Mindfulness intervention improves cognitive function in older adults by enhancing the level of miRNA-29c in neuron-derived exosomes

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Supplementary information

Supplementary Table 1

PCR primers for miRNA analysis

Gene	Sequence
miRNA-9-5p	5’-UCUUUGGUUAUCUAGCUGUAUGA-3’
miRNA-29c-3p	5’-UAGCACCAUUUGAAAUCGGUUA-3’
miRNA-124-3p	5’-UAAGGCACGCGGUGAAUGCC-3’
miRNA-125b-5p	5’-UCCCUGAGACCCUAACUUGUGA-3’
miRNA-146a-5p	5’-UGAGAACUGAAUUCCAUGGGUU-3’
miRNA-181a-5p	5’-AACAUUCAACGCUGUCGGUGAGU-3’
miRNA-25-3p	5’-CAUUGCACUUGUCUCGGUCUGA-3’
miRNA-93-5p	5’-CAAAGUGCUGUUCGUGCAGGUAG-3’
miRNA-425-5p	5’-AAUGACACGAUCACUCCCGUUGA-3’
miRNA-16-5p	5’-UAGCAGCACGUAAAUAUUGGCG-3’
snoRNA135	5’-CUAAAAUAGCUGGAAUUACCGGCAGAUUGGUAGUGGUGAGCCUAUGGUUUUCUGAAG-3’

Supplementary Table 1. Target sequences of miRNAs and snoRNA 135.

Supplementary Table 2

mRNA PCR primers for human

Gene	Direction	Sequence
DNMT3A	forward	5’-TATTGATGAGCGCACAAGAGAGC-3’
	reverse	5’-GGGTGTTCCAGGGTAACATTGAG-3’
DNMT3B	forward	5’-GACTTGGTGATTGGCGGAA-3’
	reverse	5’-GGCCCTGTGAGCAGCAGA-3’
STAT3	forward	5’-CTTTGAGACCGAGGTGTATCACC-3’
	reverse	5’-GGTCAGCATGTTGTACCACAGG-3’
BACE1	forward	5’-GCAGGGCTACTACGTGGAGA-3’
	reverse	5’-GTATCCACCAGGATGTTGAGC-3’
GAPDH	forward	5’-GTCTCCTCTGACTTCAACAGCG-3’
	reverse	5’-ACCACCCTGTTGCTGTAGCCAA-3’
RNA18s	forward	5’-GTAACCCGTTGAACCCCATT-3’
	reverse	5’-CCATCCAATCGGTAGTAGCG-3’

Supplementary Table 2. Sequence of primers for human mRNAs.

Supplementary Table 3

mRNA PCR primers for mouse

Gene	Direction	Sequence
DNMT3A	forward	5’-GCCGAATTGTGTCTTGGTGGATGACA-3’
	reverse	5’-CCTGGTGGAATGCACTGCAGAAGGA-3’
DNMT3B	forward	5’-TTCAGTGACCAGTCCTCAGACACGAA-3’
	reverse	5’-TCAGAAGGCTGGAGACCTCCCTCTT-3’
STAT3	forward	5’-CTTGTCTACCTCTACCCCGACAT-3’
	reverse	5’-GATCCATGTCAAACGTGAGCG-3’
BACE1	forward	5’-CCGGCGGGAGTGGTATTATG-3’
	reverse	5’-GCAAACGAAGGTTGGTGGT-3’
GAPDH	forward	5’-ACGACCCCTTCATTGACC-3’
	reverse	5’-CCAGTGAGCTTCCCGTTCAGC-3’

Supplementary Table 3. Sequence of primers for mouse mRNAs.

Supplementary Table 4

Immunofluorescence

Primary Antibody	Source	Dilution	Manufacture
Aβ	rabbit monoclonal	1:500	Cell Signaling
NeuN	rabbit polyclonal	1:500	Millipore

Secondary Antibody	Conjugate	Dilution	Manufacture
Rabbit IgG	FITC	1:500	Millipore
Rabbit IgG	Cy3	1:500	Jackson ImmunoResearch

Supplementary Table 4. Primary and secondary antibodies used for immunofluorescence.

Supplementary Table 5

MoCA-J Variable	Non-MBSR group (n=9) X ± SD	MBSR group (n=24) X ± SD	MBSR effect	Time effect	MBSR* time Effect
Total score					
pre	21.00 ± 3.30	21.11 ± 2.33	$F(1, 29) = 7.330$	$F(1, 29) = 13.83$	$F(1, 29) = 11.90$
post	22.55 ± 2.82	25.50 ± 2.90	$P = 0.0112$ *	$P = 0.0009$ **	$P = 0.0017$ **
Delayed recall					
pre	1.44 ± 1.89	2.44 ± 1.83	$F(1, 29) = 1.929$	$F(1, 29) = 8.577$	$F(1, 29) = 0.1410$
post	2.41 ± 1.50	3.18 ± 1.67	$P = 0.1755$	$P = 0.0066$ **	$P = 0.7100$
Visuospatial / executive					
pre	3.44 ± 1.07	2.67 ± 1.15	$F(1, 29) = 1.135$	$F(1, 29) = 0.2573$	$F(1, 29) = 16.46$
post	3.00 ± 1.31	4.00 ± 1.00	$P = 0.2954$	$P = 0.6158$	$P = 0.0003$ **
Attention					
pre	4.56 ± 0.50	4.22 ± 0.79	$F(1, 29) = 0.00337$	$F(1, 29) = 0.8882$	$F(1, 29) = 6.438$
post	4.68 ± 0.87	5.41 ± 0.65	$P = 0.9541$	$P = 0.3538$	$P = 0.0168$ *
Abstraction					
pre	1.44. ± 0.68	1.44 ± 0.68	$F(1, 29) = 0.03844$	$F(1, 29) = 0.8751$	$F(1, 29) = 0.05099$
post	1.36 ± 0.71	1.55 ± 0.72	$P = 0.8459$	$P = 0.3573$	$P = 0.8229$
Language					
pre	0.89 ± 0.57	0.89 ± 0.74	$F(1, 29) = 2.986$	$F(1, 29) = 1.161$	$F(1, 29) = 1.161$
post	1.18 ± 0.78	1.55 ± 0.89	$P = 0.0946$	$P = 0.2901$	$P = 0.2901$
Naming					
pre	2.67 ± 0.47	2.89 ± 0.31	$F(1, 29) = 1.372$	$F(1, 29) = 5.287$	$F(1, 29) = 2.306$
post	2.91 ± 0.42	2.95 ± 0.21	$P = 0.2510$	$P = 0.0289$ *	$P = 0.1397$
Orientation					
pre	5.44 ± 0.83	5.67 ± 0.47	$F(1, 29) = 7.614$	$F(1, 29) = 0.7916$	$F(1, 29) = 0.7916$
post	5.91 ± 0.29	5.91 ± 0.29	$P = 0.0099$ **	$P = 0.3809$	$P = 0.3809$

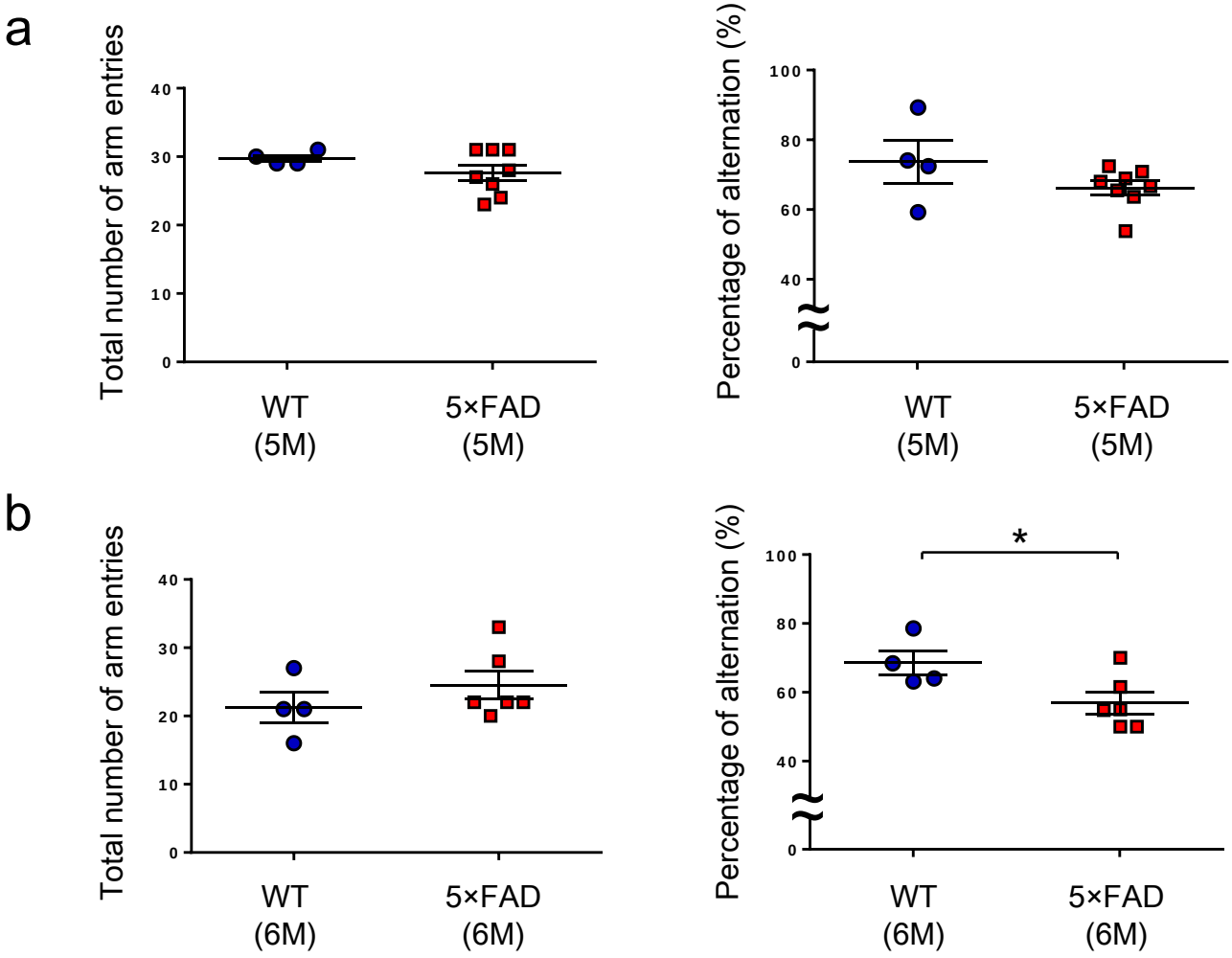
Supplementary Table 5. The results of two-way repeated measures ANOVA of MoCA-J. non-MBSR group (n = 9), MBSR group (n = 22). Values are the means **±** SD. *$P < 0.05$, **$P < 0.01$, two-way repeated measures ANOVA. The effect of MBSR, the effect of time, and the interaction effect are shown.

Supplementary Table 6

miRNA (total)	Non MBSR group (n=9) ΔCT (post) – ΔCT (pre)	*p*-value	MBSR group (n=22) ΔCT (post) – ΔCT (pre)	*p*-value
miR-9	-0.10 ± 0.64	$p = 0.6659$	-0.01 ± 0.80	$p = 0.8237$
miR-29c	0.01 ± 0.59	$p = 1$	0.05 ± 0.71	$p = 0.4826$
miR-124	-0.02 ± 0.44	$p = 0.8978$	0.01 ± 0.74	$p = 0.7024$
miR-125b	0.18 ± 0.51	$p = 0.3449$	0.16 ± 0.55	$p = 0.2756$
miR-146a	0.01 ± 0.63	$p = 0.9675$	-0.05 ± 0.59	$p = 1$
miR-181a	0.08 ± 0.77	$p = 0.7659$	0.04 ± 0.47	$p = 1$

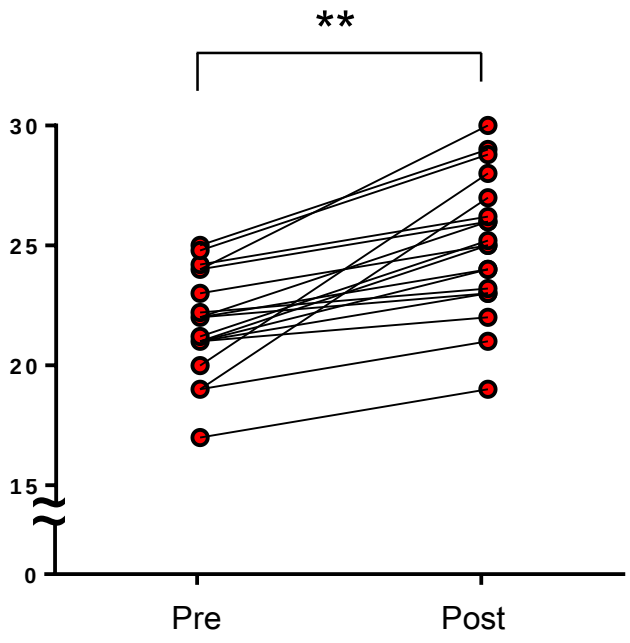
Supplementary Table 6. The changes in the expression of miR-9, miR-29c, miR-124, miR-125b, miR-146a, and miR-181a in total exosomes in each group are shown. Values are the means ± SD. non-MBSR group (n = 9), MBSR group (n = 22).

Supplementary Figure 1



Supplementary Figure 1. (a) The results of the Y maze test of 5xFAD mice at 5 months old. Total numbers of arm entries and the percentages of alternations are shown. Values are the means ± SEM. Wild-type (WT) (n = 4), 5xFAD (n = 8). (b) The results of the Y maze test of 5xFAD mice at 6 months old. Total numbers of arm entries and the percentages of alternations are shown. Values are the means ± SEM. *$P < 0.05$, unpaired *t*-test. WT (n = 4), 5xFAD (n = 6).

Supplementary Figure 2



Supplementary Figure 2. The change in the total score for the MoCA-J in 19 participants who showed a score of 25 or less before the MBSR intervention. $^{**}P < 0.01$, paired *t*-test. MBSR group (n = 19).

Supplementary Methods

Cognitive function test

Cognitive functions were assessed using the MoCA-J, which consists of visuospatial/executive, naming, memory, attention, language, abstraction, delayed recall, and orientation tasks. The total score and scores for each task were assessed before and after the intervention.

Blood collection

Blood samples were collected from participants using a vacuum blood collection tube with heparin (Terumo Corporation, Tokyo, Japan) for 10 mL, and plasma was collected 24 h later by centrifugation at 4 °C at 300 ×g for 10 min and stored at −80 °C.

Isolation of total exosomes

Isolation of total exosomes was performed according to a previous study[1]. One milliliter of plasma was thawed at 4 °C and then centrifuged at 4 °C at 1500 ×g for 10 min. Subsequently, 200 µL plasma was mixed with 400 µL thromboplastin-D (SEKISUI MEDICAL Co., Ltd., Tokyo, Japan) and incubated for 30 min at room temperature. A mixture of protease inhibitor cocktail tablets (Roche, Basel, Switzerland) and phosphatase inhibitor cocktail tablets (Roche) was then added and centrifuged at 4 °C at 3000 ×g for 30 min. After centrifugation, 600 µL supernatant was mixed with 151.2 µL ExoQuick (System Biosciences, Palo Alto, CA, USA) and incubated at 4 °C for 30 min. The samples were then centrifuged at 4 °C at 1500 ×g for 30 min. After centrifugation, all supernatants were removed, and each pellet was stored as total exosomes at −80 °C.

Isolation of NDEs

Isolation of NDEs from total exosomes was performed according a previous study[1]. Seven hundred microliters of Dulbecco's phosphate buffered saline (Thermo Fisher Scientific, Waltham, MA, USA) containing 4 µg Anti-Hu L1 cell adhesion molecule (CD171) Biotin-conjugated Antibody (eBioscience, San Diego, CA, USA) and total exosomes were mixed and incubated for 1 h at 4 °C.

After removing the supernatant, the pellets were suspended in 200 μL 0.05 M Glycine-HCl Buffer (pH 3.0) and centrifuged again at 4 ° C at 4000 × g for 20 min. The supernatant was mixed with 20 μL 1 M Tris-HCl (pH 8.0), and 100 μL of the mixture of supernatant and 1 M Tris-HCl (pH 8.0) was mixed with 25 μL 3% bovine serum albumin and 365 μL Mammalian Protein Extraction Reagent (Thermo Fisher Scientific). Total products of NDEs were stored at −80 ° C.

miRNA and mRNA isolation and quantitation

To extract total miRNA and mRNA from total exosomes and NDEs, mirVana PARIS RNA and the Native Protein Purification Kit (Thermo Fisher Scientific) were used. The extracted miRNAs were stored at −80 ° C. Fifty nanograms of miRNA was used for the synthesis of cDNA from miRNA using the TaqMan Advanced miRNA cDNA Synthesis kit (Thermo Fisher Scientific). In addition, 50 ng mRNA was used for the synthesis of cDNA from mRNA using the Omniscript RT Kit (QIAGEN, Hilden, Germany). cDNA was stored at −20 ° C. The TaqMan MicroRNA Assay protocol (Thermo Fisher Scientific) or Omniscript RT Kit (QIAGEN) was used for synthesis of cDNA. Real-time PCR of miRNA was performed with the Applied Biosystems 7500 Sequence Detection system using TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific). Real-time PCR of mRNA was performed with Applied Biosystems QuantStudio 3 and SYBR green (Thermo Fisher Scientific). To determine stable reference genes, we used the program BestKeeper. The value of Ct calculated from the expression of miR-25, miR-93, and miR-425 by BestKeeper was used as a reference gene for the evaluation of miRNA expression in total exosomes. The value of Ct calculated from the expression of miR-16 and miR-25 by BestKeeper was used as a reference gene for the evaluation of miRNA expression in NDEs. BestKeeper was also used to calculate the reference Ct from the expression of GAPDH and RNA18S to evaluate the expression of mRNAs in NDEs. After PCR, ΔCt (Ct target gene – Ct reference gene) was compared before and after the intervention. The primers that targeted miRNA and mRNA are listed in Supplementary Tables 1 and Supplementary Table 2.

Animals

We bred 5xFAD mice according to a previous study[2]. In brief, male 5xFAD mice (#034848) purchased from The Jackson Laboratory (Bar Harbor, ME, USA) were crossed with wild-type C57BL/6J females (Sankyo Lab Service Corp., Tokyo, Japan). Then we obtained the needed number of mice for experiments.

Intracerebroventricular injection of miR-29c mimic

At 4 months of age, male 5xFAD mice were implanted with a stainless steel cannula (Eicom, Kyoto, Japan), as described previously[3]. To reach the right ventricle of the brain, the cannula (0.4-mm diameter) was set at 0.4 mm posterior from the bregma, 1 mm right of the midline, and 2.5 mm deep. At 5 months of age, mice were injected with 140 pmol miR-29c mimic (5 μL) or 140 pmol negative control mimic (5 μL) through the cannula with a Hamilton syringe (0.13-mm diameter) at a rate of 1 μL/min. Both the miR-29c mimic and negative control mimic were purchased from Thermo Fisher Scientific, and miRNA complexed with Invivofectamine (Thermo Fisher Scientific) was prepared according to the manufacturer's instructions. After intracerebroventricular injection four times at 1-week intervals, the Y maze test was conducted.

Y maze test

The Y-maze (Muromachi Kikai Co., Ltd., Tokyo, Japan) is a three-arm maze with equal angles between all arms, which were 41.5 cm long and 4 cm wide with walls 10 cm high. 5xFAD mice were initially placed in one arm and allowed to move freely through the maze. The total number of arm entries and alternation behavior were recorded manually for each mouse over an 8-min period. The alternation score (%) for each mouse was calculated as [(total of alternation/total arm entries − 2)] × 100.

miRNA and mRNA isolation from the hippocampus and quantitation

After the Y maze test, excess isoflurane inhalation was carried out to euthanize the mice. After all blood was collected, brains were removed from the skull and divided into the left and right hemispheres with a scalpel. The left hemisphere was immersed in 4% paraformaldehyde for 24 h and used for immunohistochemical analysis. The right hemisphere was frozen immediately in liquid nitrogen and stored at −80 ° C for PCR analysis. The extraction of miRNA and mRNA and the synthesis of cDNA and PCR were performed as described previously[2]. In brief, the extraction of total miRNA and mRNA from the frozen right hippocampus was performed using the mirVana miRNA isolation kit (Thermo Fisher Scientific). The TaqMan MicroRNA Assay protocol (Thermo Fisher Scientific) or Omniscript RT Kit (QIAGEN) was used for the synthesis of cDNA. Real-time PCR of miRNA was performed with the Applied Biosystems 7500 Sequence Detection system using TaqMan Universal PCR Mastermix II no UNG (Thermo Fisher Scientific). Real-time PCR of mRNA was performed with Applied Biosystems QuantStudio 3 using SYBR green (Thermo Fisher Scientific). The relative expression of miRNA and mRNA was calculated using $2^{-\Delta\Delta Ct}$ using snoRNA 135 or GAPDH as an endogenous control, respectively. The primers that targeted miRNA and mRNA are listed in Supplementary Table 1 and Supplementary Table 3.

Immunohistochemical analysis

The left hemispheres were placed into 15% sucrose after immersion in 4% paraformaldehyde. The frozen brains of the left hemispheres were cut sagittally into 20-μm-thick sections and used for immunostaining, as described previously[2]. Three sections including the area of the hippocampus (1.4–2.4 mm lateral from the bregma) were chosen and incubated overnight with anti-Aβ antibody or anti-NeuN antibody at 4 ° C (listed in Supplementary Table 4). Anti-rabbit IgG FITC or Cy3-labeled antibodies were used as secondary antibodies (listed in Supplementary Table 4). DAPI (Dojindo, Kumamoto, Japan) was used for nuclear staining. Confocal laser scanning microscopy (Nikon, Tokyo, Japan) was used to obtain the images of the subiculum area.

The mean of the Aβ-positive area in a 660 × 660 μm field (one field per section × three sections) and the mean number of NeuN-positive cells in a 200 × 200 μm field (two fields per section × three sections) in the subiculum area were analyzed.

References

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