Supplemental Treatment for Huntington’s Disease with miR-132 that Is Deficient in Huntington’s Disease Brain

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Huntington’s disease (HD) is an intractable neurodegenerative disorder caused by mutant Huntingtin (HTT) proteins that adversely affect various biomolecules and genes. MicroRNAs (miRNAs), which are functional small non-coding RNAs, are also affected by mutant HTT proteins. Here, we show amelioration in motor function and lifespan of HD-model mice, R6/2 mice, by supplying miR-132 to HD brains using a recombinant adeno-associated virus (rAAV) miRNA expression system. miR-132 is an miRNA related to neuronal maturation and function, but the level of miR-132 in the brain of R6/2 mice was significantly lower than that of wild-type mice. Our miR-132 supplemental treatment, i.e., supplying miR-132 to the brain, produced symptomatic improvement or retarded disease progression in R6/2 mice; interestingly, it had little effect on disease-causing mutant HTT mRNA expression and its products. Therefore, the findings suggest that there may be a therapeutic way to treat HD without inhibiting and/or repairing disease-causing HTT genes and gene products. Although miR-132 supplement may not be a definitive treatment for HD, it may become a therapeutic method for relieving HD symptoms and delaying HD progression.

INTRODUCTION
Huntington’s disease (HD; OMIM #143100) is a dominantly inherited neurodegenerative disorder characterized by chorea movement, muscular incoordination, cognitive decline, and psychiatric problems. The responsible gene for HD is the Huntingtin (HTT) gene on chromosome 4p16.3, and aberrantly expanded CAG repeats (translated into polyglutamine tracts) in exon 1 are closely related to the onset and severity of HD. Such abnormal expansion of the CAG repeat results in unusual aggregation of the HTT protein, which is harmful to neurons in the brain. Currently, there is no definitive treatment for HD, and many efforts have been made to develop better treatments for HD.

MicroRNAs (miRNAs) are 21- to 23-nt-long small noncoding RNAs, which are processed from longer transcripts (primary miRNAs) forming a hairpin structure by digestion with a microprocessor complex containing Drosha and Dicer in the nucleus and Dicer in the cytoplasm. After processing, miRNAs are incorporated into the RNA-induced silencing complex (RISC) and function as mediators in gene silencing, which targets miRNAs partially or nearly complementary to the miRNAs. miRNAs play important roles in gene regulation through the gene silencing.

Thousands of miRNA genes have been found in animals and plants (see the microRNA database [miRBase]: http://www.mirbase.org/index.shtml). Expression profiles of miRNAs have been examined, and tissue- and stage-specific expression of miRNAs and disease-associated expression of miRNAs have been detected. Thus, miRNAs may become useful biomarkers and may provide us with clues to help better disease treatment. Regarding miRNA expression in the brain, a major change in the expression of miRNAs occurs during the first month of mouse life, which corresponds to the stage of rapid brain development. Such controlled expression of miRNAs presumably contributes to normal brain formation and maturation, and abnormal expression of miRNAs due to diseases may cause harmful effects on brain formation.

Disease-causing mutant HTT proteins adversely affect biomolecules such as proteins, mRNAs, and miRNAs in the HD brain. In this study, we examined miRNA expression in the brain of R6/2 (HD-model) mice and wild-type mice, and found that miR-132 was markedly decreased in HD mice relative to wild-type mice. Previous studies

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Thus, there may be some association between miR-132 levels and HD. We investigated miRNA expression in the striatum, a major HD focus, using an AAV miRNA expression system in this study, and we showed symptomatic improvement in motor function and lifespan of treated HD mice without suppressing disease-causing mutant HTTs.

**RESULTS**

**miR-132 Deficiency in the Brain of HD Mice**

We investigated miRNA expression in the striatum, a major HD focus, of R6/2 (HD-model) and wild-type mice by means of DNA chip technology, and found that miR-132 and its adjacent, analogous miR-21213,16 were markedly decreased in HD mice relative to wild-type mice (Table 1); note that miR-212 was considerably less than miR-132 (Figure S1). The results were confirmed by northern blotting as well (Figure 1A), and the findings were consistent with previous studies.17,18 Another HD model of YAC128 mice expressing full-length mutant HTT mRNAs showed a decrease in miR-132 in the brain,17 and more noteworthy, Johnson et al.19 indicated that miR-132 levels were decreased in post-mortem brains of HD patients. Thus, there may be some association between miR-132 and HD.

The expression of miR-132 was examined in detail by means of qRT-PCR, because a major change in miRNA expression occurs in the mouse brain during the first month after birth.12 The resultant expression profiles exhibited that the post-natal miR-132 of HD mice was expressed in the same manner as wild-type mice, but soon reached a plateau at lower levels than wild-type mice (Figure 1B). Consequently, a significant decrease in miR-132 levels occurred in HD mouse brains.

In addition to the findings, we investigated miR-132 levels in various tissues of HD and wild-type mice (Figure S2), and in human HD lymphoblastoid cell lines (Figure S3). Although the miR-132 expression levels of the examined tissues were significantly lower than that of the brain, the decreasing trend of the miR-132 level in HD as compared with normal controls appeared to be kept in the tissues examined except for the liver and kidney.

**Decrease in miR-132 Associated with Ago2**

To see whether miR-132 deficiency affected miR-132-involved gene silencing, we investigated miR-132 associated with Argonaute 2 (Ago2), which is an essential protein having endonuclease activity according to miRNA in the RISC.20 First we examined striatal Ago2 in HD and wild-type mice by western blotting. As a result, Ago2 had little difference between the two mice (Figure 2A), suggesting that there might be no difference in the ability of Ago2 itself between HD and wild-type mice. We then investigated miR-132 associated with Ago2. Immunoprecipitation with anti-Ago2 antibodies followed by qRT-PCR to detect miRNAs in immunoprecipitates was carried out (Figures 2B–2D). The resultant levels of the examined tissues were signiﬁcantly lower than that of the brain, the decreasing trend of the miR-132 level in HD as compared with normal controls appeared to be kept in the tissues examined except for the liver and kidney.

**Expression of the Target Genes of miR-132**

We examined the following miR-132 target genes: Methyl-CpG binding protein 2 (MeCP2),19 Rho GTPase activating protein 32 (p250GAP),20–22 Polypyrimidine tract binding protein 2 (Ptbp2),23 and Regulatory factor X4 (Rfx4).24 The protein levels of striatal MeCP2, p250GAP, Ptbp2, and Rfx4 were examined by western blotting. Compared with wild-type mice, MeCP2 and Ptbp2 of HD mice increased, whereas p250GAP decreased, and Rfx4 appeared to slightly decrease in HD mice (Figure 3A). The mRNA levels of Ptbp2 and Rfx4 showed little difference between HD and wild-type mice, whereas MeCP2 slightly increased in HD mice, and p250GAP decreased (Figure 3B). Based on the principle of miRNA-mediated gene silencing, a decrease in miRNAs should result in an increase in their target gene products. Accordingly, MeCP2 and Ptbp2 might be candidate genes that are affected by the shortage of miR-132 in the brain. The protein

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**Table 1. Difference in the Expression of miRNAs between R6/2 and Wild-Type Mice**

| Name*             | Signal Intensityb | R6/2 | WT  | Fold Changes |
|-------------------|-------------------|------|-----|--------------|
| mmu-miR-132-3p    | 406.7             | 1238.9 | 0.33 |
| mmu-miR-342-3p    | 132.2             | 365.6 | 0.36 |
| mmu-miR-212-5p    | 64.0              | 167.8 | 0.38 |
| mmu-miR-132-5p    | 259.5             | 643.6 | 0.40 |
| mmu-miR-766-3p    | 4.6               | 10.0  | 0.46 |
| mmu-miR-212-3p    | 175.0             | 374.3 | 0.47 |
| mmu-miR-26a-1-3p  | 3.7               | 6.6   | 0.56 |
| mmu-miR-7213-3p   | 7.6               | 13.5  | 0.56 |
| mmu-miR-299a-5p   | 48.5              | 84.9  | 0.57 |
| mmu-miR-128-3p    | 4,682.7           | 8,038.4 | 0.58 |
| mmu-miR-182-3p    | 7.6               | 12.8  | 0.60 |
| mmu-miR-34a-5p    | 278.0             | 464.7 | 0.60 |
| mmu-miR-184-3p    | 12.2              | 19.7  | 0.62 |
| mmu-miR-694-3p    | 5.3               | 8.7   | 0.62 |
| mmu-miR-465b-5p   | 5.4               | 8.5   | 0.63 |
| mmu-miR-153-3p    | 1,119.1           | 1,752.8 | 0.64 |
| mmu-miR-144-5p    | 4.0               | 6.1   | 0.65 |
| mmu-miR-205-5p    | 6.5               | 10.0  | 0.65 |
| mmu-miR-187-3p    | 318.3             | 487.9 | 0.65 |
| mmu-miR-708-5p    | 245.1             | 369.8 | 0.66 |

*10-week-old same littermate mice (male) were examined. WT, wild-type.
*The top 20 miRNAs that markedly decreased in R6/2 mouse are indicated.
*Hybridization signal intensities obtained from DNA chip analysis are indicated.
level of p250GAP appears to directly reflect its mRNA level, and Rfx4 may be similar as well.

miR-132 Supplement into the Brain of HD Mice

Because previous studies suggested that miR-132 made an important contribution to neuronal function and maturation,14,15,21,25 we conducted therapeutic trials to see what would happen to HD mice if miR-132 were supplied to the HD brains. To compensate for the shortage of miR-132 in HD brains, we constructed a viral miR-132-expression system using recombinant adeno-associated viruses (rAAVs). The constructed miR-132-expression viruses (AAV9_miR-132) and negative-control viruses (AAV9_miR-Neg) were introduced into the striatum of 3-week-old HD and wild-type mice (Figure 4A); that age was when the marked difference in miR-132 levels began to appear between the mice (Figure 1B). After virus inoculation, miR-132 levels were examined and confirmed to return to normal levels in the striatum of AAV9_miR-132-treated HD mice (Figure 4B). Consistently, the level of miR-132 associated with Ago2 markedly increased in AAV9_miR-132-treated HD and wild-type mice (Figure 4C). An increase in miR-132 was also detected in the cerebral cortex and the midbrain of the AAV9_miR-132-treated HD mice. This was probably due to the diffusion of AAV9_miR-132 viruses (Figure S4).

MeCP2 and Ptbp2, which are candidates of miR-132 targets (Figure 4D), were examined. As a result, the MeCP2 protein was reduced in AAV9_miR-132-treated mice without a decrease in MeCP2 mRNA levels (Figure S5A), compared with AAV9_miR-Neg-treated mice. Note that the decrease in MeCP2 was seen in both AAV9_miR-132-treated wild-type and HD mice. In contrast, the Ptbp2 gene products appeared to remain unchanged between AAV9_miR-132-treated and AAV9_miR-Neg-treated mice (Figure 4D; Figure S5B). Thus, the findings suggest that MeCP2 may be a target gene of miR-132 in the brain.
Symptomatic Improvement of Motor Function and Lifespan in AAV9_miR-132-Treated HD Mice

Because R6/2 mice are well known as a fulminant HD-model animal suffering from severe motor deficits and rapidly declining locomotor activity,26,27 we investigated the behavior and longevity of AAV9_miR-132-treated HD mice and also AAV9_miR-Neg-treated HD mice (as controls). Interestingly enough, miR-132 supplementation produced symptomatic improvement, i.e., rotarod and open-field test showed a definite amelioration in AAV9_miR-132-treated HD mice (Figure 5A; Figure S6). Moreover, AAV9_miR-132-treated HD mice showed a significant life prolongation relative to AAV9_miR-Neg-treated HD mice (Figure 5B) and also exhibited a slight increase in body weight (Figure S6D). Therefore, the findings strongly suggested that miR-132 recovery was effective in improving motor function of HD mice and prolonging their life, or effective in slow disease progression in HD mice.

HD mice showed a significant life prolongation relative to AAV9_miR-Neg-treated HD mice (Figure 5B) and also exhibited a slight increase in body weight (Figure S6D). Therefore, the findings strongly suggested that miR-132 recovery was effective in improving motor function of HD mice and prolonging their life, or effective in slow disease progression in HD mice.

Regarding AAV9_miR-132-treated wild-type mice, miR-132 exceeding normal levels was expressed in the brain (Figures 4B and 4C), but there was little or no difference in behavior and longevity between AAV9_miR-132-treated and AAV9_miR-Neg-treated wild-type mice (Figure 5). In addition, no significant change had been observed afterward. Thus, it is possible that a deficiency of miR-132 may have a major impact on the life of the mouse, but a surplus of miR-132 may not have.

Figure 2. miRNAs Associated with Ago2
(A) Striatal Ago2. Striatal tissues were isolated from R6/2 (HD) and wild-type (WT) mice aged 8 weeks and examined by western blotting using anti-Ago2 antibodies and Gapdh antibodies as an internal control. Numbers (#1–3) in WT and HD represent individual mice examined. (B) Western blot analysis of immunoprecipitates. Immunoprecipitates by indicated antibodies (IP) from whole brain extracts prepared from 4-month-old wild-type mice were examined by western blotting using anti-Ago2 antibodies (IB). Whole brain extracts as a source of immunoprecipitation (Input) were also examined. (C) miRNA expression in the striatum of 12-week-old HD and WT mice. Indicated miRNAs were examined by qRT-PCR as in Figure 1B. The levels of the miRNAs were normalized to the level of U6 snoRNA as a control and further normalized by the levels obtained in WT mice as 1. Data are shown as mean ± SEM (n = 3 measurements). (D) miRNAs associated with Ago2. Immunoprecipitation with anti-Ago2 antibodies from the same striatal samples as in (C) was performed, and miRNAs coimmunoprecipitated with the antibodies were examined by qRT-PCR. The levels of the miRNAs were normalized to the miR-125b levels; this is because U6 snoRNA hardly associated with Ago2 and because the miR-125b levels were similar between HD and WT mice (as shown in C). The normalized miRNA levels were further normalized by the level obtained in WT mice as 1. Data are shown as mean ± SEM (n = 3 measurements). IgG, a control IgG.

Figure 3. Expression of miR-132 Target Gene Products
The proteins (A) and miRNA levels (B) of miR-132 target genes in the striatum of R6/2 (HD) and wild-type (WT) mice at the age of 8 weeks were examined by western blotting and qRT-PCR, respectively. In western blotting, numbers (#1–3) in WT and HD represent individual mice examined. Gapdh was examined as an internal control. In miRNA analysis, the miRNA levels of the target genes were normalized to the level of Gapdh as an internal control and further normalized to the level obtained from WT mice as 1 (blue bars). Data are shown as mean ± SEM (n = 3 individual mice, **p < 0.01 by Student’s t test).
From the findings, we raised the working hypothesis that miR-132 supplement might suppress the expression of disease-causing mutant HTTs and be capable of eliminating harmful mutant HTTs from the HD brain. To address the hypothesis, we examined mutant HTT expression by RT-semiquantitative PCR. As a result, the mutant HTT level hardly changed between AAV9_mir-132-treated and AAV9_mir-Neg-treated HD mice (Figure 6). We further performed an immunohistochemical analysis for detection of mutant HTT inclusion bodies that are characteristic of HD. The results indicated that there was no significant difference in formation of inclusion bodies between AAV9_mir-132-treated and AAV9_mir-Neg-treated HD mice (Figure 7A). Western blot analysis of the striatum
also exhibited a compatible result: there was little difference in mutant HTT between AAV9_miR-132-treated and AAV9_miR-Neg-treated HD mice (Figure S7). In addition, consistent results were obtained from in vitro experiments as well using primary neurons co-expressed with exogenous mutant HTT and miR-132 (Figure 7B). Consequently, these unexpected findings suggested that miR-132 supplementation had little effect on the expression of mutant HTTs and their inclusion body formation in HD mice supplemented with miR-132.

**DISCUSSION**

**Supplemental miRNA Therapy**

Inhibition of disease-causing genes or elimination of mutant gene products is a straightforward and ideal approach for the treatment of intractable diseases caused by dominant-negative disease-causing genes such as mutant HTT genes in HD. Specific inhibition of disease-causing genes carrying nucleotide variations may be feasible by specific silencing such as disease-causing allele-specific RNAi; however, RNAi may be difficult to eliminate disease-causing gene products thoroughly from patients’ cells. For fighting against intractable diseases, it is necessary to develop other treatment approaches based on mechanisms of action different from conventional approaches, even if such approaches had the only effect of symptomatic relief. Our current study offers the suggestion that miRNAs may open up a new way of treatment for HD, i.e., miRNA supplemental therapy.

In the current study, we focused on miR-132 that markedly decreased in the brain of HD mice (Table 1; Figure 1). Our therapeutic trial using miR-132-expression AAVs showed that miR-132 supplement for reducing the deficiency of miR-132 in the HD brains conferred amelioration in motor function and lifespan of HD mice (Figure 5). In addition, it is noteworthy that miR-132 supplement hardly affected disease-causing HTT genes and their gene products (Figures 6 and 7; Figure S7); i.e., such a symptomatic improvement was gained without suppressing and/or eliminating harmful mutant HTTs. The findings provided us with a new insight into the treatment strategy for HD; that is, there may be a therapeutic approach to HD without inhibiting disease-causing mutant HTTs.

On a relevant note, Benraiss et al. recently demonstrated that a striatal transplantation of normal human glia into the brain of R6/2 mice resulted in amelioration in the HD phenotype without influencing mutant HTTs, and another recent study in which 589,306 human genomes were analyzed indicated that there were healthy individuals possessing highly penetrant, deleterious disease-causing alleles. These findings surprised us and suggested that there may be a mechanism(s) by which deleterious effects caused by harmful mutations would be alleviated without wiping out the mutations. Therefore, these studies and also our study suggested that there might be a therapeutic way to treat intractable diseases without inhibiting and/or repairing disease-causing genes and gene products.
This new strategy may further expand the versatility of the treatment approach to other diseases. Downregulation of miR-132/miR-212 was also detected in the brain of patients with Alzheimer’s disease\textsuperscript{35–37} and schizophrenia,\textsuperscript{38} other than HD.\textsuperscript{18} Therefore, miR-132 supplementation might be available and effective for the cure of such diseases in which miR-132 is deficient, whatever the causative disease genes.

In conclusion, although miR-132 supplementation may not be a definitive therapy for HD, it may become an adjunctive therapy for HD, which may help to delay the onset of disease and the progression of disease.

The current study has left a challenge; i.e., the mechanism of improvement by supplying miR-132 to the HD brain remains unknown. The AAV9_miR-132 virus appears to supply enough miR-132 to compensate for miR-132 deficiency in the HD brain (Figure 4), and the supplied miR-132 can associate with Ago2 (Figure 4C) and probably function properly. As a result, symptomatic improvement would be brought to HD mice (Figure 5). miR-132-mediated gene silencing would recover after AAV9_miR-132 virus inoculation and MeCP2 as a target of miR-132 appeared to undergo translation inhibition. McFarland et al.\textsuperscript{19} indicated that the association of Htt with MeCP2 increased in knock-in Hdh mice (another HD model), suggesting that abnormal interaction between Htt and MeCP2 may cause transcriptional dysregulation. Based on the previous study, an increase in MeCP2 in R6/2 mice might promote abnormal interaction between mutant HTT and MeCP2; thus, the decrease in MeCP2 by miR-132 supplement might reduce the aberrant interaction. As for miR-132 targets examined other than MeCP2, curiously, the relationship with miR-132 appeared to be inconsistent with the mechanism of miRNA-mediated gene silencing (Figure 3). In this regard, mutant HTTs might have some effect on certain miR-132 target mRNAs in HD mice. In any case, there is little information on the mechanism of action for amelioration of HD symptoms when miR-132 is supplied to miR-132-deficient HD mice. As a clue to the solution, authentic target genes, like MeCP2, that respond to miR-132 in HD mice may be the key molecules to understand the mechanism of action. Because miR-132 is involved in various elements related to neurons such as neuronal structure and function, it is possible that there are miR-132 target genes that have not been found yet. Among such target genes, there may be major targets contributing to amelioration in HD mice under miR-132 supplementation. To elucidate the mechanism of action for ameliorating symptoms of HD by miR-132 supplement, more extensive studies including the identification of major target genes are needed and remain to be carried out.

MATERIALS AND METHODS

Animals

R6/2 mice, which carry a mutant human HTT exon1 containing abnormally expanded CAG repeats,\textsuperscript{26} were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and maintained as described...
Identification of the mutant mice was performed by genotyping using PCR as described previously. Our sequence analysis of the PCR products indicated that the mutant mice maintained approximately 124 CAG repeats in the HTT exon1. Mice were housed, fed, and maintained in the laboratory animal facility according to the National Institute of Neuroscience animal care guidelines. All the animal experiments were performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Neuroscience. The protocols were approved by the Committee on the Ethics of Animal Experiments of the National Institutes of Neuroscience (Permit Number: 2015003).

DNA Oligonucleotides
DNA oligonucleotides used in this study were synthesized by and purchased from Sigma-Aldrich (St. Louis, MO, USA).

miRNA Expression Profile Analysis
Total RNAs were extracted from the striatum of 10-week-old R6/2 mouse (male) and littermate wild-type mouse (male). RNAs were subjected to a comprehensive miRNA expression analysis using 3D-Gene Mouse miRNA (Ver.21) chips according to the protocols supplied by TORAY Industries (Tokyo, Japan). Hybridization signals were examined by a 3D-Gene Scanner 3000 followed by quantifying with Extraction according to the manufacturer’s instructions (TORAY). Obtained miRNA expression profile data were deposited to the GEO in NCBI.

Northern Blot
Small RNA fractions were extracted from brain subregions by a mirVana miRNA Isolation Kit (Thermo Fisher Scientific) according to the manufacturer’s instructions. Aliquots of small RNA (≈0.48 μg each sample) were electrophoretically separated in 8 M urea denatured (15%) polyacrylamide gels and transferred onto positively charged nylon membranes. The membranes were hybridized with 5’-DIG-labeled miRCURY LNA miRNA detection probes (EXIQON, Velbaek, Denmark) at 65°C for 15 hr. After washing the membranes, the hybridized probes were detected by an alkaline phosphatase-conjugated anti-DIG antibody (Roche, Basel, Switzerland) and a chemiluminescent detection system.

RT-PCR
Total RNAs were extracted from brain subregions and cultured cells using a TRI REAGENT (Sigma-Aldrich) according to the manufacturer’s instructions and subjected to qRT-PCR. Real-time PCR was performed using an AB7300 Real Time PCR System (Thermo Fisher Scientific) with a FastStart Universal SYBR Green Master (Roche). PCR primers (TAKARA BIO) used are as follows (primer set IDs are indicated in parentheses): MeCP2 (MA079473), p250GAP (Arghgap32) (MA170683), Ptpb2 (MA168870), Rfx4 (MA110752), and Gapdh (MA050371).

Immunoprecipitation
Whole brain or striatal tissue was homogenized in 1 mL of ice-cold lysis buffer (10 mM HEPES [pH 7.4], 200 mM NaCl, 30 mM EDTA, 0.5% Triton X-100, 0.4 μM RNasin Plus RNase inhibitor [Promega], 1× Protease/Phosphatase inhibitor Cocktail [CST, Danvers, MA, USA]). Lysate was centrifuged at 3,000 × g for 10 min at 4°C, and the supernatant was transferred into a new 1.5-mL tube. The NaCl concentration in the supernatant was adjusted to 400 mM with 5 M NaCl, then a part of the supernatant was taken and stored as a crude lysate sample. The rest was centrifuged at 70,000 × g for 20 min at 4°C, and the supernatant was mixed with yeast tRNAs (100 μg/mL final concentration) (Sigma-Aldrich) and pretreated with 40 μL of protein A-Sepharose 4B slurry (Sigma-Aldrich) for 30 min at 4°C. After centrifugation at 12,000 × g for 2 min at 4°C, a part of the supernatant was taken and stored as an immunoprecipitation (IP) input sample, and the remaining supernatant was divided equally. The divided samples were added with anti-AGO2 IgG (2897S; CST) and a control IgG (3900S; CST), respectively, and gently mixed using a rotator overnight at 4°C. Protein A-Sepharose 4B slurry (20 μL each) was added to the treated samples and incubated further for 1 hr at 4°C with gently mixing. After incubation, the protein A-Sepharose beads were collected by centrifugation at 12,000 × g for 2 min at 4°C, washed four times with 0.4 mL of wash buffer 1 (the lysis buffer containing 0.04 U/μL RNasin Plus RNase inhibitor, 10 μg/mL yeast tRNAs, and 0.1× Protease/Phosphatase inhibitor Cocktail [1:1,000; CST]), and finally washed with 0.5 mL of wash buffer 2 (the wash buffer 1 without yeast tRNAs and RNasin Plus RNase inhibitor). RNAs co-precipitated with the protein A-Sepharose beads were extracted with TRI REAGENT according to the manufacturer’s instructions (Sigma-Aldrich). For protein extraction, the beads were treated with 2× sample buffer (125 mM Tris-HCl [pH 6.8], 2% glycerol, 4% SDS, 0.02% bromophenol blue, and 10% beta-mercaptoethanol) and boiled for 5 min followed by centrifugation at 12,000 × g for 2 min at 4°C. The
were packed into recombinant adeno-associated viruses based on BIO) according to the manufacturer injected to ligation with pW-CAG-EGFP-WPRE vector digested with the vector are indicated in Table S1. The constructed plasmid, named pMiR-Neg in this study. The pMiR-Neg plasmid encodes a short hairpin RNA just as a regular pre-miRNA, but the hairpin RNA is predicted not to target any known vertebrate gene.

For construction of expression viruses, the GFP reporter genes containing mir-132 and miR-neg in pMiR-132 and pMiR-Neg, respectively, were amplified by PCR using a PrimeSTAR HS DNA Polymerase with GC buffer (TAKARA BIO) and PCR primers. The sequences of the PCR primers used are indicated in Table S1. The PCR products were purified by a PCR & Gel purification kit (BEX, Itabashi-ku, Tokyo, Japan) according to the manufacturer’s instructions and subjected to ligation with pW-CAG-EGFP-WPRE vector digested with EcoRI and BamHI, using an In-Fusion HD Cloning Kit (TAKARA BIO) according to the manufacturer’s instructions. The resultant pW-CAG-EGFP-WPRE vectors containing mir-132 or miR-Neg were packed into recombinant adeno-associated viruses based on serotype 9 (rAAV-9) as described previously. The rAAV-9 viruses encoding mir-132 and miR-Neg, named AAV9_mir-132 and AAV9_miR-Neg, respectively, were used in this study.

**HTT Expression Plasmids**

The pEGFP-Q22 and pEGFP-Q145 plasmids that encode normal (22 CAG repeats) and abnormal (145 CAG repeats) HTT exon1 sequences, respectively, were used. In the plasmids, the HTT exon1 is fused with the EGFP reporter gene. We further constructed pDsRed-Q-Wt and pDsRed-Q-Mt plasmids, which encoded the normal and abnormal HTT exon1, respectively. In the plasmids, the HTT exon1 is fused with the DsRed-monomer gene derived from pDsRed-monomer vector (TAKARA BIO).

**Primary Cortical Neuron and Astrocyte Culture**

Mouse primary neuronal cells were prepared and cultured. In brief, mouse embryonic day 17 (E17) embryonic cerebral tissue (ICR mouse strain) was isolated, treated with 0.5% trypsin-EDTA solution containing 0.1 mg/mL DNase I (Roche) and 5 mg/mL glucose at 37°C for 20 min, dissociated by pipetting several times, and passed through a 70-μm nylon filter (DB, Franklin Lakes, NJ, USA). The dissociated neuronal cells were seeded on poly-L-lysine (Sigma-Aldrich)-coated culture plates (a cell density of $4 \times 10^3$ cells/mm$^2$) and cultured at 37°C in Neurobasal medium (Thermo Fisher Scientific) supplemented with 1% FBS (Life Technologies), 2% B27 supplement (Thermo Fisher Scientific), 1 mM glutamine (Sigma-Aldrich), and 10 μg/mL streptomycin and 50 U/mL penicillin; Wako, Chuo-ku, Osaka, Japan). Cells were grown at 37°C in a 5% CO₂ humidified chamber, and sequential passage of the cells was performed.

**Isolation of Lymphocytes from Mice Blood**

Whole blood of mice was collected by cardiac puncture using a 26G-needle syringe containing 0.1 mL of 10 mg/mL EDTA. The blood samples were transferred into 15-mL tubes and mixed with 10 mL of RBC lysis buffer (10 mM NH₄HCO₃, 144 mM NH₄Cl). Lymphocytes were collected by centrifugation at 1,600 × g for 10 min. Cells were washed four times with RBC lysis buffer and subjected to RNA extraction using a TRI REAGENT (Sigma-Aldrich).

**Lymphoblastoid Cell Culture**

Epstein-Barr virus-transformed human lymphoblastoid cell lines were cultured at 37°C in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% FBS (Japan Bio Serum, Fukuyma, Hiroshima, Japan), 110 mg/L sodium pyruvate (Wako), 4,500 mg/L D-glucose (Wako), 100 U/mL penicillin, and 100 μg/mL streptomycin (Thermo Fisher Scientific) in 5% CO₂ humidified chamber.

**Intracranial Injection of rAAV Vectors**

R6/2 and wild-type mice at post-natal day ~21 were anesthetized with somnopentyl (50 mg/kg body weight [b.w.]), and 2 μL of AAV9_mir-132 or AAV9_miR-Neg was stereotaxically injected into both sides of the striatum (~1 mm anterior to bregma, ~2 mm lateral to the midline, ~3 mm ventral to the skull surface) using a Hamilton Neuros Syringe (HAMILTON, Reno, NV, USA).

**Transfection**

Transfection of plasmid DNAs into primary cultured cells was carried out by a Lipofectamine 2000 Reagent (Thermo Fisher Scientific) according to the manufacturer’s instructions. Before transfection, culture medium was replaced with serum-free culture medium (Neurobasal medium supplemented with 2% B27 supplement, 1 mM glutamine, and 10 μg/mL streptomycin) and incubated for 2 hr at 37°C in a 5% CO₂ humidified chamber. After incubation, plasmid DNA-Lipofectamine 2000 mixtures were added into the cells. Four hours after transfection, medium was replaced with fresh, complete culture medium containing FBS.

**Rotarod Test**

Rotarod test was carried out to see the motor coordination of mice, using an Ugo Basile Rota-Rod 47600 (Ugo Basile, Comerio, Italy). The apparatus was programmed to accelerate its rod rotation from 4 to 40 rpm during 300 s. Time from when mouse was put onto the
rod of the apparatus until when the mouse fell off or cling on to the rod was recorded. Mice were subjected to three trials per day between 2:00 and 7:00 p.m., and the test was performed for the third straight day.

Open Field Locomotor Activity and Rearing Frequency

Spontaneous locomotor activity and rearing frequency of mice were examined. Mice were placed in a monitoring box (40 cm × 28 cm × 31 cm) (SUPERMEX; Muromachi Kikai, Tokyo, Japan) equipped with an activity sensor (Pyroelectric sensor PYS-001; Muromachi Kikai) and a rearing sensor (MRS-110TX-RX; Muromachi Kikai) at the top and wall of the box, respectively. Locomotor activity and rearing frequency of a mouse in the box were simultaneously measured for 15 min, and the test was performed between 2:00 and 7:00 p.m. The obtained data were analyzed by a Data Collection Program CompACT AMS Ver.3 (Muromachi Kikai).

SDS-PAGE and Western Blotting

Cultured cells and brain samples were lysed in lysis buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EGTA, 1% Triton X-100) containing 1× protease inhibitor cocktail (Protease Inhibitor Cocktail Tablets; Roche). The lysate was homogenized with a pestle and centrifuged at 3,000 × g for 10 min at 4°C and then further centrifuged at 14,000 × g for 15 min at 4°C. The resultant supernatant was collected, and protein concentration was measured by a Protein Quantification kit (DOJINDO, Mashiki-town, Kumamoto, Japan) according to the manufacturer’s instructions. Equal amounts of protein (=40 μg) were mixed with 4× sample buffer (0.25 M Tris-HCl, 40% glycerol, 8% SDS, 0.04% bromophenol blue, 8% beta-mercaptoethanol), boiled for 5 min, and separated by SDS-PAGE with 10% polyacrylamide gels. After separation, proteins were electrophotorethetically blotted onto polyvinylidene fluoride membranes (Immobilon P; Millipore, Billerica, MA, USA). The membranes were blocked for 1 hr in blocking solution (5% BSA [Sigma-Aldrich] in TBS-T buffer [Tris-buffered saline (TBS) containing 0.1% Tween 20]) and incubated with diluted primary antibodies (indicated below) in PBS containing 0.1% Tween 20 and 5% goat serum in PBS for 1 hr at room temperature and incubated in blocking solution (5% BSA [Sigma-Aldrich] in 5% goat serum [Cell Signaling Technology, Danvers, MA, USA]) for 1 hr at room temperature. The treated cells were incubated with diluted primary antibodies (indicated below) in PBS at 4°C overnight. After washing with PBS, antigen-antibody complexes were visualized by isotype-specific secondary antibodies conjugated with Alexa 488 or Alexa 594 (Molecular Probes, CA, USA). In addition, nuclear staining was also carried out with 2 μg/mL Hoechst33342 (Cell Signaling Technology) or 2 μg/mL propidium iodide (Thermo Fisher Scientific) in PBS. Stained cells were examined using a ZEISS fluorescent microscope (Axiovert 40 CFL).

For immunohistochemical analysis, dissected brain tissues were fixed with 4% PFA in PBS at 4°C overnight, incubated in 20% sucrose in PBS at 4°C overnight, embedded in O.C.T. compound (Sakura Fine-tech Japan, Koto-ku, Tokyo, Japan) on a dry ice/ethanol bath, and then cut into 15-μm-thick sections. Cryosections were treated in blocking/permeabilization buffer (0.3% Triton X-100 and 5% goat serum in PBS) for 30 min at room temperature and incubated with diluted primary antibodies (indicated below) in PBS containing 0.1% Triton X-100 and 5% goat serum at 4°C overnight. After washing with PBS, the sections were incubated with isotype-specific secondary antibodies conjugated with Alexa 488 or Alexa 594 (Molecular Probes), and examined using a Leica confocal fluorescent microscope (Leica TCS SP2). Nuclei were also stained with Hoechst33342 and examined. The primary antibodies used, as well as their dilution ratios including IDs and manufactures in parentheses, are as follows: anti-AGO2 (1/200, MAB5374; Millipore) and anti-GFP (1/500, A11122; Thermo Fisher Scientific).

Statistical Analysis

Data obtained in this study were initially evaluated by one-way ANOVA. If significant difference between data was detected by ANOVA, Tukey’s post hoc test was carried out between the data of interest. The level of statistical significance was set at 0.05.

ACCESSION NUMBERS

The accession number of the miRNA expression data used in this study is GEO: GSE100792.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and one table and can be found with this article online at https://doi.org/10.1016/j.omtn.2018.01.007.

AUTHOR CONTRIBUTIONS

H.H., M.F., and M.T. designed research; M.F., M.T., H. Fujita, T.C., H.A.P., S.W., and H.H. performed experiments; H. Furuva, M.M., K.W., T.O., Y.N., and H.H. contributed new reagents/analytic tools;
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