SILENCING MUTANT HUNTINGTIN BY RNA INTERFERENCE FOR THE TREATMENT OF HUNTINGTON DISEASE

by

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B.Sc., University of Guelph, 2003

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Medical Genetics)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

JUNE 2008

ABSTRACT

Huntington Disease (HD) is a dominantly inherited neurological disease attributed to a CAG expansion within the HD gene. The HD mutation gives rise to a polyglutamine expansion in exon 1 of the protein huntingtin (Htt). Since the discovery of the HD mutation in 1993, various HD gene mouse models have been developed to contain either fragments or full-length copies of the mutant HD gene. The existence of these HD mouse models enables focused therapeutic testing to develop potential treatments for HD. RNA interference (RNAi) therapy is a targeted gene silencing approach whereby synthetic RNA constructs are shuttled into the cell by viral vectors and used by the cell's endogenous RNAi machinery to silence a gene of interest. RNAi therapy holds promise for mutant huntingtin (muHtt) allele-specific silencing as a treatment for HD. The purpose of this thesis was to develop the tools for pre-clinical testing of RNAi-mediated gene silencing of human muHtt in the YAC128 mouse model of HD. First, AAV serotypes were compared for delivery to striatal neurons, the neurons most affected in HD. From this work AAV serotype 1 was selected as the most effective serotype for construct delivery. Second, synthetic RNAi constructs including short-hairpin RNA (shRNA) and microRNA-based constructs (miR-shRNAs) were compared for silencing of human muHtt expression *in vivo*. Here, miR-shRNAs were found to have increased gene silencing and improved tolerance in avoiding immune activation compared to shRNAs. Alternatively, the shRNAs induced dramatic immune activation and morbidity in some cases. Ultimately these findings will contribute to a pre-clinical trial in YAC128 mice investigating Htt RNAi-mediated gene silencing in the treatment of HD, which is also discussed in this thesis. This future work provides proof-of-principle for muHtt allele-specific silencing as a treatment of HD.

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LIST OF ABBREVIATIONS

AAV- adenoassociated virus

HD- huntington disease

Hdh- murine huntingtin gene

Htt- huntingtin

LV- lentiviral vectors

miR-shRNA- microRNA-based RNAi constructs

muHtt- mutant huntingtin

rAAV- recombinant adenoassociated virus vector

RNA- ribonucleic acids

RNAi- RNA interference

SCA- spinocerebellar ataxia

shRNA- short-hairpin RNA

siRNA- short-interfering RNA

Tu- transducing units

ACKNOWLEDGEMENTS

First off, I'd like to thank my supervisor, Dr. Blair Leavitt, for providing the opportunity and freedom to challenge myself in this scientific field. As a supervisor you acted selflessly in promoting my professional growth and I am very thankful for the experience of working with you. I'd also like to thank my committee members Dr. Cheryl Wellington and Dr. Louis Lefebvre for their insights and clear direction in counseling my thesis project. Further, I'd like to acknowledge the outstanding contributions of Ryan Boudreau and Beverly Davidson from the University of Iowa in beginning this work *in vitro* and providing both the tools and support through my project development.

From the lab bench I would like to thank Ge Lu for his diligence as a technician and his loyal commitment to HD research; his wisdom in science and life made for an enjoyable companionship throughout my studies. I'd also like to thank Scott Neal for his resourcefulness in the lab and his mentorship in guiding my ideas. Finally from the lab I'd like to thank all of its past and present members: Gelareh, Jenny, Austin, Kristina, Kevin, Terri, Lingling, Marion, Jeremy, Malgosia, Jacqui, Danny, and Jen W; your respectfulness as colleagues and generosity as friends shaped my experience into something truly memorable.

Lastly I'd like to extend my infinite thanks to my family: to my sister Melany, who has always been my role model in science; to my other sister Jayne, who is my strongest support; and finally to my parents, for their unconditional belief in my many aspirations.

Chapter 1: Introduction

INTRODUCTION

Huntington Disease is a dominantly inherited neurodegenerative disease with the mutant allele being inherited at a frequency of 1 out of every 2 children in affected families. Symptoms of the disease include motor abnormalities, cognitive dysfunction, and neuropsychiatric disturbances typically arising around 35-50 years of age and progressing to death approximately 15-20 years later [1]. Prevalence of the disease sits around 1:10,000 in some populations of European ancestry, with a 10-fold reduced prevalence rate for individuals of Asian or African descent [1]. To date no effective treatment exists for preventing onset or slowing progression of the disease for individuals carrying the mutant HD allele.

1.1.1 History

George Huntington definitively described Huntington chorea as a distinct disease in 1872. Although chorea, involuntary and jerky movement was described for centuries embracing many different afflictions, its inherited forms were not better delineated until the nineteenth century. Around 1832 an English physician, Elliotson, was the first to suggest its hereditary nature "when it [chorea] occurs in adults it is frequently connected with paralysis or idiotism and will perhaps never be cured. It appears to arise for the most part from something in the original constitution of the body, for I have often seen it hereditary." [2]. Years later, George Huntington described in great length his personal and detailed observations of the disease. An excerpt from his talk published in the *Medical and Surgical Reporter* on April 13, 1872 stated precisely "There are three marked peculiarities in this disease: 1. its hereditary nature; 2. a tendency to insanity and suicide; 3. its manifesting itself as a grave disease only in adult life" [3]. His vivid

account of the disease translated across much of Europe, and gave rise to the name "Huntington Chorea". While its original name is still used today, "Huntington's Disease" or "Huntington Disease" has currently gained more popular, widespread acceptance and the latter form will be used in this thesis.

1.1.2 HD clinical symptoms

The major clinical symptoms of HD stem from its neurological dysfunction affecting motor, cognitive, and psychiatric health. The appearance of an extrapyramidal movement disorder in an individual carrying the HD gene mutation or with a known family history of HD is required for the formal diagnosis of HD. Cognitive and psychiatric dysfunction may occur in HD carriers before the onset of the movement disorder, however, these changes are mild and variable across individuals with HD.

The progressive motor dysfunction seen in HD is comprised of two phases including an increase in involuntary movements, followed by a progressive decline in voluntary movements [4]. The most striking clinical feature of the disease is the involuntary movement known as "chorea" characterized by irregular, jerky movements of the face, limbs or trunk. Dystonia (sustained muscle contraction) also occurs in this stage. Abnormal voluntary movements include bradykinesia (slowed voluntary movements), rigidity, dysphagia (problems swallowing), dysarthria (speech disturbance), and gait disturbance [1]. The motor abnormalities as seen in HD are assessed using a standardized rating system known as the Unified Huntington Disease Rating Scale (UHDRS).

Subtle cognitive changes are reported to occur "presymptomatically" in HD before the development of motor dysfunction and the formal diagnosis of HD. Many early studies have alluded to learning and memory changes in gene carriers of HD [5, 6]. More recent studies evaluating larger (n=200-500) sample groups have provided confirmation of these cognitive changes [7, 8]. These presymptomatic cognitive changes

are variable and mild, only proving consistent in persons near clinical diagnosis of HD in the studies by Paulsen *et al.* (2001). In contrast, when there is a clinical diagnosis of HD, cognitive defects tend to progress at a similar rate to motor disturbances. Deficits in procedural learning [9], attention and executive function [10], memory recall [11], and reduced mental flexibility [12] are all apparent in symptomatic HD patients.

Neuropsychiatric problems are also evident in presymptomatic HD gene carriers showing increased affective disorders including depression, anxiety, and obsessive-compulsive disorder [13]. Some studies, however, have failed to repeat these findings [14] suggesting that, as is the case with cognitive changes, these affects may be subtle early on. As motor abnormalities increase in HD patients, neuropsychiatric problems can become more pronounced although highly variable in onset and severity throughout the course of the disease. Affective disorders such as depression, anxiety and irritability occur frequently, affecting 50% of patients in one study [15], while psychiatric symptoms (hallucinations, delusions) are rarely reported. Behavioural problems include loss of energy and initiative, poor perseverance and quality of work, impaired judgment, poor self-care and emotional blunting [15].

1.1.3 HD genetics

HD is a simple monogenic disease exhibiting autosomal dominant inheritance with features of anticipation, most commonly when inherited through the paternal germline. The mutation causing HD was found in 1993 by the HD Collaborative Research Group analyzing a large Venezuelan pedigree affected by the disease [16]. The HD mutation, a large CAG expansion imbedded near the 5' end of the HD gene (originally known as IT15) gives rise to a polyglutamine expansion in exon 1 of the protein huntingtin (Htt). Polyglutamine expanded mutant huntingtin (muHtt) appears to have altered physical and functional properties leading to a dominant toxic gain-of-

function. Additionally, the mutation may disrupt certain aspects of the protein's wildtype function, such as its neuroprotective role in brain, leading to a loss of function mechanism which may also contribute to HD pathogensis [17].

CAG tract length is polymorphic in both normal and HD affected individuals. A tight range, between 9 and 35 CAG repeats, exists for the normal allele in unaffected individuals while in HD carriers mutant alleles with greater than 35 CAG repeats (with upwards of 250 CAG repeats in juvenile HD [18]) is known to give rise to the disease. Longer CAG tracts are associated with an earlier age of onset and increased disease severity [19]. CAG repeat sizes between 35 and 39 are associated with very late onset, leading to reduced penetrance for this repeat range [20]. While CAG repeat sizes between 27 and 35 known as intermediate alleles are not associated with developing HD, however, they may expand into the pathogenic range during intergeneration transmission giving rise to an affected offspring [21]. Paternal transmission of the CAG repeat results in a +7.3 CAG repeat expansion on average intergenerationally [22] leading to the clinical phenomenon of anticipation. Fewer cases of maternally transmitted intergenerational expansions have been reported [23, 24]. The majority of juvenile cases of HD occur due to the paternal transmission of an expanded allele [25].

1.1.4 Neuropathology

The most dramatic pathologic feature of HD is a prominent atrophy of the caudate nucleus and putamen (collectively known as the striatum). Atrophy of the globus pallidus is also apparent, while cortical atrophy is subtle macroscopically [26]. Brain volume measurements using MRI reflect similar changes in HD brains measuring the greatest volume loss in the putamen (53%), caudate (37%), globus pallidus (41%), nucleus accumbens (41%), and amygdala (24%). Slight changes are seen in the hippocampus (9%) and cerebral white matter (13%), while regions like the cerebellum and thalamus

were relatively spared [27]. Overall it can be concluded that in HD, muHtt protein possesses a region-selective toxicity, affecting areas such as the striatum and globus pallidus early in disease course, leaving other areas of the brain relatively untouched until the end-stage of disease.

Vonsattel *et al.* (1985) established a grading system for the progressive neuropathological changes seen in HD. Analyzing 163 patients at autopsy they described 5 distinct pathological grades ranging from grade 0 early in the course of disease to grade 4 where up to 95% neuronal loss is incurred. Grade 0 brains show early 'islands' of neuronal loss and astrocytosis in the dorsoventral caudate and putamen [28]. Grade 1 brains show neuronal loss beginning in the dorsomedial 'tail' of the caudate and dorsal putamen, while neuronal loss spreads ventrally in grades 2 and 3 brains to accumulate almost 50% neuronal loss. Grade 4 brains show massive neuronal loss (up to 95%) and gliosis in the striatum with moderate gliosis in the nucleus accumbens [29]. Of the cells affected in the striatum, GABAergic medium spiny projection neurons are the most predominatly affected and form the majority of cells in the striatum [30, 31].

By light microscopy, nuclear and cytoplasmic intracellular inclusions have been identified in post-mortem human HD brain [32] and transgenic mice [33]. These inclusions, originally identified in the transgenic mice, are insoluble, ubiquitinated protein aggregates that have been shown to sequester a variety of cellular proteins. Although these inclusions have been implicated in toxicity in HD [34], other findings have suggested a protective role [35, 36] for these inclusions leaving their relevance in the course of disease pathogenesis up for debate.

1.1.5 HD mouse models

HD full-length models

Full-length models contain an expanded human HD transgene in addition to endogenous murine Hdh. One mouse line was created using a genomic yeast artificial chromosome (YAC) clone spanning the complete human HD gene, including introns and endogenous promoter (~25 kb of upstream regulatory sequence). Transgenic mice expressing full-length mutant HD cDNAs have also been generated including HD48 and HD89, expressed under a constitutive CMV promoter [37], and an inducible mouse model using a tetracycline regulated promoter in brain (PrP-tTA- 6/iFL148Q) [38].

The best-characterized and most-widely used full-length mouse model is YAC128. Of the YAC mouse lines generated, this model displayed the earliest and most pronounced phenotype stimulating its application to pre-clinical therapeutic trials. YAC46 and YAC72 mice showed relatively weak, late-onset behavioral changes beginning at 7 months of age and subtle neuropathological changes by 12 months discouraging its use in pre-clinical trials [39]. Alternatively, YAC128 shows a spectrum of measurable behavioral, cognitive, motor, and neuropatholgical changes amenable to its use in therapeutic testing. Behavioral changes occur first at 3 months of age, exhibiting first as hyperactivity and progressing to hypoactivity by 12 months. Motor deficits are evident on the rotarod beginning at 6 months of age and progressively worsen with age. Neurodegeneration of specific brain regions also develops in YAC128 mice with striatal atrophy beginning at 9 months of age and cortical atrophy arising by 12 months [40]. These quantifiable motor and neurodegenerative changes make the YAC128 line a particularly suitable model for pre-clinical therapeutic testing.

Importanty, HD full-length models replicate the selective neuropathology observed in human HD. HD48 and HD89 mice were the first full-length models reported to show selective neuronal loss in the brain suggesting that having the polyglutamine expansion within the context of the full-length Htt protein, as opposed to a N-terminal Htt fragment (see next section), is important to the selective neuronal loss seen in HD brains [37]. This phenomenon was better characterized later in YAC128 mice where neuronal loss showed its greatest effects in the striatum, with less severe neuronal loss in the cortex, and virtually no effect in regions usually unaffected in HD such as the cerebellum [40, 41]. Furthermore, an abnormal nuclear accumulation of Htt was shown to obey this same regional selectivity pattern with changes occurring as early as 2 months of age in YAC128 striatum [41].

Despite the relevance of their neuropathology to human HD, full-length HD mouse models display weaker phenotypes relative to N-terminal mouse models. This makes for longer pre-clinical trials (average 12 month trials), less dramatic outcome measurements, and wider mouse-to-mouse variability demanding larger testing cohorts (15 mice per treatment group) to reach statistical significance. Overall this increases the time and money required for each pre-clinical trial, hindering many labs from using this model for therapeutic testing.

HD fragment models

Fragment models were designed to express an N-terminal portion of the human HD gene containing an expanded CAG stretch within exon 1 in addition to endogenous murine Htt. Numerous N-terminal Htt fragments have been identified in HD brain to suggest an important pathogenic role of Htt fragments in HD. The strong neurological phenotype seen in fragment models provides interesting evidence to suggest that a

truncated N-terminal portion of mutant Htt can replicate certain aspects of the HD disease process [42]. Subsequently, a variety of HD fragment models have been generated extending over exons one and two of the htt gene to include the multiple cleavage sites (calpain, caspase, etc) concentrated around the N-terminal region of the protein [42-45].

The best-characterized N-terminal mouse models are the R6/2 and N171-82Q models, which are routinely used in pre-clinical therapeutic drug trials. The R6/2 mouse model contains HD exon 1 carrying a $(CAG)_{130}$ expansion and promoter sequence (~1kb of 5' UTR). The N171-82Q mouse was created with a slightly longer N-terminal Htt fragment than the R6 line containing 82 CAG repeats. N-terminal mouse models display the earliest disease onset and most rapid disease progression of any of the HD mouse models created. Both R6/2 and N171-82Q (line 81 and 100) exhibit HD-like features including motor deficits, severe weight loss, reduced brain weight, neuronal intranuclear inclusions, and premature death [33, 42, 44, 46-48]. In R6/2, the early onset of phenotypic abnormalities (motor deficits at 5-6 weeks) and premature death (starting at 10-13 weeks) enable the rapid completion of therapeutic trials in as few as 3 months [42]. The early and robust phenotype seen in these mice also gives rise to low inter-animal variability permitting small testing cohorts [49, 50]. Despite its advantages in pre-clinical trial design, N-terminal mouse models do not display certain neuropathological features evident in HD. For example the reduction in brain size and nuclear inclusion formation in these animals affects all CNS structures disagreeing with the predominant striatal cell loss seen in human patients [41, 42, 49]. In addition, this model does not allow the study of early pathogenesis related to the cleavage of muHtt.

Additionally a conditional N-terminal mouse model, HD94-tet, was constructed that is useful in investigating the reversibility of the disease process (by shutting down

muHtt fragment expression) at different time-points in the disease. The Tet/HD94 conditional mice were generated containing a chimeric murine/human huntingtin exon 1 fragment including 94 CAG repeats under a tetracycline regulated promoter [45]. Although not used in drug testing, these mice were critical in establishing the therapeutic potential of shutting-off the mutant HD transgene both early in disease-onset [45] and late in disease progression [51]. Even in late-stage disease progression mice showed a 25 % reduction in striatal neuronal loss and a complete recovery in motor function given a reduction in muHtt expression [51]. These mice showed promise for any therapeutic trials based on silencing the mutant HD gene.

HD knock-in mouse models

Murine and human Htt exhibit high conservation being 86% identical at the DNA level and 91% identical at the protein level [52]. As a result, multiple knock-in mouse lines were created by homologous recombination of either complete or partial human HD exon 1 containing expanded CAG repeats between 50-150 units with the mouse Hdh gene [53-58]. This model is considered the most genetically accurate mouse model of HD existing in either the heterozygous or homozygous state. Of the mouse lines created with <80 CAG repeats no neuropathological or behavioural changes were noted [53, 54, 56, 58]. Mouse lines with the largest CAG expanded tracts, >140 CAG repeats, including CAG140 and CHL2 mice exhibit the most robust phenotype of all knock-in mice and for this reason are the most thoroughly characterized mice.

CAG140 or CHL2 mice both show progressive behavior abnormalities. CAG140 mice have early hyperactivity preceding hypoactivity at 4 months of age and gait anomalies by 12 months of age. A proportion of homozygous CHL2 (Hdh (CAG)150/150) mice show inactivity, hind-limb clasping, and motor dysfunction on the

rotarod before 10 months of age. A similar proportion of heterozygous CHL2 mice show these symptoms but at a later time-point before 20 months of age [54]. Neuropathological observations of knock-in mice have revealed no visible loss of brain regions or decline in neuronal counts [54]. Most knock-in models with >80 CAG expansions have however, identified prominent diffuse nuclear Htt accumulation with age, and the subsequent formation of inclusions (ubiquitin and Htt positive) in the striatum, nucleus accumbens, and layers of the cortex [54, 55, 57]. This selective pattern of nuclear Htt accumulation parallels that seen in full-length HD mouse models confirming that the full-length expanded form of Htt is necessary for the selective pathology seen in HD.

The weak phenotype observed in these mice was initially disappointing because this model exists as the most accurate genetic model of HD. Subsequently to its earliest characterizations, however, the development of knock-in mice with larger CAG tracts has proven very useful for the investigation of the early pathogenic events in HD. Additionally the knock-in model, existing in either the homozygotic or heterozygotic state, has provided evidence for increased HD age of onset and symptomatic severity provided increased muHtt dose. These findings agree with recent human data showing increased disease severity in patients homozygotic for the mutant HD allele [59]. In terms of pre-clinical trials, while these mouse models are used for compounds testing they are not as widely used as the full-length or N-terminal mouse models.

1.1.6 Wildtype huntingtin function

While many of the models discussed above have been created with the purpose of studying the role of muHtt in HD, an alternative approach such as knocking-out the HD gene in mice is useful for studying the function of wildtype Htt. Complete loss of Hdh in mice results in embryonic lethality [60], however, this lethality can be rescued by the

presence of Htt in the extraembryonic tissue suggesting a critical role of Htt in development [61]. Adult Hdh+/- chimeras show neurological dysfunction [62], while conditional Hdh knock-outs limited to the forebrain and testis show progressive neuronal degeneration and sterility (Reiner *et al.* 2003). Together this line of evidence suggests that wildtype Htt has a functional role in the adult brain, and that a reduction of Htt levels below 50 % endogenous levels would be harmful in the adult. YAC128 transgenic mice lacking endogenous Htt (YAC128-/-) show increased motor abnormalities and decreased survival with no observable effects on striatal volume or neuronal counts [63]. This shows that wildtype Htt also plays a subtle protective role during the course of the disease to improve the motor phenotype and survival. These mouse lines illustrate the necessity of maintaining wildtype Htt's expression and prosurvival function throughout life. Therefore, RNAi therapeutic strategies aimed to silence the mutant HD allele should demand an HD allele-specific therapy in order to selectively silence the mutant HD gene.

1.1.7 Methods of gene therapy (gene replacement, gene silencing)

The possibility of altering or replacing the expression of a defective gene with gene-targeted therapy presents an obvious target for the treatment of Huntington disease and many other genetic diseases. Early gene therapy research employing gene replacement strategies in loss-of-function diseases such as severe compromised immunodeficiency (SCID), cystic fibrosis, and Duchenne muscular dystrophy have shown us the clinical potential of gene therapy as well as warned us of its possible pitfalls (reviewed in [64, 65]). SCID stands as the most successful gene therapy clinical application to date whereby a cytokine receptor γ chain gene is delivered via retroviral delivery to restore lost T cell function and the immune system. Recent clinical studies on 20 infants with X-linked SCID (SCID-X1) [66, 67] and 13 patients with Adenosine

deaminase deficiency (ADA-SCID) [68, 69] have shown successful gene replacement and T cell recovery. While these clinical outcomes are encouraging, 4 patients with SCID-X1 later developed leukaemia-like complications as a result of insertional mutagenesis by the carrier retrovirus [70]. This outcome has motivated on-going research into gene carrier vectors that do not rely on genome integration for gene expression such as Adeno-associated virus vectors (AAV) and integration deficient Lentiviral vectors (LV).

Viral-mediated gene delivery of neurotrophic factors has been tried in proof-of-principal studies in HD mouse models. Viral delivery of trophic factor genes such as brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF), and ciliary neurotrophic factor (CNTF) are proposed to counteract the neurodegeneration seen in HD by providing enhanced neuroprotection. Studies supporting this claim include neuroprotection seen after acute QA-mediated striatal neurotoxicity given pre-treatment with either rAAV-delivered BDNF or GDNF [71]. Further studies in the N171-82Q mouse model saw improvements in neuropathology and motor function with rAAV2-GDNF [72]. Alternatively, LV delivery of GDNF to the R6/2 model saw no improvement [73]. Differences between viral vectors, GDNF expression levels, or mouse models may account for the inconsistent findings here.

A second method of gene therapy, opposite in approach to gene replacement, is targeted gene silencing whereby short, anti-sense RNAs (ex. siRNAs, shRNAs, etc) are used to selectively target viral or disease-causing mRNAs for degradation in the cell (see 1.1.8 RNAi pathway in mammals). This approach would be effective for the treatment of dominant gain-of-function disorders such as HD, certain neurological disorders, viral infection, and cancers. In neurological disorders such as Alzheimers disease, siRNAs have been tested for proof-of-principal to silence genes associated with disease pathology

such as β-secretase (BACE). BACE is an enzyme known to cleave amyloid precursor protein contributing to amyloid plaque formation and Alzheimer's-related degenerative changes. LV-mediated delivery of shRNAs targeting BACE saw reduced amyloid deposition in affected brain regions of transgenic mice, with full recovery of spatial learning and memory [74]. In dominant disorders, siRNAs could be used to silence disease alleles directly precluding either gain-of-function or dominant negative mechanisms leading to disease.

1.1.8 RNAi pathway in mammals

The ability to effectively silence defective or "toxic" genes in the body holds promise for the treatment of many dominant, gain-of-function diseases. In 1998, researchers uncovered a potent pathway for regulating gene expression at the mRNA level known as RNA interference (RNAi) [75]. This pathway, which is conserved from plants to humans, uses small (~21-23 nucleotides), double-stranded RNA (dsRNA) molecules to target specific mRNAs for degradation or translational arrest.

In mammals, the RNAi pathway consists of a series of processing events (Fig. 1.1) [76]. Depending on the source of dsRNA (endogenous or exogenous), the processing events can begin in the nucleus with Drosha cleaving endogenous microRNA (miRNA) precursors, or in the cytoplasm with Dicer cleaving synthetic short-hairpin RNAs (shRNA). Ultimately, irrespective of the origin of the dsRNA, the RNAi pathway generates a double-stranded small interfering RNA (siRNA) (~21-23 nucleotides) as its final cleavage product. At its final stage in the RNAi pathway, this double-stranded siRNA is incorporated into the RNA induced silencing complex (RISC). Inside RISC, the siRNA is unwound and a single-strand (either sense or anti-sense) retained to target exactly or partially complementary mRNA transcripts for either mRNA degradation or translational repression.

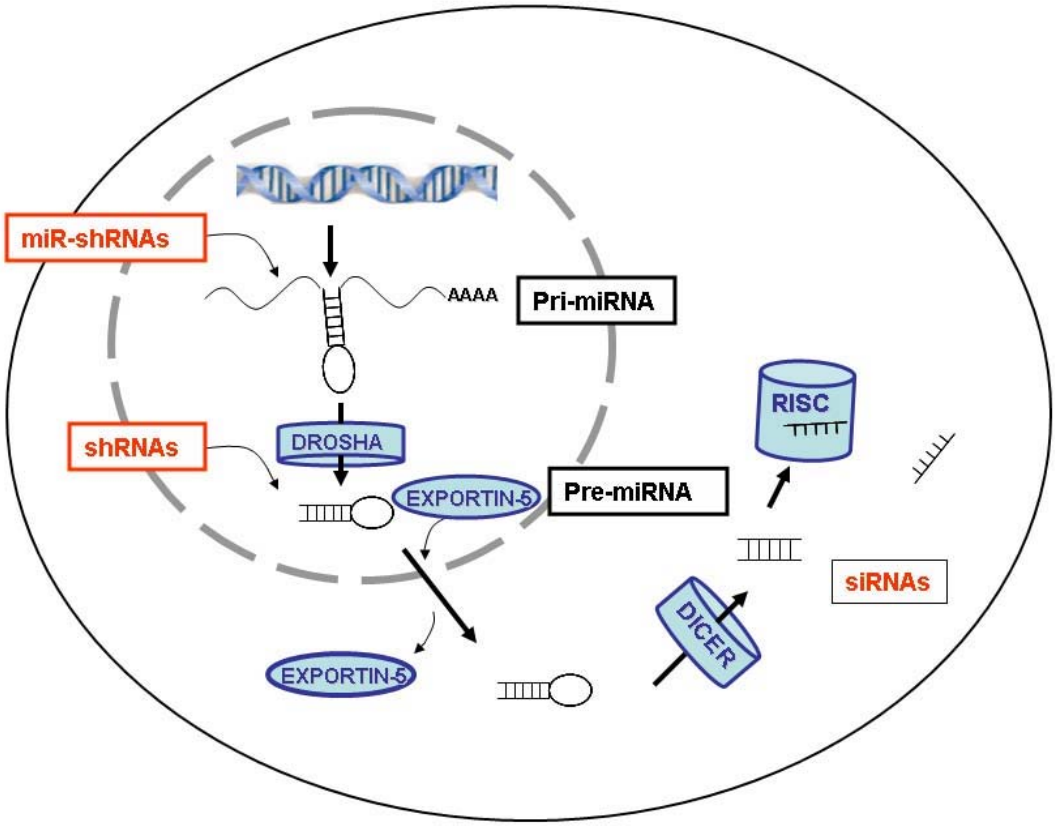

Figure 1.1: RNAi pathway in mammals

Endogenous microRNAs pass through a series of processing steps in the cell from early transcription in the nucleus to incorporation of the mature miRNA into the RISC complex. Synthetic RNAi triggers can enter this pathway at various points including early as miR-shRNAs, midpoint as shRNAs, or late as double-stranded siRNAs. For simplicity, only key enzymatic factors are depicted.

The degree of complementarity of the anti-sense RNA determines whether the RNAi pathway directs either mRNA degradation or translational repression [77]. In vertebrates, endogenous miRNAs have mismatches that permit either translation repression or mRNA degradation to occur from the 3' end of the transcript. Alternatively, synthetic siRNAs with the exact complement to its target sequence will only direct mRNA degradation from where the anti-sense RNA molecule binds the target mRNA.

The existence of the RNAi pathway in mammals has made it a very attractive mechanism for "turning off" disease-related genes using synthetic RNAi constructs. Synthetic RNAi construct designs include siRNAs [78], short-hairpin RNAs (shRNA) [79], and microRNA-based RNAs (miR-shRNA) [80], which have thus far been tried. An increased understanding of the RNAi pathway in mammals partnered with advances in the design of synthetic RNAi constructs [78, 79] continues to provide momentum to the use of RNAi-based therapeutics in disease.

1.1.9 Viral delivery

For the treatment of brain diseases, administering naked siRNA or shRNAs systemically is rather futile due to restrictions by the blood brain barrier and the rapid degradation of nucleic acids *in vivo* by endonucleases. To by-pass these limitations, direct delivery of small RNA constructs to the brain by stereotaxic injection is used. Long-term, stable expression of these small RNA constructs can be achieved by using viral vectors such as recombinant AAV.

Recombinant AAV vector (rAAV) is derived from the naturally occurring virus Adenoassociated virus (see 2.1 Introduction). rAAV has become a very popular vector for gene therapy in the brain provided its ability to transduce dividing and non-dividing cells such as neurons [81]. AAV also presents low immunogenicity and no pathogenicity in mammals proving safe for translating these trials to humans in the future [82]. Long-term expression of rAAV in mammals has also been demonstrated proving its efficacy in providing extended treatment *in vivo*. For example, rAAV has shown gene expression activity for up to 19 months in rat brain [83] and up to 6 years in primate brain [84]. Further AAV persists predominantly in an episomal form in humans [85]. This characteristic of expressing itself episomally distinguishes it from another popularly employed vector, Lentivirus. Lentivirus is derived from HIV retrovirus posing the risk of

integrating into the genome via the integrase protein. Non-specific integration can be problematic risking insertional mutagenesis and possible malignant transformations as was the case with another retrovirus [70].

Although rAAV provides many advantages as a gene therapy vector, some distinct disadvantages limit its application and use. rAAV has a small carrying limit of 4.7-5 kb [86]. This carrying capacity limits rAAVs use to delivering small genes such as BDNF and GDNF, or delivering small RNA constructs for RNAi. Another disadvantage to using rAAV is in its translation to human clinical trials. Over 80% of the human population is positive for an antibody against AAV serotype 2 [86]. This pre-existing immunity was proposed to lead to the termination of rAAV transgene expression shortly after treatment in a phase II clinical study of hemophilia B [87]. It is proposed that cell-mediated immunity targeting antigens of the AAV2 capsid directed removal of transduced hepatocytes and the decline of transgene expression [87]. This finding has motivated on-going investigation into alternative AAV serotypes, such as AAV1-8, for use in humans.

1.1.10 Gene silencing and polyglutamine disorders

Polyglutamine disorders are caused by an expanded polyglutamine tract within its associated protein and typically show widespread pathogenicity within the cell. Unfortunately, mechanisms underlying the toxic nature of these mutant polyglutamine proteins are poorly understood. RNAi provides a useful therapeutic strategy by degrading the mutant mRNA strand upstream of protein synthesis therefore removing the toxic entity causing disease.

Conditional mouse models of HD and Spinocerebellar Ataxia 1 (SCA1) have shown that shutting off the expanded polyglutamine protein, even into symptomatic onset, will recover health and neuropathology of transgenic mice. A tetracycline-

regulated mouse model of HD (Tet/HD94) carrying exon 1 of the HD gene with 94 CAG repeats was turned off at either 5 or 17 months in post-symptomatic mice [45, 51]. Both groups showed symptomatic recovery where even the later cohort saw slowed striatal neuronal loss, a 90% decrease in htt aggregate formation, and complete recovery in motor function on the rotarod. The conditional SCA1 mouse model also showed clinical efficacy in turning off the mutant gene early in disease. In this case, the SCA1 mice showed almost complete recovery in Purkinje cell pathology and motor function. Shutting the gene off late in disease saw similar but lesser effects [88]. Overall, both HD and SCA1 conditional models showed symptomatic recovery *in vivo* indicative of the potential for RNAi in these diseases.

The first *in vivo* evidence that RNAi could be feasible in dominant disorders was shown in a mouse model of SCA1 [89]. In this case, the SCA1-82Q mouse model received rAAV1 delivery of a short-hairpin RNA targeting mutant ataxin-82Q. The treatment, which only targeted 5-10 % of cerebellar Purkinje cells, saw notable improvements in neuropathology including a reduction of neuronal inclusions and reduced thinning of the cerebellum molecular layer. Further the animals showed improved motor performance.

Despite the growing *in vivo* proof that RNAi shows efficacy in treating SCA1 and other polygluatmine disorders, one limitation in the field is the ability to selectively target the disease allele while avoiding its wildtype copy. Given the existence of repetitive CAGs in both wildtype and mutant alleles, little sequence specificity distinguishes the two alleles for targeted silencing. *In vitro* siRNAs have been designed to target allelic polyglutamine expanded mutations indirectly by targeting SNPs in linkage disequilibrium with the disease mutation. One example is Machado-Joseph disease (MJD) where a C/G polymorphism distinguishes 50% of wildtype and mutant alleles in humans [90]. In this

case, siRNAs effectively targeting the C polymorphism in the mutant alleles saw selective silencing *in vitro* with modest effects on the wildtype allele [91].

1.1.11 Hypothesis and research objectives

Hypothesis: rAAV-mediated RNAi constructs will effectively silence human mutant Htt in the YAC128 mouse model of HD.

The research objectives for this project include:

- Identifying an AAV serotype for delivery to striatal neurons
- Testing RNAi constructs for silencing human mutant huntingtin expression *in vivo*
- Developing a tool for pre-clinical testing of RNAi-mediated gene silencing of human mutant huntingtin in the treatment of HD

Chapter 2: Comparing AAV1 and AAV5 serotypes for construct delivery to neurons

2.1 INTRODUCTION

AAV is potentially useful as a gene therapy vector for brain diseases due to its ability to transduce non-dividing cells [81]. AAV's predominant episomal expression does not require it to integrate into the genome providing a unique advantage to retrovirus and HIV-derived Lentivirus. Other advantages to AAV include its low immunogenicity and lack of pathogenicity in mammals [82].

Recombinant AAV vectors (rAAV) have 96% of the AAV genome removed leaving only its two inverted terminal repeats as vector backbone. Replication of rAAV is done *ex vivo* using a recombinant AAV helper plasmid to provide all the replication and packaging potential. This helper plasmid packages rAAV into a specific capsid protein to facilitate its entry into the cell [92]. To date, AAV has 11 known serotypes (AAV1-AAV11) which are distinguishable based on their capsid proteins [93].

Until recently, most research conducted using rAAV as a gene therapy vector used AAV serotype 2. The observation that AAV serotype may influence transduction prompted researchers to compare AAV serotypes for transducing specific brain regions and cell types. Early results showed distinct differences between transduction efficiency and cell tropism between AAV serotypes 1, 4, and 5 [94]. AAV5 serotype showed in some cases 5000 times better transduction than AAV2 serotype in mouse brain confirming significant serotype-related differences [94]. The ability to cross package AAV type 2 terminal repeats with different AAV capsids enabled more controlled experiments in comparing vector serotype differences using identical viral genome content. Using these new methods, both AAV1 and AAV5 serotypes showed improved

transduction efficiency relative to AAV2 serotype in different brain regions in rat [95] and mouse [96].

In this work, I compared the transduction efficiency, cellular tropism, and safety of AAV1 and AAV5 serotype delivery in mouse brain. The goal of this work was to identify an effective viral vector for RNAi pre-clinical trials in the YAC128 Huntington Disease mouse model.

2.2 MATERIALS AND METHODS

2.2.1 rAAV vectors

Recombinant AAV vectors contain reporter gene sequence flanked by AAV2 terminal repeats with all other viral genes removed. The reporter sequence includes CMV-humanized *Renilla* GFP (hrGFP)-simian virus 40 poly(a) reporter cassette for GFP fluorescence detection. Vectors are packaged inside either AAV serotype 1 or 5 capsid proteins creating unique vector constructs. These constructs are known as rAAV2/1 and rAAV2/5 respectively, however, for simplification in this study will be known as AAV1 and AAV5 to emphasize the serotype comparison being made. All vectors were prepared at the University of Iowa Gene Transfer Vector Core.

2.2.2 Surgical procedures

Bilateral striatal injections administered by stereotaxic injection to FVB/N mice at 2 months of age. Injection coordinates were 1.8 mm anterior, 0.8 mm lateral, and 3.5 mm depth using 5 uL (~10^7 Tu) per striata at an infusion rate of 250 nl/min. Brains were post-fixed in 3 % paraformaldehyde 1 month following injections and prepared as 25 μm sections for all analysis.

2.2.3 Transduction efficiency

Brain sections were quantitatively analyzed for total GFP expression area and fluorescence intensity using Metamorph imaging software. 25 μm rostral-caudal brain sections spanning 200 μm of the striatum were analyzed for each animal and AAV serotype (n=3).

2.2.4 Immunofluorescence and histology

Immunoflourescence co-localization studies were conducting using NeuN antibody (Chemicon MAB377; 1:500) for labeling neurons, GFAP antibody (DAKO Z0334; 1:3000) for labeling glia, and fluorescent-labeled secondary antibodies (1:1000 anti-mouse Alexa Fluor 594 and 1:500 anti-rabbit Alexa Fluor 594 respectively) for visualization. Cresyl violet staining was done for 3 minutes for histological analysis.

2.2.5 Statistical analysis

A Students t-test was performed to determine differences in the means between various treatment groups, and Levene's test to determine equality of variance. Differences were assumed statistically significant given they reached the 95 % confidence level. All values are represented as averages with standard error of the mean.

2.3 RESULTS

2.3.1 AAV5 serotype displays increased transduction area

Recombinant AAV vector packaged inside either AAV1 or AAV5 capsid proteins represents unique vector serotypes for transduction comparisons. FVB/N mice were striatally injected with AAV serotypes, AAV1 or AAV5, for comparison of vector distribution through the striatum. rAAV vectors were administered at equivalent titer and volume and subsequently evaluated for transduction efficiency and fluorescence intensity after 3 weeks incubation. To maximize vector distribution through the entire striatum

total injection volumes were chosen to be 5 μL. Both rAAV1 and rAAV5 showed consistent injection distributions through the striatum according to active GFP positive expression in rostral to caudal brain sections. Comparison of serotype transduction efficiency quantitatively by electronically tracing GFP positive regions found rAAV5 to have a greater transduction area through the striatum than rAAV1 (Fig. 2.1) suggesting improved distribution of rAAV5. Comparison of fluorescence intensity per unit area of injected regions did not show a significant difference between the two serotypes suggesting a roughly equivalent transduction to individual cells (data not shown).

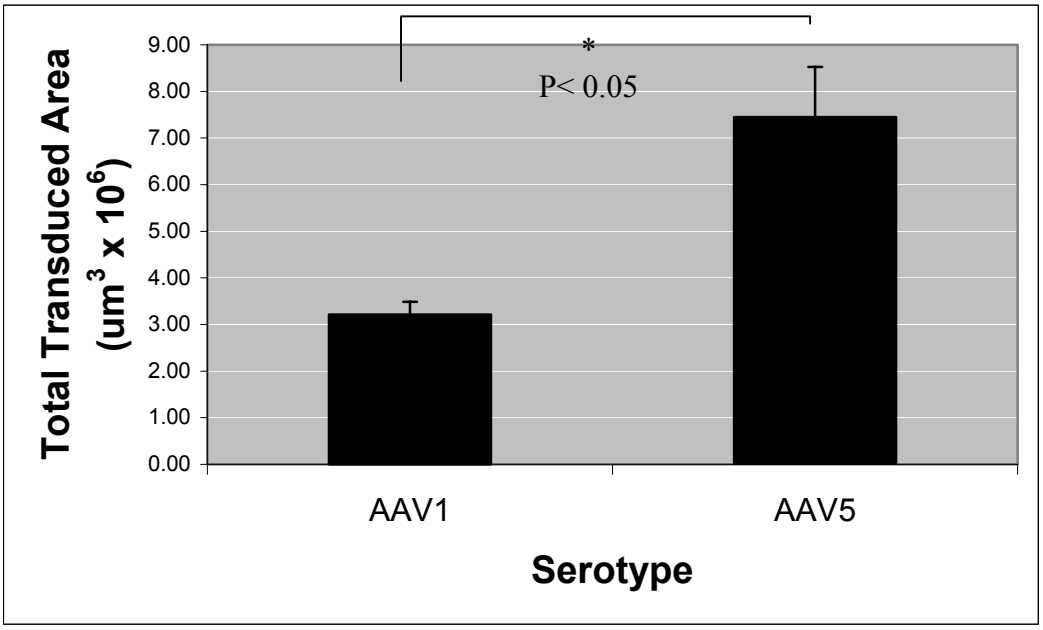

Figure 2.1: AAV5 displays increased transduction area.
Tracing total GFP positive area 3-weeks post-injection AAV5 (7.45 $\pm$ 0.62) shows an increased transduction area relative to AAV1 (3.21 $\pm$ 0.16 x 10^6) ($P<0.05$).

2.3.2 AAV1 serotype shows improved cellular tropism for neuronal cell types

AAV serotypes were compared for cellular tropism by co-localization with either neuronal or glial cell type markers. Identical brain sections were visualized for GFP-labeled virus and either red-labeled NeuN antibody to denote neuronal cell types or GFAP antibody to identify glial cell types. Image overlays showed that rAAV1

predominantly transduced neurons (Fig. 2.2A), while glial cell transduction was undetected (data not shown). Oppositely, rAAV5 showed neuronal transduction limited to marginal injection boundaries where viral expression was low (Fig. 2.2B), whereas transduction of glial cell types was more ubiquitously apparent (Fig. 2.2C). Due to an interference of NeuN labeling in areas of high rAAV5 transduction (see below), GFP positive cell types were identified according to cell morphology in these areas. In this case, GFP positive cells showed small cell bodies with a high number of processes representative of astrocyte cells.

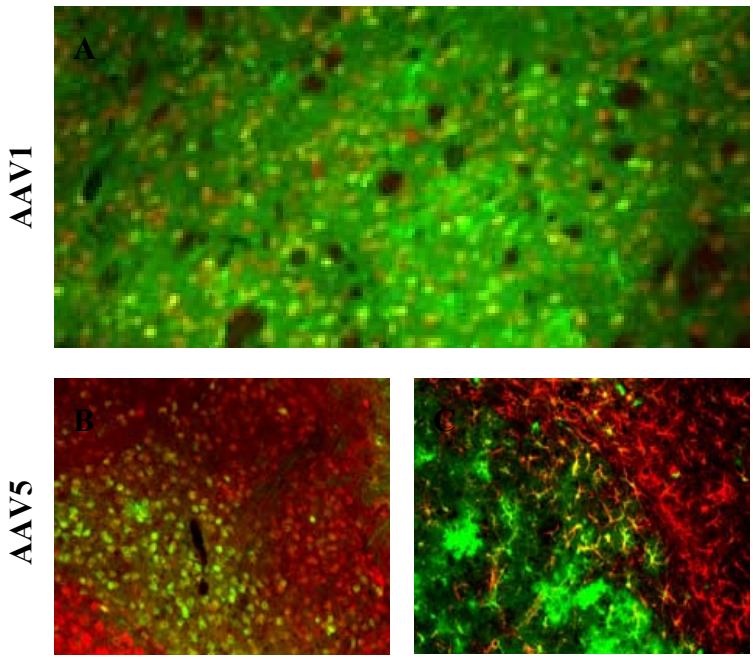

Figure 2.2: AAV1 shows improved cellular tropism for neuronal cell types.

A. Immunohistochemistry labeling neurons with NeuN antibody (red) and GFP-labeled AAV1 virus shows AAV1 predominantly transducing neuronal cell types. **B**. AAV5 shows co-localization of NeuN and AAV5-GFP only where transduction of neuronal cell types is low. **C.** Labeling of glial cells with GFAP antibody (red) shows transduction by AAV5. 20X magnification.

2.3.3 AAV5 serotype treatment induces adverse effects not evident with AAV1

Unexpectedly, areas showing high rAAV5 viral expression lacked NeuN labeling suggesting a loss of neurons in these regions or possible interference of this vector serotype with NeuN antigen expression. Cresyl violet staining of rAAV1 (Fig. 2.3C) and

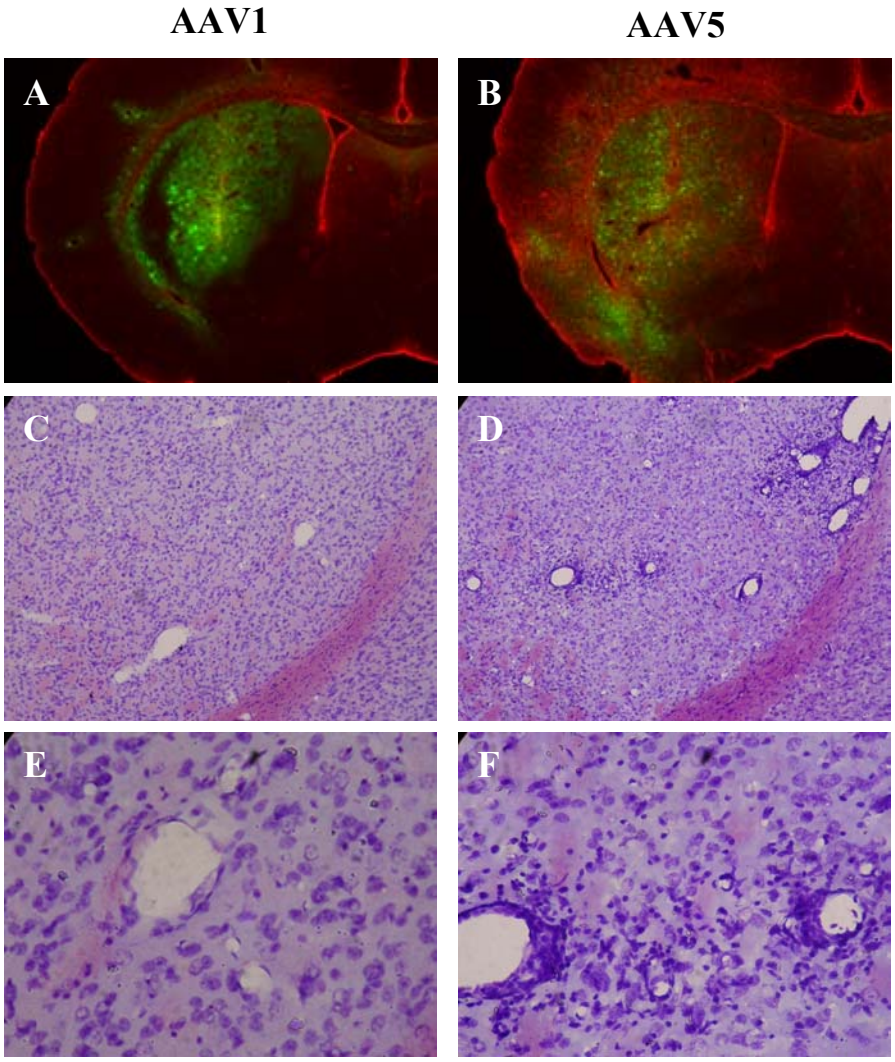

Figure 2.3: AAV5 induces adverse effects not evident with AAV1.

A, B. Labeling of reactive astrocytes with GFAP antibody (red) shows increased astrocytosis in AAV5-GFP injected hemispheres (B) relative to AAV1-GFP injected hemispheres (A). **C,E.** Cresyl Violet staining shows normal brain cellularity in AAV1 injected hemispheres at 10 X (C) and 40 X (E) magnification. **D,F.** In AAV5 injected hemispheres increased cellularity is evident around blood vessels (arrows) at 10 X (D) and 40 X (F) indicative of an immunological response.

rAAV5 (Fig. 2.3D) injected brain sections revealed normal neuron distribution and size in injected areas supporting the hypothesis that NeuN antigen expression was being affected by high rAAV5 viral load. Increased astrocytosis as indicated by increased GFAP-labeling was also observed in rAAV5 injected areas (Fig. 2.3B) in comparison to rAAV1 (Fig. 2.3A) further indicating toxicity attributed to rAAV5. Also, cresyl violet staining showed massive cellular infiltration around blood vessels in rAAV5 injected areas (Fig. 2.3F), not present in rAAV1 treated brains (Fig. 2.3E).

2.4 DISCUSSION

In this study, AAV1 and AAV5 serotypes were compared for transduction efficiency, cellular tropism, and safety in determining a proper vector for RNAi delivery to mouse striatal neurons. Results showed that AAV1 serotype appeared to be an effective and safe vector showing a high cellular tropism for striatal neurons and no obvious signs of toxicity up to 3 weeks post-injection. AAV5 serotype on the other hand proved ineffective, showing low transduction of neurons, and high transduction of glial cell types. AAV5 serotype also caused obvious signs of toxicity including astrocytosis, cellular infiltration around blood vessels, and interference with neuronal antigen expression (NeuN) in injected regions.

Two other reports similarly compare AAV serotype transduction (using AAV2 terminal repeats) in rodent brain. In rats, comparable transduction efficiency and cellular tropism was seen between rAAV1 and rAAV5 in striatum [95] with both serotypes showing relatively improved transduction relative to rAAV2. In mouse, comparison of many different serotypes including rAAV1, 2, 5, 7, and 8 saw no difference in transduction efficiency at the highest titers evaluated except for reduced transduction by rAAV2 overall [96].

Discrepancies between our results and those described in these other reports may be explained by differences in viral titer, mouse strain, injection rates, or injection volumes. The higher titer used in my experiments may have activated cellular defense pathways against rAAV5 leading to toxicity, which at lower titers may not be observed. Increasing viral dose has been previously reported to contribute to increasing toxicity of AAV serotypes in neuronal and glia cultures [97]. Mouse strain could also influence striatal sensitivity to AAV5. Differences have not been reported before for AAV serotypes, however, intrastriatal injections of neurotoxins (ie. Quinolinic Acid and Kainic acid) have revealed strain-dependent differences in susceptibility to neurodegeneration [98]. Injection parameter differences such as rate and volume are less likely to have contributed to transduction differences based on previous experiments using Adenovirus [99]. The rAAV1 was injected at a similar titer to rAAV5 and did not show adverse effects, suggesting that rAAV1 has a higher safety threshold in striatum.

Previous studies also showed predominately neuronal transduction by both rAAV1 and rAAV5 [95, 96]. In my study, rAAV1 showed the expected neuronal transduction, while rAAV5 showed a preferential transduction of glial cells over neurons and a very large area of transduction through the brain. A similar result was found in comparing AAV vector serotypes in primate brain [100]. This study found that rAAV5 gave the greatest efficiency of gene transfer in the primate brain of all the serotypes tested. Further immunohistochemistry revealed increased tropism for glial cells over neurons by rAAV5. In this previous study, it would be interesting to see if activation of "toxic" pathways accounted for the increased glial transduction efficiency by rAAV5.

Overall, I found that AAV1 serotype displays the preferred transduction of striatal neurons in our mice with a high degree of safety, whereas AAV5 serotype transduced predominantly glial cells with marked indicators of toxicity. Together, this data suggests

AAV1 serotype is a better vector than AAV5 serotype for RNAi delivery to mouse striatal neurons for future preclinical trials in YAC128 mice.

Chapter 3: Improved silencing and tolerance of miR-shRNA versus shRNA for effective knock-down of human mutant Htt in YAC128

3.1 INTRODUCTION

The ability to silence muHtt expression holds promise for the treatment of Huntington Disease. In 1998, the discovery of the RNAi pathway in *C. elegans* revealed a potent pathway for selective gene silencing in animals [75]. This pathway, conserved across species from plants to humans, presents a potential approach to selectively silence disease-related genes such as mutant HD.

Increasing knowledge of the RNAi pathway and mammalian miRNA structure has precipitated the design of synthetic RNAi constructs. Originally, siRNAs (21-23 nt double-stranded) were observed to mediate gene silencing in *Drosophila* [101, 102]. This propelled the use of siRNAs in many subsequent mammalian systems; however, the growing knowledge of endogenous miRNA processing influenced new design techniques. Synthetic shRNAs (approximately 70 nt hairpin) mimicking pre-miRNA structure were proven effective at gene silencing in mammals [103]. These transcripts originate in the nucleus like pre-miRNAs and are then processed into 21-nt RNAs by Dicer before targeting its associated mRNA. In comparing shRNAs to siRNAs, shRNAs exhibited far greater gene silencing and at a much lower dose [79] sparking their popularity. Effective *in vivo* gene targeting has been seen using shRNAs in many animal models of disease [89, 104, 105], however, recent evidence suggests that shRNAs may cause toxicity at high doses questioning the safety of this design [106].

Other researchers have designed RNAi constructs that act even earlier in the miRNA pathway creating shRNAs resembling early miRNA primary transcripts (pri-

miRNA). These modified constructs known as miR-shRNA have placed active 21-nt RNA targeting sequence onto primary miR-30 backbone including miR-30 hairpin and flanking sequences [107]. Comparing shRNA and miR-shRNA libraries, miR-shRNA sequences displayed improved processing and more efficient gene silencing [80]. To date, no analysis has been done in animal models to compare the efficacy and safety of comparable shRNA and miR-shRNA constructs making this a key question for the field.

Previously shRNA constructs (shHD2.1) targeting human muHtt were tested in a mouse model of HD [104]. This shHD2.1 construct has since been modified slightly to introduce improved strand biasing selection in creating an optimized shRNA [108]. I compared the efficacy and safety of shRNA and miR-shRNA constructs (shHD2.1 and miHD2.1 respectively) targeting identical Htt sequence in a mouse model of Huntington Disease (YAC128).

3.2 MATERIALS AND METHODS

3.2.1 Animals

YAC128 mice developed in the lab of Michael Hayden on FVB/N background containing the complete human HD gene including 128 CAG expansion and ~25 kb of upstream promoter (Fig. 3.1) [40]. All animal experiments are approved by the UBC Animal Care Committee and were carried out in accordance with our institutional animal care guidelines.

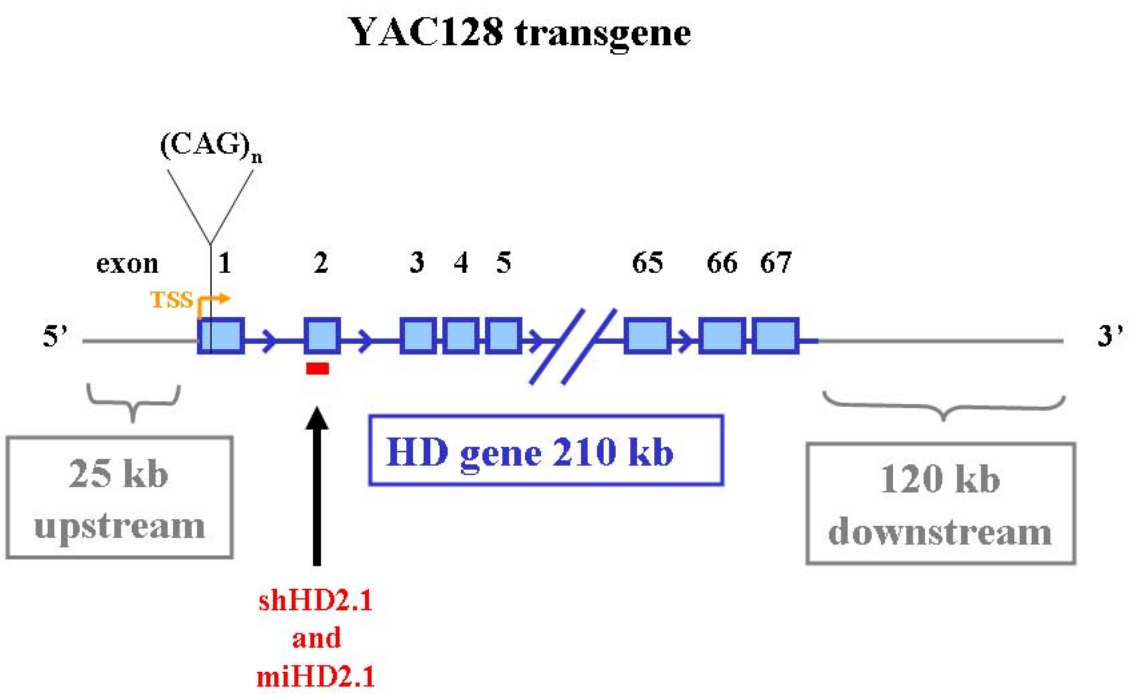

Figure 3.1: Schematic of the YAC128 transgene and HD2.1 target sites

YAC128 transgene consists of 210 kb of the human HD gene (NM_002111) including 25 kb upstream and 120 kb downstream. The HD mRNA transcript has 67 exons (light blue), with the CAG expansion existing in exon 1. The shHD2.1 and miHD2.1 constructs (red) are designed to target HD mRNA corresponding to nucleotides 416 to 436 in the human HD gene. TSS: transcription start site.

3.2.2 rAAV vectors

Recombinant AAV vectors include AAV1 serotype capsid protein combined with AAV2 inverted terminal repeats. Vectors contain either short-hairpin sequence under the U6 promoter or miR-shRNA sequence under the CMV promoter. Both vectors carry humanized *Renilla* GFP (hrGFP)-simian virus 40 poly(a) reporter cassette under the CMV promoter. Short-hairpin RNA sequences include shHD2.1 targeting exon 2 of the human HD gene (nucleotides 416 to 436) (Fig. 3.2) and negative control shBgal targeting *Escherichia coli* β-galactosidase (nucleotides 1152 to 1172) as used in [104]. MicroRNA-based sequences include miHD2.1 targeting identical sequence to shHD2.1 (Fig. 3.2) and negative control miGFP targeting eGFP (nucleotides 416 to 436). All vectors were

designed and previously tested *in vitro* by R. Boudreau from the University of Iowa. All vector constructs were synthesized at the University of Iowa Gene Transfer Vector Core.

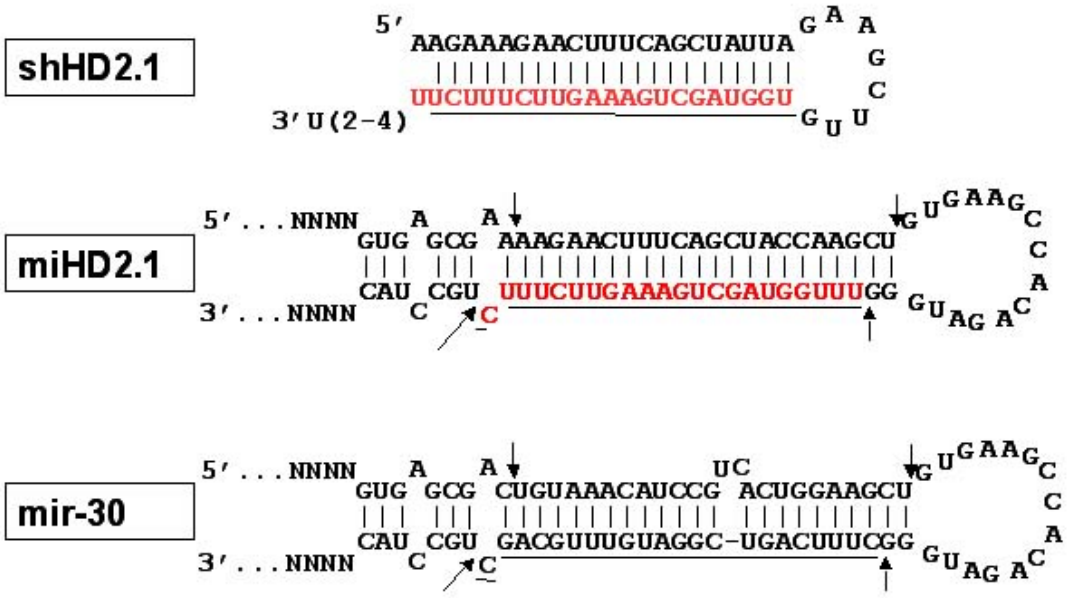

Figure 3.2: Structure of shHD2.1, miHD2.1, and endogenous miR-30.

ShHD2.1 was designed as a hairpin RNA, while miHD2.1 was designed using endogenous miR-30 sequence as its structural backbone. After cellular processing, both shHD2.1 and miHD2.1 will release similar anti-sense sequence (red) for silencing human mutant htt expression. Arrows indicate probable cleavage sites by Drosha and Dicer of the miR-based sequences.

3.2.3 Surgical procedures

Unilateral striatal injections administered by stereotaxic injection to YAC128 mice at 2 months of age. Injection coordinates were 1.8 mm anterior, 0.8 mm lateral, and 3.5 mm depth using 5 μL (shBgal or shHD2.1 ~3 x 10^8 Tu; miGFP or miHD2.1 ~7 x 10^7 Tu) per striata at an infusion rate of 250 nl/min.

3.2.4 Quantitative RT-PCR

Striatally injected brain tissue sliced into 1 mm thick coronal sections and stored in RNA-later stabilization buffer (Qiagen) for dissection. GFP positive regions were dissected out using a fluorescent microscope and identical regions in opposite

hemispheres dissected out as a control for each mouse. Total RNA isolated using the RNA mini kit (Qiagen) and cDNA made from 500 ng total RNA using the Reverse Transcriptase kit (Qiagen) both following manufacturer's protocol. qRT-PCR was performed using the SYBR Green PCR master mix (Applied Biosystems) and ABI7000 machine. Individual samples were analyzed in duplicate including control (n=3) and rAAV-treatment groups (n=8 each). Primer sequences included human Htt-specific, mouse Htt-specific, mouse DRD2, and mouse CD11b (Table 3.1).

Table 3.1: Primer sequences for huntingtin, DRD2, and CD11b.

Primer	Forward	Reverse
Human Htt	5'TCTCATTTCTCCGTCAGCAC	5'TCTGGTGGTTGATGTGATTA
Mouse Htt	5'TGGTAATGACAGTTGAGGCCC	5'CCAGGTTGTACTGCAATGGCT
DRD2	5'CATTGTCTGGGTCCTGTCCT	5'CAGGTTGGCTCTGAAAGCTC
CD11b	5'CAGGACCCACAAAACCAAGT	5'GGATGATCCCATACGGTCAC

3.2.5 Immunofluorescence and histology

GFAP antibody (DAKO Z0334; 1:3000) was used for labeling reactive astrocytes and fluorescently labeled secondary antibody (1:500 anti-rabbit Alexa Fluor 594) for visualization. Cresyl violet staining was done for 3 minutes for histological analysis.

3.2.6 Behaviour testing

Animals were monitored after rAAV injections using a modified SHIRPA test once a week for 3 weeks to identify any behavioural or physical health changes. The testing protocol was adapted from the primary screening procedure as in [109] and monitored weight, piloerection, gait or posture, motor control and coordination, novel

cage activity, response to touch, and tail-suspension testing. All parameters were scored to provide quantitative assessment.

3.2.7 Statistical analysis

A Students t-test was performed for determining differences in the mean between treatment groups and Levene's test to determine equality of variance. Differences were assumed statistically significant given they reached the 95 % confidence level. All values are represented as averages with standard error of the mean.

3.3 RESULTS

3.3.1 shHD2.1 selectively decreases human muHtt

rAAV carrying shHD2.1 and controls were injected into YAC128 transgenic mice and evaluated by qRT-PCR for human HD gene silencing. shHD2.1 selectively decreased human mutant HD mRNA by 53% ($p=0.003$) (Fig. 3.3) with no effect on endogenous murine Hdh expression relative to the sham-injected control. Unexpectedly, the short-hairpin negative control, shBgal, showed a non-specific trend for decreasing both human and murine Htt mRNA expression (Fig. 3.3). When evaluating empty rAAV control expressing GFP reporter no changes were observed in human or murine Htt mRNA expression suggesting that the shBgal-related mRNA expression changes are attributed to shRNA sequence (Fig. 3.3).

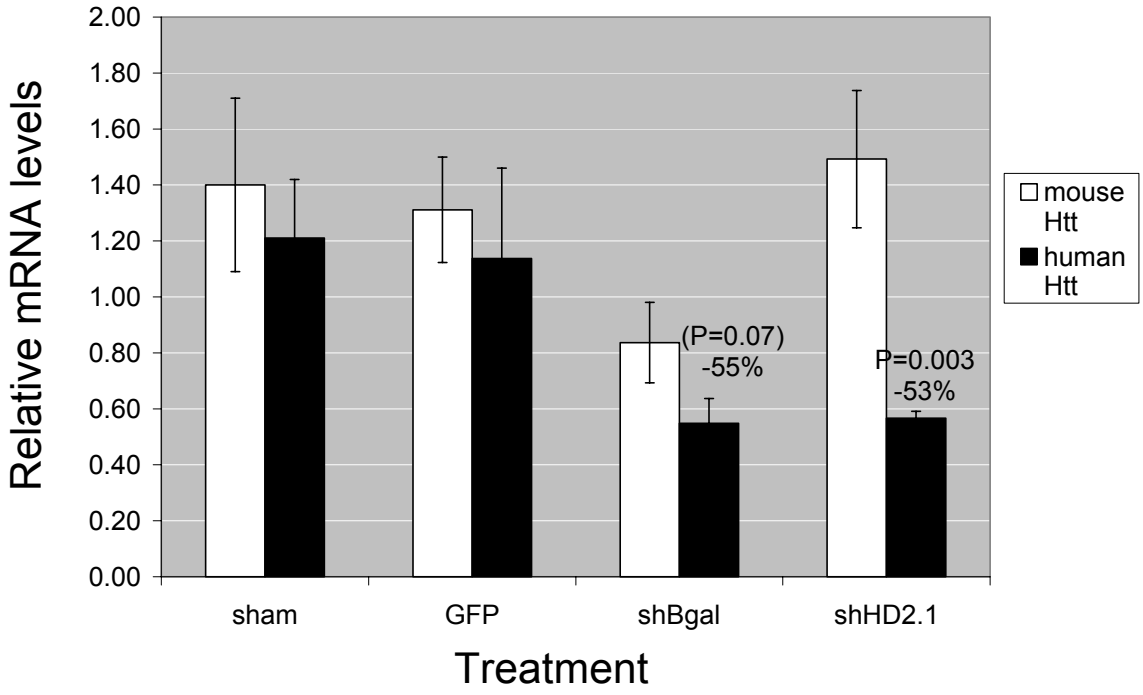

Figure 3.3: Selective silencing of human mutant htt by shRNA HD2.1.

shHD2.1 treatment selectively decreases human Htt mRNA levels by 53% (P=0.003) with no effect on mouse Htt. Negative control shBgal treatment shows non-specific effects decreasing human Htt mRNA levels by 55% (P=0.07) and a trend for decreased mouse Htt mRNA levels. These non-specific effects were not evident after AAV-GFP injection alone ruling out AAV or GFP-mediated toxicity. All mRNA values are derived from unilaterally injected mice and calculated as a ratio of injected to uninjected hemispheres to normalize for inter-animal variability. Significance is determined relative to sham injected control values. Treatment groups include sham (n=3), GFP (n=5), shBgal (n=6) and shHD2.1 (n=6).

3.3.2 miHD2.1 shows improved silencing of human muHtt

MicroRNA-based construct design was applied to the HD2.1 sequence and injected into YAC128 mice for comparison to shHD2.1 for gene silencing. MicroHD2.1 exhibited reduction of human Htt mRNA expression by 64% (p=0.01) (Fig. 3.4) with no effect on endogenous murine Htt when compared to the sham injected controls. The miGFP negative control showed no effect on either human or murine htt mRNA expression in this case.

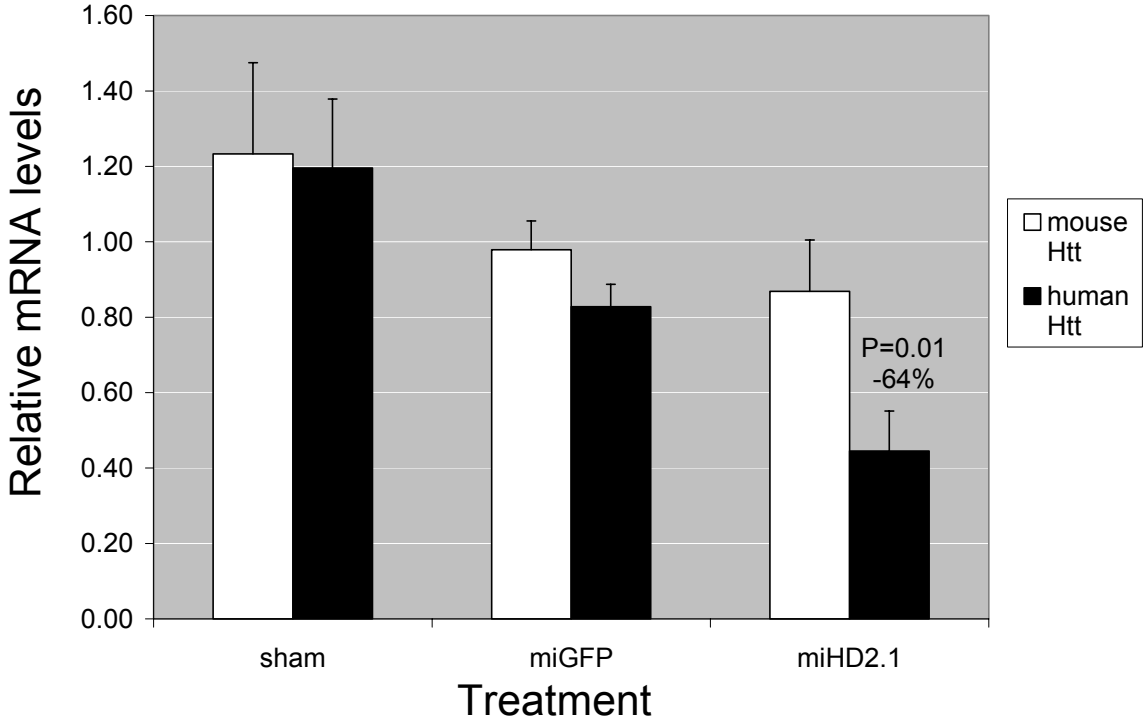

Figure 3.4: Improved silencing of human mutant htt by miHD2.1.

miHD2.1 treatment selectively decreases human Htt mRNA levels (P=0.01) by 64% with no effect on mouse Htt levels. Negative control miGFP treatment has no effect on either human or mouse Htt mRNA. All mRNA values are derived from unilaterally injected mice and calculated as a ratio of injected to uninjected hemispheres to normalize for inter-animal variability. Significance is determined relative to sham injected control values. Treatment groups include sham (n=3), miGFP (n=6) and miHD2.1 (n=6).

3.3.3 Short-hairpin constructs induce adverse effects not evident with miRNA-based treatments

Following the initial injections of short-hairpin constructs, 4 animals died out of 8 total (50%) within 3 weeks after rAAV-shBgal injection. Subsequent injections with a new preparation of shBgal construct also caused fatalities at later ages (at the same percentage). As a result, to detect any adverse effects associated with either short-hairpin or miRNA-based constructs animals were screened routinely for behavioural or non-specific mRNA expression changes as indicators of toxicity.

In the modified SHIRPA behaviour screen, shRNA-treated mice displayed abnormal trunk curling and limb clasping in the tail-suspension test (Fig. 3.5B). All of the

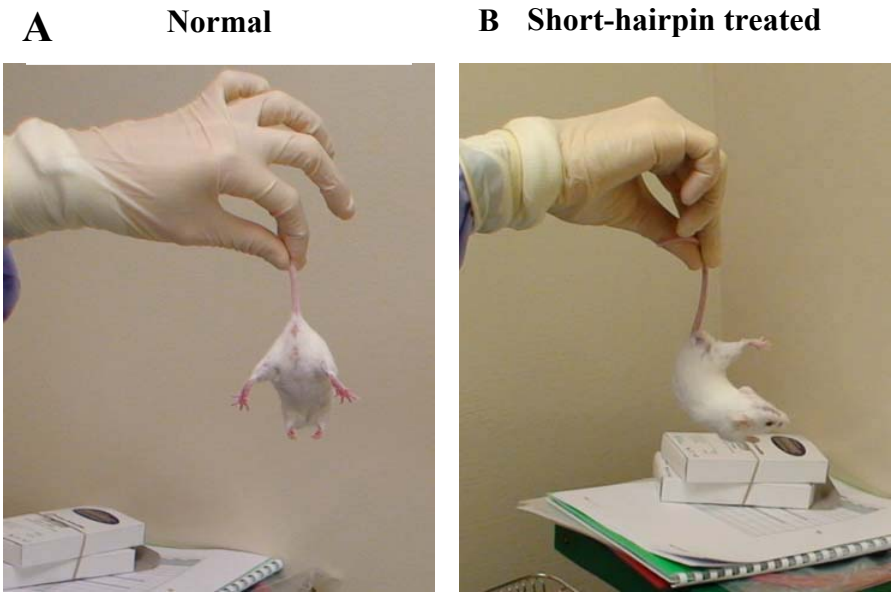

Figure 3.5: Behavioural changes associated with shRNA treatment not evident after miRNA or empty AAV-GFP treatment.

A. Animals injected with AAV-GFP, miGFP, or miHD2.1 show normal splay upon tail suspension. **B.** Animals injected with either shBgal (8/8) or shHD2.1 (3/7) display abnormal trunk curling and limp grasping upon tail suspension.

shBgal-treated mice displayed this abnormal behaviour (8/8), while only a portion of the shHD2.1-treated mice showed this abnormality (3/7). None of the miRNA-treated mice, either miGFP or miHD2.1, displayed any abnormal behaviours in the screen (data not shown).

Non-specific mRNa expression changes were evaluated using "indicator genes" to reveal changes in specific cell types including CD11b for microglia and DRD2 for neurons. Up-regulation of CD11b was observed in shRNA-treated mice, shBgal (408%; p=0.006) and shHD2.1 (338%; p=0.03) (Fig. 3.6A). A subtle trend for increased CD11b

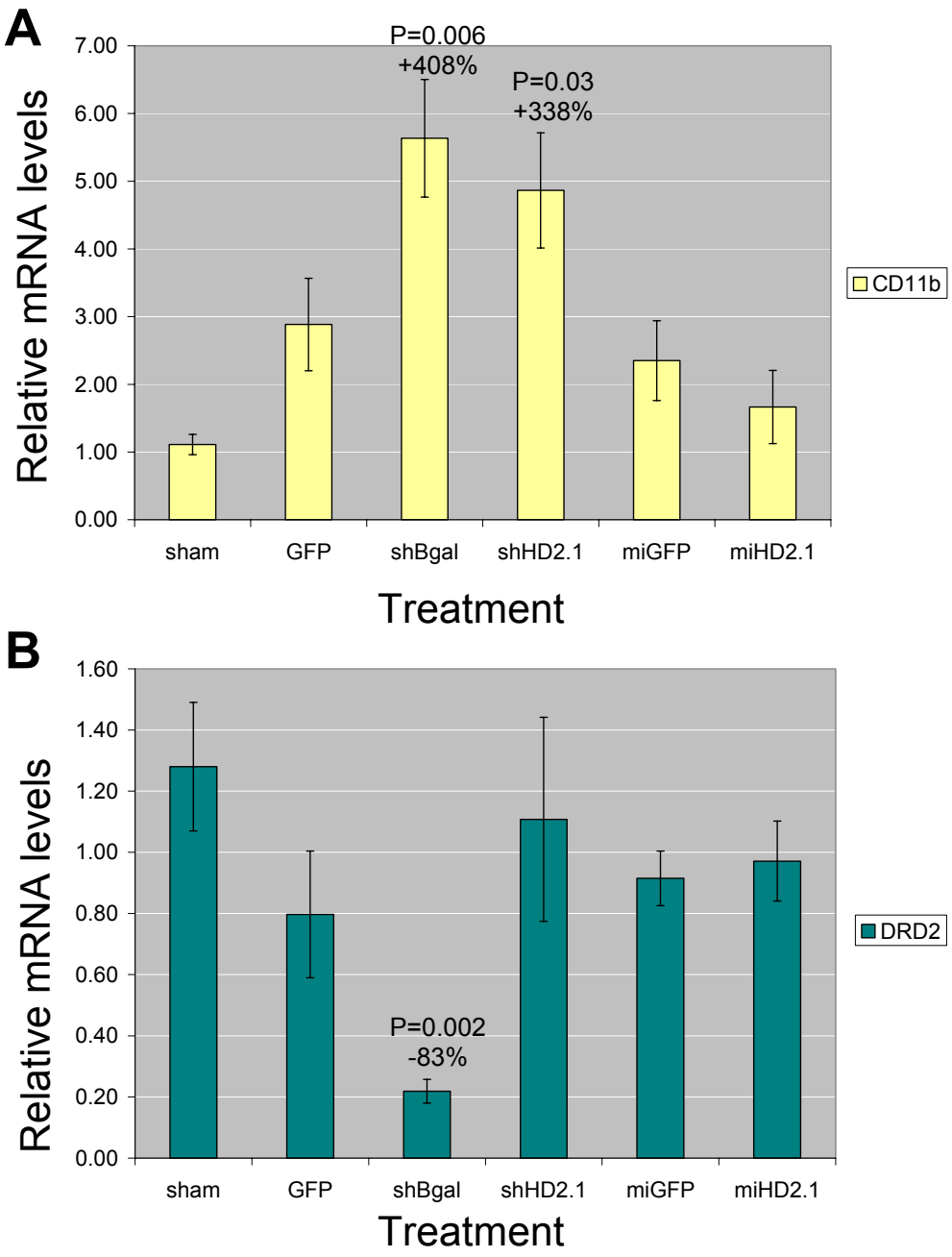

Figure 3.6: Non-specific gene changes after shRNA treatment not evident after miRNA or empty AAV-GFP treatment.

A. shBgal (408 %; P= 0.006) and shHD2.1 (338 %; P= 0.03) exhibit increased microglial activation marker CD11b relative to sham injected controls according to qRT-PCR. miRNA constructs miGFP and miHD2.1 show comparable CD11b levels to empty AAV-GFP alone. miGFP, miHD2.1, and empty AAV-GFP CD11b levels are not statistically different from sham-injected controls. **B.** Striatal DRD2 expression is unaffected by shHD2.1, miGFP, and miHD2.1 small RNA constructs relative to sham and empty AAV-GFP treatment. Conversely shBgal constructs significantly reduce DRD2 expression (-83%; P= 0.002). All mRNA expression values are represented as ratios of injected to uninjected striata to normalize per mouse. Treatment groups include sham (n=3), GFP (n=5), shBgal (n=6), shHD2.1 (n=6), miGFP (n=8), and miHD2.1 (n=8).

was observed for miGFP or miHD2.1-treated mice, similar to the increases seen when injecting empty rAAV-GFP; however these changes were statistically insignificant relative to changes seen in sham-injected controls. When evaluating changes in neuronal DRD2 levels, shBgal induced a dramatic decrease in DRD2 expression levels (-83%; p=0.002) with no other treatment groups having an effect relative to the sham-injected controls (Fig. 3.6B).

As a follow-up to the mRNA expression changes seen in shRNA-treated mice neuropathological indicators were evaluated to see if the differences could be accounted for by neuropathological changes. GFAP-labeling revealed increased astrocytosis in the injected hemisphere of shBgal and shHD2.1-treated mice (Fig. 3.7). Further cresyl violet staining revealed a dramatic loss of neurons in the injected hemisphere of shBgal treated mice (Fig. 3.8A) relative to both its respective uninjected hemisphere and shHD2.1-treatment (Fig. 3.8B). A summary of the toxicity findings can be found in Table 3.2.

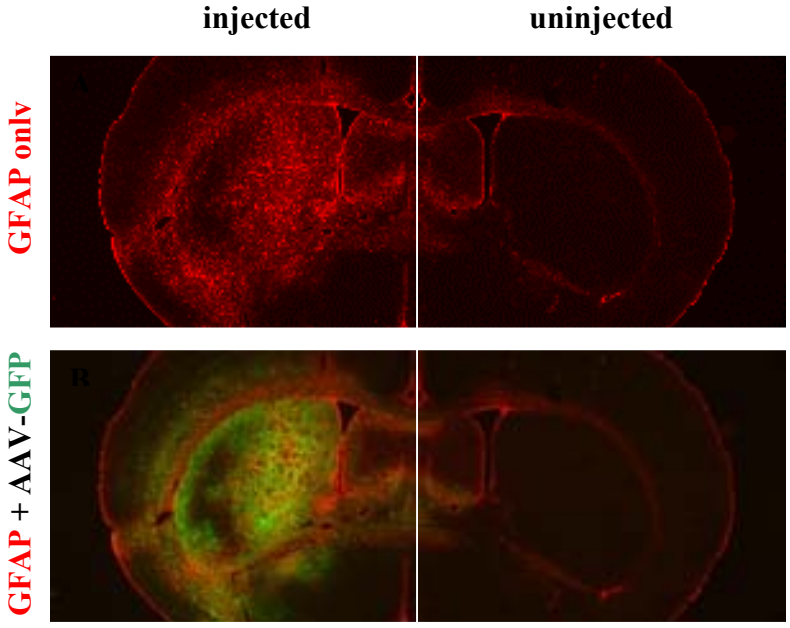

Figure 3.7: Astrocytosis evident after shBgal and shHD2.1 treatment.

Immunohistochemistry labeling GFAP (red) showed increased astrocytosis localized to shBgal (n=3) and shHD2.1 (n=3) injected hemispheres. **A.** Representative image showing increased GFAP labeling in shHD2.1 unilaterally injected brain (2.5X magnification). **B.** Same brain section as in A. showing GFAP co-localizing to regions of shHD2.1 GFP labeled virus.

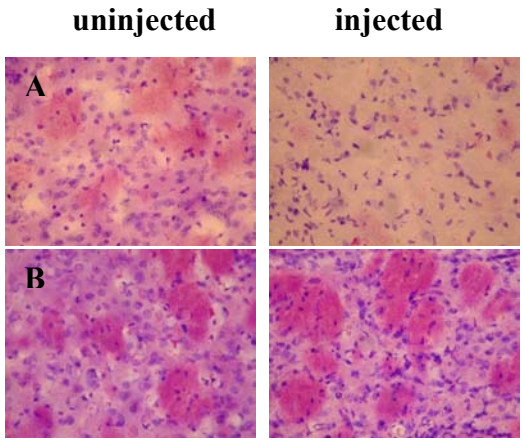

Figure 3.8: Neuronal integrity is affected after shBgal treatment.

A. Cresyl violet staining reveals neuronal loss at shBgal injection sites in animals displaying overt toxicity (weight loss, death). **B.** Neuronal loss is not evident at shHD2.1 injection sites although cresyl violet staining does reveal increased glia at these sites. Arrowheads identify areas of neuronal loss, while arrows identify glial cells. Images show injected versus uninjected striatal hemispheres at 20X.

Table 3.2: Summary of behaviour, neuropathology, and fatalities associated with short-hairpin and miRNA treatments.

	AAV-GFP	shBgal	shHD2.1	miGFP	miHD2.1
Tail-suspension	Normal	Curling; Limb grasping	Curling; Limb grasping	Normal	Normal
Neurons	Normal	Neuronal loss	Normal	Normal	Normal
Glia	Normal	Activated microglia; astrocytosis	Activated microglia; astrocytosis	Normal	Normal
Fatalities	None	4/8	None	None	None

3.4 DISCUSSION

This study found that miHD2.1 constructs showed improved gene silencing at 64% human muHtt silencing relative to the 53% seen by shHD2.1 in YAC128 mice. It is important to note that miHD2.1 constructs were injected at 10-fold less viral titer relative

to shHD2.1 and yet still showed better performance. These results agree with preliminary tissue culture experiments that demonstrated increased gene silencing by miHD2.1 relative to shHD2.1 (personal communication B. Davidson). These preliminary experiments also showed that lower dosages of miHD2.1 were required for effective silencing (personal communication B. Davidson). Together these data confirm improved silencing using miR-shRNA construct design both *in vitro* and *in vivo*.

Differences in cellular processing of RNAi triggers provide a plausible mechanism for the increased gene silencing seen by miHD2.1. Similar observations were made comparing large-scale libraries employing both shRNA and related miR-based design strategies [80]. In testing these constructs in 293 cells, miR-shRNAs showed 12 times more processed 21-nt RNA than related shRNA constructs suggesting improved processing by Dicer in constructs containing miRNA backbone. An increased processing rate of miHD2.1 in neurons would allow for greater gene silencing overall, and may explain my finding of increased silencing by miR-shRNA with lower titer than shRNA.

My work showing increased silencing by miHD2.1 provides the first *in vivo* example that miR-shRNA constructs exhibit enhanced performance over related shRNAs in silencing a disease gene. Others have shown similar improvements using miR-shRNAs in cellular models of disease such as Leukemia. In one case, combination miR-shRNAs showed improved Bcr-Abl oncogene suppression and slowed leukemic cell growth previously unaffected by shRNA constructs [110]. My work and the work of others provide evidence to support miR-based RNAi as a novel treatment strategy.

Our experiments, while showing marked differences between miR-based and shRNA constructs with respect to gene silencing, also revealed important safety considerations in comparing miR-shRNAs and shRNAs in an animal model. These experiments showed that both shHD2.1 and the shBgal negative control induced dramatic

upregulation of CD11b indicative of toxicity. The presence of clasping on the tail suspension test indicated unilateral brain dysfunction. Consistent with striatal injury the negative control construct, shBgal, induced a more severe toxicity in some animals developing neuronal loss, shrunken brain hemispheres, weight loss, and fatality within as little as 2 weeks in some cases. The mice treated with miR-shRNAs either miHD2.1 or miGFP showed no behavioural or neuropathological signs of toxicity.

The fatality seen in our short-hairpin negative-control mice was similar to another report showing fatalities in mice after delivering high titer shRNA to the liver [106]. Several of the mice treated with high dose shRNA vector showed weight loss and died within one month of treatment comparable to our results. In testing 49 distinct rAAV-shRNA vectors and empty vector components, these authors found that at high doses 36 shRNAs caused liver injury, with 23 ultimately causing death. This said that the fatality was a generalized response to high dose shRNAs. Mechanistically, the authors suggested that high levels of shRNAs saturated cellular RNAi pathways, specifically exportin-5, and competed with endogenous miRNAs for shuttling out of the nucleus.

In my study, shBgal and to less of an extent shHD2.1 may have saturated cellular RNAi pathways interfering with endogenous miRNA export and processing. This cellular disruption could upregulate cell defense pathways (as with shHD2.1) and in more severe cases lead to neuronal death and fatality (as with shBgal). At first our results were very surprising given shBgal negative-control had been previously tested in the N171-82Q mouse model of HD [104]. Subsequent repeat injections with new viral preparations showed consistent toxicity in our mice ruling out the possibility of viral contamination. Further, testing shBgal in another mouse strain, C57BL/6J, showed no adverse affects (unpublished) suggesting a very unexpected increased sensitivity of the FVB/N strain to shRNA-related toxicity. Ongoing work in the lab is investigating the possibility that

endogenous brain-specific miRNA processing may be disrupted by high dose shRNAs in YAC128 mice. MiHD2.1 may not show these same toxicological effects *in vivo* due to the improved processing of miRNA-based constructs as in Silva *et al*. (2005) allowing effective silencing with lower titers. Other groups have also shown that shRNAs compete with endogenous miRNAs *in vitro* and that miR-shRNAs do not even at higher doses [111]. The authors confirm the finding of Grimm *et al*. (2006) and show that some of this effect is caused by saturation of exportin-5, although they show that other factors may be involved in the toxicity as well (they propose RISC).

I have generated data demonstrating increased efficacy and tolerance of miHD2.1 over shHD2.1. This work lays the groundwork for HD pre-clinical trials using the miHD2.1 construct. It also provides growing evidence to the field in support of miR-shRNA construct design. The surprising toxicity seen in YAC128 mice using shRNA constructs provides a striking brain-related example of the deleterious effects of shRNAs. Further studies are investigating the potential interference of shRNAs on endogenous miRNA pathways in the brain, to better understand the brain's sensitivity to RNAi therapy for future therapeutic studies.

Chapter 4: Summary and conclusion

This thesis explored rAAV vector serotypes and RNAi constructs in the YAC128 mouse model of HD. Initially, AAV serotypes were compared for transduction to striatal neurons in choosing an appropriate viral vector for gene delivery. Subsequently, rAAV-delivered RNAi constructs, using either shRNA or miR-shRNA construct designs, were compared for human muHtt gene silencing in YAC128 mice. Overall this work determined that rAAV1-mediated miHD2.1 constructs were effective in silencing human muHtt in the YAC128 mouse model of HD. Ultimately this work contributes to a future pre-clinical trial testing RNAi-mediated gene silencing of human muHtt as a treatment for HD.

Initially this report compared the transduction of AAV1 and AAV5 serotypes for use as an RNAi vector in YAC128 mice. My results showed that AAV1 serotype displayed neuronal tropism, substantial coverage through the striatum, and immune tolerance proving it to be an effective vector for construct delivery. Alternatively, AAV5 showed high glia transduction and activation of immune response making it an ineffective vector for construct delivery in YAC128. Comparing these results to others investigating AAV serotypes [95, 96] my results characterized toxicity never before reported using AAV5. Either the higher viral titers used in my experiments or a FVB/N strain-specific sensitivity to AAV5 could explain the AAV5-related toxicity observed. Interestingly this toxicity was not observed with AAV1 serotype at a similar titer. Overall, this work concluded AAV1 serotype vectors to be an effective and safe delivery method for RNAi constructs in YAC128 mice.

After determining AAV serotype 1 to be an appropriate viral vector for gene delivery, a shRNA construct targeting human muHtt for gene silencing, shHD2.1, was

tested in YAC128 mice. An initial version of the shHD2.1 construct was designed by my collaborators at the University of Iowa (B. Davidson) and previously shown to be effective in the N171-82Q fragment model of HD. An optimized strand-biased version of shHD2.1 was then tested in our YAC128 mice. This construct was expected to show improved silencing of human muHtt based on previous *in vitro* work (personal communication B. Davidson), but instead triggered neuroinflammatory changes and morbidity in YAC128 mice. In addition the negative-control shRNA, shBgal, previously tested in the N171-82Q model showed neuro-immunological activation, off-target gene silencing, morbidity, and fatality in YAC128 mice. I showed that this shRNA-related toxicity was not attributed to vector or GFP expression, was reproducible with fresh viral preparation, and hence showed remarkable similarities to the shRNA toxicity reported by Grimm *et al*. (2006). From this I concluded that shRNA constructs may not be useful for RNAi *in vivo* and tested miRNA-based constructs targeting identical regions of human muHtt as shHD2.1.

In vitro work by our collaborators and others had shown that miR-shRNAs were more potent inducers of gene silencing and at lower dosages. My work confirmed these findings *in vivo* showing increased human muHtt gene silencing using miHD2.1, with no evidence of immunotoxicity or morbidity. These results concluded miHD2.1 to be a more potent and safer construct for human muHtt gene silencing in YAC128 mice, and present a novel RNAi therapeutic approach to be tested in an animal model of HD.

4.1 SIGNIFICANCE

This work contributes to the development of Htt allele-specific RNAi approaches in treating HD. The *in vivo* testing of rAAV vectors is important to this pursuit because the vector is critical in delivering the construct to the appropriate cell type and must

prove safe at high viral titers in moving from pre-clinical to human clinical trials. AAV serotype 2 has recently proven safe and effective in phase I human clinical trials for Parkinson's disease [112]. This work showed no adverse events related to rAAV-mediated gene delivery in advanced-stage Parkinson's disease patients. Additionally, rAAV-mediated glutamic acid decarboxylase (GAD) gene delivery coincided with improved motor scores and brain metabolism showing a positive therapeutic effect. This finding is promising for the eventual translation of gene therapy work using rAAV in other neurological diseases such as HD. My work contributed to further testing of alternate AAV serotypes, AAV1 and AAV5, in a rodent model based on their improved transduction efficiency relative to AAV serotype 2 [95, 96]. The toxicity observed with AAV5 serotype suggests a dose-related toxicity that must be considered in future trials with this serotype. For these serotypes to progress into human clinical trials more rigorous dose-response testing in non-human primates would be required.

My work also contributes to the development of Htt allele-specific RNAi approaches in exploring promising new RNAi construct designs. Understanding which construct design shows the strongest selective gene silencing, with minimal side effects, is crucial in detecting positive therapeutic effects in pre-clinical animal model testing. Given the time and number of animals required for pre-clinical trials in YAC128 mice, I wanted to ensure we had the most promising construct in moving ahead into the pre-clinical testing phase. My results showing improved silencing and safety of miHD2.1 contributes a new RNAi construct in moving forward to pre-clinical testing.

The translation of RNAi therapies to human clinical trials currently demands a greater reassurance of safety before employing this treatment strategy. The issue of off-target effects and interference with endogenous miRNA pathways are crucial impediments to clinical progress. The toxicity seen with shRNA constructs in this study

and others warn against the use of this particular construct design. Oppositely the miR-shRNA construct, miHD2.1, proved more effective and safer than shHD2.1 in YAC128 mice at early time-points. This work then merits further long-term investigation of miHD2.1 constructs in YAC128 mice, and as well suggests greater attention to the gene therapy field in exploring miRNA-based constructs in developing RNAi therapies.

4.2 FUTURE DIRECTIONS

The improved performance of miRNA-based constructs *in vitro* (personal communication B. Davidson) and in my *in vivo* analysis provides strong evidence to support the use of this therapeutic strategy in the YAC128 mouse model of HD. I am currently in the process of expanding my findings to a larger pre-clinical trial in YAC128 mice testing the rAAV1-mediated miHD2.1 RNAi approach. (refer to Chapter 5). I predict that given the significant gene silencing and tolerance of miHD2.1 constructs, these animals will show a significant therapeutic effect. This pre-clinical trial will be the first *in vivo* RNAi therapeutic study to use miR-based RNAi constructs. This will help identify the therapeutic efficacy of using a miR-based RNAi approach. This RNAi therapeutic study will also be unique in using a full-length (YAC128) mouse model of HD. Previous RNAi pre-clinical trials have used fragment models of HD (see 5.1 Introduction). Using the YAC128 model provides unique advantages to studying gene-targeted therapeutic approaches in that it contains the complete human HD gene. In designing RNAi constructs this enables greater possibilities in optimizing siRNA target sequences. If the miR-based RNAi approach proves effective in YAC128, siRNA target sequences throughout the HD gene can be compared for gene-silencing efficacy in future studies. Use of the YAC128 model also provides phenotypic advantages over fragment mouse models in the selective striatal neuronal loss observed. Although a milder

phenotype overall, the striatal neuronal loss in YAC128 provides a measurable endpoint to reveal a treatment's neuroprotective properties. Ultimately, a neuroprotective treatment could translate to humans in treating the massive striatal neuronal loss seen in HD patients.

Chapter 5: Future directions

Pre-clinical trial using miRNA-based RNAi for the therapeutic treatment of HD

5.1 INTRODUCTION

HD is a devastating neurological disease attributed to an expanded CAG tract within the HD allele. Synthetic small RNA molecules provide a powerful means to silence the mutant HD gene through a natural RNAi pathway used by endogenous microRNAs. Initial experiments in a conditional HD mouse model showed early promise for RNAi-based therapeutics in the treatment of HD. These experiments found that turning off the mutant HD allele early or late after symptomatic onset improved aggregate formation, neuronal loss, and motor function in the Tet/HD94 conditional HD mouse model [51, 113].

The growing knowledge in the field of RNAi-based treatments has since motivated researchers to test alternate approaches for the effective knockdown of a disease gene. Different groups have tried sleeping beauty transposons [114], shRNAs [104, 115, 116], and lipid-conjugated siRNAs [117] for silencing muHtt in HD mouse models. Thus far, shRNAs have shown the most promising results with consistent improvements seen across different HD mouse models. After shRNA-mediated treatment, the N171-82Q mouse model saw reduced aggregate formation and full motor recovery on the rotarod at 18 weeks [104]. The R6/1 mouse model saw reduced aggregate formation and 60 % improvement in rear-paw clasping [115]. While these results are promising for its treatment efficacy, cases of shRNA-associated toxicity reported in HD mouse models [115] and other reports [106] have breathed caution to the community about the eventual translation of this approach to human patients.

An alternate but similar approach to shRNA-based therapy is the development of small RNA constructs that more closely resemble early microRNA transcripts. These modified transcripts known as miR-shRNAs place the RNAi targeting sequence onto an endogenous miRNA backbone. Most commonly, the primary-miR-30 backbone has been used, including miR-30 hairpin and flanking sequences [107]. In comparing shRNA and miR-shRNA derived construct libraries, miR-shRNA sequences displayed improved processing and more efficient gene silencing [80]. This would suggest that lower dosages of miR-shRNAs than shRNAs are required for effective gene silencing. Others have also shown *in vitro* that shRNAs exert a competition with endogenous miRNAs that miR-shRNAs at even higher dosages do not [111]. Together this suggests improved performance given a miRNA construct design.

Previous work in our laboratory has shown that a miRNA-derived construct, miHD2.1, showed improved muHtt silencing and a higher safety tolerance *in vivo* than shRNA designed constructs in the YAC128 mouse model of HD. The purpose of this current work is to test the pre-clinical efficacy of miHD2.1 in ameliorating the HD phenotype in YAC128 mice. It is hypothesized that decreasing muHtt protein early in disease progression will lead to an improvement in behavioural and neuropathological phenotypes in YAC128 mice.

5.2 MATERIALS AND METHODS

5.2.1 Animals

YAC128 mice developed in the lab of Michael Hayden on FVB/N background containing the complete human HD gene including 128 CAG expansion and ~25 kb of upstream promoter (refer to Chapter 3, Fig. 3.1) [40]. Wildtype and transgenic animals will be assigned to testing cohorts as specified in Table 5.1. For an overview of study

design refer to Fig. 5.1. All animal experiments are approved by the UBC Animal Care Committee and will be carried out in accordance with our institutional animal care guidelines.

Table 5.1: Experimental design for pre-clinical therapeutic trial.

	Pre-Clinical Trial		**Gene Silencing**	
Treatment	**Wildtype**	**Transgenic**	**6 months**[a]	**12 months**[a]
Sham	17	15	4	4
miGFP	17	15	6	6
miHD2.1	17	15	6	6
TOTAL	51[b]	45	16	16

[a]all YAC128 transgenic
[b] more wildtype than transgenic mice included to compensate for 10% of wildtype mice developing SID epileptic phenotype by 12 months of age

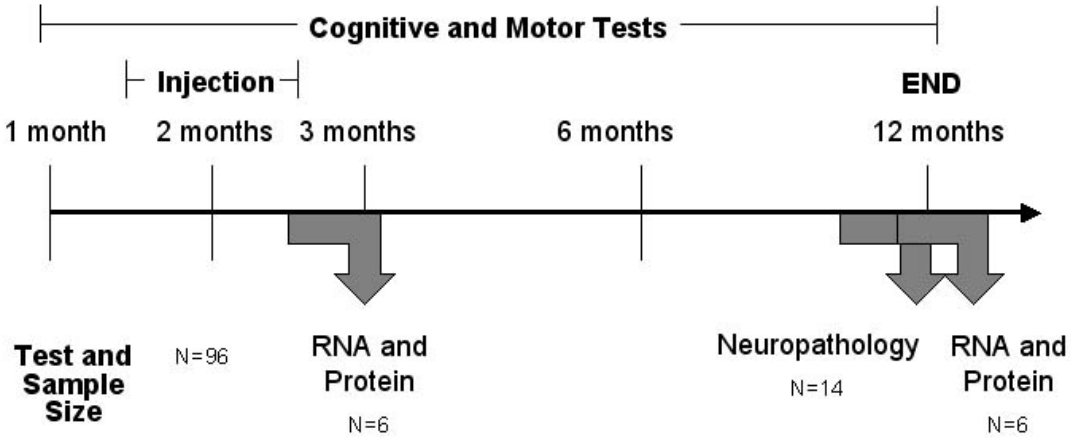

Figure 5.1: Timeline of miRNA-based RNAi pre-clinical trial in YAC128.

5.2.2 rAAV vectors

Recombinant AAV vectors include AAV1 serotype combined with AAV2 inverted terminal repeats. Vectors contain miRNA-based RNAi sequence and a humanized *Renilla* GFP (hrGFP)-simian virus 40 poly(A) reporter cassette both under the CMV promoter. MiHD2.1 sequence includes siRNA target sequence for exon 2 of the human HD gene (nucleotides 416 to 436) integrated into endogenous miR-30 backbone including miR-30 hairpin and flanking sequences (refer to Chapter 3, Fig. 3.2). The negative control construct uses a similar miR-30 backbone with siRNA targeting eGFP (nucleotides 416 to 436). The Vector Core at the University of Iowa produces all vectors.

5.2.3 Surgical procedures

Bilateral striatal injections will be administered by stereotaxic injection to animals at 2 months of age. Injection coordinates are 1.8 mm anterior, 0.8 mm lateral, and 3.5 mm depth using 5 μL (miGFP or miHD2.1 ~7 x 10^7 Tu) per striata at an infusion rate of 250 nl/min. For qRT-PCR experiments, animals will be unilaterally injected only.

5.2.4 Quantitative RT-PCR

Striatally injected brain tissue will be sliced into 1 mm thick coronal sections and stored in RNA-later stabilization buffer (Qiagen) for dissection. GFP positive regions will be dissected out using a fluorescent microscope and identical regions in opposite hemispheres dissected out as a control for each mouse. Total RNA will be isolated using the RNA mini kit (Qiagen) and cDNA made from 500 ng total RNA using the Reverse Transcriptase kit (Qiagen) both following manufacturer's protocol. qRT-PCR will be performed using the SYBR Green PCR master mix (Applied Biosystems) and ABI7000 machine. Primer sequences include human Htt-specific, mouse Htt-specific, mouse DRD2, and mouse CD11b (refer to Chapter 3, Table 3.1).

5.2.5 Behaviour and motor control testing

Two-month old animals will be trained on rotarod (UGO Basile) for 3 trials per day over 3 consecutive days. Animals will then be tested on rotarod and open-field (San Diego Instruments) to establish a phenotypic baseline before treatment. Post-treatment, animals will be tested in open field to measure activity level changes at 3 and 12 months of age. Motor co-ordination and balance will be monitored using accelerating rotarod every 2 months until 12 months of age. The rotarod will be set to accelerate from 5 revolutions per minute (rpm) to 40 rpm over 4 minutes, and performance will be measured as the total running time on the rotarod to a maximum of 300 s per trial. Rotarod scores are the average of 3 trials executed 2 hours apart. All testing will occur in the light cycle.

5.2.6 Neuropathological assessment

At 12 months of age mice will be euthanized and assessed for neuropathology. Animals will be deeply anesthetized and transcardially perfused with 3% paraformaldehyde in phosphate-buffered saline (PBS). Brains will be post-fixed for 24 hours in 3% paraformaldehyde, weighed, and then further fixed in 25% sucrose solution before cutting. Coronal sections are prepared as 25 μM sections on a cryostat (Microm HM 500M, Richard-Allan Scientific, Calamazoo, MI, USA) and stored in PBS before analysis. Coronal sections (25 μm) spanning the striatum (200 μm total) will be incubated in NeuN antibody solution (1:100 Chemicon) for neuronal detection, and biotinylated anti-mouse antibody (1:200 Vector Laboratories), ABC Elite kit (Vector Laboratories) and diaminobenzidine (Pierce) for signal amplification and visualization. Stereoinvestigator software (Microbrightfield) will be used for striatal volume and neuronal count analysis. Striatal volumes will be determined by tracing the perimeter of the striatum in serial sections, while neuronal counts will be determined by counting

neurons within a 550 μm x 550 μm grid of 25 μm x 25 μm counting frames spaced evenly throughout the striatum using a 100x objective lens. To assess neuronal cross-sectional area, a single neuronal section from each animal will be incubated with Alexa488-conjugated NeuN antibody (MAB377X, Chemicon) for neuronal detection and visualization. Each section is then mounted and analyzed using Neurolucida software (Microbrightfield) to trace the perimeter of clearly defined neurons within a 550 μm x 550 μm grid of 25 μm x 25 μm counting frames.

5.3 EXPECTED OUTCOMES

This study will add additional proof-of-principle evidence to validate microRNA-mediated muHtt allele-specific silencing as a treatment of HD. Specifically this study will use microRNA-based RNAi constructs (miHD2.1) to silence human muHtt in YAC128 mice and will monitor behavioural and neuropathological outcomes to assess therapeutic efficacy. I predict that decreasing muHtt protein early in disease progression will lead to an improvement in behavioural and neuropathological phenotype in YAC128 mice. The miHD2.1 construct has thus far been shown to selectively silence the human muHtt gene decreasing mRNA expression levels by ~80 %. It is expected that this gene suppression will translate to a comparable decrease in human muHtt protein resulting in a positive therapeutic outcome. I am currently confirming the effect on muHtt protein in YAC128 mice before commencing the pre-clinical trial.

RNAi-mediated suppression of the mutant Htt gene is also expected to show broad improvements in both motor function and neurodegeneration in YAC128 animals. Previous RNAi studies targeting a fragment of mutant Htt have detected phenotype changes including motor improvements in genetic models of HD [104, 115] and slowed neuronal loss in rapid viral HD models [117, 118]. Using the YAC128 mouse model

provides the advantage of evaluating both motor function and neuronal loss simultaneously given its slower disease progression. The existence of the full-length human muHtt gene in YAC128 also provides a more clinically relevant scenario in studying gene-targeted therapeutic approaches.

5.4 POTENTIAL PITFALLS

Potential obstacles to proving miHD2.1 therapeutic efficacy in YAC128 mice include problems with long-term rAAV expression, inadequate gene suppression, or limitations in viral spread to affected brain regions.

Sustained viral and miR-shRNA expression throughout the duration of the experiment is ideal in showing a positive therapeutic effect in YAC128 mice. An adaptive immune response has been shown to silence rAAV vectors [87] and shRNAs *in vivo* [106]. This immune response is typically activated immediately within a few weeks after vector or shRNA delivery. Provided the vectors and miR-based constructs that I used showed no immediate immune activation up to 3-weeks post-injection as assessed by qRT-PCR and immunohistochemistry (refer to Chapters 2 and 3), it is unlikely for vector or construct silencing to occur in the preclinical trial. Further, AAV1 serotype has been tried in other mouse trials showing tolerance and gene expression up to 1 year post-injection [119].

A second obstacle could be inadequate Htt gene silencing by the miHD2.1 construct. Inadequate protein suppression is of concern given Rodriguez-lebron *et al*. (2005) showed discrepancies between Htt mRNA (-78%) and protein (-28%) suppression by shRNA constructs. For this reason it is expected that protein analysis be completed before commencing the pre-clinical trial with the expectation that 50% protein suppression is observed. Further, once the trial has commenced necessary checks have

been put in place to monitor Htt gene silencing by quantitative RT-PCR periodically through the trial. This ensures that should the construct or vector be silenced it will be known before in-depth behaviour and neuropathological analysis is completed.

A third obstacle to proving therapeutic efficacy of miHD2.1 would be an inadequate spread of the virus through the brain. The striatum is the predominantly affected region in the brain showing massive neuronal loss in HD patients and for this reason it is standard to target this area in treatment. Presently rAAV1 transduces nearly ~50-100 % of the striatum with variability highly dependent on the skill of the surgical technician. To minimize this risk in the pre-clinical trial the same surgical technician will be used for all rAAV injections. A small body of research, however, suggests that HD-related dysfunction may also reside within cortical pathways affecting trophic synthesis and supply to the striatum [120, 121]. With this in mind a therapy delivered intrastriatally would be ineffective on cortical dysfunction and additional brain regions could potentially be targeted in future studies.

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Appendix: Animal care protocol

The University of British Columbia

Animal Care Certificate

Application Number: A05-1777

Investigator or Course Director: Blair R. Leavitt

Department: Medical Genetics

Animals Approved: Mice FVB/N 300

Start Date: **October 26, 2005** Approval Date: **December 21, 2005**

Funding Sources:

Funding Agency:	Canadian Institutes of Health Research
Funding Title:	Laboratory for experimental therapeutics in animal models of human disease
Funding Agency:	Merck Frosst Canada Inc.
Funding Title:	Mouse Genomics
Funding Agency:	High Q Foundation
Funding Title:	TREAT-HD: Testing of Experimental Therapeutics in Transgenic Model of HD: Trials in Mouse Models of HD
Funding Agency:	Migenix Inc.
Funding Title:	SA: Work Study No. 06-005
Funding Agency:	Huntington Society of Canada
Funding Title:	Centre for Experimental Therapeutics in Animal Models of Human Disease
Funding Agency:	Huntington Society of Canada
Funding Title:	The Centre for Experimental Therapeutics in Animal Models of Human Disease
Funding Agency:	High Q Foundation
Funding Title:	The translational research on excitoxicity to accelerate therapeutics for Huntington disease (TREAT-HD) research UBC
Funding Agency:	Huntington Society of Canada
Funding Title:	The Centre for Experimental Therapeutics in Animal Models of Human Disease
Funding Agency:	NCE: National Centers of Excellence
Funding Title:	Global transcription analyses of melocular pathways underlying neurodegenerative diseases
Funding Agency:	High Q Foundation
Funding Title:	CRA: TREAT-HD: Translational Research on Excitotoxicity to Accelerate Therapies for Hungtington's Disease.
Unfunded title:	N/A

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility

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