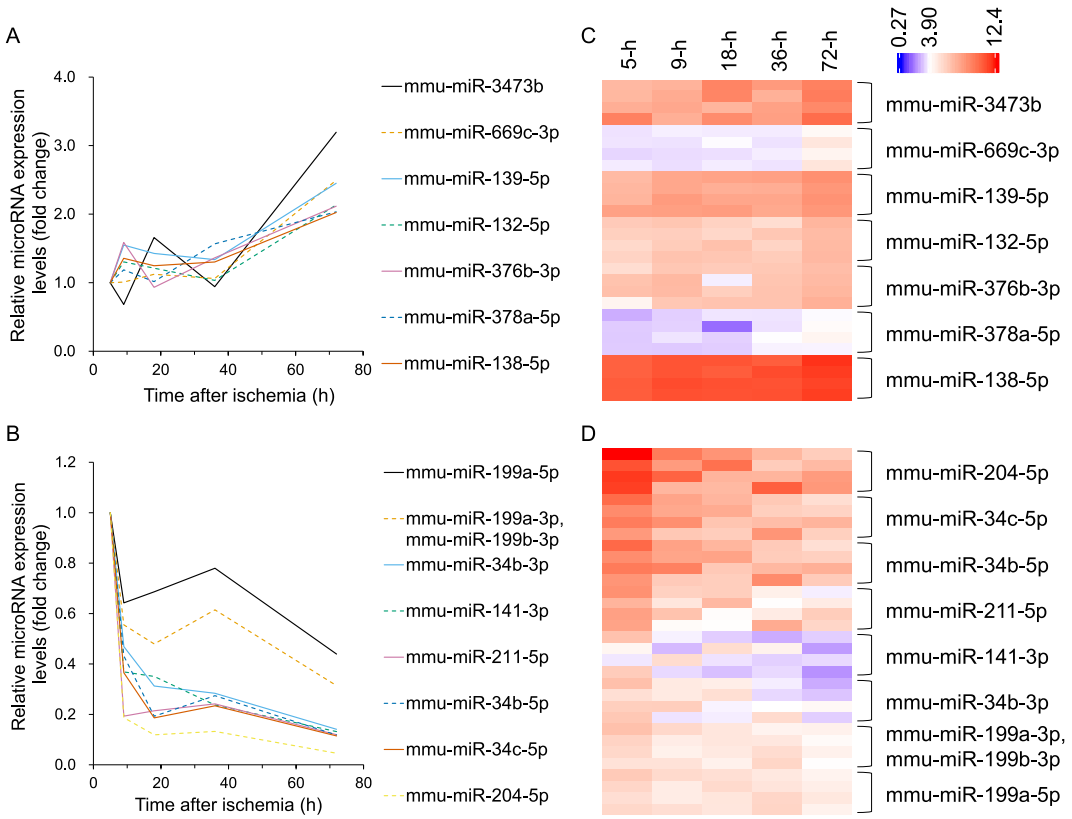

Fig. 5. Trends of expression levels of (A, C) significantly monotonically increasing and (B, D) significantly monotonically decreasing microRNAs in the hippocampus associated with cerebral ischemia or neuropsychiatric disorders. The fold change was defined as the ratio of the signal intensity at 5 h to that at each time point after ischemia (5, 9, 18, 36, and 72 h, n = 4 per group). In the heatmaps (C, D), the columns and rows indicate time after ischemia and microRNAs, respectively. The expression levels of the microRNAs were $\log_2$-transformed and assigned in the same color code used in Fig. 4. White indicates median of all values used in the heatmap of all data (Fig. 4), blue indicates lower expression, and red indicates higher expression.

volume after MCAO, whereas downregulation by antagomir decreased the effect of preconditioning [15]. In addition, the downregulation of miR-204-5p in a mouse model of myocardial ischemia/reperfusion (IR) injury exacerbated myocardial damage [16], suggesting that miR-204-5p may play a common role in ischemic changes both in the brain and myocardium. In a non-ischemic disease model, miR-204-5p was downregulated in the hippocampus of a rat model of depression induced by chronic unpredictable stress, whereas overexpression of miR-204-5p reduced oxidative stress, neuroinflammation, and depression-like symptoms [17]. MiR-204-5p has been implicated in post-stroke depression as well as cerebral ischemia. In addition, downregulation of miR-204-5p in cerebrospinal fluid has been reported in patients with genetic frontotemporal dementia [18], suggesting that vascular dementia as a complication of cerebral infarction and dementia due to neurodegeneration may have a common mechanism.

4.1.2. MiR-34c-5p

MiR-34c-5p has been reported to have anti-ischemic effects similar to those of miR-204-5p and is downregulated in MCAO-treated rat brain and OGD-treated cell models. MiR-34c-5p is reported to be involved in inflammation and apoptosis and shows anti-ischemic effects through overexpression [19]. Since transient cerebral ischemia in gerbils could induce apoptosis in neurons in the hippocampal CA1 region [10], we thought that the anti-apoptotic effects of miR-34c-5p may make it a useful therapeutic candidate. A relationship between miR-34c-5p and post-stroke epilepsy has also been suggested. In patients with drug-resistant epilepsy as well as in rat models of epilepsy, miR-34c-5p was downregulated, accompanied by hippocampal neuronal loss and worsening neuroinflammation in the rat model [20].

4.1.3. MiR-34b-5p

Some reports indicated that miR-34b-5p may also have anti-ischemic effects [21], whereas others suggested that it exacerbates the damage caused by ischemia [22]. In an ischemic white matter injury model, the administration of a drug that restores white matter function upregulated miR-34b-5p expression in the optic nerve of OGD-treated mice [21]. In contrast, in a rat model of common carotid artery occlusion (CCAO), administration of a miR-34b-5p mimic impaired cell viability and increased apoptosis [22].

4.1.4. MiR-211-5p

MiR-211-5p is another miRNA with potential protective effects against cerebral infarction and post-stroke depression; it also shows anti-apoptotic effects. Reportedly, miR-211-5p is downregulated in the blood of patients with stroke [23], MCAO rat brain [23,24], OGD neurons [23], and the OGDR model of pheochromocytoma-12 (PC12) cells [24]. Upregulation of miR-211-5p in MCAO rats could reduce cerebral infarct size and improve neurological deficits [23,24]. However, another study showed that miR-211-5p was upregulated in the MCAO rat brain [25], indicating the need for future follow-up studies. MiR-211-5p is downregulated in the hippocampus of a rat model of chronic stress-induced depression. Additionally, upregulation of miR-211-5p in the hippocampus reduces neuronal apoptosis and alleviates depression-like behaviors [26]. MiR-211-5p is also downregulated in a rat model of spinal cord injury; its upregulation by agomir alleviates neuronal apoptosis and inflammation [27].

4.1.5. MiR-141-3p

Our results showed that miR-141-3p was downregulated after cerebral ischemia in gerbils, in contrast to a previous study reporting upregulated expression in a mouse MCAO model [28]. MiR-141-3p was also found to be downregulated in a cardiomyocyte hypoxia/reoxygenation model; cardiomyocyte apoptosis was reduced by its overexpression [29]. These contrasting findings highlight the need for further studies.

4.1.6. Other downregulated miRNAs (miR-34b-3p, miR-199b-3p, and miR-199a-5p)

Various miRNAs related to cerebral ischemia were also found to be downregulated as a consequence of ischemic manipulation. As previously reported, the upregulation of miR-34b-3p alleviated apoptosis in brain vascular endothelial cells after OGDR [30]. MiR-199b-3p is downregulated by OGD processing in mouse hippocampal neurons and associated with apoptosis [31]. However, contrasting observations have been reported regarding the dysregulation of miR-199a-5p. Some studies have reported its downregulation in rats with cerebral infarction and protective effects by miRNA mimics administration [32], whereas other reports revealed upregulation in MCAOR mice [33] and rats [34] and protective effects by inhibiting miR-199a-5p.

4.1.7. MiRNAs downregulated earlier than cognitive decline and neuronal cell death

As described above, several of the miRNAs downregulated in this study are expected to have anti-ischemic effects when upregulated. In particular, expression levels of miR-204-5p, miR-34c-5p, miR-211-5p, miR-34b-3p, and miR-199b-3p were remarkably reduced at 9 h time point compared with 5 h post-ischemia, suggesting that upregulation immediately after ischemic stroke onset may be effective and further assessment is required. In addition to the protective effect on cerebral infarction itself, effects on post-stroke depression, dementia, and epilepsy may also be expected.

4.2. Monotonically upregulated miRNAs

4.2.1. MiR-3473b

Our results indicated that miR-3473b expression levels were remarkably increased at 72 h compared with 36 h after transient ischemia. MiR-3473b is upregulated in the cortex and striatum of MCAO-treated mice; the downregulation by antagomir reduces inflammatory factors and infarct volume [35]. In addition, miR-3473b antagomir inhibited microglial activation in the substantia nigra in a mouse model of Parkinson's disease [36]. These results suggest a possible proinflammatory role of miR-3473b.

4.2.2. MiR-669c-3p

A previous study concluded that miR-669c-3p expression remains unchanged 1 d after MCAO treatment in mice but increases significantly 3 days later [37]. This change over time is consistent with our results. The same study also reported that ischemic damage was reduced. In addition, sensorimotor function was improved by the overexpression of miR-669c in the striatum before cerebral ischemia. MiR-669c has an anti-ischemic effect and may be induced by ischemia.

4.2.3. MiR-139-5p

In the present study, miR-139-5p was found to be upregulated at 72 h vs. 36 h, in contrast to previous reports, which suggested that miR-139-5p is downregulated by cerebral ischemia. MCAO treatment of mice and OGD treatment of neurons downregulated miR-139-5p, whereas administration of ginsenoside Rd before ischemia increased miR-139-5p expression in a concentration-dependent manner, neuronal pyroptosis was decreased, thus reducing ischemic brain damage [38].

4.2.4. MiR-132-5p

Similarly, miR-132-5p was markedly upregulated between 36 h and 72 h post-ischemia recovery time points. However, in a previous study, it was found to be upregulated 1 d after transient cerebral ischemia in gerbils [8]. A total of 6 min of bilateral CCAO (3 $\times$ 2-min occlusions) was performed, which is a longer ischemic duration compared with our 5-min occlusion. The miRNA changes may

have occurred earlier due to the higher degree of cerebral ischemia.

4.2.5. MiR-376b-3p

MiR-376b-3p is upregulated in hypoxic stress of vascular pericytes in the rat brain [39] and in the urinary exosomes of amyloid-β-accumulated mice [40]. This may contribute to the impairment in the blood-brain barrier caused by cerebral infarction and future cognitive decline.

4.2.6. MiR-378a-5p

MiR-378a-5p expression appears to start increasing between 18 h and 36 h after ischemia in our experiment. According to Sun et al., miR-378a-5p remained unchanged 1 d after transient ischemia in gerbils but was upregulated 7 days later [8], which is consistent with our results. In addition, it has been shown that OGD treatment of hippocampal neurons in newborn rats upregulates miR-378a-5p in a time-dependent manner from 24 h to 48 h; knockdown with an inhibitor promotes cell viability and reduces apoptosis [41].

4.2.7. MiR-138-5p

However, different results have been reported for miR-138-5p. miR-138-5p is reportedly downregulated in MCAO-treated rats. Furthermore, upregulation of miR-138-5p reduces inflammation and improves neurological function [42], whereas overexpression of miR-138-5p in MCAO-treated mice alleviates neuronal damage [43]. However, its upregulation in patients with ischemic stroke has also been reported [44].

4.2.8. MiRNAs upregulated earlier than cognitive decline and neuronal cell death

The miRNAs that were monotonically upregulated in this study have been reported to have various functions, such as proinflammatory and anti-ischemic effects. In particular, the upregulation of some miRNAs occurs before the beginning of neuronal cell death. However, as previously described, neuronal cell death progresses from 36 h to 72 h after ischemia. Therefore, distinguishing whether the miRNA upregulation during this period is due to ischemia itself or changes associated with cell death is impossible. Of the miRNAs that were upregulated in this study and reported to be associated with brain ischemia, only miR-378a-5p began to upregulate within 36 h after ischemia.

4.3. Limitations

This study has several limitations. First, we did not perform an evaluation earlier than 5 h after ischemia. However, if the recovery time after surgery is too short, the evaluation of cognitive function may be inaccurate due to residual anesthesia or surgical invasion. Our results showed no significant difference in cognitive function between the sham and 5 h after surgery groups. Second, it has not been verified whether altered miRNAs could have therapeutic effects immediately after ischemia onset. Conducting future experiments to restore dysregulated miRNAs at an earlier phase than the cognitive decline and neuronal cell death is necessary. In other words, we can induce upregulation to the downregulated miR-204-5p, miR-34c-5p, miR-211-5p, miR-34b-3p, and miR-199b-3p, and conversely, downregulation to the upregulated miR-378a-5p, to the brains of gerbils immediately after global cerebral ischemia. Upregulation can be achieved by administering miRNA mimics, whereas downregulation can be achieved by administering antisense miRNA oligonucleotides, locked nucleic acid anti-miRNAs, small molecular miRNA inhibitors, and miRNA sponges [45]. This will allow us to confirm whether dysregulation of miRNAs could be restored and whether neuronal cell death and cognitive decline might be ameliorated after this treatment. Experiments are also needed to verify the findings in comparison with therapeutic intervention. For example, edaravone, a free radical scavenger, is a neuroprotective reagent that we previously reported its therapeutic effect on cerebral ischemia in gerbils [46]. Comparing the changes in miRNA expression after administering edaravone to gerbils with cerebral ischemia with the present results, we could verify whether the miRNAs identified in this study were related to therapeutic intervention. Third, we have not evaluated the dysregulation of long non-coding RNAs [47] and messenger RNAs involved in epigenetic regulation other than miRNAs, and the changes in the expression levels of their target proteins. Fourth, reverse transcription quantitative polymerase chain reaction (RT-qPCR) experiments have not been conducted. Although the microarray used in this study has quantitative properties, RT-qPCR is needed for more accurate miRNA quantification. These lacunae need to be further investigated in future studies.

5. Conclusions

This study revealed temporal changes in miRNA expression in the hippocampus immediately after 5 min of transient cerebral ischemia in gerbils. Most of the significantly monotonically downregulated miRNAs showed a large decrease from 5 h to 9 h after ischemia, whereas cognitive decline and neuronal cell death in the hippocampal CA1 region progressed from 36 h to 72 h. Regarding significantly monotonically upregulated miRNAs, one miRNA that is known to be associated with ischemia initiated upregulation within 36 h after ischemia. Restoration of these dysregulated miRNAs might be a potential therapeutic target for cerebral infarction and be used before neuronal death occurrence, requiring further assessement in future studies.

Data availability statement

Data will be made available on request.

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Ethics statement

This study was approved by the Animal Committee of Kagawa University Faculty of Medicine (approval number: 21655-2).

CRediT authorship contribution statement

Yasuhiro Hamada: Writing – original draft, Visualization, Validation, Methodology, Conceptualization, Data curation, Formal analysis, Investigation. **Tadayuki Takata:** Writing – review & editing, Validation, Methodology, Investigation, Data curation, Conceptualization, Funding acquisition. **Hisakazu Iwama:** Data curation, Formal analysis, Visualization. **Rie Kawakita:** Investigation. **Wakako Nonaka:** Investigation. **Kazushi Deguchi:** Supervision, Resources. **Hideki Kobara:** Supervision, Resources. **Asahiro Morishita:** Validation, Supervision. **Osamu Miyamoto:** Validation, Resources, Methodology. **Takehiro Nakamura:** Validation, Resources, Methodology. **Toshifumi Itano:** Methodology, Project administration, Supervision, Validation, Writing – review & editing. **Tsutomu Masaki:** Supervision, Funding acquisition, Resources.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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