RNA interference improves motor and neuropathological abnormalities in a Huntington’s disease mouse model


Materials and Methods

Plasmids and Adenoassociated Virus (AAV) Construction. Myc-tagged HD-N171-82Q was expressed from a pCMV-HD-N171-82Q plasmid (20). PCR (Pfu DNA polymerase, Stratagene) was used to amplify the U6 promoter along with shRNAs targeting human htt (shHD2.1; Fig. 1A), eGFP (shGFP) (22), or Escherichia coli β-galactosidase (base pairs 1152–1172; shLacZ). PCR products were cloned, sequenced, and inserted into pAAV.CMV. hrGFP, which contains AAV serotype 2 inverted terminal repeats, a CMV-humanized Renilla GFP (hrGFP)-simian virus 40 poly(A) reporter cassette, and sequences used for homologous recombination into baculovirus (23). Recombinant AAV serotype 1 vectors were generated as described in ref. 23. AAV titers were determined by using quantitative PCR and/or DNA slot blot analysis and were 5 x 10¹² vector genomes per ml.

Animals. All animal studies were approved by the University of Iowa Animal Care and Use Committee. HD-N171-82Q mice were purchased from The Jackson Laboratory (20, 24) and maintained on a B6C3F1/J background. Hemizygous and age-matched WT littermates were used for the experiments, as indicated.

Northern Blot Analysis. HEK293 cells were transfected (Lipofectamine 2000, Invitrogen) with pCMV-HD-N171-82Q and plasmids expressing shHD2.1, shGFP, or shLacZ at shRNA-to-target ratios of 8:1. Forty-eight hours after transfection, RNA was harvested by using TRIzol reagent (Invitrogen), and 10 µg was assessed by Northern blot analysis (NorthernMax, Ambion) using probes to human htt or human GAPDH. Band intensities were quantified by using a Storm 860 phosphorimager and IMAGEQUANT 1.2 software, both from Molecular Dynamics.

Abbreviations: HD, Huntington’s disease; htt, huntingtin; RNAI, RNA interference; shRNA, short hairpin RNA; AAV, adenoassociated virus; hrGFP, humanized Renilla GFP.

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For in vivo studies, total RNA was isolated from hrGFP-positive striata. Thirty micrograms of RNA was run on 15% polyacrylamide/urea gels, transferred to Hybond-N+ membranes (Amersham Pharmacia), and then probed with 32P-labeled sense oligonucleotides at 36°C for 3 h, washed in 2× SSC at 36°C, and exposed to film.

**Western Blot Analysis.** HEK293 cells were transfected as described with shHD2.1 or shGFP singly or in combination with pCMV-HD-N171-82Q. Forty-eight hours later, cells were lysed to recover total protein. Western blots were incubated with anti-myrc (1:5,000; Invitrogen), anti-full-length human htt (1:5,000; MAB2166, Chemicon), or anti-human β-actin (1:5,000; Clone AC-15, Sigma) followed by horseradish peroxidase-coupled goat anti-mouse or goat anti-rabbit secondary antibodies (1:20,000 and 1:100,000, respectively; Jackson Immunochemicals, West Grove, PA). Blots were developed by using ECL-Plus reagents (Amersham Pharmacia). For evaluation of transduced brain, 3-week-old mice were injected as described, and protein was harvested from striata 2 weeks later. Twenty-five micrograms of protein was run on SDS/PAGE as described, transferred to nitrocellulose, and then probed with antibodies to detect human htt (1:500, mEM48; a gift from X. J. Li, Emory University School of Medicine, Atlanta) and mouse prion protein (1:5,000, Chemicon). Secondary antibody incubations were performed as described above.

**Quantitative RT-PCR.** In vitro shRNA dose-response. HEK293 cells were transfected with 0 (mock), 10, 100, or 1,000 ng of either shLacZ or shHD2.1, and RNA was harvested 24 h later. After DNase treatment (DNA-Free, Ambion), random-primed, first-strand cDNA was generated from 500 ng of total RNA by using TaqMan reverse transcription reagents (Applied Biosystems) according to the manufacturer’s protocol. Assays were performed on a sequence detection system by using primers and probe sets specific for human htt and mammalian rRNA (Prism 7000 and TaqMan 2X Universal Master Mix; Applied Biosystems). Relative gene expression was determined by using the relative standard curve method.

**In vivo htt mRNA expression.** Striata were dissected from 5.5-month-old mice, snap-frozen in liquid nitrogen, and pulverized. cDNA was generated as described above. Relative gene expression was assayed by using TaqMan primer/probe sets specific for human htt and mammalian rRNA or TaqMan Assays-by-Design primers/probes specific for mouse htt (mHdh, Applied Biosystems). All values were calibrated to contralateral, un.injected striata. Human htt detection, shHD2.1 samples, n = 8 striata; shLacZ, n = 7; un injected, n = 4. Mouse htt detection, injected HD samples, n = 4; uninjected samples n = 2.

**AAV Injections.** AAV injections were performed in 4-week-old mice by using the following parameters (coordinates are reported with respect to the bregma): striatal, 0.5 mm anterior, 2.5 mm lateral, 2.5-mm depth, 5 μl per site, 250 nl/min infusion rate; cerebellar, 0.1-mm depth, 1 μl per site, 250 nl/min infusion rate.

**Behavioral Analysis.** Stride length measurements. Mice injected bilaterally at 4 weeks of age were analyzed at 4 months of age. Analyses were performed as described in ref. 25, with some modifications. Specifically, mice were allowed to walk across a paper-lined chamber (100 cm × 10 cm with 10-cm walls) and into an enclosed box. Mice were given one practice run and were then tested three times to produce three separate footprint recordings, totaling 42 recordings each for front and rear footprints per mouse. Measurements were averaged, and data were presented as box plots. ANOVA with Scheffe’s post hoc test was performed to determine statistical significance. Uninjected mice, n = 4; injected WT, n = 3; injected HD-N171-82Q, n = 6 mice per group.

**Rotarod performance test.** Two separate experimental cohorts of mice were injected at 4 weeks of age and tested on the rotarod (model 7000, Ugo Basile, Varese, Italy) at 10 and 18 weeks of age as described in ref. 26. Data from trials 2–4 for each day are presented as means ± SEM. Uninjected WT, n = 6; shLacZ WT, n = 5; shHD2.1 WT, n = 6; un injected HD-N171-82Q, n = 5; shLacZ HD-N171-82Q, n = 10; shHD2.1 HD-N171-82Q, n = 11.

**Immunofluorescence.** Forty-micrometer free-floating coronal sections from mice (n = 5 per group) were stained with mEm48 antibody (1:500; 24 h, 4°C), followed by AlexaFluor 568-labeled goat anti-mouse secondary antibody (1:200; 4 h, room temperature; Molecular Probes). Sections were mounted onto slides and covered in Gel/Mount (Biomedica, Foster City, CA), and images were captured by using fluorescent microscopy with either a DM RBE (Leica) or confocal microscope (Zeiss) equipped with a charge-coupled device camera (SPOT RT, Diagnostic Instruments, Sterling Heights, MI).

**Results**

**shHD2.1 Reduces Human htt Expression in Vitro.** In vitro screening was used to identify effective shRNAs directed against a CMV promoter-transcribed HD-N171-82Q mRNA, which is identical to the pathogenic truncated htt fragment transgene present in HD-N171-82Q mice (20). Hairpin constructs targeting sequences in human exons 1–3 were evaluated by cotransfection. One htt-targeted shRNA, shHD2.1 (Fig. 1A), reduced HD-N171-82Q mRNA and protein levels by ~85% and ~55%, respectively, compared with control shRNA-treated samples (Fig. 1 B and C).

To test whether shHD2.1 could silence endogenous full-length human htt expression, HEK 293 cells were transfected with plasmids expressing shHD2.1 or shGFP. ShHD2.1, but not control shRNAs, directed gene silencing of endogenous htt mRNA and protein (Fig. 1 D and E).

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Neuronal inclusions in HD-N171-82Q striata are variable. Inclusions may be present in 10–50% of all striatal neurons in different end-stage HD-N171-82Q mice (20). In contrast, robust and widespread EM48-positive inclusions are present in cerebellar granule cells by ∼3 months of age (see ref. 20 and Fig. 3), and cerebellar HD-N171-82Q mRNA levels are ∼8-fold higher, compared with striatum (quantitative real-time RT-PCR, data not shown). This high-level cerebellar expression is partially attributable to the transcriptional profile of the prion promoter driving HD-N171-82Q transgene expression (20). Cerebellar inclusions are not typically found in the brains of adult-onset HD patients. However, cerebellar pathology has been reported in juvenile-onset HD cases, which are the most severe forms of the disease, and interestingly, in Hdh140 knock-in mice as early as 4 months of age (14, 27–30). The abundant inclusions in HD-N171-82Q cerebellar neurons provide a second target for assessing the effects of AAV.shHD2.1 on target protein levels. Direct cerebellar injections were performed in a separate cohort of mice, and HD-N171-82Q expression was examined by immunofluorescence. The data in Fig. 3 show that shHD2.1 reduces HD-N171-82Q expression as measured by EM48 immunoreactivity in transduced (hrGFP-positive) cells, whereas AAV.shLacZ does not. Together, the data show that AAV.shHD2.1, but not control AAV.shLacZ, reduces mutant htt expression and prevents the formation of the disease-associated neuronal inclusions.

Striatal Delivery of AAV.shHD2.1 Improves Established Behavioral Phenotypes. The effects of shRNA treatment on established behavioral deficits and animal weight were tested. RNAi directed to striatum did not normalize the notable weight differences between HD-N171-82Q and WT mice (shHD2.1-injected, 22.7 ± 3.8 g; shLacZ, 22.6 ± 2.8 g), compared with age-matched WT mice (shHD2.1, 26.3 ± 0.4; shLacZ, 27.3 ± 5.8), confirming that intracerebral injection confines RNAi therapy to the site of application (20, 26). However, significant improvements in stride length measurements and rotarod deficits were noted.

Stride length and rotarod tests were performed on uninjected mice, and mice were injected bilaterally with either AAV.shHD2.1 or AAV.shLacZ into the striatum. As shown in Fig. 4A, HD-N171-82Q mice display significantly shorter stride lengths than WT mice, consistent with prior work (14, 25, 31). Gait deficits in AAV.shHD2.1-treated HD-N171-82Q mice were significantly improved, compared with AAV.shLacZ-treated (improvements for front and rear strides, 13% and 15%, respectively; \( P < 0.0001 \)) and uninjected HD-N171-82Q mice (front and rear strides, 14% and 18%, respectively; \( P < 0.0001 \)). Gait improvements did not fully resolve; all HD-N171-82Q groups remained significantly different from their age-matched WT littermates. There was no effect of AAV.shLacZ or AAV.shHD2.1 expression on the stride lengths of WT mice.

The accelerating rotarod test was used to confirm the beneficial behavioral effects of RNAi targeted to the mutant human HD allele (20). Mice were left uninjected or were injected bilaterally into the striatum with either AAV.shLacZ or AAV.shHD2.1 at 4 weeks of age, followed by rotarod analyses at 10 and 18 weeks of age (Fig. 4B). By 10 weeks, uninjected and AAV.shLacZ-injected HD mice showed impaired performance, compared with all other groups, and continued to demonstrate significantly reduced performance over the course of the study (\( P < 0.05 \), relative to all other groups). Importantly, HD mice treated with AAVshHD2.1 showed dramatic behavioral improvements, compared with control-treated HD mice (\( P < 0.0008 \)) (Fig. 4B). AAV.shLacZ-treated HD mice showed a 22% decline (\( P < 0.005 \); ANOVA), whereas AAV.shHD2.1-treated HD mice displayed a modest, nonsignificant 3% drop in rotarod performance between 10 and 18 weeks of age. The partial normalization of rotarod deficits in HD mice injected with
AAV.shHD2.1, compared with WT mice, was consistent with the gait analyses.

We found no significant difference in stride length or decline in rotarod performances over time, in WT mice left untreated or injected with shRNA-expressing AAVs (Fig. 4). We did note differences in rotarod performance between uninjected and injected WT mice at the 10-week time point, however. Importantly, this difference in rotarod performance resolved by 18 weeks of age. These data suggest that there was some detrimental effect of direct brain injection on rotarod performance from which the mice recovered over time. Although these data do not rule out the possibility that RNAi expression may cause potentially detrimental off-target effects on a cellular or molecular level, they do suggest that RNAi expression in mammalian brain had no overt negative impact on motor behavior (Fig. 4).

**Discussion**

We show that motor and neuropathological abnormalities in a relevant HD mouse model are significantly improved by reducing striatal expression of a pathogenic htt allele by using AAV1-delivered shRNA. Our laboratory previously showed that RNAi can improve neuropathology and behavioral deficits in a mouse model of spinocerebellar ataxia type 1 (26), a dominant neurodegenerative disorder that affects a population of neurons distinct from those degenerating in HD.

The shHD2.1 hairpin sequence was developed before studies describing optimal shRNA design (32–35). Review of the hairpin sequence according to currently understood rules suggests that our intended guide strand may not be preferentially loaded into the RNAi-inducing silencing complex. However, shHD2.1 did reduce htt expression in vivo and in vitro, and importantly, our...
Northern blot analysis data suggest that the processed active guide strand was protected by the RNAi-inducing silencing complex in vivo. It is possible that shRNAs that more closely follow currently understood guidelines for processing in vitro may further improve correction of HD-associated phenotypes in vivo.

Striatal delivery of AAV.shHD2.1 had no effect on weight loss in HD-N171-82Q mice. The underlying cause of weight loss in mice and humans is unclear but may be an indicator of the systemic nature of the disease irrespective of, or indirectly related to, brain involvement (36). It has been suggested that expanded htt may cause hypothalamic dysfunction leading to weight loss in HD patients and HD-N171-82Q mice (37, 38). The inability to normalize weight differences in this study may be attributed to the focal striatal delivery of AAV.shHD2.1. It is possible that normalization of weight differences and greater improvements in motor function could be accomplished by a broader injection regimen.

Prior work demonstrated an essential role for htt in embryogenesis and postnatal neurogenesis (39–42). However, the effect of partial reduction of normal htt expression in adult, postmitotic neurons in vivo is unknown. In the current study, shHD2.1 reduced expression of a mutant, disease-causing human htt transgene but had no effect on normal mouse htt expression because of sequence differences between mouse and human genes. In HD patients, shHD2.1 would be expected to reduce the expression of both the mutant and normal htt alleles. Thus, RNAi as a therapy for HD will require addressing two important issues. First, can adult striatal neurons tolerate and benefit from partial nonselective knockdown of both the normal and disease alleles? Our data show that HD-like symptoms can be improved by partial reduction of mutant htt expression, suggesting that complete elimination of mutant allele expression may not be required. Second, disease allele-specific silencing will require identification and testing of disease-linked polymorphisms, one of which has been identified in exon 58 (43). This proof-of-principle work in the HD-N171-82Q-transgenic model provides evidence that disease allele-specific silencing can be accomplished in vivo.

In summary, we show that RNAi can dramatically improve HD-associated abnormalities, including pathological and behavioral deficits, in a mouse model of HD. Our data suggest the feasibility of treating HD by directly reducing mutant htt gene expression by using RNAi and support its general applicability to treating other dominant neurodegenerative disorders.

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Fig. 4. AAV.shHD2.1 improves behavioral deficits in HD-N171-82Q mice. (A) The box plot shows that the bilateral striatal delivery of AAV.shHD2.1 improved stride length in HD-N171-82Q mice. HD mice had significantly shorter stride lengths, compared with WT mice. AAV.shHD2.1 mediated significant gait improvement, compared with control-treated HD mice. *P < 0.0001; ANOVA with Scheffé’s post hoc test. (B) Bilateral striatal delivery of AAV.shHD2.1 significantly improved rotarod performance in HD-N171-82Q mice. Only AAV.shLacZ-injected and uninjected HD-N171-82Q mice declined significantly with time. Data are means ± SEM.