Supplemental information

Methods

MicroRNA lentivector constructs

Lentivector constructs encoding either precursor microRNA-7a (pre-miRNA-7a), anti-microRNA-665 (miRZip665), scrambled control sequences or empty lentivector together with pPACKH1 packaging plasmids were purchased from SBI (Systems Biosciences; SBI, Mountain View, CA). pPACKH1 lentivector plasmid is a mixture of three plasmids; one encoding *Rev* gene, one encoding *Gag* and *Pol* genes, and one encoding *VSV-G* gene and comprised HIV-based third-generation lentiviral expression system.

In brief, the stem-loop pre-miRNA-7a (Accession: MI0000641) with an additional 200-400 base pairs (both upstream and downstream) flanking genomic sequence was cloned into the pCDH-CMV-MCS-EF1α-GreenPuro cDNA expression lentivector (SBI; Cat No: CD513B-1), which drives the expression of pre-miRNA-7a under constitutive CMV promotor. Upon transduction, the pre-miRNA-7a processed into the mature microRNA-7a in the host to upregulate the expression of microRNA-7a-5p. The similar lentivector (i.e. pCDH-CMV-MCS-EF1α-GreenPuro cDNA expression lentivector) without any scrambled sequence was used as a control, which has been widely considered as a reliable lentivector control in previous studies (1, 2).

To knockdown the microRNA-665 expression, the mature anti-microRNA-665 sequence (accession: MIMAT0012844) was cloned (in reverse complimentary manner) into the pGreenPuro (CMV) shRNA miRZip expression lentivector (SBI; Cat No: SI505A-1) under H1 RNA polymerase III promoter. Upon transduction, the expressed single-stranded short hairpin RNA (shRNA) transcript folds into the stem-loop (or hairpin) structure that after processing by the DICER enzyme, preferentially produces an anti-sense microRNA, which is a stably expressed interfering RNA (or anti-microRNA) that binds to the endogenous microRNA-665 to downregulate its expression and antagonize its function. The shRNA template was rationally

designed to be asymmetric, ensuring that the sense strand does not contain the endogenous miRNA sequence and enabling accumulation of the anti-miRNA. The similar lentivector (i.e. pGreenPuro shRNA lentivector) encoding scramble sequence (MZIP000-PA-1) was used as a non-target control, which has been successfully demonstrated as a reliable shRNA lentivector control in various studies (3-5).

Sequences of the precursor (or stem-loop) and mature microRNA of both microRNA-7a and microRNA-665 are provided in supplementary table S1.

Lentiviruses production and titration

Lentivector constructs transformation and propagation: All lentivector DNA constructs were subjected to the bacterial transformation by using Stbl3 E. Coli strain (Invirogen, Cat No: C7373-03) as per manufacturer instructions. Transformed bacteria were plated on LB-ampicillin agar plate overnight at 37^0C. The next day, 1 single bacterial colony was randomly picked and inoculated in LB-ampicillin media to propagate the constructs which was purified from bacterial pellet using HiSpeed Plasmid Midi kit (Qiagen, Cat No: 12643). To check the plasmid integrity, the NEBcutter V2.0 – New England Biolabs online tool was used to find the digestion sites and digested DNA fragments were run on the agarose gel.

Transfection: The lentiviral vectors were packaged into the pseudolentiviral particles by transfecting HEK-293 cells. HEK293 cells were maintained at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 100 units/mL of Penicillin/Streptomycin (Life Technologies). In brief, $4x10^6$ HEK-293 cells were plated in a petri dish and next day cells were transfected with the lentivectors plasmid DNA along with the pPACKH1 packaging plasmids using TransIT-293 transfection reagent (Mirus) according to the manufacturer's instructions. Following 48 and 96 h of transfection, cell culture media containing viral particles were collected, which were centrifuged and then filtered to remove cellular debris. The filtered viral media was concentrated approximately 100-fold by ultracentrifugation at

25,000 rpm for 90 min at 4^0C and the precipitate was resuspended in artificial cerebrospinal fluid (aCSF) and then aliquoted in small volume to store at -80^0C.

Lentivirus titer: Lentiviral vectors were titrated by fluorescence detection of GFP-positive 293 cells in the flow cytometer after 48 h of infection of cells with serial dilutions of virus. The lentiviral titers were in the range of 2×10^8 - 1×10^9 infectious units/ml.

MicroRNA expression by quantitative real-time PCR (qPCR)

A 10 ng of total RNA was reverse-transcribed into the cDNA using a microRNA-specific stem-loop RT primer with TaqMan microRNA reverse transcription kit (Applied Biosystems) in a 15-µl RT reaction at 16^0C for 30 min, 42^0C for 30 min followed by enzyme inactivation at 85^0C for 5 min. The resulting cDNA was amplified by real-time PCR in a 1:15 ratio, using the microRNA-specific PCR primers (forward and reverse) and TaqMan MGB probe with TaqMan universal master mix II (Applied Biosystems) in 20-µl PCR reaction mix. Samples were run in a 7900 HT standard real time PCR detection system (Applied Biosystems) consisting of 40 cycles of 15 sec at 95^0C for denaturation and 60 sec at 60^0C for annealing and extension, preceded by a 10 min enzyme activation step at 95^0C. The expression of each microRNA was defined from the threshold cycle (Ct), and relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method after normalization with reference to the expression of U6 snRNA endogenous control.

Gene expression by quantitative real-time PCR (qPCR)

A 0.5 µg of total RNA was reverse-transcribed to cDNA using high-capacity cDNA reverse transcription kit (ThermoFisher) in a 20-µl RT reaction at 25^0C for 10 min, 37^0C for 120 min followed by enzyme inactivation at 85^0C for 5 min. A 50 ng of cDNA samples was used for determination of expression levels of selected target genes by quantitative real-time PCR using TaqMan fast advanced master mix with TaqMan assays. Samples were run in a 7900 HT standard real time PCR detection system (Applied Biosystems), consisting of 40 cycles of 1 sec at 95^0C for denaturation and 20 sec at 60^0C for annealing and extension, preceded by a 2 min enzyme activation step at 95^0C. The threshold cycle value was taken as the fractional cycle

number at which the emitted fluorescence of the sample passes a fixed threshold above the baseline. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative differences between experimental and control groups as a fold change in gene expression after normalization with reference to the expression of beta-actin endogenous control.

Immunofluorescence staining

Rats were anesthetized with an intraperitoneal injection of ketamine/xylazine (75 mg/kg ketamine with 5 mg/kg xylazine), the heart were exposed, and the rats were intracardially perfused with 0.01 M phosphate-buffered saline (i.e. 1x PBS, pH 7.4) through the left ventricle, followed by 4% paraformaldehyde in 1x PBS for tissue fixation. The brains were then dissected out and immersed in 30% sucrose in 1x PBS for 18–36 h (until tissue sinks) at 4°C to cryoprotect the tissues. Brains were flash frozen on dry ice and stored at −80 °C until use. Sections were permeabilized by incubating in 1x PBS containing 0.3% triton X-100 for 10 min and washed again with 1x PBS 3-times for 5 min. Non-specific binding was blocked by incubating the sections with 10% goat serum for 1 h at room temperature. Following washing in 1x PBS 3-times, free-floating sections were then incubated with mouse monoclonal anti-GFP (1:200; Santa Cruz Biotechnology) and rabbit monoclonal anti-NeuN (1:200; Millipore) or rabbit monoclonal anti-GFAP (1:200; Abcam) in 10% goat serum at 4°C overnight. The following day, the sections were washed thoroughly with 1x PBS 3-times, and incubated with goat anti-mouse Alexa Fluor 488 (1:1000, Life Technologies Corporation) and goat anti-rabbit Alexa Fluor 546 (1:1000, Life Technologies Corporation) in 10% goat serum for 1 h at room temperature. The sections were washed again with 1x PBS 3-times and mounted using Vectashied mounting medium (Vector Laboratories, Inc., CA). The staining was visualized under fluorescence microscope (Olympus Fluoview FV1000) using the Fluoview software (Olympus FV10-ASW, version 4.2b).

Luciferase reporter constructs containing microRNA-7a-5p target site

The 3'-untranslated region (3′-UTR) of *Gabra1* gene containing predicted rno-miR-7a-5p target site was amplified from rat brain VMH genomic DNA. A 25 ul PCR reaction mix was run in a Mastercycler Nexus Thermal Cycler (Eppendorf) consisting of 35 cycles of 15 sec at 95^0C for denaturation and 30 sec at 55^0C for annealing and 15 sec at 68^0C for extension, preceded by a 5 min initial denaturation step at 95^0C. The amplified DNA fragment (~100 bp) was analyzed onto the 1.5% agarose gel using a 100bp ladder, which was extracted in a 20 ul elution volume using Qiaquick gel extraction kit (Qiagen). Following gel extraction, the DNA fragment was digested with XbaI enzyme to create sticky ends and then passed through Microspin G-50 column in phenol-chloroform mix for purification.

Table S1. Precursor (or stem-loop) and mature microRNA sequences of microRNA-7a-5p and microRNA-665

MicroRNAs	Precursor (or stem-loop) sequences	Mature sequences
rno-miR-7a-5p	5'-UGUUGGCCUAGUUCUGUGUGGAAGACUAGUGAUUUUGUUGUUUUUAGAUAACUAAGACGACAACAAAUCACAGUCUGCCAUAUGGCACAGGCCACCU-3' (Accession: MI0000641; Chromosome 17)	5'-UGGAAGACUAGUGAUUUUGUUGU-3' (Accession: MIMAT0000606)
rno-miR-665	5'-GAACAGGGUCUCCUUGAGGGGCCUCUGCCUCUAUCCAGGAUUAUCUUUUUAUGACCAGGAGGCUGAGGUCCCUUACAGGCGGCCUCUUACUCU-3' (Accession: MI0012606; Chromosome 6)	5'-ACCAGGAGGCUGAGGUCCCUUA-3' (Accession: MIMAT0012844)

Figure S1

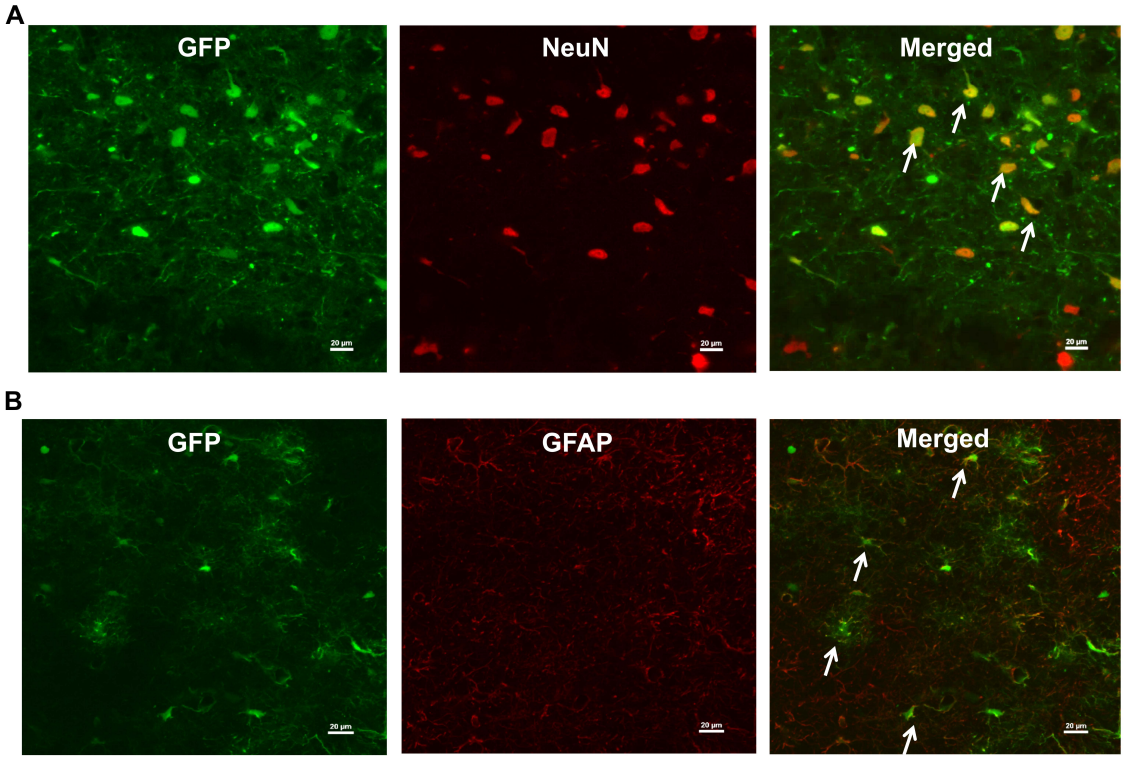

Figure S1. Lentivirus-mediated microRNA-7a-5p overexpression occurs in both neuronal and non-neuronal cells. Immunofluorescent staining images reveal the co-localization of virally-delivered GFP with either **(A)** NeuN (Neuronal marker) or **(B)** GFAP (Astrocytic marker) in miR-7a lentivirus-treated control rat brain sections (green for GFP, red for NeuN or GFAP and yellow/orange for dually-stained cells noted by white arrow, scale bar: 20 μm).

References

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