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Unexpected off-targeting effects of anti-huntingtin ribozymes and siRNA *in vivo*

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Abstract

Gene transfer strategies to reduce levels of mutant huntingtin (mHtt) mRNA and protein by targeting human Htt have shown therapeutic promise *in vivo*. Previously, we have reported that a specific, adeno-associated viral vector (rAAV)-delivered short-hairpin RNA (siHUNT-2) targeting human Htt mRNA unexpectedly decreased levels of striatal-specific transcripts in both wild-type and R6/1 transgenic HD mice. The goal of this study was to determine whether the siHUNT-2-mediated effect was due to adverse effects of RNA interference (RNAi) expression in the brain. To this end, we designed two catalytically active hammerhead ribozymes directed against the same region of human Htt mRNA targeted by siHUNT-2 and delivered them to wild-type and R6/1 transgenic HD mice. After 10 weeks of continuous expression, these ribozymes, like siHUNT-2, negatively impacted the expression of a subset of genes in the striatum. This effect was independent of rAAV transduction and specific to the targeting of a unique sequence in human Htt mRNA. After consideration of the known potential RNAi-specific toxic mechanisms, only cleavage of an unintended RNA target can account for the data reported herein. Thus, long-term rAAV-mediated RNAi in the brain does not, in and of itself, negatively affect striatal gene expression. These findings have important implications in the development of therapeutic RNAi for the treatment of neurological disease.

Introduction

The underlying cause of Huntington's disease (HD) is the inheritance of a copy of the gene encoding Htt with an expanded polyglutamine-encoding CAG repeat located within the 5' end of the coding region (HDCRG, 1993). The mutant huntingtin protein (mHtt) is expressed during development through adulthood, causes neuronal dysfunction, and ultimately cell death of neurons in the striatum and neuropathology is present to a varying extent in other regions of

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the brain (Schilling et al., 1995; Sharp et al., 1995a; Sharp et al., 1995b). The development of motor, cognitive and psychiatric symptoms of the disease, like neuronal cell loss, is slow and progressive, generally affecting HD patients in mid-adulthood. The disease is inevitably fatal after a period of worsening symptoms. Although, there are a number of pharmacological treatments that have shown promise at reducing symptoms and cellular pathology and increasing survival in transgenic HD mice (Chen et al., 2000; Dedeoglu et al., 2002; Ferrante et al., 2002; Ferrante et al., 2000; Ferrante et al., 2003; Ferrante et al., 2004; Ona et al., 1999; Van Raamsdonk et al., 2005), treatment of HD patients is very limited.

The precise function of normal Htt and the abnormal function acquired by the mutant form of the protein are not fully understood (Cattaneo et al., 2005; Li and Li, 2004). It is clear that continued expression of mHtt is required for disease progression as conditional knock-out mice do not display HD-like motor symptoms after mHtt expression is blocked (Yamamoto et al., 2000). This observation demonstrated that lowering mHtt levels would likely be beneficial for the treatment of HD. Several groups, including ours, have now shown that it is possible to reduce the levels of mHtt mRNA post-transcriptionally using a variety of strategies that include antisense oligonucleotides, DNA enzymes, ribozymes and siRNAs in culture cells (Boado et al., 2000; Chen et al., 2005; Hasholt et al., 2003; Nellesmann et al., 2000; Omi et al., 2005; Park et al., 2004; Yen et al., 1999).

In vivo, anti-Htt short-hairpin RNA molecules delivered via recombinant adeno-associated viral vectors (rAAV) to the striatum of three different lines of transgenic HD mice have shown efficacy at reducing mHtt levels and benefit in terms of reducing cellular pathology and decreasing motor symptoms (Harper et al., 2005; Rodriguez-Lebron et al., 2005; Wang et al., 2005). Because AAV-based gene therapies for various neurodegenerative disorders are showing promise in early stage clinical trials (Mandel and Burger, 2004), it is possible that HD patients may, in the foreseeable future, be treated with AAV-delivered anti-Htt siRNAs. As such, investigation of the positive and negative effects of siRNAs directed against mHtt is warranted.

During the course of our investigation of the effect of anti-mHtt siRNAs, we observed that one of two siRNA molecules mediating RNAi of mHtt *in vivo* had the unanticipated effect of lowering levels of several, non-targeted transcripts expressed in the striatum of the R6/1 mouse. Because expression of mHtt negatively regulates DARPP-32, preproenkephalin and some other genes expressed specifically in the striatum (Bibb et al., 2000; Luthi-Carter et al., 2000; Menalled et al., 2000), we anticipated that reducing the expression of mHtt via RNAi would lead to an increase in levels of these transcripts. Although the two siRNAs, designated siHUNT-1 and siHUNT-2, both effectively lowered mHtt mRNA and protein levels in cell culture and *in vivo*, siHUNT-1 expression in the R6/1 striatum increased mRNA levels of DARPP-32 and preproenkephalin, as predicted, while expression of siHUNT-2 led to a marked reduction in the levels of these transcripts in R6/1 mice (Rodriguez-Lebron et al., 2005). Importantly, siHUNT-2 negatively affected the levels of DARPP-32 and ppENK transcripts in wild-type littermate control mice. Wild-type littermates do not contain the human mHtt sequence targeted by siHUNT-2 suggesting that siHUNT-2 siRNA was having a non-specific, off-target effect.

Several mechanisms have been proposed for siRNA-induced untoward effects (Jackson and Linsley, 2004). Over-expression of short-hairpin RNAs can saturate the cellular RNAi machinery, blocking the nuclear export and processing of endogenous miRNAs required for proper cellular development and maintenance (Yi et al., 2005; Grimm et al., 2006). In addition, siRNAs can stimulate protein kinase R and the 2', 5' oligo A pathway inducing an interferon-response (Judge et al., 2005; Kim et al., 2004; Sledz et al., 2003). Unbiased or passenger-strand biased loading of RISC can also lead to non-specific, off-target gene silencing. Lastly, guide-

strand RISC-dependent cleavage of an mRNA other than the intended target can occur (Jackson et al., 2003; Judge et al., 2005; Lin et al., 2005). The first two potential mechanisms are siRNA-specific and mostly sequence independent. The last two are solely dependent on sequence-specific cleavage of mRNA and, therefore, the same siRNA-mediated unintended effects would predictably be observed using RISC-independent mRNA cleavage methods such as ribozymes. In this study, we asked whether the effects induced by siHUNT-2 (Rodriguez-Lebron et al., 2005) were caused by a sequence-dependent off-targeting mechanism. Our experimental approach consisted of testing two different catalytically active ribozymes designed to cleave human Htt at the same site targeted by the siHUNT-2 siRNA. The studies revealed that analyzing several transcripts in addition to the intended target can expose important and otherwise undetectable differences between equally effective siRNAs.

Results and Discussion

In vitro activity of HD6 and HD7 ribozymes

SiHunt-2 was designed to target a unique region of human Htt mRNA between the ATG initiation coding and the extended CAG repeat [(Rodriguez-Lebron et al., 2005); Fig 1A, grey area]. Within the sequence of human Htt mRNA targeted by siHUNT-2, there are two GUC triplets corresponding to potential ribozyme cleavage sites. We designed two ribozymes, HD6 and HD7, that would anneal to the sequences flanking each GUC site and catalyze the cleavage of human Htt mRNA (Fig 1A, boxes). The siHUNT-2 and ribozyme target sequences within the 5' coding region of human Htt mRNA and the two-dimensional representations of HD6 and HD7 hammerhead ribozymes are shown in Fig. 1A. To determine the *in vitro* kinetic properties, HD6 and HD7 ribozymes and their cognate 13-mer RNA targets were incubated in cleavage reaction buffer and aliquots were removed at intervals over a 3 hr period. The products were fractionated by denaturing polyacrylamide gel electrophoresis and the relative intensity of the target and cleavage products were determined by densitometric analysis. Both HD6 (Fig. 1B, upper panel) and HD7 (Fig. 1B, lower panel) converted greater than 95% of the input target to product within the first 5 min of the reaction. Multiple turnover kinetic analysis demonstrated that the K_m and k_{cat} (turnover number) of HD6 were 1 μM and 6.6 min^{-1} , respectively. HD7 had the same K_m value as HD6 but had a higher k_{cat} value of 14 min^{-1} . Saturation was achieved in these analysis and all calculations were based on non-linear regression plots (data not shown).

To determine whether HD6 and HD7 could access target sites within a longer transcript containing an expanded CAG repeat, which has the potential to form extensive secondary structure, HD6 and HD7 ribozymes were incubated in cleavage reaction buffer with an *in vitro* transcribed 1.2 kb mRNA synthesized from a cloned human Htt cDNA fragment (Fig. 1C). The radio-labeled RNA synthesized from the cloned template was denatured, allowed to re-fold and incubated in the presence of HD6 or HD7. Aliquots of the reaction were removed at time 15, 30 and 60 min, subjected to denaturing polyacrylamide gel electrophoresis and autoradiography, and analyzed for the generation of cleavage products and the decrease in input target RNA. The control reactions were incubated for 60 min in the absence of HD6 or HD7. Specific cleavage products of the expected size were observed 15 min after the start of each reaction. HD7 had a faster rate and exhibited greater efficacy at cleaving the 1.2 kb mRNA than HD6. Despite the apparent difference in rate of cleavage, both ribozymes could access their target in a biologically relevant RNA transcript.

Generation of recombinant rAAV vectors expressing HD6 and HD7 anti-human Htt ribozymes

HD6 and HD7 ribozymes were cloned in rAAV vector backbones (Fig. 2A). The rAAV vector backbone contained the intronic sequence derived from the chicken β -actin (CBA) gene placed between the CBA promoter and the ribozyme cloning site. The inclusion of the intron at this

position stimulates transport of the ribozyme RNA to the cytoplasm (Zhou et al., 2000). An internal self-cleaving hairpin ribozyme was located downstream of the ribozyme cloning site. This internal hairpin ribozyme catalyzed the cleavage of the primary RNA transcript such that the anti-human Htt ribozymes were liberated from the remainder of the mRNA and could access the human Htt target mRNA. The rAAV vector also contained a copy of the coding sequence for GFP with an internal ribosome entry site (IRES). A SV40-derived polyadenylation signal was present downstream of the hairpin ribozyme and IRES-GFP. The polyadenylated primary transcript produced from the CBA promoter included either the HD6 or HD7 hammerhead ribozyme and GFP, which were separated post-transcriptionally by the action of the internal hairpin ribozyme. The control vector produced a primary transcript that matured after self-cleavage into GFP mRNA.

HD6 and HD7 reduce levels of mHtt mRNA in HEK293 cells

HEK293 cells were transiently co-transfected with a plasmid expressing exon 1 of human Htt with an extended CAG repeat [pCMV-R6/1; (Rodriguez-Lebron et al., 2005)] and the plasmids expressing HD6 or HD7 and GFP or control vector expressing GFP alone. Northern blot analysis of RNA isolated 48 hr after transfection demonstrated that HD6 and HD7 decreased the levels of mHtt mRNA by approximately 60% when a 1:10 ratio of pCMV-R6/1 to ribozyme vector was employed (Fig. 2B). Expression of the GFP-only control vector did not effect levels of mHtt mRNA. These analyses demonstrated that HD6 and HD7 could bind to and catalyze the cleavage of mHtt mRNA in cultured cells. Previously, we demonstrated that siHUNT-2 lowered pCMV-R6/1 mRNA and protein levels in cell culture to a similar extent (Rodriguez-Lebron et al., 2005).

In vivo expression of rAAV5-HD6 and HD7

We generated rAAV serotype 5 (rAAV5) viral vectors expressing HD6 or HD7 with an IRES-GFP or rAAV5 expressing GFP alone. Six week-old mice were divided into experimental groups [rAAV5-HD6 (R6/1 n=7), rAAV5-HD7 (R6/1 n=8) and rAAV5-GFP (R6/1 n=8)] and injected unilaterally at two different striatal sites in the right hemisphere while the uninjected left hemisphere served as an internal control. Ten weeks post-surgery, the animals were euthanized and the brains were removed for analysis. This experimental design was used to allow for comparison between the previous experiments using rAAV5-siHUNT-1, rAAV5-siHUNT-2, rAAV5siRho (Rodriguez-Lebron et al., 2005) and the current study. Intrastriatal delivery of rAAV5-HD6, -HD7 and -GFP resulted in wide-spread transduction of the mouse striatum as evidenced by *in situ* hybridization using a probe to detect GFP mRNA that was produced from the viral vector within the same primary transcript as the HD6 and HD7 ribozymes (see column labeled GFP, Fig. 3). Examination of cresyl violet-stained sections derived from rAAV5-treated mice revealed no obvious cell death or altered morphology of the striatum (data not shown). The extent of transduction and the relative intensity of the hybridization signal of the mRNA expressed from each of the rAAV5 vectors were comparable among all mice (data not shown).

Animals that received rAAV5-HD7 ribozyme in the right striatum had lower levels of high molecular weight mHtt protein in the injected compared to the uninjected striatum demonstrating that HD7 was active *in vivo* (Fig. 4). Importantly, there was no difference in the levels of mouse Htt among R6/1 and wild-type mice or between the injected and uninjected striata (data not shown). Levels of mHtt decreased by approximately 30% in rAAV5-HD7-treated striatum compared to the contralateral uninjected striatum (Fig. 4; $F[1,7] = 6.04$, $p < 0.05$, one-way ANOVA). Comparable reductions in the levels of mHtt were observed in rAAV5-siHUNT-1 and rAAV5-siHUNT-2 treated animals (Rodriguez-Lebron et al., 2005). We did not observe a difference in the levels of mHtt protein between the untreated or rAAV5-HD6-treated striatum, which was likely due to a reduced efficiency of mRNA knock-down by

HD6 (in agreement with our data obtained *in vitro*) combined with the dilution of the sample with protein derived from tissue that had not been transduced by rAAV5-HD6 (data not shown).

HD6 and HD7 increase NGFI-A and decrease DARPP-32 expression

The expression of a particular subset of mRNAs is altered by the expression of mHtt protein early in the progression of HD in humans and in several transgenic mouse models of the disease (Sugars and Rubinshtein, 2003). The physiologic consequences of this presumptive transcriptional dysregulation remain to be determined, although such changes may contribute to brain dysfunction and eventually to neuronal cells loss observed in HD. The levels of these specific mRNAs, therefore, can be used as a measure of the impact of mHtt. Originally, we hypothesized that treatments that effectively reduce mHtt levels *in vivo* would slow the rate of loss of specific mRNAs. siHUNT-1 delivered by rAAV5 reduced levels of mHtt mRNA and protein and led to a small increase in levels of DARPP-32 and ppENK mRNA in transduced striatal neurons of R6/1 mice (Rodriguez-Lebron et al., 2005). This rAAV-siRNA did not affect the constitutive levels of these mRNAs observed in age- and treatment-matched wild-type mice (Rodriguez-Lebron et al., 2005). In contrast, rAAV5-siHUNT-2 reduced levels of DARPP-32 and ppENK in transduced neurons of both wild-type and R6/1 mice (Rodriguez-Lebron et al., 2005), suggesting that this shRNA had a non-target specific effect on gene expression in addition to lowering levels of mHtt mRNA and protein.

To determine the effect of rAAV5-HD6 and rAAV5-HD7 ribozyme treatment on striatal gene expression, we employed *in situ* hybridization using probes specific for NGFI-A, DARPP-32, ppENK, PDE10A, PDE1B, D2 receptor and β -actin mRNA. We observed that the rAAV5-GFP-only (UF11) control vector did not alter the relative levels of mRNA encoded by any of these genes in the transduced region of the striatum relative to adjacent non-transduced regions of the ipsilateral striatum or the uninjected contralateral striatum (Fig. 3). In contrast, it appeared that the mRNA levels of DARPP-32, ppENK, PDE10A, PDE1B, and D2 receptor in R6/1 and wild-type mice were lower in the regions of the striatum transduced by rAAV5-HD6 and rAAV5-HD7 (Fig. 3). These data demonstrated that transcriptional regulation of these particular genes were negatively affected by HD6 and HD7 in wild-type and R6/1 mice and that the effect was co-localized with transduced cells. Of particular interest, rAAV5-HD6 and -HD7 vectors appeared to increase levels of NGFI-A mRNA relative to the uninjected hemisphere for both genotypes of mice (Fig. 3). Previously, we did not observe an increase in NGFI-A transcript levels when siHUNT-1 or siHUNT-2 was administered to wild-type or R6/1 mice (Rodriguez-Lebron et al., 2005). Importantly, increased NGFI-A expression indicates that transduced striatal neurons remained transcriptionally competent and viable throughout the experimental timeline. This observation, together with the robust GFP expression argues against a non-specific global suppression of striatal transcriptional activity.

We then sought to determine whether co-expression of HD6 and HD7 would lead to a more pronounced effect on the levels of these transcripts. Six week-old R6/1 and wild-type mice were injected with rAAV5-HD6 and rAAV5-HD7 administered on two successive days (HD6 +HD7) (R6/1 n=8; wild-type n=4). Ten weeks post-surgery the animals were euthanized and the transcript levels determined by *in situ* hybridization. We observed that the combined effect of both ribozymes administered sequentially to wild-type and R6/1 mice was similar to the observed differences in gene expression when both ribozymes were delivered independently to individual animals (Fig. 3). These data suggest that the negative effect associated with the expression of HD6 and HD7 is potentially mediated by the sequence-specific cleavage of an mRNA that is common to both of these ribozymes.

We repeated these experiments using rAAV serotype-2 viral capsids to control for virus-induced effects. In addition, a catalytically active ribozyme directed against alpha 1 antitrypsin (AAT) was used as a control for the effects of ribozyme over-expression. *In situ* hybridization

to detect AAV2-encoded GFP mRNA demonstrated that intrastriatal expression of HD6, HD7 and AAT expressing vectors resulted in intense but focal transduction (Fig. 5). Levels of NGFI-A mRNA appeared to increase and DARPP-32 mRNA levels appeared to decrease in the region of the striatum directly transduced by AAV2 in both wild-type and R6/1 mice, which indicated that the effects of HD6 and HD7 on gene expression were related to relative transduction and not due to surgery or general delivery of AAV vectors.

Previously, we had determined that rAAV5-siHUNT-2, but not rAAV5-siHUNT-1 or rAAV5-siRho control vector decreased levels of DARPP-32 and ppENK mRNA (Rodriguez-Lebron et al., 2005). Using tissue generated from the experiments described in (Rodriguez-Lebron et al., 2005), we extended our *in situ* analysis of the effects of siHUNT-1 and siHUNT-2 on mRNA levels of NGFI-A, PDE10A, PDE1B, D2 receptor and β -actin and included these data in the quantitative analysis of mRNA levels. The optical density of the hybridization signal for each probe was determined in the rAAV5-transduced regions of the striatum and the contralateral striatum for each animal treated with rAAV5-GFP (R6/1 n=8), rAAV5-HD6 (R6/1 n=7), rAAV5-HD7 (R6/1 n=8), rAAV5-HD6 and -HD7 (R6/1 n=8; wild-type n=4), siHUNT-1 (R6/1 n=12; wild-type n=6), and siHUNT-2 (R6/1 n=10; wild-type n=5). As expected, the levels of each message, except β -actin, were lower in the untreated striatum of all R6/1 mice compared to age-matched wild-types (data not shown). The ratio of the hybridization signal in the rAAV5-transduced and contralateral striatum of each animal was determined and the mean ratios (+ one standard error) are shown in Fig. 6. There was a statistically significant increase in the levels of NGFI-A mRNA in R6/1 mice that received rAAV5-HD6 and rAAV5-HD7 and in R6/1 and in wild-type mice that received both rAAV5-HD6 and -HD7 (Fig. 6). Addition of both ribozymes did not have additive effects on NGFI-A induction. We did not observe an increase in NGFI-A when siHUNT-1 or 2 were administered (Fig. 6). In fact, the decrease in the low constitutive levels of NGFI-A in the striatum of R6/1 compared to levels observed in wild-type mice was further reduced by administration of siHUNT-2. The lack of NGFI-A induction in wild-type and R6/1 mice via administration of siHUNT-1, siHUNT-2, rAAV2-AAT, rAAV2-GFP and rAAV5-GFP (UF11) control vectors indicated that NGFI-A induction was not caused by transduction and long-term expression of rAAV2 or rAAV5 serotype viral vectors but was specifically related to the expression of HD6 and HD7 ribozymes. The reason for the difference in NGFI-A induction between ribozyme and siHUNT-2-expressing viral vectors is currently unresolved.

Altered transcriptional activity is related to target sequence and not rAAV transduction or sustained overexpression of ribozymes or shRNAs

As reported previously, there was a statistically significant increase in DARPP-32 and ppENK mRNA in R6/1 mice treated with rAAV5-siHUNT-1 (Rodriguez-Lebron et al., 2005). These observations indicated that siHUNT-1, by lowering levels of mHtt, had the positive effect of increasing levels of DARPP-32 and ppENK mRNA. The observation that expression of DARPP-32, ppENK, PDE10A, PDE1B, and D2 mRNA is not negatively affected by rAAV5-siHUNT-1, rAAV5-GFP or rAAV2-GFP vectors indicates that rAAV2 or rAAV5-mediated transduction and long-term transgene expression do not lead to changes in the steady-state levels of these transcripts in the mouse striatum.

The HD6-, HD7- and siHUNT-2-mediated effects on gene expression are independent of reduction in the levels of mHtt, as wild-type mice do not express the human Htt target and there is no cleavage target for these molecules within the mouse Htt mRNA. Importantly, of the mRNAs tested, levels of DARPP-32 and ppENK were increased by expression of siHUNT-1 and knock-down of mHtt expression in R6/1 mice. PDE1B, PDE10A and D2 receptor mRNA levels did not increase when R6/1 mice were treated with siHUNT-1. Although we observed a change in the intensity of the hybridization signal in the HD7-treated striatum of R6/1 mice

for DARPP-32 and ppENK (Fig. 3) these differences were not statistically significant ($p > 0.01$, Fig. 6). The lack of a significant HD7-induced alteration of striatal DARPP-32 or ppENK mRNAs suggests that the observed net DARPP-32 and ppENK mRNA levels were the product of increases due to knock-down of mHtt and decreases due to the off-target effect. It is of interest that levels of a subset of transcripts (DARPP-32 and ppENK) that progressively decrease in HD can be rescued by reducing mHtt levels using RNAi or ribozymes while others (PDE1B, PDE10A and D2), which have also been shown to decrease in HD, are unaffected by either of these two treatments (see Fig 7 for a summary of these results). One potential explanation is that the former may be more responsive to slight decreases in mHtt levels while the latter require earlier intervention to prevent secondary changes that regulate their expression. Alternatively, greater knock-down of mHtt could be required in order to reverse the loss in steady-state mRNA levels.

Recently, other groups have shown the potential for unintended RNAi-induced toxicities (Grimm et al 2006). The mechanisms responsible for these effects include saturation of RNAi machinery, immuno-stimulation, unbiased loading of the RISC complex and sequence-specific off-targeting (Hornung et al 2005; Birmingham et al 2006; Grimm et al 2006). The results obtained in this study demonstrate that the first two mechanisms, which are siRNA-specific, did not contribute significantly to the unintended loss of a subset of striatal transcripts in the mouse brain. Furthermore, since both HD6 and HD7 targeted the same sequence as the guide-strand in the siHUNT-2 shRNA (Fig 1A), it is unlikely that improper loading of the passenger strand into RISC played a significant role in the induction of the observed negative effects. Thus, based on common effects of HD6, HD7 and siHUNT-2 on the expression of a group of striatal-specific genes and the observation that co-expression of HD6 and HD7 did not result in additive effects, it appears that the HD6 and HD7 ribozymes and the siHUNT-2 shRNA guide strand direct the cleavage and degradation of mHtt mRNA and of an additional unintended mRNA molecule.

Originally, the human Htt mRNA sequences targeted by siHUNT-1, siHUNT-2, HD6 and HD7 were compared to known human cDNA and human genomic DNA sequences using standard similarity algorithms. We did not find any sequences, other than the human Htt sequence, that had significant similarity to the sequences targeted by either siHUNT-1, siHUNT-2 or the two ribozymes. The short sequence, which spanned the nucleotides in the immediate vicinity of both ribozyme cleavage sites and the sequence that would anneal to the 5' end of the active strand of siHUNT-2 in an active RISC complex (see Fig 1A), has sequence similarity with a mouse EST (Accession number, AK04381). This cDNA was identified during a wide scale screen of the mouse transcriptosome (Carninci et al., 2005; Katayama et al., 2005). Based on alignment, AK04381 may be a variant of zinc ring finger protein 13 and could possibly be a common target for HD6, HD7 and siHUNT-2. It is also possible that HD6, HD7 and siHUNT-2 alter the abundance of an, as yet, unidentified mRNA. The observation of the larger than expected number of expressed antisense transcripts that may play important roles in the control of gene expression in the mouse increases the likelihood RNA molecules encoded by what was predicted to be the non-coding strand of genes could also be a target for siHUNT-2, HD6 and HD7 or for that matter any of the siRNAs that are being developed and tested for therapeutic use. Identification of the mouse RNA targeted by siHUNT-2, HD6 and HD7, although intriguing, will be challenging since expression of these molecules leads to a decrease in a number of transcripts, many of which do not contain potential common cleavage sites. A comprehensive analysis of sequences that are common to these three molecules could help derive new targeting sites to validate or invalidate potential gene targets. This, however, is currently beyond the scope of our studies.

Therapeutic RNAi holds great promise due to its inherent specificity. This study illustrates that sequence-dependent *in vivo* off-target effects of siRNA molecules can occur and should be

considered when developing gene transfer-based RNAi therapies. Detection of these off-target induced changes in mRNA levels may require systematic analyses of transcriptional activity. It should be pointed out that HD7 (this study) and siHunt-2 (Rodriguez-Lebron et al., 2005) can inhibit the expression of human Htt *in vivo* and could therefore have been considered as components of a gene therapy for HD. Whether the off-target effects we have identified in the context of the mouse transcriptosome disqualify these inhibitors from further consideration remains to be established.

Materials and Methods

In vitro activity of hammerhead ribozymes

HD6 and HD7 anti-human Htt ribozymes and their cognate 13-mer target sequences were purchased as RNA oligonucleotides (Dharmacon Inc., Boulder, CO). The 1.2 kb human Htt mRNA was generated by *in vitro* transcription as described in (Yen et al., 1999). *In vitro* cleavage reactions included a 1:10 molar ratio of ribozyme to radio-labeled target RNA oligonucleotide or *in vitro* transcribed human Htt cDNA in a buffer containing 10 mM MgCl₂ and 20 mM Tris-HCl. Reactions were carried out at 37°C. Aliquots of each reaction were removed and the reaction terminated by adding the cleavage reaction to an equal volume of EDTA-containing denaturing loading dye. The products were fractionated by denaturing polyacrylamide gel electrophoresis. Densitometry was used to determine the relative amount of target and cleavage product at each time point.

Cloning of ribozyme-expressing plasmids and viral vector production

Complementary DNA oligonucleotides corresponding to the coding and non-coding strands of HD6 and HD7 were allowed to anneal and cloned into the *NsiI/HindIII* sites of pTR-UF12 using standard methods. Culture, transfection and post-transfection analysis of HEK293 cells were performed as described in (Rodriguez-Lebron et al., 2005) using 1:5 and 1:10 molar ratios of pCMVR6/1 and plasmids expressing HD6 or HD7. Forty-eight h after transfection, cells were harvested for northern blot analysis of levels of transfected pCMV-R6/1 RNA and endogenous β -actin mRNA.

The plasmids for HD6, HD7 and UF11 were packaged into rAAV vectors at the Gene Therapy Center, University of Florida (Zolotukhin et al., 1999; Zolotukhin et al., 2002). The number of copies of encapsulated DNA/ml (particle titer) (rAAV5-HD6 = 8.04×10^{13} ; HD5-HD7 = 5.36×10^{13} ; rAAV5-UF11 = 6.60×10^{12}) was determined by quantitative PCR.

Animals and Experimental Groups

Transgenic R6/1 HD mice were originally obtained from Jackson Laboratories and maintained in a colony at Dalhousie University. The housing, genotyping and care of mice are described in (Hebb et al., 2004). All animal care, handling, and surgical protocols were in accordance with the guidelines established by the Canadian Council on Animal care and were approved by the Carleton Animal Care Committee at Dalhousie University. Every effort was made to minimize the number of animals used in this study and their discomfort. R6/1 and wild-type mice were divided into experimental groups as described in the results.

Surgical Procedures and analysis of brain tissue

Anesthesia was induced and maintained using isoflurane and mice were devoid of pain reflex throughout the entire surgical procedure. Prior to regaining consciousness, all mice received a single injection of the long-lasting analgesic ketoprofen (2 mg/kg). Mice (6–7 week-old) received intrastriatal injections of ribozyme-containing or control rAAV suspended in phosphate-buffered saline (PBS) or PBS alone at a dose of 2 μ l/site and an infusion rate of 1

µl/min. Mice were allowed to survive for 10 weeks following surgery. The methods used to deliver rAAV constructs, coordinates of injection and methods for harvest, western blot analysis and *in situ* hybridization analysis of brain tissue have been previously described in detail (Rodriguez-Lebron et al., 2005). High-molecular weight human huntingtin was detected using a 1:500 dilution of Hum-1 antibody (Rodriguez-Lebron et al., 2005). Protein loading was normalized to levels of β-tubulin (1:2000 MMS435P; CONVACE). The distribution of GFP produced from the rAAV constructs was analyzed by direct fluorescence of fresh frozen tissue. Following *in situ* hybridization using probes listed in (Rodriguez-Lebron et al., 2005), sections were stained with cresyl violet for histological analysis (MacGibbon et al., 2002). The sequence of the probe used to detect GFP mRNA expressed from rAAV2 vectors was 5' GCGGAGCGGGGCACGGGGCGAAGGCAGCG 3'. Radio-labeled tissue sections were exposed to film for a period of 2 days to 3 weeks. Multiple exposures were obtained to ensure that the signal had not saturated the film.

Statistical Methods

The quantitative data in this study were analyzed for statistical significance using parametric tests with attempt to keep the experiment-wise error at $p < 0.05$. One way analysis of variance was used to assess significance for the western blot data in figure 4. For the quantitative data presented in figure 6, mean group values of the ratio of the hybridization signal in the injected and control striata for each animal are presented. One-way ANOVA followed by Bonferroni-Dunn post-hoc tests were used to determine statistical significance among treatment groups.

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Figure 1.

(A) Schematic diagram showing the sequences of the 5' coding region of human Htt mRNA targeted by HD6, HD7 and siHUNT-2 (grey area) and two dimensional hammerhead structures of HD6 and HD7. The initiation codon is in bold font. (B) Short radio-labeled RNA targets (13-mer) were incubated *in vitro* with either HD6 (top panel) or HD7 (bottom panel) ribozymes and product formation was monitored at the indicated times using denaturing poly-acrylamide gels. (C) Cleavage of a 1.2 kb human Htt transcript by HD6 (left panel) and HD7 (right panel) demonstrated that both ribozymes could access their target site in the context of a biologically relevant RNA transcript. HD6 (left panel) or HD7 (right panel) were incubated in the presence of the 1.2 kb transcript for either 15 (lanes 2 and 7), 30 (lanes 3 and 8) or 60 minutes (lanes 4 and 9). Lanes 1 and 6 correspond to a 60 min incubation time in the absence of ribozyme and lane 5 represents an overnight incubation of HD6 and the 1.2 kb target. In B and C the open

arrow heads indicate the radio-labeled target and the filled arrow heads indicate the radio-labeled cleavage product.

Figure 2.

(A) Schematic diagram showing the predicted cellular processing of the ribozyme-containing expression cassette. This vector was used to transiently express active HD6 and HD7 ribozymes in cell culture and to generate the AAV particles needed for *in vivo* studies. IRES, internal ribosome entry site; Rbz, coding region for hammerhead ribozyme; Self-cleaving Rbz, internal hairpin ribozyme that post-transcriptionally separates the hammerhead ribozyme and GFP mRNA; GFP, coding region for enhanced green fluorescent protein; ITR, inverted terminal repeat. (B) HD6 and HD7 dose-dependently decreased the levels of overexpressed human Htt mRNA in culture. HEK293 cells were transiently co-transfected with a plasmid expressing the 5' end of human mHtt in a 1:5 (lanes 1, 3 and 5) or 1:10 (lanes 2, 4 and 6) ratio with plasmids expressing either GFP (lanes 1 and 2), HD6 (lanes 3 and 4) or HD7 (lanes 5 and 6). Total RNA was isolated 48 hr post-transfection, and the levels of mHtt and β -actin mRNA were determined by northern blot analysis. The film was over-exposed to show the mHtt mRNA that remained after treatment with HD6 and HD7.

Figure 3.

rAAV5-HD6, rAAV5-HD7 and rAAV5-siHUNT-2 negatively affected transcription in both wild-type and R6/1 mice. The rAAV5 treatment and genotype is indicated on the left of each row of sections. The probes used for *in situ* hybridization analyses are indicated above each column of sections. The sections shown for each probe were isolated from an individual animal and are representative of all animals in the treatment group.

Figure 4.

Levels of mHtt protein were decreased by rAAV5-HD7 in R6/1 mice. The mean levels of Hum1-immunoreactive high-molecular weight mHtt protein for the left (uninjected) and right (rAAV5-HD7) striatum were determined by western blot analysis and expressed as a mean +SEM. The levels of mHtt in the HD7-treated striata was ~70% of that observed in the untreated control striata (n = 4, * = p < 0.05).

Figure 5.

rAAV2-HD6 and rAAV2-HD7 were expressed and negatively effected transcription in wild-type and R6/1 mice. The control ribozyme rAAV2-AAT was injected into the left striatum of each mouse and rAAV2-HD6 (HD6) or rAAV2-HD7 (HD7) ribozymes were injected into the right striatum as indicated on the left of each row of sections. The genotypes of the mice are also indicated on the left. Coronal brain sections were allowed to hybridize with probes for the GFP mRNA expressed from the rAAV2 vector, NGFI-A and DARPP-32. The probe used for *in situ* hybridization analysis is indicated above each column of sections.

Figure 6.

(A–F) Quantitative analysis of the ratio of the hybridization signal (optical density) indicating relative mRNA levels of indicated transcripts following administration of rAAV5-HD6 (HD6 rbz), -HD7 (HD7 rbz), GFP controls (GFP), HD6 and HD7 (HD6+HD7 rbz), siHUNT-1 and siHUNT-2 to R6/1 mice and HD6 and HD7 (wt+mix rbz), siHUNT-1 (WT-siHunt1) and siHUNT-2 (WT-siHunt2) to wild-type mice. The optical density of the hybridization signal for each animal was measured in the left (uninjected) striatum and right (rAAV5-injected) striatum and expressed as a ratio. The histograms show mean group ratios for each transcript as indicated above each graph and the error bars indicate one standard error. *, $p < 0.01$ difference (Fisher's

PLSD) between the treatment group and control animals receiving rAAV5-UF11 expressing GFP only (GFP).

Figure 7.

Schematic summary of the data demonstrating ribozyme- and siRNA-induced off-targeting. This figure contains an over-view of the transcriptional changes that occur after expression of each individual siRNA or ribozyme as indicated. The far right column summarizes the simple conclusion to be drawn from the particular pattern of the ribozyme- or siRNA-induced changes in striatal transcription. These schematics are intended illustrate how the disparate results lead to the conclusion of off-targeting.