CHARACTERIZATION OF THE CAG 140 MOUSE MODEL OF HUNTINGTON’S DISEASE AND THE EFFECTS OF RAAV DELIVERED SHRNA AND HGFP

By

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To my family, who have helped me through it all
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hGFP  Humanized Green Fluorescent Protein
HOM  Homozygous
HSV  Herpes Simplex Virus
Htt  Human huntingtin gene
IHC  Immunohistochemistry
KA  Kainic acid
KI  Knock-In
KO  Knock-Out
mRNA  messenger RNA
Het  Heterozygous
Hom  Homozygous
NeuN  Neuronal Nuclei
NF-κB  nuclear factor kappa-light-chain-enhancer of activated B cells
NGFI-A  Nerve Growth Factor Inducible A
NIH  National Institute of Health
NIIs  Neuronal Intranuclear Inclusions
NMDA  N-methyl-D-aspartic acid
nTG  Non-Transgenic
PBS  Phosphate Buffer Solution
PCR  Polymerase Chain Reaction
PDE10a  Phosphodiesterase 10a
PDE1b  Phosphodiesterase 1b
PKA  Protein Kinase A
ppENK  preproenkephalin
QA  Quinolinic acid
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<td>RNAi</td>
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<td>RPM</td>
<td>Revolutions Per Minute</td>
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<td>SCA</td>
<td>Spinocerebellar ataxia</td>
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<td>shRNA</td>
<td>Short Hairpin RNA</td>
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<td>siRNA</td>
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<td>TBP</td>
<td>Tata Binding Protein</td>
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<td>Yeast Artificial Chromosome</td>
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CHARACTERIZATION OF THE CAG 140 MOUSE MODEL OF HUNTINGTON’S DISEASE AND THE EFFECTS OF RAAV DELIVERED SHRNA AND HGFP

By

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Huntington’s disease (HD) is a fatal neurodegenerative disease caused by a polyglutamine expansion in the huntingtin gene. Currently there is no treatment or cure for HD which afflicts approximately 3 to 8 individuals out of 100,000. Since the discovery of the huntingtin gene in 1993, several rodent models have been generated to study HD. Here we examine the behavioral and histopathologic characteristics of one HD mouse model (CAG 140) consisting of 140 CAG repeats knocked into the 5’ mouse huntingtin gene. Rearing, rotarod and gait behavioral analysis were performed once a month starting at 2.5 months out to 18 months. Histopathological examinations, including mRNA transcriptional analysis and neuronal intranuclear inclusion (NIIs) quantification, were performed on a cross-sectional cohort of mice starting at 3 months of age and examined out to 18 months. We found significant behavioral and histopathological abnormalities in the knock-in mice when compared to non-transgenic mice.

The work performed here shows that the CAG 140 mouse is a suitable model for HD studies. The CAG 140 model exhibit aspects of the HD in a more protracted natural history compared to other murine HD models. As seen in other HD models and in
humans, the transcriptional down-regulation in the knock-in mice as they age is one of the best characteristics of this model. In addition, the progressive NII s increase over time could be used as marker of the disease progression.

We explored the use of rAAV delivered RNAi as a potential therapeutic for the disease by using a shRNA targeting the expanded \textit{htt} allele. While knock-down of mutant \textit{htt} mRNA reduced the number of NII s, mice also displayed significant transcriptional dysregulation. Additionally, the non-specific shRNA and Green Fluorescent Protein (GFP) controls showed significant reductions in striatally enriched mRNA transcripts. Further investigation into the effects of the GFP controls indicated a significant loss of DARPP-32 protein, apparent loss of NeuN positive cells and an increase in glial fibrillary acidic protein (GFAP) positive astrocytes in the injected side. Our work here indicates GFP may not be the best reporter transgene and other less toxic reporters might need to be examined.
Huntington’s Disease

Huntington’s disease (HD) is a fatal progressive neurodegenerative disease characterized by the gradual development of sporadic, involuntary motor movement called chorea. Other HD symptoms include depression, dementia and personality changes as well as other motor skill abnormalities including clenching of the teeth (bruxism), and gait and ocular motor abnormalities (1-8). While HD is most associated with sporadic motor movement; rigidity, ataxia and slowed movements (bradykinesia) can also be seen in the later stages (2, 9). Symptoms early in the disease are slight and usually include chorea and the ocular motor deficits. As the disease progresses, motor skills continue to deteriorate and more cognitive and psychiatric abnormalities manifest themselves (10). The onset of symptoms usually occurs in 30 to 50 year olds and the symptoms progress with the patients’ age. Histopathologically, HD mainly affects the medium spiny neurons in the striatum (11, 12). In addition, progressive cortical thinning (13-18), striatal atrophy (19-21), and striatal specific transcriptional dysregulation (22-26) are also seen in HD patients. The main cause of death, which usually occurs within 10 to 20 years after diagnosis is pneumonia, primarily due to the eventually bed ridden state the patients reach in the later stages of the disease (27-29).

Approximately 3 to 8 out of 100,000 individuals are estimated to have HD in the United States. The prevalence of HD individuals ranges from 1 in 100,000 individuals in some European countries and Japan to as high as 7 in 1,000 in the Lake Maracaibo region in Venezuela (30-32). However, as noted by Spinney and Rawlins (33, 34), the prevalence may in fact be higher than what has been claimed.
In 1993, the Huntington’s Disease Group discovered a single gene, *huntingtin* (*htt*), located on human chromosome 4 was directly linked with the occurrence of HD. The group found that in the first exon of *htt*, the tri-nucleotide CAG (which encodes for the amino acid glutamine) repeat region is abnormally expanded in HD patients (35). Normal individuals have between 6 and 35 CAG repeats whereas those with HD have ≥36 repeats. There are reports of individuals with 36 to 39 repeats that do not exhibit any clinical manifestations (36, 37), but these are rare cases. The age of HD symptom onset cannot be predicted but the development of symptoms tends to be inversely related to the number of CAG repeats in huntingtin (38-40). HD patients typically have one expanded copy and one normal copy of the *htt* gene, but in some rare cases they have two expanded copies (41, 42). Disease progression in the homozygous patients is much faster than the heterozygous HD patients (42) though the severity of the disease does not appear to be any greater in the homozygous individuals compared to heterozygotes (43, 44).

Lengths of CAG repeats ranging from 27-35 repeats have been shown to be unstable and as certain cells proliferate, CAG repeat regions can either expand or shrink. If this CAG expansion occurs in germ line cells, these mutations can be passed on to the individual’s children and potentially manifest into symptomatic HD (37, 45, 46). Triplet expansion is not limited to germ lines and has been reported in somatic cells, for example in the striatum (47).

There are a number of other CAG repeat diseases that have been collectively called polyglutamine diseases. These polyglutamine diseases include a number of the spinocerebellar ataxias (SCA types 1, 2, 3, 6, 7 and 17), spinobulbar muscular atrophy
(SBMA) also called Kennedy’s disease, Dentatorubropallidoluysian atrophy (DRPLA) and HD. All of the diseases have a motor function component which may include ataxia, chorea or dystonia and some like, HD, SCA 1, SCA-17 and DRPLA have cognitive deficits associated with the disease (48-52). Because all of these disorders are caused by the same expansion of a polyglutamine tract, it is thought that similar pathological pathways occur in each disease. If a treatment or therapy can be designed and successfully implemented in one expanded polyglutamine disorder, the same therapeutic concept might be able to be applied to the other diseases.

**HD Pathology**

Huntingtin is ubiquitously expressed in all cells throughout the body, but the central nervous system (CNS) and particularly the basal ganglia and cortex appears to be primarily affected (53-55). Other tissues and organs such as testes (53, 54, 56, 57) and muscle (58-60) have displayed various adverse functional and physiological problems due to mutant htt. While the testes and muscles do appear to be affected by the huntingtin expansion, the primary cause of the HD symptoms stem from the dysfunction in the basal ganglia.

The basal ganglia consists of the striatum (caudate and putamen), globus pallidus (external and internal segments), substantia nigra and the sub-thalamic nucleus. These structures are responsible for coordinating voluntary movements and suppressing unwanted involuntary movements. In the basal ganglia circuitry, there are two main pathways involved in the process of motor movement; the direct and indirect pathways (see Figure 1-1). The direct pathway involves neurons containing D1 receptors (called D1 neurons, which contain enkephalin) and the indirect pathway involving neurons with D2 receptors (called D2 neurons which contain substance P) (61-64).
**Direct Pathway**

In normal functioning basal ganglia, the D1 neurons have inhibitory projections to the globus pallidus internal segment (GPI). The GPI has inhibitory projections to the thalamus which, in turn, has excitatory projections to the motor cortex. Overall, activation of the D1 receptors results in the excitation of the motor cortex. Disruption of the direct pathway by the D1 neuronal loss or decreased D1 neuron activation induces more inhibitory signals being sent to the thalamus, leading to less excitatory input to the motor cortex and, subsequently, suppression of motor function (65-68). A diagram of the pathway can be seen in Figure 1-1.

**Indirect Pathway**

In the indirect pathway, striatal medium spiny neurons bearing D2 receptors project to the globus pallidus external segment (GPe), which, in turn, will generate an opposite net result to the D1 pathway. The GPe has inhibitory projections to both the GPI and the subthalamic nucleus. The subthalamic nucleus subsequently has excitatory projections to the GPI. Overall, in the indirect pathway, D2 receptor activation causes motor cortex inhibition. Thus, D2 neuronal loss or decreased D2 activation promotes more excitatory input to the motor cortex (65-68)

**Cellular Signaling in the D1 and D2 Neurons**

D1 receptor activation increases cyclic adenosine monophosphate (cAMP) levels inducing activation of protein kinase A (PKA) which phosphorylates a highly enriched protein in the medium spiny neurons of the striatum called dopamine- and cAMP-regulated phosphoprotein of 32 kDa (DARPP-32) (69-75). DARPP-32 modulates various downstream neuronal targets, such as phosphotase protein (PP-1) inhibition (76-79). The D2 receptors, when activated, cause a decrease in cAMP which will, in
turn, reduces the concentration of phosphorylated DARPP-32 (71, 74, 80). The downstream signaling of D2 activation is therefore the opposite of what is seen in D1 activation.

In addition to the D1 and D2 receptors, medium spiny neurons also express other receptors that help modulate the cAMP signaling pathways and subsequent regulation of DARPP-32 and PP-1 such as cannabinoid type one (CB1) and adenosine (A<sub>2a</sub>) receptors (81, 82). Activation of CB1 receptors without D2 receptor activation on a D2 neuron decreases cAMP levels similar to D2 receptor activation alone (83). However, when both CB1 and D2 receptors are co-activated, there is an increase in cAMP accumulation (84). Adenosine receptors are expressed specifically on D2 neurons and upon activation induces cAMP accumulation similar to activation of D1 receptors (81). Adenosine activation may potentially be a mechanism that is directly counteracting the D2 activation in the D2 neurons (85).

Two phosphodiesterases (PDE) that are found to be enriched in the striatum also regulate cAMP and DARPP-32. Both PDE10a and PDE1b have been found in the striatum (86-88), and have been shown to catalyze the hydrolysis of cAMP and other cyclic nucleotides (89, 90). These two proteins play a role in the activation of DARPP-32 and the subsequent signaling pathways of the basal ganglia.

**HD and the Striatal Pathways**

Reinier et al. determined that HD affected the two striatal projection neuron populations neurons differently (91). Ensuing studies demonstrated that HD preferentially causes cellular dysfunction in the D2 neurons resulting in a stronger motor cortex activation (65-68). This strong motor cortex activation induces the characteristic chorea observed in HD patients. D2 subtype neurons are the first striatal
projection neuron population to be damaged and lose their inhibitory signaling in HD. As the disease progresses, the D1 neurons also become damaged generating the rigid ataxia associated with the end stages of the disease (91, 92).

As a marker for D2 neurons, enkephalin mRNA and protein levels were shown to be reduced in HD patients (24, 25, 63, 64, 91, 93, 94). The D2 receptor, CB1 and other striatal-specific gene transcripts have also been reported to be reduced in patients (23, 95-97). Recent mRNA microarray studies demonstrated that the expression of a significant number of transcripts and proteins were abnormally dysregulated in early stage HD patients compared to healthy individuals prior to D1/D2 neuronal degeneration. Differences in specific mRNA and protein levels before and after neuronal degeneration suggest that these genes and/or proteins can be used as pathological markers for HD progression (98, 99).

**Transcriptional Factors Associated with Huntingtin**

Decreased mRNA described in the previous section suggests transcriptional dysregulation in HD patients which might indicate that the mutant htt affects gene transcriptional regulation. One of the first transcriptional factors associated with htt was CREB binding protein (CBP). Although no direct interaction has been determined between CBP and wild type htt, various groups have shown mutant htt interacts with CBP in vivo and in vitro (100-104). Steffan et al. concluded mutant htt appears to bind to CBP while Shimohata and Dunah found that TATA-binding protein (TBP) associated factor II130 (TAFII130), a co-activator for CREB transcriptional activation, can bind to long stretches of glutamines, which are found in mutant htt. They also showed TAFII130 and TBPs in inclusion bodies of another human poly-glutamine disease spinocerebellar ataxia type 3 (SCA3).
Another study examining the CBP-regulated transcriptional pathways, found nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is down regulated in the presence of mutant htt (105) Interestingly, NF-κB p50 can directly bind to the huntingtin protein via the several HEAT (huntingtin, elongation factor 3, protein phosphatase 2A, TOR1) motifs contained in huntingtin (106). Further evidence for NF-κB regulation by huntingtin was observed by Marocora et al. who showed that wild-type huntingtin helped regulate the transport of activated NF-κB from the neuron synapse to the nucleus (107). In the study Marcora et al., mutated huntingtin reduced the speed at which the neuron transported the NF-κB from the synapses (107). The group suggested huntingtin associated protein -1 (HAP-1) might be the intermediate between NF-κB and huntingtin and the transport to the nucleus is hampered by the poly-glutamine expansion in mutant huntingtin (107). The same group previously has shown that HAP-1 interacts with NeuroD, another neuronal-specific transcription factor. The interaction appears to be modulated by huntingtin itself and helps with the activation of NeuroD (108).

Wild-type htt has been shown to sequester the transcriptional repressor factor REST/NRSF (repressor element silencing transcription factor/neuron-restrictive silencing factor) to the cytoplasm (109). Wild-type htt’s ability to sequester a transcriptional repressor would results in an increase of downstream transcripts, mutant htt appears to lose the ability to sequester REST/NRSF and allows larger quantities of the repressor to enter the nucleus. The net effect is a down-regulation of a variety of transcripts, many of which are important in neuronal development, signaling and other transcriptional regulation (110, 111).
In addition to the transcriptional factors mentioned above, Sp1 (102, 112), p53 (100, 113), NCoR (114, 115), and PGC-1α (116, 117) are other transcription factors that have been shown to have their functions altered by the presence of mutant htt. Regardless of htt function, the fact that mutant htt does associate with transcription factors is an important feature when examining the effect of the expanded polyglutamine mutation in mutant htt. These mutant htt-related aberrant transcriptional associations have radical downstream effects that could be the ultimate cause of the disease.

In the current rodent models of HD, which will be discussed later in this chapter, there is clear evidence of transcriptional down-regulation. DARPP-32 (118-120), ppENK (119, 121), D2 (119, 122), CB1 (123-125) and PDE10a (22, 26) as well as many other (119), have all been shown to be down regulated in various mouse models. More importantly, these transcriptional abnormalities in the models of HD recapitulate what is seen in the human disease (126). Transcriptional and translational dysregulation could have major implications in HD pathology and the behavioral abnormalities of HD.

**Neuronal Intranuclear Inclusions**

Neuronal intranuclear inclusions (NIIs) were first discovered in the striatum of the R6/1 HD mouse model by Davies et al. in 1997 (127). Using an antibody (clone mEM48) against the amino-terminal of the mutant htt protein, a distinct nuclear staining pattern was observed in the R6/1 brain sections. The inclusion bodies that were stained with the EM48 antibody were found to be inside the nucleus and were dubbed neuronal intranuclear inclusions or NIIs. In addition to the discovery and localization of the NIIs, ubiquitin staining was co-localized to the same inclusion bodies. Later the same year,
DiFiglia found these NIIs in human post-mortem patients and has subsequently been used as a pathological marker of HD (128).

**HD Models**

The first attempts to generate a suitable animal model to study HD focused on inducing HD symptoms via injections of excitotoxic chemicals to induce neuron lesions. One of the first excitotoxic chemicals used in an attempt to simulate the characteristic human HD symptoms and/or pathology in rats was kainic acid (KA). When injected in the striatum of rats, KA induces an axon sparing lesion which causes a variety of motor and behavioral abnormalities similar to characteristic HD symptoms documented in human patients (129-131). However KA also induces non-specific cellular degeneration of the olfactory, cortex, hippocampus and other limbic structures which is not typical to human HD pathology (132, 133).

In 1986, another excitotoxic reagent quinolinic acid (QA), a N-methyl-D-aspartic acid (NMDA) receptor antagonist, was first characterized by Beal et al. (134). QA-induced lesions cause rodents to display behaviors and pathology that more accurately imitates HD compared to other excitotoxic chemicals (134). In contrast to KA, QA lesions does not induce excessive non-specific cytotoxicity and does not affect the striatum somatostatin- and neuropeptide Y-expressing medium spiny neuron subpopulations, which are usually retained in HD patients (135-137).

Since QA lesions can cause HD-like behavior in rats and it is expressed endogenously in both human and rodent brain tissues, it was hypothesized that elevated QA levels could be the possible cause of HD (138). Later studies determined that although QA is present in HD patient tissues, QA expression is not up-regulated in comparison to normal individuals (139-141). While QA-induced lesions facilitated the
study of the HD, the discovery of the causative gene in HD, *huntingtin*, allowed for genetic models of HD to be developed which more accurately model the disease.

**Mouse Models of HD**

Various HD rodent models have been developed to help understand the mammalian cellular pathways involved in HD. There are three general types of rodent models; the knock-outs, transgenic and knock-in models. All three models have been extensively used to study HD and as well as to test therapeutics. A general overview of a few of the most commonly used mouse models of HD are described below.

**Knock-Out Mouse Models of HD**

The mouse homolog of the huntingtin gene, *hdh*, is approximately 90% homologous with human *htt* which suggests that the two homologs likely share similar functions in both species (142). Therefore studying mouse *hdh* in HD murine models may elucidate how wild-type human *htt* functions and potentially the role of mutant *htt* in HD. Hdh knock-out (KO) models were generated to determine whether the lack of a functional wild-type *hdh* can induce HD-like symptoms. Duyao et al. generated a null *hdh* mutant in 1995 by deleting the 4<sup>th</sup> and 5<sup>th</sup> exons of the *hdh* gene. Homozygote Hdh(−/−) null mice died around embryonic day 7.5 but heterozygote mice were not affected by the deletion (143). Similarly Nasir et al. inserted a neomycin resistance cassette into the *hdh* 5<sup>th</sup> exon to create a truncated Hdh mutant (~20 kDa vs. the normal ~340 kDa). The homozygote truncated Hdh mutation was also embryonic lethal. Homozygote truncated Hdh mice died anywhere from 7.5 to 12.5 days and like the Duyao null *hdh* mutant mice, the heterozygote mice were viable. (144). However the heterozygote mice from Nasir study displayed phenotypical differences from nTG mice which were not reported in the heterozygotes from the Duyao et al. study. Heterozygote
mice from the Nasir et al. report were more active in both light phases and their memory was impaired, as tested by the Morris-water maze (144).

Another Hdh KO mouse model was developed by Zeitlin et al., in which the promoter through the first intron of the hdh gene was replaced with the neomycin resistance cassette, similar to how Nasir generated a truncated hdh mutant. Just like the previous KO models, homozygous mutant mice were embryonic lethal at approximately embryonic day 7.5. Disputing Nasir but confirming Duyao, the heterozygote mice in the Zeitlin study did not appear to have any phenotypical differences when compared to the nTG (145).

Conditional Knock-Outs

The insertion of either a human htt fragment or the expansion of the native hdh gene in mice has been invaluable in the study of HD. Just as useful has been the study of conditional knock-outs where the reduction of hdh or the removal of the wild-type hdh can be controlled. Dragatis et al. in 2000 developed a cre-lox system to remove the hdh only in the brain and after neo-natal development (146). The cre gene is under the tissue specific promoter CamK2a and only activates post-natally in the forebrain (147). When the hdh gene was turned off by the cre-lox system, the gross morphology of the brains is similar to the nTG littermates up until 4 months where neuronal degeneration is observed in the cortex. Further neuronal degeneration is observed in these mice at approximately 8 months in the hippocampus, striatum, amygdala and cortex. Behaviorally these mice showed the classical HD clasping phenotype at about 2 months, and wire rod hanging and cage top rotation motor skill tasks showed abnormalities at around 3 to 4 months of age (146). Near or complete loss of huntingtin, as this study shows, is detrimental.
Transgenic Mouse Models of HD

Transgenic models have either the full length human huntingtin or a certain fragment of the human gene inserted into the mouse genome, with either the normal or an expanded CAG repeat region. The insertion is in addition to the endogenous hdh gene. Examples of these transgenic mouse models are described below. The R6, N171-82Q and the YAC transgenic mice models are among the most widely used in the field.

Tetracycline regulated transgenic

Yamamoto et al. generated a tetracycline regulated transgenic HD mouse model (148). Here a tetracycline responsive element is placed in control of an htt transgene with 94 repeats. When tetracycline is introduced to the diet of the mice, the transgene is turned off. Transgenic huntingtin mice, when administered tetracycline, showed a decrease in the hind limb clasping phenotype and aggregations in striatal tissue was reduced in the treated animals. In particular the tetracycline feed mice showed an increase in striatum size compared to the mice not feed tetracycline (148). While the tetracycline mouse line is conditional knock-out model and not an ideal model for HD, it was utilized as a method of determining if removal of mutant htt would alleviate all symptoms of the disease. In this study, reductions of the mutant htt appear to alleviate the HD like symptoms.

R/6 model

The first transgenic mouse models of HD was developed in 1996 by Mangiarini et al. (149). The R6 models contain the 5’ of the untranslated region, the first exon, and 262 base pairs of the first intron of the htt which is inserted into the mouse genome. The entire human transgene is driven by the htt promoter. There are two main lines
generated in this fashion that are commonly used, the R6/1 and the R6/2 which have 116 and 144 repeats respectively placed into the CBA x C57BL/6 mouse background. The gene expression levels of the R6/1 and R6/2 in relation to the endogenous mouse huntingtin are 75% and 35% respectively. Cognitive deficits are observed at about 4 weeks in R6/2 mice and at 10 to 12 weeks in the R6/1 mice (150-153). These R6 mice show resting tremors, clasping behavior when held by the tail, gait abnormalities and other motor skill deficits occurring approximately at 5 to 9 weeks in the R6/2 mice and 8 and 9 weeks in the R6/1 line (149, 154). The pathological hallmark of HD, NIs were found first in the R6 lines and has since been one of the most utilized histopathological marker in models of HD as well as in humans (127, 155-158). Transcript levels, similar to what is seen in humans, are dysregulated in the R6 mice (22, 26, 124, 125, 159, 160).

**N171-82Q**

N171-82Q model uses the prion promoter that creates the first 171 amino acid residues of htt. The expanded glutamine repeat region contains 82 repeats and causes a progressive neurodegenerative disease similar to HD (157). Phenotypically these mice did not show any major differences until approximately 12 weeks of age. At 12 weeks, the transgenic mice’s weight plateaus unlike their nTG counterparts who continual gain weight. Like the R/6 mice, subsequent studies have shown large amounts of gliosis and neuronal apoptosis that appear at around 12 weeks (161). Tremors are evident at 16 weeks and hypoactivity is observed at around 20 weeks of age (157). Behaviorally the N171-82Q mice showed little differences to the nTG control mice in rotarod latency to fall or beam walking tasks out to 12 weeks where after the mice progressive accelerating rotarod deficits (157, 162). Gait abnormalities, in
particular the stride length of both front and rear paws, is significantly shorter at 16 weeks (163). The N171-82Q mice have an shortened life span and usually die at ~20 to 24 weeks of age (157).

**YACs**

The same year the Bates R/6 mouse models were being developed, Hodgson et al. (164) developed a full length model of HD in the mouse. Using yeast artificial chromosomes, the group inserted the full length human HD gene into the mouse. Localization and expression of the transgene was similar to \( hdh \) and these YAC mice usually have one or two transgene copies inserted into the genome. Three YAC mice were developed that contained 18, 46 and 72 CAG repeats in the YAC transgene (164, 165).

The full length expanded gene inserted into the mouse genome displays neuronal degeneration in the 72 CAG repeat line. The degeneration observed was anywhere from 4 to 40% medium spiny neurons at approximately 12 months of age. There was no obvious degeneration in the YAC18 or the YAC46 lines. Histopathologically the YAC72 mouse line shows an increase of htt N-terminal staining in the nucleus. Behaviorally, these mice showed early hyperactivity and late hypoactivity (164, 165). A larger poly-glutamine mouse was created in 2003 with a 128 CAG insertion. The YAC128s showed significantly early onset of the hyperactivity starting at 3 months compared to the 7 month onset of the YAC72 mice. Twelve month old YAC128 mice showed an average of 18% neuronal loss (166).

**Knock-Ins**

The Knock-in models have portions of the human \( htt \) replacing the corresponding mouse \( hdh \) homolog. Another version of a knock-in is the artificial expansion of the
endogenous hdh polyQ region. The knock-in models, therefore, have the ability to have a heterozygote and a homozygote genotype.

Initial studies of human htt knock-in mice by Levine et al. created a model of HD by replacing the 5' portion of the hdh gene with the corresponding human portion of the htt gene. The 5' portion of the hdh exon 1 starting at 18 base pairs before the polyglutamine region into the first intron was replaced with the corresponding human htt. Two lines were generated with either 71 CAG repeats or 94 repeats. The CAG 71 mice did not show any behavioral or histopathological abnormalities associated with HD in mouse models. The CAG 94 line, however, showed hyperactivity at approximately 2 months and hypoactivity at 4 months. Microaggregates show up at 6 months and progress to NIs at 18 months. The CAG 94 knock-in model also showed cellular dysfunction but no noticeable cellular loss (121, 167, 168).

The same group later created a knock-in model with 140 repeats (169). Similar to the previous models with 94 repeats, there is an early hyperactivity followed by a decrease in activity when compared to their nTG litter mates. Aggregates (NIs) are present in the striatum, cerebellum, cortex and olfactory tubercles of the CAG 140 mouse model at approximately 4 months of age. More recent studies have shown that homozygote CAG140 mice had behavioral abnormalities such as open field and running wheel activity deficiencies at select time points early in the course of the disease and slight sex differences in home cage activities. The later studies also showed DARPP-32 protein reductions and cortical gliosis that start at 12 months along with striatal gliosis starting around 23 months (170, 171).
Striatal electrophysiological properties in the CAG 140 mice exhibit similar properties to the R6 mouse line. These include lower spontaneous excitatory postsynaptic currents (EPSCs) and higher spontaneous inhibitory postsynaptic currents (IPSCs) in the medium spiny neurons of the striatum (172). Pre-frontal cortex electrophysiology examinations have revealed that the CAG 140 mice tend to have no significant differences in spontaneous firing rates or bursting activity when compared to their nTG counterparts, unlike the R6 mice which showed significant increases (173). However, the same study found that cortical spike synchrony was diminished in both the CAG 140 and the R6 mice. The group suggested that some of the phenotypical motor differences observed between the R6 and the CAG 140 mice could be due to differential spontaneous firing and bursting activities but that the similar asynchronous firing may contribute to the striatal signaling abnormalities observed in both mice (173). The CAG 140 model will be the model used in our studies and will be further characterized in the first part of the work done here.

Another knock-in models includes the \( Hdh^{(CAG 150)} \) model and was created by Lin et al. and showed a similar phenotype to other full length knock-in HD models (174). Behaviorally, both heterozygous and homozygous \( Hdh^{(CAG 150)} \) mice showed lower home-cage activity, clasping phenotype, gait abnormalities and NII. A longitudinal study of the \( Hdh^{(CAG 150)} \) has shown that the majority of measurable behavioral phenotypes are not significant till around 70 to 100 weeks of age. Rotarod deficits, beam walking abnormalities and clasping behavior were all observed between 70 and 100 weeks. Histopathologically the \( Hdh^{(CAG 150)} \) mice show an increase in NII at around
Shelbourne et al. expanded the CAG region in hdh to 72 or 80 repeats and the resulting mice exhibited social abnormalities such as higher aggression (176). The Shelbourne mice did not show any significant histological difference other than a 10-15% reduction in overall brains size when compared to their wild-type littermates (176). Inclusion bodies associated with HD appear in the 80 CAG expanded hdh model at approximately 15 months (176). Both the 72 and the 80 CAG repeat mice showed early aggression compared to their nTG littermates.

In a similar fashion to the mice generated by Levine and Menalled created a chimeric hdh/htt expanded CAG knock in mice that either expressed 92 or 111 repeats (177). These Q92 and Q111 knock-in mice show the hallmark aggregates, starting at as diffuse staining and progress to full NII inclusions. Full NIIIs were evident starting at 40 weeks in the Q111 mice. Both the heterozygote and the homozygote mice in the 111 repeat model have shown abnormal gait which consists of a shortening of the stride length and an increase in stride width at 96 weeks (178). Like other knock-in mice, life span was not drastically affected (178).

**Gain of Toxic Function**

HD pathology is generally thought to be caused by a gain of toxic function by mutant htt. Aberrant transcriptional factor binding (100, 102-104, 112, 113, 115-117, 179) would suggest that mutant htt has gained an additional role that would bind these transcriptional factors more tightly than wild-type huntingtin. Furthermore, heterozygous mice generated from the knock-out mice created by Zeitlin and Dauyo (143, 145) and the viability of homozygous knock-in mice (168, 169) suggest that a lack of wild-type
huntingtin is not the cause of HD. If the mutant htt did not perform the wild-type functions, then both the heterozygous knock-out mice and homozygous knock-in mice would not be viable and would be embryonic lethal, as seen in the homozygous KO mice. If HD is caused by a gain of toxic function in the mutant htt, then removing the toxic protein should alleviate the disease.

**Adeno Associated Virus (AAV)**

**Basic Biology**

Adeno-Associated Virus (AAV) is a single stranded DNA (ssDNA) parovirus which requires a helper virus, either a herpes virus or adenovirus, to assist in its replication. AAV is able to infect both dividing and non-dividing cells. Although AAV can incorporates its genome into that of the host cell, it does not cause any known disease and stimulates only a weak immune response. Wild-type AAV genome is approximately 4.7 kilobases and encodes two genes, cap and rep (180). The cap gene contains three open reading frames for the capsid proteins VP1, VP2 and VP3 (181). The rep gene is responsible for the viral genome replication and encodes for the proteins, Rep78, Rep68, Rep52 and Rep40. Two promoters and alternative splicing creates the four polypeptides from just rep gene. The adenovirus proteins E1A, E4 and E2A help facilitate the AAV genome transcription, gene regulation and translation respectively together with the 4 AAV rep proteins, Rep78, Rep68, Rep52 and Rep40 (180, 181).

At the 5'- and 3’-ends of the AAV genome are palindromic repeat regions referred to as inverse terminal repeats (ITRs) which act as the start sites for DNA replication and packaging signals (182). The 3’ ITR acts as the primer for replication, allowing DNA polymerase to begin synthesis of the second DNA strand. After replication of the AAV
genome, the ITRs help in the loading of the genome into the viral capsid, if the ITRs are not present or lost, genome packaging can not occur (180).

**Serotypes**

There are many different types of AAVs serotypes that have been discovered over the years (183-185). Most of these serotypes can be divided into groups or ‘clades’ that are related to one another by their similar genetics. Gao defined a clade as a group of three or more AAVs that were phylogenetically similar. As of now, there are 6 clades and a variety of independent clones (183). Each of the AAVs in a particular clade has little or no serologic cross-reactivity to other clades. Thus if a new AAV is discovered and does not have serological cross-reactivity to any of the known clades, it would be considered an independent clone. If two more clones were found to be serologically related to this virus, a new clade could be identified.

**Receptor Binding and Entry**

Two receptors are responsible for AAV infectivity, one for cell surface binding and the second for endocytosis. AAV2 was shown to bind to a cell surface protein in permissive cell lines and the entry could be abolished by trypsinization of the culture (186). Further studies determined that the proteins involved in particle binding were glycosylated and led groups to determine that the proteoglycan, heparin sulfate proteoglycan, was responsible for AAV2 binding to cell surfaces (186-188).

The co-receptor for endocytosis is not a single receptor in AAV2. Human fibroblast growth factor receptor 1 (FGFR1) (186), αVβ5 integrin (189, 190), hepatocyte growth factor receptor (c-Met) (191), integrin α5β1 (192), and laminin (193), have all been implicated as co-receptor of AAV2 binding and subsequent internalization. Similarly there are a wide variety of co-receptors for the various serotypes that have been
isolated (189, 193-197) PDGF\(\alpha\) and \(\alpha2,3\) N-linked sialic acid are co-receptors for AAV 5 (194, 195); \(\alpha2,3\) N-linked sialic acid and \(\alpha2,6\) N-linked sialic acid are co-receptors for AAV 1 and 6 (196); and laminin has been found to be the co-receptors for AAV 2, 3, 8 and 9 (193).

After the internalization and creation of an early endosome, the virus particle exposes nuclear localization signals on the VP1 and VP2 subunits of the capsid (198). The exact route to the nucleus is currently still under debate and various groups have suggested passage to lysosomes or to the Golgi apparatus (181, 199). Once the particle reaches the nucleus, whether uncoated or not, the ssDNA genome of the AAV needs to be synthesized into dsDNA in order to produce viable gene expression.

rAAV

The use of AAV as a potential vector in gene therapy was not fully explored until it was determined that the capsid protein could be provided in \textit{trans} and yield a viable virus particle that does not replicate after infection (200). The \textit{cap} gene was replaced with a neomycin cassette, and a plasmid containing the \textit{cap} gene was introduced separately into the culture. The resulting virus produced expressed the resistance gene when culture cells were infected (200). The only components necessary in \textit{cis} from the original AAV genome are the 145 base pairs ITRs. The \textit{cap} and \textit{rep} genes can be provided in \textit{trans} to produce a viable recombinant AAV (rAAV) with a transgene cassette between the ITRs (182). Production of rAAV took a step further when the helper proteins from Ad were also provided in \textit{trans} in another plasmid (201). The additional plasmid containing the necessary helper proteins negates the need for using an actual helper virus (201). The most current and effective production of rAAV have
come from using baculovirus or HSV helper proteins on separate plasmids rather than using the Ad helper proteins (202-205).

**AAV Pseudotyping**

Since production of rAAV has been streamlined for the AAV2 serotype, various pseudotypes of the virus have been made. Utilizing the ITRs in the genome of the AAV2 genome, these pseudotypes have the cap proteins of the desired serotype but use the rep protein from the AAV2 virus (206). The subsequent production is exactly the same described in the previous section. To differentiate between these different types the nomenclature, rAAV 2/n, is sometimes used. The ‘n’ here depicts the capsid type. Cell tropism of the pseudotyped rAAVs is determined by its capsid identity.

**Neuronal Targeting**

One of the first examples of neuronal AAV tropism was performed by Davidson et al. in 2000 (207). Here, the group examined the AAV serotypes 2, 4 and 5 and found that AAV 2 and 5 transduced striatal neurons effectively while AAV4 transduced ependymal cells. Burger et al. in 2004 compared AAV 1, 2 and 5 and found that while AAV 2 does appear to infect a wide variety of brain structures including the striatum, thalamus, substantia nigra and spinal cord, both AAV 1 and 5 give a greater transduction efficiency and distribution (208). These studies along with numerous others have greatly expanded the known tropisms in the brain of the widely used AAVs (185, 207-212).

To date, there are approximately 10 main serotypes being used in studies infecting various regions of the brain and each serotype has a varying degree of expression depending on the region being infected. AAV 1, 2 and 5 can all infect the striatum, but the degree of transduction is not the same. The degree of transduction
may also vary from species to species. For instance, in mice rAAV5 infectivity is overall
greater degree than rAAV8 but in rats rAAV8 appears to infect neurons to a greater
extent than rAAV5. Table 1-1 give the relative hierarchy of serotype infectivity in the
rodent CNS of AAV 1, 2, 5, 8, 9 and 10 (207-209, 211-214).

RNAi

Fire et al. in 1998 discovered that injecting both sense and anti-sense RNA
corresponding to endogenous mRNA resulted in a reduction of the corresponding
protein (215). This phenomenon, called RNA interference (RNAi), has become an
important tool in the field of biology. The importance of RNAi discovery was recognized
with the awarding of the Nobel Prize to both Andrew Fire and Craig Mello in 2006.
Currently there is a wide use of variations of the RNAi, such as short interfering RNA
(siRNA), short hairpin RNA (shRNA) and microRNAs (miRNA). Each of these versions
degrades the corresponding mRNA but the processing of the different forms varies
slightly.

There are two main pathways associated with RNAi, the siRNA pathway and the
miRNA pathway. Both reduce the production of protein but in slightly different ways.
 siRNA pathway causes protein reduction by degradation of the mRNA while the miRNA
pathway inhibits translation of the mRNA by creation of P-bodies. Below are brief
descriptions of both pathways.

siRNA Pathway

The siRNA pathway begins with dsRNA that has been introduced to the cell by a
virus or other artificial means. Dicer, an endonuclease, cleaves longer dsRNA (>25nts)
into shorter ~21 nucleotide siRNA fragments with 2 nucleotide 3' overhangs (216-218).
In mammals and C. elegans only one Dicer is responsible for cleaving these long
dsRNA into the smaller siRNAs (219, 220). Other species such as *Drosophila* and many plants, multiple Dicer or Dicer-like proteins help in the processing of dsRNA (220-222).

After Dicer cleaves the long dsRNA into ~21 nucleotide siRNAs, the catalytic protein Argonaute is recruited and forms the RNA induced silencing complex (RISC) (223). The Argonaute protein is comprised of 2 basic functional regions, the PAZ (224-226), and the PIWI regions (227-229). The PAZ region loads the 3’ overhang of the siRNA into the RISC in part (224, 225). The PIWI domain is considered to be an RNAse H like domain (230) and helps in the degradation of the passenger strand of the siRNA (231-234). After the degradation of passenger strand, the PIWI domain facilitates in the degradation of the mRNA (228, 229). The target mRNA is cleaved by the PIWI domain of Argonaute between the base pairs corresponding to the 10th and the 11th nucleotides of the siRNA (235, 236) A simplified pathway can be seen in Figure 1-2.

**miRNA Pathway**

Normally, miRNAs are encoded in the genome of organisms and are transcribed into an ssRNA which form large secondary structures. These long ssRNAs, called pri-mRNA, produce large stem and loop structures (See Figure 1-2). In the nucleus, Drosha with the help of DGCR8, cleaves the pri-miRNA at certain points in the stems to produce smaller stem-loop structures called pre-miRNAs (237-240). After Drosha/DGCR8 cleavage, the pre-miRNA is exported out of nucleus and into the cytoplasm the by the RanGTP-dependent Exportin 5 (241-243).

Once in the cytoplasm, the pre-miRNA is cleaved at the stem loop base by the endoribonuclease protein, Dicer, and converted to the final miRNA (216, 217). From here the process is similar to the siRNA with one exception at the end. miRNAs are normally not 100% complementary to their target mRNAs and these mismatches can
result in translational repression rather than mRNA degradation. In addition, in mammals, miRNA binding sites are typically in the 3’ UTR of mRNA, rather than in the coding regions. The RISC containing the mismatched miRNA can bind with the corresponding mRNA and form P-bodies where the mRNA can either be degraded or sequestered (244-248). If the miRNA does not have mismatches, the mRNA will be degraded as seen in the siRNA pathway.

**shRNAs**

One form of commonly used RNAi is shRNA which consists of one continual RNA strand with a stem and a loop, similar to miRNAs. The stem is what contains the sense and anti-sense portions of the RNAi. The shRNA construct can be constitutively expressed when placed after an appropriate Pol III promoter and can be introduced to a cell as plasmid or viral vector. Similar to miRNAs, the shRNAs are generated in the nucleus and transported out by Exportin-5 (241). Unlike the miRNAs however, the shRNA enters siRNA pathway at Dicer and the loop is cleaved off approximately 22 nucleotides from the 3’ terminus of the stem (249). The resulting siRNA enters the RISC and subsequently degrades its target mRNA (250, 251).

**RNAi considerations**

One problem of RNAi is the ‘off-targeting’ phenomena. An RNAi molecule that is meant to knock-down a transcript may inadvertently reduce expression of another mRNA. The inadvertent off targeting has primarily to do with the target sequence itself. Because the siRNA and shRNAs that are incorporated into the RISC are approximately 21 nucleotides long, it is quite likely to have some sequence homology to another mRNA that was not intended to be targeted. Lewis et al. found that the nucleotides from 2-7 in the 5’ portion of the anti-sense strand, called the seed sequence, are the primary
sequences used by the RISC to suppress translation and lead to RNA turnover in the miRNA pathway (252). The smaller seed sequence greatly increases the likelihood of potential off-targeting. Not surprisingly, Jackson et al. found multiple transcripts that contained the complementary regions to seed sequence of one siRNA were reduced regardless of the siRNA or shRNA concentration or delivery method (253). Work performed since the seed sequence discovery has indicated that the region is a requirement for target recognition (254, 255). While the seed sequence may allow for other mRNAs with a complement to the region to be down regulated, nucleotides 9-11 appear to assist mRNA recognition (256).

**Hypothesis**

In order to better understand the progression of the CAG 140 mouse model, we tested the hypothesis that the insertion of 140 CAG repeats in the mutant huntingtin allele in the CAG 140 mouse model would elicit behavioral and histopathological differences. With the use of RNAi and the ability to specifically knock-down one allele in the CAG 140 mouse model we also hypothesized that reductions of the mutant huntingtin would lead to improvements in the CAG 140 phenotype.
Figure 1-1 Schematic diagram of the indirect and direct pathways found in the Basal Ganglia. Image modified from Yin et al. 2003 (257).
Figure 1-2 Diagram of RNAi pathways in a mammalian cell. Image modified from diagram in Cullen 2005 (258)
Table 1-1 Differences in infectivity rates of AAV serotype between rats and mice

RAT: AAV 10 ≥ AAV 9 ≥ AAV 8 ≥ AAV 1 ≥ AAV 5 ≥ AAV 2
MOUSE: AAV 10 > AAV 9 > AAV 7 ≥ AAV 5 ≥ AAV 8 = AAV 1 > > AAV 2
CHAPTER 2
MATERIALS AND METHODS

Animals and Tissue Preparation

Ten week old CAG140 knock-in mice on the C57/BL6 background strain (169) were used for behavioral and histological experiments (a kind gift of Scott Zeitlin). A portion of the first exon of the hdh gene was replaced with the human equivalent (169). The knocked-in region spans from 18 base pairs upstream of the CAG repeat region through the 100 base pairs of the first intron and introduces approximately 140 CAG repeats (169).

Genotype determination was performed by PCR analysis after isolation of genomic DNA from tail snips. PCR primers for the nTG gene are as follows: F- 5’ACGCATCCGCCTGTCAATTCTG 3’ and R- 5’ CTGAAACGACTTGAGCGACTC 3’. Primers for the knock-in gene are F- 5’ GCCCGGCATTCTGCACGCTT 3’ and R- 5’GAGTACGTGCTCGCTCGATG 3’. An initial 5 minute 94°C was followed by 36 cycles of 30 seconds at 94 °C, 30 seconds at 65 °C and 1 minute at 72 °C. Following the last cycle a final elongation step was performed at 72 °C for 7 minutes. PCR samples were run on a 2 % agarose gel and genotype was determined by either the presence of the nTG band at 534 base pairs and/or the knock-in band at 287 base pairs.

For all behavioral experiments 8 nTG mice, 13 heterozygous, and 4 homozygous mice were tested. In addition, mice at 3, 6, 9, 18, and 24 months of age were used for the in situ hybridization study as well as the immunohistochemistry (IHC) quantification of inclusion bodies and cortical thickness. The number of mice used in these cross-sectional studies varies and are indicated in the appropriate figure legends. No gender differences were observed in the study.
Mice were euthanized with a pentobarbital overdose (>150mg/kg), the brains were removed and stored at -80°C until further processing. All appropriate housing and handling procedures were followed in accordance with the Institutional Animal Care and Use Committees at the University of Florida.

**Plasmid and rAAV Preparations**

siHUNT1 and siHUNT2 were developed previously by Rodriguez-Lebron et al. (259). The shRNAs were originally designed to target the R6/1 transgene and in the CAG 140 model, only siHUNT2 targets the knock-in htt gene. The siHUNT1 shRNA targets the sequence: 5’GCCGCGAGTCGGCCCGAGGC 3’ which originally targeted the human 5’ UTR region which is not present in the CAG 140 mouse strain. siHUNT2 targets the sequence 5’ GGCCTTCGAGTCCCTCAAGTCC 3’ which is immediately upstream of the CAG repeat region in the human portion of the knock-in htt gene. The shRNAs were cloned into the rAAV pSOFF-H1p-hrGFP vector backbone with BgIII/HindIII restriction enzyme sites. The shRNAs expression is driven by the human RNase P H1 promoter while the hrGFP gene is driven by the herpes simplex virus thymidine kinase promoter. hrGFP was originally cloned from cDNA encoding the humanized *Renilla reniformis* GFP (Stratagene, La Jolla, CA, USA). Control rAAV5-GFP was cloned using the UF-11 vector backbone containing the hGFP created by Zolotukhin et al. (260). The hGFP transgene is driven by the CMV enhancer/ CBA promoter.

rAAV viral production was performed by the University of Florida Powell Gene Therapy Center Vector Core Facility as described previously (261). Viral titers ranged from 1.7 to 5.7 x 10^{13} vg/ml.
**Intrastriatal Surgical Injections of rAAV Vectors**

Mice were anesthetized with isoflurane by inhalation. After the mice were anesthetized, hair was shaved from their heads and a 2 mg/kg intraperitoneal injection of Mannitol (stock solution 30% v/v) was given approximately 15 minutes prior to the stereotaxic injections. Mice were then placed in a stereotaxic frame (Kopf Instruments, Tujunga, CA) for intracranial injections. The anterior-posterior (AP) and medial-lateral (ML) stereotaxic coordinates (AP +1.0, ML +1.8 and AP +0.4, ML +2.1) were measured from bregma with an empirically determined flat skull. A burr hole was drilled in the skull at the desired coordinates determined from bregma and the dorsal-lateral stereotaxic coordinates was then measured from the dural surface (DV -3.3 and DV -3.4 respectively). A glass micropipette was fitted onto a 10 µl Hamilton microsyringe and both were attached to a continuous infusion system (Carnegie Medicin, Sweden) to inject the viral vector. 2 µl of the rAAV was administered at the two coordinates indicated above. The rAAV was injected at a rate of 0.5 µl/min and the needle was allowed to stay in place for 1 additional minute. Following the 5 minutes, the needle was retracted 1mm for an additional 4 minutes. After a total of 9 minutes the needle was removed entirely. Two injections sites in the right striatum were used for the unilateral histological studies and 2 injections sites in both striatal regions were injected for the behavioral studies.

All appropriate housing, handling, and surgical procedures were followed in accordance with the Institutional Animal Care and Use Committees at the University of Florida.
Rearing Behavior

nTG, heterozygous, and homozygous mice were individually placed in 1 L beakers in the dark and videotaped for 10 minutes. Testing was performed at the end of the light cycle for the mice. The tapes were viewed by an individual who was ignorant of the mouse genotype whom determine the number of times the mice reared up and touched the side of the beaker with their front paws. Rears that did not include a front paw touch were not counted. Average number of rears per minute was calculated. The rearing task was performed once every two weeks initially and then once a month when the mice were 4 months old.

Rotarod

The accelerating rotarod (Columbus Instruments, Columbus, OH) started at 5 RPM and increased in speed at 0.3 RPM per second. The mice were allowed to stay on the apparatus until they fell off. The time that each mouse fell off was noted. The test was performed 4 consecutive times per day over 3 consecutive days. The maximum speed was then calculated from the latency to fall. A minimum of 1.5 minutes between each trial was allowed. We were unable to perform the rotarod test in the 15 month year old mice.

In addition, we measured the latency to fall off the rotarod at two different constant speeds. The constant speed rotarod had two speeds. Starting at 10 RPMs, the mice were allowed to continue to run on the rod until they fell or until 60 seconds elapsed. The test was performed again at 18 RPM. The test consisted of two consecutive 10 RPM - 18 RPM switches over two consecutive days following the accelerating rotarod task described above. A minimum of 1.5 minutes between each trial was allowed. We were unable to perform the rotarod test on the 15th month.
Gait Analysis

A gait apparatus with a runway of 50 cm by 10 cm wide with 10 cm high walls was used to test gait. Each mouse was initially placed in a dark goal box for 5 minutes with a Froot Loop™. The mouse was then taken out of the goal box, shown the well lit runway corridor and then placed back into the dark goal box for 1 minute. Next, the front paws of the mouse were painted blue and the rear paws painted orange with non-toxic water-based paint. White paper was laid down along the corridor floor. The mouse was then placed at the opposite end of the corridor from the goal box and allowed to return to the goal box on its own volition. Based on the marked colored foot prints, the distance between the front and rear paws (gait width), and the distance between the front footprints of the consecutive steps (gait length) were measured. In subsequent tests, mice were only allowed in the goal box for 1 minute before their feet were painted.

In Situ Hybridization

Fresh-frozen mouse brain tissue was sectioned at 14µm on a cryostat. Approximately 25 slides with 4 to 5 sections per slide for each animal were used for both IHC (see next section). *In situ* hybridization was performed on representative coronal mouse brain sections (bregma +1.70 to -0.50) mounted on slides by using radio-labeled (\(^{33}\)P) gene-specific antisense oligonucleotide probes. The probes targeted dopamine and cyclic AMP-regulated phosphoprotein with molecular weight 32 kDa (DARPP-32), pre-pro-enkephalin (ppENK), phosphodiesterase 10a (PDE10a), phosphodiesterase 1b (PDE1b), dopamine receptor type 2 (D2), cannabinoid receptor type 1 (CB1), neuronal growth factor I-A (NGFi-A, also known as zif-268), dynamin and β-actin. The methods employed for *in situ* hybridization and hybridization signal quantification have been described previously in detail (22, 26, 119, 124, 259).
Quantification of Transcripts

To determine the relative mRNA transcript levels, optical density (OD) was calculated using the Quantity One analysis software from Bio-Rad (Hercules, CA). Background intensity was subtracted and the resulting values were normalized to β-actin transcript levels. Heterozygote and homozygote percentages were determined based on nTG levels. For analysis after rAAV5 injections, transcripts from the injected side were compared with those from the uninjected side.

Immunohistochemistry

Slides for IHC staining were washed 3 times with PBS, fixed with 4% paraformaldehyde for 15 minutes. The slides were then washed 3 times with PBS, and then the samples were treated with 3% (v/v) hydrogen peroxide and 10% methanol solution for 10 minutes. Subsequently a blocking step was performed using 7.5% (v/v) natural horse serum (NHS) and 0.1% Triton-X100 solution for 2 hours at room temperature. Primary mouse antibodies anti-htt mEM48 (MAB5374, Millipore Billerica, MA, 1:2000 dilution), anti-NeuN (MAB377, Millipore, 1:1000 dilution), and anti-GFAP (mouse anti-GFAP MAB360, Millipore, 1:1500 dilution) were diluted in 1% NHS and 0.1% Triton-X100. Primary antibody solutions were applied to the slides overnight at 4°C using Hybriwells™ (Grace Bio-Labs, Bend, OR). After washing slides with PBS, they were incubated in biotinylated horse anti-mouse secondary antibody with 1% NHS and 0.1% Triton-X100 for 2 hours at room temperature. ABC solution from the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) was applied to prime the secondary antibodies on the tissue for an hour before NovaRed (Vector Laboratories) visualization was performed. Slides were then allowed to dry overnight and an alcohol dehydration procedure was performed before coversliping the slides.
Near infra-red (IR) staining was preformed to quantify the DARRP-32 protein levels in the striatum of the injected mice. A similar fixing and blocking procedure was performed as described previously without the hydrogen peroxide/methanol incubation step. Slides were incubated with antibodies against DARPP-32 (Rabbit anti DARPP-32 EP720Y, Cambridge MA, 1:7,500 dilution) and GFP (Goat anti-GFP ab5450, Abcam, 1:1000 dilution) with 1% natural donkey serum (NDS) and 0.1% Triton-X100 was applied to the slides overnight at 4°C using Hybriwells™ (Grace Bio-Labs, Bend, OR). After washing the slides with PBS, they were incubated in 1% NDS, 0.1% Triton-X100 and donkey anti-rabbit and donkey anti-goat IR- 2nd antibodies at a 1:500 dilution (Donkey anti Goat 800 CW, 926-3324 and Donkey anti-rabbit 680 CW, 926-32223, Li-Cor Lincoln, NE) for 2 hrs at room temperature. Slides were then allowed to dry overnight and an alcohol dehydration procedure was performed before coverslipping the slides and collecting data with the Li-Cor IR Odyssey scanner.

Quantification of Inclusion Bodies and NeuN Positive Cells

Using the program Stereo Investigator (MBF Bioscience, Williston, VT) striatal regions of the brain sections were outlined using the optical fractionator function. Using approximately 300µm separation between each stop in the setup parameters, a representative number of inclusion bodies were counted in one brain section. Five sections, left and right, were counted for the initial quantification of the CAG 140 model. Two sections, left and right, were quantified for the therapeutic assessment following rAAV injections. Average number of inclusions per stop was calculated from at least three animals per analysis group. Because each section was 14 µm thick a true unbiased stereological estimation of NII's or NeuN positive cells was impossible. However, this sampling method yielded an estimated of NII and NeuN cell density
identically for each animal. To avoid reporting the estimated numbers which may not be truly representative of actual NII numbers, we normalized each animal to the mean of the 6 month NII density in order to give an index of percentage increase over the sixth month time point in the initial quantification for the characterization. A percentage of the uninfected striatal region was calculated for the animals in the AAV injections studies.

We calculated the percentage decrease on the injected side from the uninfected side for the NeuN positive cells. For analysis after rAAV5 injections, injected striatal regions NII levels / NeuN positive cells were compared with un.injected striatal regions.

**Cortical Thickness**

Measurements of cortical thickness were determined using the Quantity One program from Bio-Rad. The average thickness of 3 sections from the left and right hemispheres was determined from the anti-dynamin *in situ* hybridization films. Animals with less than 2 sections available to measure were not included in the average.

**Qualitative Analysis of GFAP Staining**

Qualitative analysis of the GFAP+ cells was performed by an individual who did not have knowledge of the genotype, age or injection location. Astrocytic-like cell bodies were assessed from the anti-GFAP stained sections and the score for each animal was given as follows; no observable astrocytic bodies: 0 (-), few astrocytic bodies present: 1 (+), moderate number of astrocytic bodies present: 2 (++) and high number of astrocytic bodies present: 3 (+++). After all animals were examined, the average score for injection-side or control-side and genotype was calculated.

**Statistical Analysis**

One-way or Two-Way analysis of variance (ANOVA) was performed where applicable. Two-way ANOVA was followed by Bonferoni/Dunn *post-hoc* tests. All
longitudinal data were analyzed using repeated measures ANOVA. In these repeated measures analysis, no post hoc comparisons between groups at individual time points was performed due to the lack of a time X genotype interaction in all cases. Statistics and Graphs were generated in Graph Pad Software (Prism, La Jolla, CA) or Statview 5.1 (SAS Institute, Inc. Cary, NC). P–values of <0.05 were accepted to be significant.
CHAPTER 3
CHARACTERIZATION OF THE CAG 140 MOUSE MODEL OF HUNTINGTON’S DISEASE

Introduction

Since the R6 and other HD models that are transgenic and have both the expanded 5’ portion of the human mutant htt gene and the endogenous mouse hdh alleles, these models do not perfectly match the genetics of HD. To more accurately model HD, various knock-in models have been developed. These models have replaced one of the hdh alleles with either a fused human/mouse mutant htt chimera (168, 169, 177, 178) or simply expanded the CAG region of the mouse hdh gene itself (174). The knock-in mouse models, like the transgenic mouse models, have been created with various lengths of the expanded CAG region (168, 169, 174, 177, 178). These knock-in mice tend to have a longer life span than the transgenic with a slower progression towards neuropathology seen in the models (157, 165-169, 177, 178).

Menalled et al. (169) have developed a mouse knock-in model characterized by 140 CAG repeats. This CAG 140 model contains a small portion of the human gene, starting from 18 base pairs upstream of the CAG repeat region through 100 base pairs of the first intron and was initially characterized by the Menalled group (169) The CAG 140 model further characterized by Dorner and Hickey (170, 171). To date, all characterizations of the CAG 140 mouse were restricted in the timing of the behavioral testing and the pathological aspects examined. Thus, here, we have undertaken a longitudinal study of the CAG 140 model to provide a comprehensive picture of the progression of disease in this mouse model. Additionally, the initial CAG 140 characterization studies were performed with the homozygous mice (169-171). No study, to date, has directly compared the non-transgenic (nTG), heterozygote and the
homozygote genotypes. To this end, we performed monthly rotarod, rearing, and gait behavioral analysis as well as a cross-sectional analysis of transcripts and NII levels at 3 month intervals for approximately 18 months in nTG, heterozygote and homozygote CAG140 mice. The longitudinal and cross-sectional study reported here expands the characterization of the CAG140 mouse model phenotype and provides a comprehensive set of dependent variables that can be used for future testing of therapeutics in the CAG 140 model.

**Results**

**Behavioral Abnormalities in HD Mice**

**Rearings**

We placed individual mice in a 1 L beaker for 10 minutes in the dark and the number of rears was recorded. From the beginning the nTG mice reared significantly more times than the knock-in mice. nTG mice averaged 2.74 rears per minute while both heterozygote and homozygote averaged 1.36 time per minute (Figure 3-1). There was no difference in rearing between the heterozygote CAG140 and homozygote CAG140 mice. While there was a significant difference between the knock-in and the nTG mice, this difference was not progressive.

**Rotarod**

We used two different rotarod tests to determine if the CAG140 mice exhibited a deficiency in motor skills. The accelerating rotarod started at a speed of 5 RPM and increased in speed by 0.3 RPM each second (Figure 3-2A). nTG mice reached an average max speed of 21.3 RPM before falling, whereas the heterozygote and homozygote reached 16.8 and 17.2 RPM, respectively. The maximum speed that the nTG mice were able to perform was significantly higher than either the heterozygous or
the homozygous CAG 140 mice. There was no significant difference between the maximum rotarod performance speeds of the heterozygous or homozygous CAG 140 mice.

The constant speed rotarod performance was tested at two different speeds, 10 RPM and 18 RPM (Figure 2B and 2C). While the accelerating rotarod showed a difference between the genotypes, these tests showed no difference between nTG and either the heterozygous CAG140 or the homozygous CAG140 mice. Each of the genotypes showed considerable variability at each time point. On the 10 RPM constant speed rotarod the nTG, heterozygous and homozygous stayed on an average of 50.9 seconds, 43.7 seconds and 47.2 respectfully. The 18 RPM constant speed rotarod showed the nTG, heterozygous and homozygous stayed on an average of 31.8, 19.4 and 22.7 respectfully.

Gait analysis

Like the rearings, gait analysis was performed once a month. The results for both stride length and stride width did not indicate any overall difference between any of the genotypes (Figure 3-3). The ratio between the two measurements did not show any significant differences either.

Expression of the CAG140 Allele Leads to Transcriptional Anomalies in the Mouse Brain

In situ hybridization of transcripts was performed on nTG, heterozygote and homozygous mice to ascertain the transcriptional changes associated with the expression of the CAG140 allele of hdh. The striatally enriched transcripts examined here were DARPP-32, ppENK, PDE10a, PDE1b, D2, CB1 and NGFi-A.. β-actin was used to normalize the other transcripts. We examined 3 month old mice through 19/20
months at intervals of approximately 3 months. We examined the homozygous mice only out to 12 months.

Transcriptional levels of heterozygote compared to nTG (Figure 3-4) started to decrease at 6 months in four transcripts (PDE1b, NGFiA, CB1 and ppENK). We saw a transcript reduction of approximately 20% when compared to nTG transcript levels at 9 months with the exception of ppENK and DARPP-32 which showed a 7 % increase and a 15 % decreases respectively. Transcripts continued to decrease over time and eventually by 19/20 months the transcripts were approximately reduced by 30% compared to controls. The transcript with the greatest reduction levels compared to nTG was PDE10a which decreased by 36%, while ppENK transcript levels decreased the least, by14%, at the final time point. Dynamin transcript levels did not show any differences in the heterozygous mice at any point.

Similar to heterozygous CAG 140 mice, homozygote transcript levels (Figure 3-5) began to decrease at 6 months. At 12 months, the average transcript reduction was 35% with the greatest reduction being CB-1 at 56%, and the lowest reduction being DARPP-32 at 18% when compared to nTG mice transcript levels. For comparison, heterozygous mice at 12 months have an average reduction of 22% across the transcripts while homozygote mice at 12 months transcripts reduced by an average of 35% compared to nTG. Dynamin transcript levels did not show any differences in the homozygous mice, similar to what was observed in the heterozygous mice.

**Older CAG140 Mice Exhibit an Increase in NII s**

Sections from each genotype at the various ages were stained with EM48 for semi-quantification of the NII s. Figure 3-6 shows the percentage increase over the baseline levels of NII s at 6 months. 6 months was the first time NII s became evident in
any of our mice, and we used 6 months as our normalization baseline. Similar to the heterozygous mice, the homozygote NII s showed an increase over time compared to the 6 month baseline but these differences did not reach statistical significance. nTG mice, as expected, did not show significant inclusion staining at any time point (Figure 3-9 C).

Significant increase in the percent of NII s seen above baseline did not occur until 15 months for the heterozygous mice. At 19/20 months the heterozygous mice had 5.3 inclusions per 1mm² equating to 8.8 times as many inclusions compared to the 6 month time point. There was no significant difference between heterozygote and homozygote inclusion counts. For an example of the NII stain see Figure 3-9.

**Cortical Thickness**

Using the sections probed for dynamin from the *in situ* hybridization experiments, we measured the cortical thickness. Cortical thinning has been recently found to decrease in HD patients (13-18) and because dynamin transcript signal was found to be located primarily in the cortex, it provided a reasonable metric of cortical dimensions. When compared to the nTG animals, the cortices of the heterozygous and homozygous CAG 140 mice showed a significant difference across the genotypes (Figure 3-7; ANOVA, p=0.0001).

Using NeuN staining, the number of neurons in this cortical region were counted and showed no significant decrease in the homozygotic mice (Figure 3-9 D). Thus, a neuronal population decline is unlikely to explain the loss of cortical thickness observed here. In Figure 3-9 B, the region that was counted is outlined.
Weight

In order to determine if there was abnormal weight gain in our CAG 140 mouse model, we examined the cross sectional weight of our colony. In our behavioral group, we did not have enough of each gender to calculate a proper average. In Figure 3-8 shows there was no overall weight gain or loss differential between the nTG and the afflicted mice. Mice were placed into two month groups in order to have enough mice at certain time points to be significant.

Discussion

Behavioral and pathological abnormalities in HD mouse models range greatly in severity. The more severe transgenic R6/2 line developed by Mangiarini et al. (149), exhibits a clasping phenotype and motor skill deficits as early as 5 weeks and usually die from the disease at 13 weeks (149, 154). The R6 mice lines show some similar histopathology features to the human disease, in particular, the presence of the NIIIs at 5 weeks are (262) evidence of the similar pathology of HD (127, 156). Transcriptional dysregulation is another hallmark of HD and is evident in the R6 mice (22, 26, 118, 120, 124, 125, 159, 160). Another commonly used transgenic model is the N171-82 line developed by Schilling et al. (157) which develops motor abnormalities, including clasping, at 16 weeks and dies at approximately 30 weeks (162). The N171-82 mice also produce the NIIIs at 6 ½ months and transcriptional irregularities are evident as well (119, 121, 263, 264). While both of these transgenic models have been used extensively for the study of HD, they may not represent the correct genetics of HD. Both the R6 and the N171 transgenic models have the expanded human htt gene in addition to the endogenous mouse hdh.
Recently developed knock-in models have the advantage of modeling the human disease by having one or two copies of the gene, as would be the case in the HD. When the present study started, there was very little information on the time course of the disease in the knock-in CAG140 mouse model. The model was initially characterized by Menalled et al. (169) and more recently studied by Dorner and Hickey (170, 171), but each of these characterizations examined only a few select time points and only examined homozygous mice. Rotarod deficits were observed at 4 months, slight rearing behavioral differences detected at 6.5 months in a novel cage environment, and night time running wheel differences were detected at 4, 6 and 8 months (170). These studies, while examining differences between nTG and the CAG 140 mice at selected time points, did not investigate both heterozygous and homozygous mice. In the present study, we examined the behavioral characteristics over a longitudinal time course in one set of mice, a corresponding time-matched time course of striatal specific transcripts, and NIIs in a separate cross-sectional study. The present study also examined both the heterozygous and the homozygous CAG 140 mice which to date have not been compared in a single study. The CAG 140 mice do not exhibit the shortened lifespan like the other more widely used models such as the R6/1, and thus allow for a more extended examination of the HD progression.

**Behavioral Abnormalities**

Behaviorally, the CAG 140 model exhibited slight and subtle overt differences between afflicted and nTG animals. There were no obvious gait, size or activity differences between the genotypes by simple observations. However, behavioral testing revealed a relatively mild pathological phenotype. As early as 3 months, we observed a significant difference in the rearing behavior in the knock-in mice (Figure 3-1). In HD
patients, individuals have been reported to have slight motor, language and cognitive skill deficits that precede the major HD symptoms and could be an early indication of the disease (265-268), and it is not inconceivable that CAG 140 rearing behavior may be a manifestation of similar subtle early or developmental deficit in HD mice. While other models have shown gait abnormalities, no significant differences were observed in our study. Neither gait width and gait length (Figure 3-3) nor the ratio between the two was significantly different from nTG (data not shown).

In HD, motor and cognitive skills progressively worsen as the individual ages (8, 269, 270). In the CAG 140 knock-in mouse model examined here, there was little to no progressive decline in the behavioral tasks. Rearing and rotarod behavioral tasks showed a large significant deficit when comparing nTG mice to the knock-in mice, but this deficit did not increase as the mice aged. In fact, for the rearing behavior, nTG mice approached the knock-in rearing numbers towards the end of this study. In our rearing behavior task it is possible that the mice, both the nTG and the knock-in mice, may show some habituation to the task and this habituation may explain the nTG’s slow decrease in rearing behavior. We attempted to limit this habituation effect by spacing the tests by one month. The lack of a progressive behavioral deficit in the CAG140 mouse model is a drawback of the model but does not preclude the possibility that other behavioral paradigms might uncover a progressive deficit especially in light of the progressive neuropathological deficits uncovered in this study (see below).

**Transcriptional Dysfunction**

In humans with HD, as well as other mouse models of HD, striatal specific transcript and protein levels are known to be altered. We examined striatal DARPP-32, ppENK, PDE10a, PDE1b, D2, CB1, and NGFi-A transcripts because of various reports
that have shown these transcripts are altered in HD (22-26, 118, 119, 121, 124, 125, 263). The transcripts examined here, are all enriched in the striatum and have a role in cell signaling to varying degrees (63, 64, 82, 91, 271-274). Both D2 and CB1 receptors, when activated, help to regulate the intracellular levels of cAMP (74, 75, 80, 83, 84) which in turn regulates the phosphorylation of the striatal specific protein DARRP-32 (74, 75, 80). PDE10a and PDE1b are also striatal specific and have been shown to hydrolyze cAMP (275-279). Studies regarding upstream binding sites of the gene that codes PDE10a revealed that there is a NGFiA binding site which suggests that NGFiA could help regulate the transcription of PDE10a (22). The interconnections between these striatally enriched transcripts highlight their functional significance especially since mutant htt has been associated with decreases in these transcripts.

Indeed, just as seen in the transgenic R6 mouse model (22, 26, 118, 120, 124, 125, 159, 160), various other mouse models of HD (119, 121, 263, 264), there was an age-dependent decrease of striatal specific transcripts in the CAG 140 model. There was a decrease of mRNA levels in heterozygous CAG140 mice starting at 6 months of age and a reduction of nearly 20% in most transcripts by 9 months of age. The trend for reduced striatal specific transcripts in CAG140 mice continued as the mice age and at 19/20 months an average reduction of 30% was observed. Homozygous mice showed a reduction of 20% already by 6 months, and at 12 months, the homozygous mice showed a reduction of approximately 35%. The difference between the heterozygous and the homozygous striatal transcript levels observed at 12 months may indicate a gene dosing effect of the two expanded knock-in htt alleles present in the homozygous mice compared to the single copy in the heterozygotes. The decrease in relative mRNA
of these various transcripts in the CAG140 mice corroborated what has been seen in HD as well as what has been observed in other mouse models of HD (22, 26, 119, 125, 126, 159).

**Neuronal Intranuclear Inclusions**

Another well established pathological component in HD and HD models, in addition to transcript dysregulation, is the presence of the NII. To date, there have been few attempts to accurately quantify NII in HD mouse models. In the present study, we have used an unbiased sampling method to determine NII density over the life-span of the CAG140 mouse. We normalized the NII densities to the six month time point which was the first age where we could detect significant NII. Visualizing these data demonstrated an age related progression in CAG140 mice (Figure 3-6).

Wild-type htt has been shown to associate with various transcription factors and that association is altered when mutant htt is present (100, 105-110). It would follow that the function and or availability of the transcription factors to perform their tasks might be altered if sequestered by the insoluble precursors to the NII or in the NII themselves. Our observations support the hypothesis that transcription factor segregation due to mutant htt could in turn decrease mRNA levels (Figures 3-3 and 3-4). We see significant decrease of transcripts before a significant increase of NII in the CAG 140 mice. This may suggest sequestering of transcriptional factors before the actual formation of distinct inclusion bodies (250, 280, 281). While sequestering of transcription factors may account for our early behavioral data, from our data we cannot conclude conclusively that this is the cause of the behavior in the CAG 140 model.
Cortical Thinning

Cortical thinning has recently been examined during the progression of HD and a number of studies have determined via functional magnetic resonance imaging (fMRI) that as the disease progresses in humans cortical areas begin to thin or atrophy (13, 15, 16). The CAG 140 mouse model displayed an overall significant difference between the genotypes (ANOVA, p = 0.0001) (Figure 3-7). nTG mice also showed a significant decline in the cortical thickness over time (1 way- ANOVA, p = 0.01). The nTG cortical thinning could be attributed to aging which has been seen in normal human aging (282-284). Here, the CAG 140 mice might reach a threshold of cortical thinning, and the nTG mice reach that point at a later stage. Examining neuron density at 9 and 12 months of the homozygous mice, did not reveal a consistent decrease in the number of neurons (Figure 3-9 D). This lack of NeuN staining suggests that atrophy or loss of cell types other than neurons might potentially be responsible for the observed cortical thinning. The implications of such cortical thinning in the CAG 140 mice can not be determined from the present study. More precise learning behavioral tasks may provide a clue as to the effects of such cortical loss.

While a non-invasive method of determining transcript and/or NIIIs in humans has not been developed, post-mortem studies have indicated that, in all likelihood, a progressive decrease of transcripts and a progressive increase of NIIIs occurs in HD. These histopathological characteristics make the CAG 140 mice a good model to test early intervention to HD and their affects at a cellular level. Cortical thinning may provide a real-time measurable progressive pathological marker for the CAG 140 mice. Experimental therapeutics that help return the various transcript levels to normal levels, reduce or eliminate the NIIIs associated with HD, or help stem the associated cortical
thinning in HD could be tested first in the CAG 140 mouse model. Additionally we have found a series of behavioral tests that show long-term, quantifiable, non-progressive deficits when compared to nTG.

In conclusion, the CAG140 knock-in mouse model of HD has many aspects that are similar to the human disease. Not all of the aspects mimic the human disease perfectly but the histopathological characteristics do recapitulate what is observed in the human disease and would provide good benchmarks for determining the efficacy of drugs and treatments. The progressive decreases in transcripts and cortical thinning as well as the increase in NIs over time allow us to gauge treatments at various points in the disease. Determining the ideal window for treatment is especially important in HD since it is purely a genetic disorder and early intervention in the human disease is possible.
Figure 3-1 Longitudinal rearing behavior of the CAG140 knock-in mouse model of Huntington’s disease shows a deficit in heterozygous and homozygous mice. Mice were videotaped in a 1 L beaker for 10 minutes in the dark starting every two weeks for the first four testing periods then once a month after. nTG mice reared significantly more than heterozygous and homozygous mice (ANOVA; p<0.0001). There was no difference between heterozygous and homozygous mice (p>0.05) (N : nTG= 8, Het= 13, Hom= 4).
Figure 3-2 Latency to fall of the CAG140 mouse model on the accelerating and constant speed rotarod. A) CAG 140 mice (heterozygous and homozygous) displayed rotarod performance deficits beginning at 11 months of age (ANOVA; P<0.05). There was no difference between the performance of the heterozygous and the homozygous mice. B) 10 RPM constant speed rotarod testing revealed no differences between nTG and CAG 140 mice. C) All genotypes performed similarly in the 18 RPM constant speed rotarod test, although performance at this task was generally reduced with age (ANOVA; p<0.001) (N : nTG= 8, Het= 13, Hom= 4).
Figure 3-3 Stride length and width gait analysis for the CAG 140 mouse model of Huntington’s disease. Gait analysis for each mouse was done once a month. Stride length was measured as the distance between subsequent front foot steps. Stride width was measured as the horizontal distance between the placement of the front paw and the rear paw. All measurements were performed from the center of the paw pad. Only the last homozygous time point was significant for the stride length ($p<0.001$) Overall no significant difference was observed in either analysis (ANOVA $p>0.05$) ($N : nTG= 8$, Het= 13, Hom= 4).
Figure 3-4 Striatal mRNA transcript in situ $^{33}$P hybridization of heterozygote CAG140 knock-in mouse model of Huntington's disease. The striatal area of the heterozygous mice was analyzed for optical density after mRNA transcript radioactive in situ hybridization in relation to the nTG mRNA transcripts after normalization to β-actin mRNA transcript optical density. An overall transcriptional down-regulation was observed in A) D2, B) DARPP-32, C) ppENK, D) PDE1b, E) CB1, F) PDE10a and G) NGFiA (ANOVA; p<0.001) In D2, DARPP-32 and PDE1b (A, B and D) the transcriptional down-regulation begins at 9 months and continues through the last age group. In CB1 and PDE10a transcripts (E and F), reductions begin earlier at 6 months continue to be significant through the course of the cross sectional analysis. ppENK and NGFiA (C and G) showed variable but overall transcriptional down-regulation. Asterisk represent significant differences between heterozygote transcript levels and the nTG transcript levels at the corresponding age (p<0.05) (N: 3 Months: nTG=6, Het =9, 6 Months: nTG=7, Het= 5, 9 Months: nTG=7, Het=6, 12 Months: nTG=3, Het=12, 15 Months: nTG=11, Het=12, 19/20 Months: nTG=12 Het=13).
Figure 3-5 Striatal mRNA transcript in situ 33P hybridization of homozygote CAG140 knock-in mouse model of Huntington's disease. The striatal area of the homozygous mice was analyzed for optical density after mRNA transcript radioactive in situ hybridization in relation to the nTG mRNA transcripts after normalization to β-actin mRNA transcript optical density. An overall transcriptional down-regulation was evident similar to what was observed in the heterozygous mice, in A) D2, B) DARPP-32, C) ppENK, D) PDE1b, E) CB1, F) PDE10a and G) NGFia transcripts (ANOVA; p<0.001) All transcripts except D2 and DARPP-32 showed significant reduction starting at 6 months (C-G). Overall transcriptional differences between heterozygous and homozygous mice are significant (ANOVA; p<0.01) Asterisk represent significant differences between homozygote transcript levels and the nTG transcript levels at the corresponding age (p<0.05) (N: 3 Months: nTG=6, Hom=6, 6 Months: nTG=7, Hom=11, 9 Months: nTG=7, Hom=7, 12 Months: nTG=3, Hom=8).
Figure 3-6 Quantification of Neuronal Intranuclear Inclusions (NIIs). NIIs were quantified using a sterologically based sampling regime. Counts were estimated identically for each group and normalized to the 6 month age group. 6 months was chosen because NIIs were first detected at this age group. At 6 months there is no significant difference between nTG background staining and heterozygote NII numbers (p>0.05). A significant increase in NIIs is evident as the mice age (One-Way ANOVA; p<0.0001). Starting at 15 months there is a significant difference in the percent of inclusion bodies over the 6 month baseline. (N: 6 Months: nTG=3, Het= 4, Hom=3, 9 Months: nTG =3, Het= 5, Hom=3, 12 Months: nTG=3, Het= 4, Hom=4, 15 Months: nTG= 4, Het= 3, 18/19 Months: nTG= 3, Het= 5).
Figure 3-7 Cortical thickness of the CAG 140 knock-in mice decrease over time compared to the nTG mice. Using the Dynamin in-situ film images of the mice examined previously, cortical thickness of the nTG, heterozygous, and homozygous mice were measured in millimeters using the Quantity One program. There is an overall significant difference between the nTG and the knock-in mice (ANOVA; p = 0.0001) (N: 3 Months: nTG=6, Het=9, Hom=6, 6 Months: nTG=7, Het=5, Hom=11, 9 Months: nTG=7, Het=6, Hom=7, 12 Months: nTG=3, Het=12, Hom=8, 15 Months: nTG=11, Het=12, 19/20 Months: nTG=12 Het=13).
Figure 3-8 No significant weight differences in the CAG 140 mouse model. Weights of our CAG 140 mouse colony showed little differences in the weight of either the male or the female mice in any of the genotypes. Male nTG only showed slightly lower weights at the earliest time point ($p<0.01$), but this did not appear to be a permanent weight difference. Females showed one time point at 5-6 months of age where the nTG was significantly increased ($p<0.001$), but this returned to the other weights in the following time points. Overall there was no weight difference in either the males or the females across the ages examined here (ANOVA, $p>0.05$) (Male N: 1-2 months; nTG = 7, Het= 15, Hom=9, 3-4 months; nTG = 5, Het= 12, Hom= 9, 5-6 months; nTG = 13, Het= 9, Hom= 10, 7-8 months; nTG=19, Het=13, Hom=5, 9-10 months; nTG= 7, Het= 6, Hom= 9; Female N: 1-2 months; nTG = 5, Het= 21, Hom= 13; 3-4 months, nTG= 11, Het= 5, Hom= 8; 5-6 months, nTG= 13, Het= 12, Hom= 7; 7 to 8 months, nTG= 10, Het= 10, Hom= 10; 9-10 months nTG= 13, Het=12, Hom= 4).
Figure 3-9 Representative images of NIIIs and neuronal NeuN quantification

A) Representative images of a (i)nTG, (ii)heterozygous, and (iii)homozygous striatal area containing NIIIs at 12 months. Arrows show examples of NIIIs. Scale bar represents 25μm

B) Representative image of cortical region that was counted (black box) and measurement of cortical thickness (white arrow). Scale bar represents 250μm. Levels were adjusted (A and B) for better visualization in this figure.

C) Homozygous progression of NIIIs as a percent over the 6 month baseline.

D) NeuN positive cells average per 1mm². 9 month and 12 month old nTG and homozygous mice were stained with NeuN to determine the total cortical neurons in the given area seen in B). CC: Corpus Collosum.
CHAPTER 4
RAAV5-SHRNA TREATMENT OF THE CAG 140 MOUSE MODEL

Introduction

As discussed in the introduction, HD pathology is generally thought to be caused by a gain of toxic function by mutant htt. Various group have demonstrated that mutant htt associates more closely with transcription factors such as CBP than the wild-type htt (100, 102-104, 112, 113, 115-117, 179). Additionally studies by Zeitlin and Dauyo have demonstrated that mice with only one copy of huntingtin, mice are phenotypically normal (143, 145). If the disease is caused of a loss of one of the copies of huntingtin, then the heterozygous knock-out mice, generated by Zeitlin and Dauyo would have shown HD like symptoms. Lastly, with the generation of knock-in mice and the viability of the homozygous mice from these lines (168, 169), it can be concluded that mutant htt still retains its ability to perform some wild-type htt functions, but also gains a toxic function. If HD is caused by a gain of toxic function in mutant htt, then removing the toxic protein should alleviate the disease.

As described in the introduction, RNAi is a useful tool in specifically targeting mRNA for degradation, which, in turn, reduces protein levels. Various groups have attempted to reduce the mutant htt transgene in the R6/1 and N171-Q82 mouse lines with some success (163, 259, 285). Others have introduced a mutant htt gene by viral vector and showed pathological similarities to HD that were alleviated by the introduction of siRNAs (286, 287). Both of the transgenic and the viral-mediated introduction of HD studies showed behavioral and histopathological improvements. However, the transgenic and viral induced models of HD still contain both endogenous copies of hdh. A knock-in model of the disease is a closer genetic approximation of the
disease, and the results from a reduction of the mutant htt in the knock-in model would be more suitable gauge of therapeutic benefit in humans.

Our lab previously designed two shRNAs that target the R6/1 HD model (259). siHUNT1 and siHUNT2 effectively reduced the mRNA of mutant htt in the R6/1 mouse line. siHUNT1 targets a region in the human untranslated region (UTR) and not in the mouse untranslated region. In the development of the CAG 140 mouse model, the UTR and 5' region of the hdh was left intact, allowing us to use siHUNT1 as a control (169). We decided to use siHUNT2 as our experimental shRNA, because it targets the region immediately upstream of the CAG repeat region in our CAG 140 mouse model. The 18 base pairs immediately upstream of the CAG repeat region is a human sequence and is different enough to allow for specific targeting (169, 259).

However, there appears to be some off-targeting effects with siHUNT2 in the background line, CBA x C57BL/6, of the R6/1 transgenic mice where siHUNT2 was originally targeted to (169). While effectively reducing the mRNA and protein levels in culture, siHUNT2 showed some down-regulation of DARPP-32 and ppENK mRNA levels in vivo after 10 weeks after injection in the R6/1 mice ((259)). The CAG 140 knock-in have a slightly different background strain (129Sv x C57BL6) (259, 288), and this difference could potentially produce less toxic side effects after siHUNT2 administration. We decided that the slightly different mouse background and a longer term expression of siHUNT2 might be able to show if the reductions of the mutant protein would be of therapeutic value despite the potential off targeting effects previously seen in the R6/1 mouse line.
After the CAG 140 characterization, we concluded that 10 weeks of age would be an appropriate time point to start a treatment in this model. While there will be rearing behavior differences at ten weeks, this was the earliest point at which we could do surgery on the animals before the onset of transcriptional down-regulation and the appearance of NIs. This early therapeutic intervention would allow us to demonstrate that RNAi intervention maybe possible. Because the disease has histopathological progressive traits and later intervention may prove inefficient, if improvements are detected after early intervention in the CAG 140 model, it is possible later term intervention may also be beneficial.

**Results**

**Behavioral Analysis**

Five heterozygous mice were injected bilaterally with the rAAV5-siHUNT2 vector along with three GFP control injected heterozygous mice for behavioral analysis. Six weeks after injections, at 4 month of age, the mice were tested for rearing behavior (Figure 4-1 A). Significant increases in the average number of rearings were observed at both the 4 month and 5 month ages after siHUNT2 injected mice. The increase in average rearing was significantly above the uninjected heterozygous averages. The rearing average reached slightly above the nTG rearing averages that was reported previously (chapter 3). GFP animals did not show significant improvements in rearings compared to the uninjected heterozygous mice. When the rearing behavior testing was performed at 9 months and later, we did not see the same improvements. The rearing averages of the siHUNT2 injected mice returned to about the same levels as the heterozygous uninjected mice. Similarly the GFP injected animals showed no difference to the heterozygous uninjected animals.
No significant difference in the bilaterally injected siHUNT2 mice was detected on the accelerating rotarod task (Figure 4-1 B). Early time points showed no significant differences between the injected and uninjected heterozygous mice (p>0.05). The rotarod behavior is consistent with our earlier observations in chapter 3 that showed no significant differences in the heterozygous mice and the nTG until 10 months. At the 12 month age point, the non transgenic mice could stay on the device at a significantly higher speed than all the heterozygous mice (p<0.05)

**Transcriptional Analysis for rAAV5- siHUNT1 and siHUNT2siHUNT2 Mice**

Ten week old mice were injected with rAAV-5 siHUNT1 or siHUNT2 and were analyzed for transcriptional effects at 14 weeks and 26 weeks post-injection. GFP control animals were also injected at 10 weeks, but the results will be described in chapter 5. Figure 4-2 (siHUNT1) and 4-3 (siHUNT2) displays the results from the radioactive *in situ* hybridization as a comparison between the uninjected and the injected striatal regions.

In the siHUNT1 injected mice, which should not target either the endogenous or the mutant huntingtin mRNA, there was significant decreases in the injected side when compared to the uninjected striatal regions (Figure 4-2). Starting at 14 week post injection of nTG mice showed a significant decreases on the injected side when compared to the uninjected side (Figure 4-2 A, ANOVA, p<0.001). The transcriptional down-regulation continued in the nTG 26 week post-injection shown in Figure 4-2 A (ANOVA, p<0.0001). Examining the transcript down-regulation over time revealed no significant differences between the 14 and 26 week time points (ANOVA, p>0.05).

Heterozygous mice also showed a similar decrease in transcripts at 14 weeks post-injection (Figure 4-1 B ANOVA, p<0.001). These decreases observed in the
heterozygous mice are below the normal disease related decreases reported in chapter 3. No significant difference between the transcripts was evident at the heterozygous 26 week time point (Figure 4-2 B; ANOVA, p>0.05). The apparent increase in transcript levels from the 14 to 26 week time points in the heterozygous mice was significant (siHUNT1 HET transcripts 14 weeks vs. 26 weeks, ANOVA, p<0.0001).

Homozygous transcripts in mice injected with siHUNT1 also showed significant decreases at 14 weeks (Figure 4-2 C; ANOVA, p<0.0001) and at 26 weeks (Figure 4-2 C; ANOVA, p<0.005). ppENK mRNA transcript in the homozygous mice at 26 weeks post injection was the only transcript that showed any increase above the disease level transcript levels. The homozygous siHUNT1 26 week post-injection ppENK transcript levels were significantly above the uninjected side by 26% (t-test p<0.01) but still significantly below the uninjected 26 week uninjected nTG ppENK levels (t-test, p<0.05).

Figure 4-3 depicts the results from the siHUNT2 injected mice which targets the mutated huntingtin mRNA and showed an overall decrease in transcriptional levels compared to the uninjected side. Again, these transcriptional decreases are in relation to the disease related decreases reported in chapter 3. siHUNT2 caused significant transcriptional down-regulation at the 14 week time point (Figure 4-3 ANOVA, p<0.0001) and at the 26 week time point in nTG mice (ANOVA, p<0.05). Comparing the 14 week transcript levels to the 26 week transcript levels after injections with siHUNT2, shows that there was a significant increase (siHUNT2 nTG 14 week vs. 26 week; ANOVA, p<0.05).

Heterozygous mice did not appear to be significantly affected by the injections of siHUNT2 at either post-injection time points (Figure 4-3 B; ANOVA, both p>0.05).
siHUNT2 injected homozygous mice showed significant mRNA transcript reductions at 14 weeks (ANOVA, p<0.001) but at the 26 week time point massive atrophy was observed in half of the homozygous striatums. Because the transcript levels could not be accurately calculated due to the atrophy, only a small number of animals could be quantified and no significant difference in transcripts were observed in the 26 week siHUNT2 injected homozygous mice (ANOVA, p>0.05)

There was no significant difference between any of the siHUNT1, siHUNT2 or GFP transcripts level except for the heterozygous mice at 14 weeks. At 14 week time point siHUNT1 showed a significantly lower level of transcripts than the GFP injected animals (ANOVA, p<0.05). At all the other time points and genotypes there were no significant differences between the vector injections.

**NII decreases with shRNA treatment**

NIIIs were quantified by counting the number of observed NIIIs seen in the given field after a sterologically based sampling at 100x and has been described in chapter 3. siHUNT1 and siHUNT2 injections of heterozygous mice at 14 weeks showed no overall reductions in the amounts of NIIIs compared to the uninjected striatal regions of the heterozygous mice (Figure 4-4 A; ANOVA, p>0.05). Heterozygous mice at the 26 week time point showed an overall significant reductions in NIIIs with injections of siHUNT1 and siHUNT2 (Figure 4-4 B; ANOVA, p<0.01). Similarly, the homozygous injected mice showed significant overall NII reductions after both shRNA injections at the 14 and the 26 week post-injection time points (Figure 4-4 C and D ANOVA, both p<0.01). These reductions of NIIIs in the injected striatum are in despite of the fact that siHUNT1 does not target either mutant *htt* or *hdh*. GFP injections did not reduce the number of NIIIs on the injected side significantly in either genotype or at either time points.
Discussion

We hypothesized that the reduction of the mutant huntingtin protein via RNAi would improve some of the observed abnormalities seen in our characterization of the CAG 140 model. As described in the introduction, Yamamoto et al. created a mouse model of HD which had the mutant huntingtin fragment in a tetracycline controlled promoter (148). Addition of tetracycline lowered the expression of the mutant huntingtin and the HD neuropathology and HD behavior observed previously in the mouse was rescued. Harper et al. and Rodriquez-Lebron et al. have both successfully reduced the mutant huntingtin fragment from both the N171-82Q and R6/1 transgenic mouse models of HD by rAAV mediated RNAi delivery and showed neuropathological and behavioral improvements (163, 259). Other studies have introduced mutant htt into either a mouse or rat by a viral method and successfully rescued the resulting HD phenotype with either siRNAs or shRNAs (286, 289, 290) With these studies, we theorized that reductions in mutant huntingtin in a knock-in model of HD could show similar neuropathological and behavioral improvements.

Our allele-specific shRNA, siHUNT2, was designed by our lab and targets a region immediately upstream of the CAG repeat region. As a control we had another shRNA, siHUNT1, that in the CAG 140 model does not target either the knock-in allele or the endogenous hdh (259). The siHUNT2 had previously shown off-targeting; however the region that it targets is specifically human in the expanded CAG allele and is the only region that can be specifically targeted for allele-specific knock-down (259, 288). The siHUNT1 shRNA did not show any significant off-target in the R6/1 mice previously and should have been a suitable control shRNA in the CAG 140 model (259).
Behavioral Improvements

Bilateral injections of the rAAV5-siHUNT2 vector was performed in heterozygous mice in order to determine if any behavioral therapeutic effect was evident despite the transcriptional irregularities. The injections were performed at the same time as the unilateral injections (10 weeks) and showed early improvements in rearing behavior 6 weeks and 10 weeks post-injection (Figure 4-1 A). While these results are from a small cohort of animals (5 siHUNT2 and 3 GFP), the slight improvement in rearing suggests that RNAi mediated knock-down of mutant htt may provide some therapeutic benefit in the early months after injection. Behavioral improvements were not evident later at 26 weeks post-injection or beyond. Rotarod motor performance at 6, 26 and 38 weeks post-injection showed no overall improvement in the siHUNT2 and GFP injected mice compared to the uninjected heterozygous mice (Figure 4-1 B).

While we observe some early behavioral improvements, these come at a time (10 weeks post-injection) immediately before we see transcriptional dysregulation, in our unilateral siHUNT2 injections (discussed below). It is possible that the improvements detected at the 6 and 10 week post injection-time points come prior to significant down-regulation of the transcripts in the CAG 140 knock-in mice. The fact that we see little improvements at later time points after we observe siHUNT2 induced transcriptional down-regulation may indicate that such transcriptional dysregulation would abrogate any behavioral improvements generated by knock-down of the mutant huntingtin. With the present data we can not make any concrete conclusions about the connection between the transcript levels and the slight behavioral improvements seen at the early time points after injection. Behavioral analysis was not performed on the R6/1 mice with siHUNT2 so it is impossible to say if a similar early behavioral improvement could be
elicited in the R6/1 mice with the siHUNT2 shRNA before the transcriptional irregularities are seen.

**Transcriptional Dysregulation**

Despite the fact that previous work with our shRNA constructs indicated that the siHUNT1 should have been a suitable control, as it did not have any noticeable off-targeting effects (259, 288), we have found that there was further down-regulation of the disease related striatal transcripts as reported in chapter 3. The direct correlation between the further transcriptional down-regulation caused by siHUNT1 or siHUNT2 and any neuropathology or behavior can only be speculative. In work previously done by Rodriguez-Lebron et al from our lab had utilized the R6/1 mouse line that contained the full first exon of the mutant *htt* fragment inserted into the mouse genome (149) and both siHUNTs targeted the transgene (259). The viral constructs generated by Rodriguez-Lebron et al was a rAAV5 vector with a titer at approximately 1 to 5 x 10^{13} vg/ml, which matches our construct and titers in this project (259). The R6/1 mice were injected with both the siHUNT vectors and transcript and NIs were examined at 10 weeks post-injection, approximately one month prior to our histopathological examinations (259). The mouse model differences along with the longer rAAV5-siHUNT experimental time points may explain some of the discrepancies seen between the studies. In particular, the extended time of shRNA exposure may explain the transcript down regulation. A longer experimental time course in the R6/1 mice may produce similar transcriptional results as seen here.

As characterized in chapter 3, there are mRNA transcript decreases in the heterozygous and homozygous mice over time and it is possible that reductions beyond the normal mRNA transcript decreases could have further detrimental outcomes.
However, because we do not see further behavioral deficits (Figure 4-1) after injections of siHUNT2 it is hard to gauge exactly what the consequences of further transcriptional down-regulation would be. siHUNT2 was not examined for behavioral improvements in the previous work done by Rodriguez-Lebron (259). siHUNT1 bilateral injections for behavior showed delayed onset of the clasping behavior that is characteristic of the R6/1 mouse line (259).

Various groups have determined that shRNAs may themselves be toxic in vitro as well as in vivo (285, 291-297). Grimm et al. showed that over expression of shRNAs in mice liver caused lethality (291). Investigating further, a large number of the native regulatory miRNAs in the liver were significantly reduced which was directly correlated with the morbidity of the mice (291). The reduction in the miRNAs, after shRNA administration, has been theorized to be caused by an overloading of the Exportin-5 complex that helps regulate the export of both shRNAs as well as miRNAs (241, 294). The over expression of shRNAs creates an accumulation of pre-miRNAs that can not be handled properly by Exportin-5 which can lead to cellular death (241, 294).

McBride et al. showed the potential for shRNA toxicity in the CAG 140 mouse model (292). Using three different shRNAs targeted towards the hdh and the mutant htt, the group showed two of the three shRNAs tested had micogliai (Iba-1) activation and down-regulation of DARPP-32 protein. Examining the amount of anti-sense and sense sequences present, McBride showed that the microglial activation and protein reductions were linked to the amount of mature anti-sense sequence in the neurons (292). Lower levels of mature anti-sense showed less toxicity in mouse brains. We did not examine our shRNA injected mice for microglial activation, and this would be a good
future examination to determine if our shRNAs toxicity in CAG 140 mice is caused by
the same mechanism reported in the McBride paper. In our work, despite siHUNT1 not
being targeted to known sequence in the CAG 140 model, we see transcriptional down-
regulation. If, as suggested by McBride (292), the levels of the anti-sense sequence are
at a high enough level, the fact that siHUNT1 doesn’t target anything may become
irrelevant. Our observed transcriptional down-regulation might be a result of high mature
anti-sense levels.

McBride, and later Boudreau, demonstrated that the shRNA mediated toxicity
could be ameliorated by placing the RNAi cassette into a miRNA backbone (292, 295).
Boudreau’s study showed a similar phenomena to Grimm (291) and Yi’s (294) work that
showed significant reductions in endogenous miRNAs if shRNAs are used. When
shRNAs are placed in a miRNA backbone, the endogenous miRNA levels were restored
and as in McBride’s study, observed toxicity was reduced (292, 295). Bourdeau and
McBride’s work would suggest that placing our siHUNT1 and siHUNT2 constructs into
miRNA backbones may help eliminate the observed toxicity.

Gene dosing could also be playing a role in the effectiveness of the shRNA
treatment here. At any one time point, the only variable in the groups is the genotype.
When comparing transcript reductions across genotypes, there are some slight
significant differences. In particular transcripts from the 26 week old mice treated with
siHUNT1 showed significantly less reductions than either the nTG or the homozygous
mice (ANOVA, p<0.001). In the siHUNT2 injections at 14 weeks, nTG transcripts were
reduced further than the heterozygous ones (ANOVA, p<0.05). In both of these cases
the heterozygous mice appear to be less effected than their nTG counterparts. It is
possible that heterozygous mice, for some unknown reason, are not affected by the shRNA toxicity as much as their nTG or homozygous counter parts.

**NII Reductions**

In both the siHUNT1 and siHUNT2 injected mice, the number of NIIIs were significantly reduced. Previous work with either siHUNT1 or siHUNT2 did not quantify the NIIIs past 10 weeks post-injection, and Rodriguez-Lebron showed that a control shRNA and GFP did not reduce the amount of NIIIs after 10 weeks in the R6/1 line while both siHUNT1 and siHUNT2 reduced the NIIIs considerably (259). The work performed here does not refute the previous work; however it suggests that the reductions seen in the R6/1 model may not have been solely due to reductions of mutant htt. siHUNT1, which does not target any known transcript in the CAG 140 mice, induced NII reductions at 26 weeks indicating that something other than mutant *htt* knock-down is altering the levels of NIIIs. If the R6/1 study had gone out further it is possible the control shRNA may have shown some similar toxic attributes that we see here in the siHUNT1 and siHUNT2 shRNAs.

As mentioned previously, shRNAs have been shown to potentially be toxic (285, 291-297). Our work here with NII reductions may help support that notion. Since both siHUNT1 and siHUNT2 reduce NIIIs considerably but only siHUNT2 actually targets the mutant *htt* it is suggestive that any presence of shRNAs could contribute to the reduction of the inclusion bodies. The mechanism by which shRNAs would reduce inclusion bodies is unknown. Thus, reductions of NIIIs by shRNA treatments may not be indicative of any actual therapeutic benefit.
Compounding Factor

Despite our best efforts to have proper controls, we did not have an appropriate viral injection control. The GFP control in our experiments was the hGFP designed by Zolotukhin et al. (260), a humanized *Aequorea* GFP and the GFP in the siHUNT vectors is a humanized version of the *Renilla* GFP. In addition to the GFP discrepancy the promoters that drive each GFP are different. This GFP differences creates a problem when evaluating our work performed with the siHUNT vectors since we do not have proper controls to determine if the GFP is contributing to the observed effects. We can not definitively conclude that humanized Renilla GFP has no effect outcome measurements. The transcriptional down-regulation can not be definitively declared to be the result of shRNA toxicity solely. In the next section we will address the hGFP issue as stand alone problem.
Figure 4-1 Slight behavioral improvements after rAAV5-siHUNT2 injections are evident in Rearing behavior but not in the Accelerating Rotarod. A) Early rearing behavior improvements in siHUNT2 injected mice. The siHUNT2 injected mice showed a significant increase in rearing behavior compared to the rearing behavior observed previously in the heterozygous mice (p<0.05). siHUNT2 injected mice reached nTG rearing levels (p>0.05). Examining the heterozygous mice at 9 months the improvement did not continue (p>0.05) B) 12 month Accelerating Rotarod behavior showed no improvement. Early time points (4 and 9 months) did not show differences between any of the groups (p>0.05), consistent with earlier reports where no early accelerating rotarod deficits are noticed. At 12 months, all heterozygous mice were significantly different than the uninjected nTG (p<0.05).
Figure 4-2 In situ $^{33}$P hybridization of striatally enriched transcripts 14 and 26 weeks after rAAV5-siHUNT1 injections. The striatal area of the rAAV5-siHUNT1 injected mice was analyzed for O.D after mRNA transcript radioactive in situ hybridization, normalized to β-actin mRNA density and reported as percent of the uninjected region. A) 14 week and 26 week nTGs were both significantly reduced (ANOVA, p<0.001 and p<0.0001). B) Heterozygous transcripts at 14 weeks (ANOVA, p<0.001) but not at 26 weeks (p>0.05) were significant reduced compared to uninjected sides. C) Homozygous 14 week and 26 week transcripts had an overall reduction compared to uninjected (ANOVA, p<0.0001 and p<0.005). Heterozygous transcripts showed a significant increase over time (ANOVA, p<0.001). ppENK homozygous 26 week transcript level was significantly greater than uninjected side (t-test, p<0.01) but did not reach nTG ppENK levels at the same age (t-test, p<0.05) (nTG: 14 week N=5, 26 weeks post-injection N=7, HET: 14 week post-injection N=6, 26 week N=7, HOM: 14 week N=7, 26 week N=5). Asterisk = significant difference between injected and injected, + = indicates significantly higher than the 14 week time point.
Figure 4-3 In-situ $^{33}$P hybridization of striatally enriched transcripts 14 and 26 weeks after rAAV5-siHUNT2 injections. Values are percent of the uninjected striatal region. A) nTG 14 and 26 week transcript levels showed an overall reduction compared to the uninjected side (ANOVA, p<0.0001, and p<0.05). B) Heterozygous 14 26 week transcript levels did not show significant reductions (ANOVA, p>0.05). C) Homozygous 14 week transcripts were overall significantly lower (ANOVA, p<0.001) but homozygous 26 week transcripts were not reduced (ANOVA, p>0.05). nTG CB1 transcript increased significantly from 14 weeks to 26 weeks (p<0.05) nTG:14 N=7 (PDE10a, PDE1b N=5; NGfiA N=4)), 26 weeks N=6 (D2, PDE10a and NGfiA N=5), HET: 14 week N=5 (D2 N=4), 26 week N=7 (D2, PDE10a and CB1 N=6), HOM: 14 week N=6 (DARPP-32 and ppENK N= 7, D2 PDE10a and PDE1b N=5), 26 week N=3. Asterisk = significant difference between injected and injected, + = indicates significantly higher than the 14 week time point. nTG= non-transgenic, HET= heterozygous, HOM = homozygous.
Figure 4-4 Reductions of NIIs are evident after shRNA injection into the striatum of CAG 140 heterozygous and homozygous mice. A) Heterozygous 14 week siHUNT1 and siHUNT2 injections did not show any significant difference between injected and uninjected. B) Significant difference was evident when comparing injected to uninjected in the heterozygous 26 week post injections (ANOVA, p<0.01). C) Homozygous 14 week injections showed a significant reductions after shRNA injections (ANOVA, p<0.01) as did the D) Homozygous 26 weeks post-injections (ANOVA, p<0.001) siHUNT1 (HET: 14 week N=5, 26 week N=5; HOM: 14 week N=5, 26 week N= 4) siHUNT2 (HET: 14 week N=5, 26 week N=6; HOM: 14 week N= 4, 26 week N= 3) GFP (HET: 14 week N= 6, 26 week N=6; HOM: 14 week N= 3, 26 week N=5).
CHAPTER 5
LONG TERM EFFECTS OF RAAV5-HGFP INJECTIONS IN THE CAG 140 MOUSE MODEL

Introduction

The GFP protein discovered by Shimomura et al. (298) has become widely used in biological studies as a way to monitor intracellular protein trafficking and localization, cellular migration and proliferation, and to assess the transduction efficiency of plasmids and viral vectors in vitro and in vivo. GFP is a highly stable (299, 300) and relatively long lasting protein with a half-life of ~26 hours in vitro (301). GFP is one of many different types of fluorescent proteins that are used in the biological fields. Other fluorescent proteins include dsRED, mCherry, yellow fluorescent protein (YFP) and cyan fluorescent protein (CFP) which are all derivatives of the original GFP, or derived from other organisms such as Discosoma striata and the sea corals Anthozoa (302-306). There are over 38 variants of the monomeric fluorescent proteins and over 19 dimeric or trimeric fluorescent proteins and many more are being generated to facilitate in the need for tracking and localization of multiple intracellular proteins and process (for a comprehensive review see Chudakov et al. 2010 (307)).

Humanized GFP (hGFP) is a modification of the original Aequorea victoria GFP developed by Zolotukhin et al. which modified the Aequorea victoria jellyfish GFP cDNA sequence to allow for a more efficient translation into protein (260). Different organisms translational machinery recognizes certain codons more frequently than others (308-310), and by changing these codons to match appropriately with the mammalian translation preferences, Zolotukhin et al. designed an GFP cDNA that was more efficiently translated into protein. The ‘humanized’ modifications produced 70-fold more signal than the original GFP (260).
We have used the hGFP in our rAAV viral preparations for many years as a control for viral infection as well as in our experimental vectors to monitor the localization of the viral transduction (208, 259, 288, 311-315). hGFP is an ideal protein marker because it is more efficiently translated into protein and the peak emission (509 nm) spectra is the same as the original GFP (260, 298, 316). Additionally, hGFP can be stained with *Aequorea* GFP or eGFP antibodies to determine the full extent of the rAAV-hGFP transduction.

During shRNA work in the previous section, we found that the rAAV5-hGFP had negative effects on the levels of mRNA transcripts that are striatal specific. These mRNA transcript reductions were surprising and alarming because our lab, as well as many other labs, have used rAAV-hGFP as a control for years. The implications that at least there is transcriptional dysregulation when using rAAV-hGFP might call into question a number of studies where such transcriptional dysregulation may have had a direct impact on the results. Thus, we decided to further investigate the rAAV-hGFP effect by looking at various histopathological differences between the injected and uninjected side of the brain.

**Results**

**Transcriptional Down-Regulation in GFP injected Mice**

Striatal rAAV5-hGFP Injections in 10 week old nTG, heterozygous and homozygous mice produced transcriptional down-regulation at 14 and 26 weeks post-injection (Figure 5-1). The nTG mice injected with rAAV5-hGFP displayed significant reductions in D2, DARPP-32, ppENK, PDE10a, PDE1b and CB1 transcripts at the 14 week post-injection compared to the uninjected side (Figure 5-1 A; ANOVA, p<0.001). At the 24 week post-injection time point, transcripts were also significantly reduced.
(Figure 5-1 A; ANOVA, p<0.0001). There was no significant difference between the transcripts at 14 and 26 weeks in the nTG injected with hGFP (ANOVA, p>0.05). The heterozygous mice did not display any significant reductions in mRNA transcripts at the 14 week post-injection time point (Figure 5-1 B; ANOVA, p >0.05). Heterozygous striatal mRNA transcript levels were reduced significantly compared to the uninjected sides at the 26 week post-injection (Figure 5-1 B; ANOVA, p<0.05). Like the heterozygotes, homozygous mice displayed no significant reductions in the mRNA transcript levels at 14 weeks (Figure 5-1 C; ANOVA, p>0.05) but at the later 26 week post-injection, significant reductions in comparison to the uninjected side were evident (Figure 5-1 C; ANOVA, p<0.0005). An example of the transcript reduction from our rAAV-hGFP injections can be seen in Figure 5-2. DARPP-32, ppENK and PDE10a transcript levels are clearly reduced on the injected side (right side of images).

**DARPP-32 Protein Reductions**

Reductions of the mRNA levels suggested there could be significant protein level reductions as well. We examined the protein levels of DARPP-32 in rAAV5-hGFP injected mice to determine if the mRNA reductions observed above resulted in protein level reductions. Examining the nTG, there was significant reductions in DARPP-32 protein after the injection of rAAV5-hGFP. Figure 5-3 A and B depicts an example of a 26 week post-injection nTG mice stained for GFP and DARPP-32. Clear reductions of DARPP-32 protein staining were visible on the injected side(Figure 5-3 A and B). Using the Odyssey Near-infrared (IR) imaging system, we were able to calculate a 40% relative reduction of DARPP-32 protein in the injected vs. the uninjected striatal regions at both 14 and 26 weeks of transgene expression (Figure 5-3 C; ANOVA, p <0.05). Despite DARPP-32 mRNA reductions in the heterozygote and homozygous mice at 26
weeks, we did not see a significant reduction of DARPP-32 protein (Figure 5-3 D; ANOVA, p > 0.05).

**Total Striatal Cell Count**

Cresyl violet staining was performed in order to determine the total number of cells in the striatum. Stereological like sampling was performed in the exact same manner as NeuN cell counts to determine the number of cells in the uninjected and the injected mice. No significant difference was observed between the two striatal regions counted (Figure 5-3; p > 0.05)

**NeuN Positive Cell Reductions**

NeuN is a nuclear protein antibody stain that is specific for post-mitotic, differentiated neurons (317). Using NeuN IHC, we attempted to determine if neuronal loss was evident. Quantification of the NeuN stain, using the stereological like technique described in the methods section, showed a significant reduction overall as well as at each of the time points in the number of NeuN positive neurons of nTG mice (Figure 5-5 D; ANOVA, p < 0.0001, asterisk = p < 0.001). Reductions in the homozygous mice were not as significant but still showed considerable decreases (Figure 5-5 D, ANOVA, p < 0.001, asterisk = p < 0.01). This reduction in NeuN staining was easily detectable in photomicrographs. Figure 5-5 A shows a low power magnification of an nTG 24 week post-injection mouse. Higher power images of uninjected and injected striatal regions can be seen in Figure 5-5 B and Figure 5-5 C. There was no significant difference in the number of NeuN stained neurons between the uninjected sides of the nTG and homozygous at either 14 or 26 weeks post-injection (Figure 5-5 E; ANOVA, p > 0.05).
Astrocytic Activation

The astrocyte specific marker, glial fibrillary acidic protein (GFAP), was used to determine if there was an increase in the number of activated astrocytes in the injected striatum of the nTG mice. Figure 5-6 indicates that there was an increase in the observable number of activated astrocytes as seen by GFAP positive staining at low magnification (Figure 5-6 A). At higher magnification (Figure 5-6 C) activated astrocytes can be identified by cell morphology.

Qualitative analysis of the staining of GFAP was performed by an individual that did not know the injection type or age. Relative amounts are qualitatively assessed by a scale ranging from 0 to 3. The qualitative analysis shows an overall increase in the amount of GFAP cells in the injected striatum when compared to the uninjected side (Table 5-1).

Discussion

In the course of the shRNA portion of the project we utilized the rAAV-hGFP as our control injection. However, when examining one of our experimental outputs, *in situ* hybridization of mRNA transcripts, we noticed that control rAAV-hGFP reduced mRNA transcript levels significantly. The implication of a widely used control that causes significant experimental differences is concerning because it may call into question any experiment where hGFP has been used. We investigate here some of the histopathological abnormalities that are occurring with the rAAV5-hGFP injections.

**mRNA Transcript Down-regulation After rAAV5-hGFP Injections**

rAAV almost exclusively transduces neurons and has rarely been shown to transduce other cell types in the brain (208, 212, 214, 318). We used the serotype 5 of rAAV and have not attempted to alter the tropism of the virus, so neurons should be the
only target of our viral vector. The striatal transcripts investigated in this study are neuronal specific and therefore the reductions in these transcripts are likely due to the rAAV5-hGFP viral injection. In all three genotype (nTG, heterozygote and homozygote) as well as most of the time points (14 and 26 week post-injections) we detected transcriptional down-regulation, similar to what was seen in the siHUNT injections described earlier in chapter 4. Similarly, we did not see a significant change in transcript levels in any genotype over time (ANOVA, p>0.05)

NGFiA appeared to be the only consistently transcript that was not down regulated (See Figure 5-1). A recent study by Beck et al. in 2008 has shown that NGFiA can be regulated in astrocytes in and around a glial scar after an ischemic injury by astrocytes (319). While we did not induce an ischemic injury and the Beck study was examining the NGFiA protein levels, not mRNA transcript levels, it is suggestive that our NGFiA transcript levels could in fact be due in part to non-neuronal cells (319). We have no way to determine if the NGFiA transcript levels we are measuring are from neurons or from other types of cells in the striatum. If non-neuronal cells are up-regulating NGFiA after our injections, it may explain why we do not see a loss of NGFiA.

**Medium Spiny Neuron Population and Markers**

The significant decreases in mRNA transcripts after injection of rAAV5-hGFP suggested the possibility that the protein levels associated with the transcripts might also be decreased. Staining of the DARPP-32 protein, one of the transcripts that decreased in almost all of the genotypes and post-injection times, showed a significant reduction in DARPP-32 protein when compared to the uninjected side (Figure 5-3 B and C). Our DARPP-32 staining looked at the total reductions of DARPP-32, not just the activated phosphorylated forms. Such alterations in the DARPP-32 total concentrations
could impact downstream transcriptional machinery as DARPP-32 plays an important
role in gene regulation (71, 77, 320, 321). While we did not investigate the protein levels
of the other translational down regulated genes, if they are reduced in a similar manner
as DARPP-32, there could be significant consequences in cellular signaling.

Figure 5-3 B shows a remarkable reduction of DARPP-32 staining in the nTG
section and such reductions could be due to neuronal loss. Staining for a neuronal
specific marker would tell if the rAAV5-hGFP injections are causing neuronal death, or
simply causing cellular dysfunction characterized by the transcriptional and translational
dysregulations seen in Figure 5-1 and 5-3. One widely used neuronal marker that
specifically labels post-mitotic and differentiated neurons is NeuN (317). When we
stained the rAAV5-hGFP injected mice we observed reductions in NeuN positive cells in
the injected striatum compared to the uninjected striatum (Figure 5-5). Not only were
there fewer cells overall, the cells that were positive on the injected side showed lighter
staining when compared to the uninjected side. These reductions in NeuN may suggest
there is neuronal loss after rAAV5-hGFP injections.

Despite the evidence that there might be neuronal cell loss in our rAAV5-hGFP
injected animals, there have been a few reports that have suggest that NeuN reductions
may not indicate neuronal loss (322, 323). We still saw GFP staining in the regions of
the most significant DARPP-32 loss. Unfortunately, we were unable to stain for other
neuronal markers such as MAP-2 or Tau which would help indicate if neuronal loss was
occurring. If in fact the NeuN reductions do not indicate neuronal loss, then NeuN
protein reductions are potentially another translationally dysregulated protein resulting
of the injections of rAAV5-hGFP.
The protein that the NeuN antibody binds to is called Fox3 and is part of the Fox RNA splicing family (324). The fact that NeuN (Fox3) is involved in RNA splicing may be important in the transcriptional down-regulation that we have seen with our injections. The Fox proteins enhance RNA splicing when the Fox binding element (UGCAUG) is present in introns (324-329). The amino acid region in Fox-1 that binds the UGCAUG intronic sequence is homologous to a region in the Fox 3 protein, suggesting Fox 3 could bind the same intronic region and help enhance RNA splicing (324, 330). Underwood et al. showed that the protein levels of Fox 1 and Fox 2 directly affect both mRNA and protein levels of RNA sequences that contain the intronic Fox binding element in neurons (329). If NeuN/Fox 3 is being down-regulated by hGFP then it would not be surprising that downstream transcripts could be adversely affected as well. Zhang et al. found a number of potential binding sites for the Fox 1 and Fox 2 RNA splicing proteins. A search of the database generated from Zhang’s study revealed that PDE10a has one predicted splice variant regulated by Fox1/2 (331). While it has not been confirmed as of 2008, it is intriguing that one of the transcripts decreased in our studies has potential alternative splicing derivatives controlled by a protein (NeuN/Fox3) that is down regulated. If NeuN/Fox3 function is somehow being altered by the presence of hGFP, the splicing that is necessary for proper mRNA processing would be effected. NeuN/Fox3 down-regulation might be one explanation for the transcriptional dysregulation we are observing in our hGFP injected mice.

**GFAP Activation after rAAV5-hGFP Injections**

Single stereotaxic injections into the brain have shown some increase in astrocytic activation by GFAP staining (314, 315) but such effects are usually restricted to the needle tract. Peden et al. showed that striatal rAAV re-administration to the opposite
side in rats showed considerable increases in astrocyte activation (314) but no significant astrocytic activation was observed in their single administration studies. Peden also showed that the transgene, hGFP, showed little apparent toxicity because repeated administrations of the same hGFP cassette with a different serotype showed no reductions of hGFP expression and little immune response, either astrocytic or microglial.

Our results here show that 14 weeks and 26 weeks after a single rAAV5-hGFP injection we saw relatively large astrocytic activation on the injected side of the brain not confined to the needle tract. The two studies by Peden et al. showed single injections have little GFAP activation, but both studies only examined rats at 4 to 8 weeks post injection. Not only did the study not go past 8 weeks, Peden et al.’s injections were done in rats, not mice. It is possible that there is a species dependent variable in the astrocytic response to the rAAV5-hGFP injection.

McBride et al. have examined microglia (Iba-1 positive staining) at 16 weeks post injection of an GFP vector using the CAG 140 heterozygous mice but showed no significant increases (292). Though, the hrGFP was used in their studies rather than the hGFP that we are using here. It would still be useful to know if any astrocyte activation was present at the same time in the McBride study. A more robust study of both astrocytic and micglial activation in long term injected mice would be prudent to determine if hGFP, or GFP in general, has any effect.

**Conclusions**

The results of the work done here, demonstrate that there was dramatic negative effects following rAAV5-hGFP injections into mice. These toxic side effects were apparent at 14 and 26 weeks post-injection in our nTG and also in our CAG 140
homozygous mice. Transcriptional down-regulation, DARPP-32 protein reductions, NeuN reductions, and GFAP activation were all evident in the rAAV5-hGFP injected mice. These results possible indicate that the rAAV5-hGFP injections have potential long term toxic side effects and further investigation is warranted to determine the exact extent and role either rAAV5 or hGFP may have in mice long term.
Figure 5-1 Transcriptional down-regulation of striatally enriched mRNA transcripts 14 and 26 weeks following rAAV5-hGFP injections. The striatal area of the rAAV5-hGFP injected mice was analyzed for optical density and normalization to β-actin. Values shown are percent of the uninjected striatal region. A) nTG transcripts 14 and 26 weeks post-injection showed significant reductions compared to uninjected side (ANOVA, p<0.0001 and p<0.0001). B) Heterozygous transcripts 14 weeks (ANOVA, p>0.05) did not show significant differences from uninjected sides while 26 weeks post-injection were significantly reduced (ANOVA, p<0.05). C) Like the heterozygous transcripts homozygous transcripts were not reduced at 14 weeks (ANOVA, p>0.05) but were at 26 weeks (ANOVA, p<0.0005) nTG:14 week post-injection N=6 26 weeks post-injection N=8 HET: 14 week post-injection N=5 (D2 N=3), 26 week post-injection N=7 (DARPP-32 N=6 ppENK N=5), HOM: 14 week post-injection N=5, 26 week post-injection N=5) asterisk = significant differences between injected and injected.
Figure 5-2  Example of $^{33}$P radioactive in situ mRNA hybridization of various transcripts and verification of the hGFP transcript. DARPP-32, ppENK and PDE10a transcript reductions are clearly seen in the images above. Injection side is evident by the presence of hGFP transcript seen on the right side of the far left image. The pattern shown here is also evident in the other transcripts shown in Figure 5-1.
Figure 5-3 Total DARPP-32 protein reductions after rAAV5-hGFP injection. A) Representative section of GFP stained 28 week old rAAV5-hGFP injected mouse with a near IR antibody. B) Same section stained with a near IR antibody for DARPP-32. Injected side is shown on the right. C) Quantification of total DARPP-32 from the uninjected and injected striatal regions showed an overall reduction by ANOVA (ANOVA, p <0.05). 14 week post injection N=5, 26 week post-injection N=4, D) Het and Hom DARPP-32 quantification of DARPP-32 at 26 weeks post-injection. No significant differences in the Het and Hom mice individually or overall was observed (ANOVA, p>0.05).
Figure 5-4 Quantification of total striatal cells shows no significant difference following rAAV5-hGFP injections in nTG mice. Cresol violet staining shows no significant differences between uninjected and injected striatal regions of nTG mice. Total cells were quantified using a sterologically based sampling regime. Bars represent the percentage of the uninjected side. (14 weeks post-injection N=3, 26 weeks post-injection N=4).
Figure 5-5 NeuN staining reductions after rAAV5-hGFP injections. A) Low power representative image of an nTG 14 weeks post-injection, scale bar = 1mm B) High power representative images of uninjected and C) injected of image in A). D) Quantification of NeuN positive cells in the nTG animals injected with rAAV5-hrGFP have an overall reduction compared to the uninjected side (ANOVA, p<0.0001) as well as in the homozygous injected mice (ANOVA, p<0.001) (nTG: 14 weeks post-injection N=4, 26 weeks post injection N=4; HOM: 14 weeks post-injection N=3, 26 weeks post injection N=5; asterisk = p<0.001). E) Average NeuN positive cells per 1mm$^2$ from uninjected side of nTG and homozygous mice. NeuN positive cells were quantified using a sterologically based sampling regime. All asterisk represent significant differences from corresponding uninjected sides. Scale bar = 50 μm.
Figure 5-6. GFAP positive staining 26 weeks after rAAV5-hGFP injections in a nTG mouse. A) Low magnification of a 14 week post-injection nTG mouse injected with rAAV5-hGFP. Scale bar = 1 mm. B and C) show the highlighted region in A). B) High magnification of the uninjected side and C) High magnification of the injected side. Scale bar= 50 μm.
### Table 5-1 Qualitative analysis of GFAP positive labeled cells in the striatum

<table>
<thead>
<tr>
<th>Genotype</th>
<th>14 Weeks Post-Injection</th>
<th>26 Weeks Post Injection</th>
</tr>
</thead>
<tbody>
<tr>
<td>nTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uninjected</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Injected</td>
<td>+++/++++</td>
<td>++</td>
</tr>
<tr>
<td>Homozygote</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uninjected</td>
<td>+</td>
<td>+/-</td>
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<tr>
<td>Injected</td>
<td>+++/++++</td>
<td>++</td>
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CHAPTER 6
DISCUSSION

Characterization of the CAG 140 HD Mouse Model

Overview of CAG Characterization

The CAG 140 mouse model characterized in this project is one of the many models that have been developed to study HD (145, 146, 149, 157, 164, 165, 168, 178). At the start of this project, little was known about the long-term behavioral or histopathological progression of the HD-like symptoms in CAG 140 model. In order to determine if this HD knock-in mouse line was a viable and representative model of HD, a long term study was needed. Here we have shown that there are aspects of the CAG 140 model that are similar to HD and previous HD mouse models. Similarities include the disease related transcriptional down-regulation and the progressive increase of NIIs. In the CAG 140 model, we observed significant rearing behavioral and rotarod deficit; however, these were not progressive.

We started our behavioral testing at 10 weeks of age and found there was a difference in the number of rearings in a novel environment between the nTG and the afflicted mice. The knock-in hypoactivity is congruent with what was seen before in Menalled’s initial study of CAG140 mice (169). Gait abnormalities were observed in the knock-in mice at 12 months in Menalled’s study, but in the work performed here, neither the heterozygous nor the homozygous mice displayed gait abnormalities compared to the nTGs. Unlike what is observed in the R6, N171 and other transgenic models, we did not detect any abnormal clasping behavior at any point during the course of these studies. Additionally, we did not observe any significant overall weight differences(Figure 3-8). This is contrary to what has been described previously in the
CAG 140 mice over a similar time course. In the Dorner study (171), female knock-in mice weighed significantly less than their nTG counterparts.

RNA transcript analysis of this model showed disease related down-regulation in a variety of striatal specific genes. Over time, CAG 140 mice displayed a down-regulation of a variety of striatal specific transcripts which is congruent with both humans and all other HD mouse models (23-25, 63, 64, 91, 93-97). The mRNA transcriptional down-regulation began between 6 to 9 months and after the onset of rearing behavior deficits. The progressive decreases in the mRNA transcripts did not correlate with the rearing behavioral abnormalities observed. The transcriptional changes seen here may not be associated with the behavioral abnormalities and suggests that other factors are contributing to the observed behavior.

There is a progressive increase in the density of striatal NII's in the CAG 140 mice, which are present in both humans as well as the other mouse models of HD (127, 128, 157, 168, 169, 178). We began to observe NII's at approximately 6 months in the CAG 140s, which is about the same time we began to see transcript down-regulation. Numerous groups have shown that mutant htt has an effect on the function of various transcriptional factors (104-108, 110, 111), and, in some cases, transcriptional factors have been found to be co-localized to inclusion bodies (105). Previous characterizations of the CAG 140 mice has shown that NII's begin to form earlier at approximately 4 months, but the discrepancies between these studies and ours might be due to the fact we are missing the actual window of the NII's between our 3 month and 6 month age groups.
In the work performed here, we observed progressive increases in NIIIs but we did not see progressive behavioral abnormalities. Like the transcript levels discussed above, the presence of NIIIs may have no real bearing on the behavioral aspects in this mouse model. There is some evidence that instead of being detrimental, the NIIIs may in fact be beneficial. Two such studies have found that NIIIs are a protective aspect of HD, as the decreases in NIIIs causes higher neuronal death (332) while increased NII formation showed greater neuronal survival (333). The latter study by Arrasate in 2004 was supported by a recent study in 2010 by Miller et al. that showed a direct quantitative relationship between neuronal survival and the formation of NIIIs in an cell culture model of HD (334). When an NII is formed, the soluble mutant huntingtin decreases dramatically and the neuronal survival time increases. Those cells that did not form NIIIs but did contain the mutant protein died much sooner than the NII containing neurons (334). From these studies, NIIIs as a measure of disease progression or therapeutic value may not be useful.

In conclusion, while there is no progressive behavioral phenotype in the CAG 140 mice from the tasks performed here, these mice do display progressive histopathological abnormalities that mimic similar data from other HD models as well as from the human disease (23-25, 62-64, 91, 93, 94, 96, 97, 127, 128, 157, 168, 169, 178). Additionally, the CAG 140 model may be a more genetically accurate model for HD than other transgenics, because they have the correct gene dosing associated with HD. The R6, N171-Q82, YACs and virally generated models of HD, still contain both copies of the endogenous hdh, and as such, may represent confounded HD models. The knock-in models of HD, including the CAG 140 mice, have the correct gene dosing
and exhibit the phenotypes that have been classically associated with HD. Both the transgenics and the knock-in models of HD have been invaluable to the study of HD.

**Future Directions of Study in the CAG 140 HD Model**

Despite the work performed here and previous work on the CAG 140 model, more characterizations could be performed. Cognitive and psychological abnormalities are present in the human disease and might be present in the CAG 140 mouse model as well. Detailed cognitive testing such as the Morris water-maze task or the recently designed ‘What Where When’ task might be warranted to test such potential abnormalities (335, 336). De Vito et al. designed the ‘What Where When’ task to test a rodent’s ability to remember if an object is novel, the location of that object spatially, as well as when the object was placed in its original location (336). The ‘What-When-Where’ task as might give invaluable information about the learning and memory impairments that might be present in the CAG 140 mice. Moreover, a longitudinal study of these tasks might show if the mice showed an initial inability to learn the task or a gradual impairment as the mice age.

Our transcriptional analysis of the mice is an important, but equally as important would be a protein profile of the CAG 140 mice. An analysis of the potential protein down-regulation in this model would be another important analysis. DARPP-32, as an example, has been shown to be down regulated in other HD mice models (118, 120) as well as in this model by Hickey et al. (170). It would be invaluable to determine which proteins are being down-regulated and at what time the down-regulation occurs.

Various groups have demonstrated that cortical thinning is progressive in HD and is one method of detecting the progression of the disease in humans (13, 15-18). In our hands, some slight cortical thinning differences between the nTG and the knock-in mice
were observed. Slight cortical thinning is congruent with the previous studies on thinning in humans with HD (13-18, 337). Longitudinal studies in the R6 and YAC mice using MRI have shown not only cortical but other morphological changes as the mice age (338-340). Such longitudinal studies of cortical thinning in the knock-in mice models would be more beneficial and potentially more significant than the cross-sectional study performed here.

**Therapeutic Treatment in the CAG 140 HD Model**

**Huntingtin Knock-Down**

Previous work showing that the reductions of mutant htt in transgenic lines can improve the HD associated phenotypes (148, 163, 259). In particular, two studies, Harper et al. and Rodriguez-Lebron et al., showed shRNA mediated knock-down of the mutant htt showed improvements in both behavior and some neuropathological aspects of the transgenic mouse models (163, 259). These three studies showing reductions of mutant htt in transgenic models of HD lead us to investigate the possibility that knock-down of the mutant htt in a knock-in model would elicit the same improvements. Our use of shRNA mediated knock-down of mutant htt in the CAG 140 knock-in model did not show the same improvements observed previously in the transgenic models of HD.

Despite the NII reductions seen in the siHUNT2 injected mice, we did not detect an improvement in the striatal mRNA transcript levels but rather we saw additional decreases in the disease-related striatal specific transcripts. Additionally, we observed a similar pattern in our siHUNT1 shRNA, which does not target the mutant htt present in CAG 140 mice. Not only did our shRNA control show NII reductions and further transcriptional down-regulation, but our GFP control injections also showed transcriptional down-regulation. Slight behavioral improvements were seen early in
rearing behavior after injections with siHUNT2, but these improvements did not continue and returned to the uninjected heterozygote averages. Because of the disconnect between any long term overt behavioral improvements and the further transcriptional down regulation, we can not definitively conclude that reductions in NII$s$ are either beneficial or detrimental.

Homozygous CAG 140 mice 26 weeks after siHUNT2 injection showed massive striatal atrophy in half of the injected animals. The only copy of $htt$ present in these mice is the expanded mutant $htt$ and the observed atrophy suggests total loss of huntingtin maybe detrimental. The mice that did not show siHUNT2-induced atrophy did not have reductions in transcripts below that of the disease related reductions. These non-atrophied siHUNT2 26 week post-injection homozygous mice did have significant reductions in NII$s$. It is impossible to conclude any therapeutic advantage when half of the animals treated with the shRNA show massive atrophy and the half show no real improvement other than a reduction in the NII$s$.

Based on our siHUNT2 26 week data, that showed atrophy, and previous work done on hdh reductions (146), complete reduction of striatal $htt$ may not be the best approach. Dragatis et al. in 2000 showed total reductions of hdh in the brain via a cre-lox system displayed significant neuronal loss and some behavioral abnormalities (146). Our homozygous mice injected with the siHUNT2 would be a localized version of the Dragatis study, and the massive atrophy could be equivalent to the neuronal loss seen in the Dragatis mice. The atrophy seen in half of our homozygous mice injected with siHUNT2 at 26 weeks may support the Dragatis study that reported complete reduction of $htt$ is potentially toxic. It should be noted though that the Dragatis study reduces all
hdh in the CNS of the mice. This total reduction would not be realistic in a human gene-therapy treatment as of right now. There is no current method of transducing the entire CNS with a vector that would reduce all mutant htt or htt. However, the Dragatis is a good proof of concept study that should be considered when examining complete knock-down of mutant htt.

Two recent studies by Drouet and Boudreau show that endogenous reduction of hdh in addition to the reduction mutant htt via RNAi is tolerable in contrast to the Dragatis hdh knock-down (285, 287). In the Drouet paper, the mutant htt was introduced via lentivirus injections to the striatum (287) and Boudreau used the N171-Q82 transgenic HD model (285). The RNAi knockdown of mutant htt and hdh in both of these latter studies yielded some transcriptional abnormalities (285, 287) but showed an overall significant improvement in both NIs as well as the behavioral phenotype. While these two studies show some promise for non-allele specific siRNA targeting in HD, it should be noted that in a heterozygous mice knock-in mice, there is half as much endogenous hdh as there is in a transgenic model (one copy of hdh vs two copies). Reducing the hdh protein levels in a transgenic mouse line to 50% would be equivalent to the normal hdh levels in a heterozygous knock-in mouse. A similar 50% reduction of hdh in a heterozygous knock-in mouse would be equivalent to a 25% reduction in a transgenic mouse model. The fact that both models used in the Drouet and Boudreau studies have two copies of the endogenous hdh, creates a gene dosing issue that may confound the interpretation of the RNAi treatments. To study the complete removal of all huntingtin further, a knock-down of both the endogenous and the mutant HD allele specifically in the striatum of a rodent knock-in
model would be of great benefit and would determine if significant knock-down of \textit{htt} and \textit{mtt} would be of any benefit. McBride et al. attempted this approach by injecting their shRNAs that target both \textit{hdh} and \textit{mtt} into the heterozygous CAG 140 model, but their shRNAs proved to be toxic and showed microglial activation along with DARPP-32 protein reductions. Converting their shRNAs into miRNAs, McBride et al. showed the toxicity could be reduced, but no further studies into the effectiveness of their miRNAs on HD pathology or behavior in the CAG 140 mice was performed (292).

\textbf{Future Direction and Studies}

As discussed previously in chapter 4, shRNA might be toxic \textit{in vivo} (285, 291-293, 295) and if the shRNA toxicity is playing a role in the transcript down-regulation, it might be overshadowing any therapeutic effect due to the mutant \textit{htt} knock-down. shRNA toxicity appears to be alleviated by using miRNA backbones (285, 292, 295) or by using a different promoter that produces less of the shRNA (341). While Giering showed that by changing the RNA Pol (III) promoter, that is normally used to drive shRNAs, with the RNA Pol (II) promoter reduced the observed toxicity when used in the liver (341). This is promising for the use of shRNAs in general but there has not been an attempt in neurons to show the same amelioration. Additionally, Giering et al. did not examine the possibility of off targeting due to the imprecise start transcriptional start site associated with the Pol (II) promoter. As recently as 2010, groups have still been utilizing the Pol (III) promoter and seeing shRNA toxicity in neurons (293).

Our work here does not utilize either the miRNA backbone or the Pol (II) promoter system that would modulate the amount of RNAi being produced by neurons. These two issues may explain the potential siHUNT1 toxicity that we observe. While we can not explain the exact mechanism by which siHUNT1 is causing such toxicity, it maybe due
to over loading the endogenous miRNA pathways described by Grimm and Boudreau (291, 295). To determine if the observed toxicity in our study is due to the overloading of the miRNA pathways, siHUNT1 could be converted to a miRNA form and tested for transcriptional dysregulations. siHUNT1 should not target either the mutant or the normal allele and the effect of the shRNA vs. miRNA could then be examined directly. Once a less toxic, and ideally non-toxic, method of RNAi production is designed, the effectives of huntingtin knock-down can be examined without complications.

There are approximately 45 to 50% of individuals with polymorphisms in the mutant huntingtin allele that could be specifically targeted by designing custom a RNAi (342, 343). It would be important to determine if there is a single target sequence in htt that could be used which would both reduce the mutant htt enough to relieve the effects of HD, but leave enough normal htt so neurons can function normally. Such an RNAi sequence would target everyone afflicted with the disease would be much easier and more cost effective than designing specific sequences to target an individual whose sequence is unique. Unfortunately the exact role of huntingtin is still a mystery and the threshold levels of normal huntingtin are unknown.

Compounding Variables

Convection Enhanced Delivery

Mannitol has been shown in the clinic to help alleviate brain swelling (344-346) by allowing water to move from the brain towards the blood stream to relieve the pressure. Various groups, including our lab, have demonstrated that this osmotic pressure differential can greatly increased the viral transduction area if mannitol is given prior to intracranial injections (347-351). This method of increasing viral spread has been named convection enhanced delivery (CED) and was used in this study.
Increasing the volume of transduced cells in the brain would be ideal, as long as no adverse effects arise from the transgene or the virus itself. Small negative effects caused by the transgene and or virus, might be increased substantially due to the larger transduction volume if CED is used. Additionally, the introduction of the differential osmotic pressure between the CNS and the circulatory system might allow for immunological peptides (such as the viral particle) to flow from the brain to the periphery, after breaking the dura during our stereotaxic injections, were there is a greater probability of an immune response would be elicited.

We observed an increase in astrocytic proliferation in our mice on the injected side. This increased astrocytic activity could be part of an immune response. However, because we do not observe an exaggerated astrocytic proliferation in the uninjected hemisphere, the mannitol injections by themselves are not the cause of the astrocytic response. It is most likely the combination of the mannitol and stereotaxic injection that cause the astrocytic response. Further studies into the possibility that mannitol contributes to an inflammatory/immunological response would be necessary if such osmotic CED is to be used in the future.

**Transgene Toxicity**

**GFP toxicity**

There have also been a number reports of GFP toxicity *in vitro* (352, 353) as well as *in vivo* (213, 354-358). Klein et al. in 2006 showed that after injections of rAAV8-GFP into the substatia nigra (SN) there was loss of tyrosine hydroxylase positive cells but no substantial cellular loss due to the presence of the GFP (213). Klein’s work is congruent with our studies in that we still see both the mRNA transcript of hGFP and hGFP antibody staining even though both DARPP-32 and NeuN protein staining are reduced.
Cellular dysfunction might be a better explanation than cellular death. While Klien’s study did not show NeuN loss, as was observed in our study, the loss of tyrosine hydroxylase positive cells in the SN suggests a similar process might be happening (213).

One study in 2009 and two recent study in 2010 showed that GFP may become toxic if it is placed behind a strong promoter. In 2009 Ulusoy et al. used rAAV5 at varying titers in the SN to test the dose optimization for various shRNAs, and found that at high titers ($10^{12}$ and $10^{13}$ vg/ml), their hGFP control showed cellular loss (359). Beltran et al. saw that hGFP was possibly the cause of cone cell loss after delivery of rAAV5-hGFP vectors to the retina of canines (355). When placed behind either the CBA promoter, or the a human G-protein-coupled receptor protein kinase 1 promoter (hGRK1) at various high titers ($10^{12}$ and $10^{13}$ vg/ml), hGFP showed retinal atrophy and detachment. The mouse opsin promoter (mOP) was also examined but did not show the same cellular atrophy at any of the titers examined. Another study by Sawada et al. injected P0 wistler rat pups with a lentiviral vector containing GFP driven by a strong murine stem cell virus promoter (MSCV) and showed that after three weeks, Purkinje cells had significant morphological and electrophysiological abnormalities (354). PBS injections and lentiviral vector injections containing a GFP driven by a weaker Purkinje specific promoter did not show any noticeable abnormalities. While the group showed significant neuronal abnormalities after the MSCV driven GFP injections, no microglial activation was observed (354).

These three studies support the evidence in our work here that rAAV5-hGFP injections to the striatum of mice may have some toxic effects. In both the Ulusoy and
the Beltran study, the rAAV5-hGFP (created by the UF-11 vector), as well as titers used (10^{12} to 10^{13} vg/ml) match what was used in our mice (rAAV5- hGFP at 1.69 \times 10^{13} vg/ml). The fact that the same vector construct and titer that we used in our study showed similar toxicity in another species and at another injection site gives the work performed here more credence. It is possible that GFP driven by a strong promoter and or high viral titers containing GFP may lead to cellular toxicity.

**Double transgene toxicity**

Krestel et al. in 2004 generated a mouse line that expressed both eGFP and β-galactosidase at the same protein levels and showed significant neuropathology and cellular death in neurons (356). Significant pathology was evident in these double transgenic mice which included aggregates, large glial proliferation, as well apoptotic markers (cleaved caspase-3). Mice also exhibited muscle weakness compared to nTG littermates and died within 1 month of birth. As a comparison, eGFP transgenic mice that express double the amount of eGFP compared to the eGFP/β-galactosidase mice do not show the same phenotype except for aggregations of eGFP. β-galactosidase transgenic mice also do not show such a dramatic phenotype as was noted by Krestel et al. (356, 357). The group suggested that the expression of two exogenous proteins expressed in together cause a synergetic effect leading to the toxicity. The recent development of the *Brainbow* mice helps to support the concept that fluorescent proteins themselves are not toxic. No reports so far have come out suggesting that the multiple fluorescently colored mice have any neuropathological abnormalities (360).

In our rAAV5-hGFP, construct there is a neomycin resistance gene downstream of the hGFP. The neomycin gene was originally placed in the UF-11 plasmid construct for cell selection in *in vitro* studies. If the neomycin gene is being expressed along with
hGFP (and the shRNAs for that matter) we maybe seeing a similar effect to what Krestel observed in their double transgenic mice. The glial proliferation is an interesting similarity between our rAAV5-hGFP injections and the Krestel double transgenic mice and might indicated a similar toxic process. Removing the neomycin resistance gene that is present in our UF-11 vector would be a good first step in trying to reduce toxicity.

**Concluding Remarks**

At the end of the study, the hypothesis regarding a phenotypical difference between the nTG and the knock-in mice proved to be correct. There is a behavioral and histopathological difference between the nTG and the knock-in mice. Our second hypothesis that RNAi could reduce the phenotypical differences by reducing the mutant huntingtin was not fully proven. Because of potential shRNA and or GFP toxicity we can not definitively state that RNAi can rescue the CAG 140 knock-in mice.

In conclusion, long term expression (14 to 26 weeks) of shRNAs and hGFP by rAAV delivery has shown signs of neuronal toxicity. RNAi and transgene expression levels, whether generated by the promoter or the efficiency of the viral delivery method, is an important factor to consider in the future. CED is a useful tool to maximize the area transduced by rAAV or other viral vectors, but if such enhancements in transduction lead to toxic protein and or RNAi overload in neurons, such methods might need to be re-evaluated. As the gene-therapy field generates newer and more efficient rAAV serotypes effects seen in this study may become more common.

GFP will probably not be a factor when designing a product for human use, but the use of GFP as a control has been a cornerstone of biology for years and any toxicity due to GFP may call into question past research. Similarly, if shRNAs themselves are
directly causing toxicity, results from past shRNA studies could be called into question and less toxic substitutes, such as miRNA, may need to replace them
REFERENCES


BIOGRAPHICAL SKETCH

Aaron Coates Rising was born in Lexington, Kentucky on April 23rd, 1982 to James and Leah Rising. He spent the first five years of his life in a small town in eastern Kentucky named Irvine. At the age of five, Aaron moved with his parents and his 3 year old brother, Andrew, to Saudi Arabia. Living overseas for 17 years in Saudi Arabia allowed for many exotic travel experiences and adventures.

Aaron came back to the United States for the last three years of high school and attended St. Geroge’s School in Newport, Rhode Island. After graduation Aaron went to Carnegie Mellon University in Pittsburgh, Pennsylvania where he studied biology. Working in two different labs, Aaron got experience in immunohistochemistry, microscopy and basic neuroscience skills. Receiving his B.S in biology and minors in business administration and chemistry he went on to graduate school.

In 2004 Aaron began his graduate career at the University of Florida. In 2005 Aaron joined Dr. Ron Mandel’s Lab where he worked on the projected presented here. In addition to working with Dr. Mandel, he has worked closely with Dr. Alfred Lewin in the Molecular Genetics and Microbiology Department at the University of Florida as well as Dr. Elieen Denovan-Wright in the Pharmacology Department at the University of Dalhousie in Halifax, Nova Scotia, Canada.