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Jennifer Webster Bradford Date

The role of glia in Huntington's disease pathology

By

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The role of glia in Huntington's disease pathology

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An abstract of
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Abstract

The role of glia in Huntington's disease pathology

By Jennifer Webster Bradford

Huntington's disease (HD) is the most common of nine inherited polyglutamine diseases, and is caused by an expansion of the polyglutamine tract (>36 repeats) in the N-terminus of the huntingtin (htt) protein. Although mutant htt is ubiquitously expressed throughout the body, little is known about how it impacts non-neuronal tissues. Previous work in our lab has demonstrated that glial cells are negatively affected by the presence of mutant htt, and their dysfunction could contribute to disease progression. Glial cells are essential components of the central nervous system, and play crucial roles in neuronal function and cell-cell communication, but little is known about how mutant htt in glia contribute to HD pathology. To this end, we generated two different HD mouse models that express N-terminal human mutant htt that is controlled by the glial fibrillary acidic protein promoter (GFAP), which drives gene expression in astrocytes. This dissertation describes the results from behavioral and molecular characterization of these novel mouse models. These studies found that expression of mutant htt in astrocytes was sufficient to produce a progressive, late onset HD phenotype that included body weight loss, decreased rotarod ability and early death, when compared with wild type littermates or control transgenic mice. Molecular analysis aimed at uncovering the possible mechanisms underlying these phenotypes revealed that the HD mice expressing mutant htt in astrocytes had glutamate transporter defects. It was found that the glutamate transporter defects were due to improper binding of mutant htt to the Sp1 transcription

factor, which resulted in decreased gene expression. These results challenge the classic cell autonomous view of neurodegeneration by implying an important role for glia in HD pathology. These findings also suggest that treatment of astrocytes might be a viable target in developing new HD therapies.

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Chapter 1

General Introduction

This chapter includes discussions previously published as Bradford JW, Li SH and Li XJ. 2010. Polyglutamine toxicity in non-neuronal cells. *Cell Research*. 20(4):400-407.

1. The polyglutamine disease family

There are at least nine inherited neurodegenerative diseases that are caused by an expansion of a polyglutamine (polyQ) tract, including Huntington's disease, spinocerebellar ataxia 1-3, 6, 7 and 17, spinobulbar muscular atrophy, and dentatorubropallidoluysian atrophy (Li and Li, 2006; Zoghbi and Orr, 2000). The polyQ disease mutations result from a glutamine (CAG) trinucleotide repeat expansion in unique genes that do not share homology. Despite the lack of homology in the disease proteins, these diseases do share many common pathological features. First, symptoms of these diseases usually appear at midlife and progressively worsen until death, some 15-20 years later. A juvenile or early onset form also exists for these polyQ diseases and is usually associated with more than 60 CAG repeats. Longer repeat sizes are associated with an earlier age of disease onset. Second, these diseases all show aggregates or inclusions formed by mutant proteins in the brain, which are a pathological hallmark of the polyQ diseases. It remains debated as to whether aggregates are toxic or have a protective role, but they do reflect the accumulation of mutant polyQ proteins in cells. Third, expression of these mutant proteins is generally widespread throughout the body, but selective degeneration is seen in specific brain regions in each polyQ disease. Although these diseases cause neuronal degeneration, evidence is growing that expression of mutant proteins in non-neuronal cells could also contribute to polyglutamine disease pathology (Martin et al., 2008; van der Burg et al., 2009).

1.1 Spinocerebellar ataxias

This group of polyQ neurodegenerative disorders includes six (SCA 1, 2, 3, 6, 7, and 17) identified diseases (Zoghbi and Orr, 2000), which compose about 50% of all

families affected by spinocerebellar ataxia (SCA) (Soong and Paulson, 2007). These diseases are all dominantly inherited and characterized by cerebellar degeneration, which results in ataxia, speech difficulties, dementia, and eventually death (Zoghbi and Orr, 2000). SCA1 is caused by a polyQ expansion of >43 repeats in the *ataxin-1* gene (Chung et al., 1993). SCA2 is caused by the *ataxin-2* gene containing >37 polyQ repeats (Imbert et al., 1996). SCA3, also called Machado-Joseph disease, is the most common form of the ataxias and occurs when the disease protein ataxin3 has >50 repeats (Warrick et al., 2005). The SCA6 gene encodes a voltage-dependent calcium channel (*CACNA1A*), which is a membrane channel protein that contains a polyQ domain that expands to cause SCA6 (Kordasiewicz et al., 2006; Soong and Paulson, 2007). SCA7 results from the expansion of the CAG/polyQ repeat (>37 units) in the *ataxin7* gene (Stevanin et al., 1998). One interesting point about SCA7 is that expression of ataxin-7 is high in peripheral tissues, such as heart, skeletal muscle and pancreas, and lower in the brain. Also, SCA7 is associated with cone-rod dystrophy retinal degeneration (La Spada et al., 2001). SCA17 occurs when there is an expansion of 42 or more repeats in the polyQ tract of the TATA-box binding protein (TBP), which plays an essential role in transcriptional regulation. In addition to the critical role that TBP plays in cellular function, SCA17 also shows selective neurodegeneration associated with cerebellar atrophy, in particular, Purkinje cell death (Friedman et al., 2007).

1.2 Spinobulbar muscular atrophy and dentatorubropallidoluysian atrophy

Two other polyQ diseases, spinobulbar muscular atrophy (SBMA) and dentatorubropallidoluysian atrophy (DRPLA), have also been well characterized. SBMA, also known as Kennedy disease, occurs when there is a polyQ expansion of 36 or more

repeats in exon1 of the androgen receptor (AR) gene (Sobue et al., 1989; Suzuki et al., 2008). SBMA is the only X-linked, recessively inherited disease in the polyQ neurodegenerative disease family, and primarily affects males (Tanaka et al., 1999). This disease is characterized by motor neuron loss in the spinal cord and brain stem, and leads to a slowly progressing disease with onset in middle age (Soukup et al., 2009). Symptoms of SBMA include muscular atrophy and weakness, particularly of the limbs and facial muscles, reduced male fertility, and death that often occurs due to pneumonia (Sperfeld et al., 2002; Suzuki et al., 2008). Interestingly, AR expression is lower in the central nervous system (CNS) than in cardiac and skeletal muscle, skin, and prostate (Tanaka et al., 1999).

DRPLA is a dominantly inherited neurodegenerative disease that occurs when the atrophin-1 protein has an expanded polyQ tract containing >48 repeats (Yamada et al., 2006; Yu et al., 2009). DRPLA transgenic mice exhibit symptoms including ataxia, chorea, and dementia (Schilling et al., 1999b). Although atrophin-1 is a transcriptional regulator and is expressed ubiquitously in different types of cells (Yu et al., 2009), DRPLA leads to degeneration in only select neuronal populations in the cerebellum (Schilling et al., 1999b).

1.3 Huntington's disease

Huntington's disease (HD) was first described by the New York physician George Huntington in 1872, and is the most common polyQ disorder with an incidence of around 1 in 10,000 people of Caucasian descent, but also occurs in other populations. Huntington's disease is inherited in an autosomal dominant manner and exhibits disease anticipation mainly through the paternal line. HD is caused by an expansion of a polyQ

tract, which is located in the N-terminal region of the HD protein, huntingtin (htt). The polyQ tract is naturally polymorphic in humans, and individuals having more than 36 CAG repeats will develop HD (The Huntington's Disease Collaborative Research Group, 1993; Kremer et al., 1994). Symptoms present in midlife and usually consist of body weight loss, psychiatric problems, cognitive deficits, and movement disorders (chorea), which are followed by death that occurs 10-20 years after onset. The juvenile form of HD occurs when the polyQ tract expands to 60 or more CAG repeats, and disease onset occurs before 20 years of age (Turmaine et al., 2000). It has been known for some time that the age of disease onset inversely correlates with repeat length, so that individuals with longer repeats will develop symptoms earlier than those with shorter repeats. Even though the gene encoding the disease protein was identified in 1993 (The Huntington's Disease Collaborative Research Group, 1993), and extensive work has been performed on HD, there is no cure and few effective treatments have been developed.

Htt is normally a cytoplasmic protein (~350 kD) and is essential for cell survival (Dragatsis et al., 2000). This is evident because htt knock-out mice are embryonic lethal by day 8.5, which indicates a critical role for htt early in development (Duyao et al., 1995; Nasir et al., 1995). While the primary function of htt has yet to be elucidated, it probably plays a role in cellular trafficking and also functions as a scaffold protein (Harjes and Wanker, 2003; Li and Li, 2004). Although normal and mutant htt is ubiquitously expressed, selective neurodegeneration occurs early (begins in grade 1) in the medium spiny neurons (MSNs) of the striatum (Vonsattel et al., 1985). Other brain regions such as the deep layers of the cortex, the hypothalamus, and hippocampus also undergo neurodegeneration, which becomes widespread in the late stages of HD

(Vonsattel et al., 1985). While the reason behind the selective degeneration remains unknown, many ideas have been proposed to help explain this phenomenon. One explanation is that the MSNs are more sensitive to mutant proteins because they have lower ubiquitin-proteasome system (UPS) activity as they age than other cells in the CNS (Tydlacka et al., 2008). Certain environmental influences in the striatum may also make this region susceptible to mutant htt toxicity. It is well known that MSNs are innervated by glutamatergic axons from the neocortex, which release large amounts of the excitatory neurotransmitter glutamate onto the dendrites of the MSNs. Being surrounded by high levels of glutamate could make these cells inherently more sensitive to damage by excitotoxicity, which is caused by constant activation of glutamate receptors, like the NMDA receptors that are located on MSN dendrites. Overstimulation of glutamate receptors results in high intracellular levels of calcium, which can cause neuronal dysfunction and eventually death (Sattler and Tymianski, 2001). Understanding why neurodegenerative diseases selectively target a particular region, like the striatum in HD, would be helpful in developing specific therapies that could prevent or delay damage to the targeted area.

Expanded mutant htt misfolds and produces an abnormal protein conformation which is subsequently cleaved to form pathogenic N-terminal htt fragments. Because of this unique property of mutant htt, extensive studies have been performed to identify the proteolytic cleavage sites. These studies have identified that various proteases, including calpains and caspases, can degrade mutant htt to generate N-terminal htt fragments that contain the polyQ repeat (Gafni and Ellerby, 2002; Kim et al., 2001; Lunkes et al., 2002; Qin and Gu, 2004; Wellington et al., 2002). The fragments have the ability to enter the

nucleus and form aggregates (inclusions), while the majority of full-length mutant htt remains in the cytoplasm (DiFiglia et al., 1995; DiFiglia et al., 1997; Sharp et al., 1995). Studies involving mouse models and in vitro work have clearly shown that smaller N-terminal mutant htt fragments are more toxic than longer fragments or the full length protein (Mangiarini et al., 1996; Saudou et al., 1998). As no nuclear localization signal has been found in htt, passive transport or htt complexing with a nuclear factor like CBP may be responsible for entry of small mutant fragments into the nucleus (Hackam et al., 1998; Xia et al., 2003).

It is evident that only N-terminal mutant htt is able to form aggregates, which can accumulate mainly in the nucleus and neuropil (axon and dendrites) (DiFiglia et al., 1995; Gutekunst et al., 1999; Sharp et al., 1995) (**Fig. 1.1**). At the beginning stages of disease, neuropil aggregates are widespread in areas not associated with early degeneration, like the cortex, and are infrequent in the striatum, which experiences early neuronal loss (Gutekunst et al., 1999). As HD progresses, nuclear aggregates become equally abundant as neuropil aggregates, although larger, and may represent htt accumulation in disease resistant cells. While aggregates are a hallmark of HD, they have never been directly linked to toxicity, and cells might form them as a protection mechanism against soluble mutant protein (Saudou et al., 1998).

The accumulation of soluble mutant htt fragments into aggregates may cause the inactivation of its toxic function, and may prevent it from aberrantly binding to nuclear proteins. The nuclear localization of soluble N-terminal mutant htt can lead to abnormal binding of mutant htt to various transcription factors, which subsequently affects transcriptional expression (Beal and Ferrante, 2004; Li and Li, 2004). Microarray studies

have shown that approximately 2% of genes in HD patients and mice have altered gene expression profiles. Most of the altered genes have decreased expression levels and include neurotransmitter receptors and genes involved in calcium and retinoid signaling pathways (Luthi-Carter et al., 2000). It was found that many of the genes with altered expression contained Sp1 transcription factor binding sites. A series of discoveries later found that expanded mutant htt interacts strongly with Sp1 (Dunah et al., 2002), and that soluble mutant htt binds more strongly to Sp1 than the insoluble (aggregated) form (Li et al., 2002). Overexpression of Sp1 can even rescue HD toxicity (Dunah et al., 2002). Because Sp1 is involved in the transcription of many genes, mutant htt could have a widespread impact on expression levels by altering Sp1 function. Sp1 is just one of the many transcription factors that is known to aberrantly interact with mutant htt and more studies on this topic might lead to a better understanding of the toxic role N-terminal fragments play in the nucleus.

1.4 Huntington's disease mouse models

Beginning in the mid 1990's, many different HD genetic mouse models have been created to aid in the study of HD. The first HD transgenic mouse model to be characterized was the R6/2 model, which ubiquitously expresses exon 1 htt with 115-150 CAG repeats, under the human htt promoter (Mangiarini et al., 1996). The well characterized R6/2 mouse displays neurological phenotypes beginning at 8 weeks, which include decreased motor skills, tremors, shuddering, stereotypic grooming, brain weight loss and a lifespan of only 3-4 months (Davies et al., 1997). Although this mouse model has a progressive neurological phenotype, including the presence of neuronal nuclear

inclusions prior to 4 weeks of age, no obvious neurodegeneration is seen until the very end of life (Mangiarini et al., 1996).

Another well characterized HD mouse model is the N171-82Q transgenic mouse that expresses truncated mutant htt containing the first 171 N-terminal amino acids and 82 glutamine repeats (Schilling et al., 1999a). The transgene is driven by the mouse prion promoter, which drives expression primarily in neurons (Schilling et al., 1999a). These mice exhibit progressive neurological phenotypes consisting of loss of coordination, tremors, ataxia, abnormal gait, and body weight loss within 90 days of age and live only around 4-5 months (Andreassen et al., 2001; Schilling et al., 1999a).

A mouse model expressing full length htt with an expanded repeat would theoretically be the most accurate representation of HD, and thus, the *Hdh* knock-in (KI) and yeast artificial chromosome (YAC) transgenic mice were generated. The *Hdh* KI mouse has 72-80Q knocked into the endogenous *Hdh* mouse allele (Shelbourne et al., 1999), and the YAC model contains a full length human htt allele with 72 CAG repeats (Hodgson et al., 1999). Although these full-length mutant htt expressing mouse models should be accurate models of HD, these mice fail to show robust HD phenotypes. Even a KI mouse model expressing 150 repeats fails to show most of the characteristic phenotypes associated with HD (Lin et al., 1999). The YAC transgenic HD mice display more obvious behavioral phenotypes, but can live as normally as WT mice (Hodgson et al., 1999). Despite the lack of overt neurological phenotypes, these full length models do produce mutant htt nuclear inclusions and reactive gliosis is present in their brains.

Although the models mentioned above have provided invaluable clues into HD pathogenesis, many of them fall short in recapitulating the well characterized

neurodegeneration seen in HD patients (Li and Li, 2004). This fact has led to the view that cell dysfunction rather than overt cell loss probably leads to the observed phenotypes in HD mouse models. Moreover, early studies have been primarily focused on neuronal expression of mutant htt despite the fact that the majority of cells in the brain are glia. Thus, models that allow researchers to study mutant htt specifically in glia and peripheral tissues will help establish the role of mutant htt outside of neurons. This approach may help us identify whether symptoms of HD such as weight loss, high number of deaths due to cardiac failure, and high incidence of diabetes are due to the direct influence of mutant htt in peripheral tissues or are secondary effects of neurodegeneration. As glia are so important to proper CNS function, models that specifically express mutant htt in glia are particularly helpful for one to understand glia contribution to the CNS pathology in HD. In addition, information gained in these types of studies will help advance therapeutic approaches toward HD pathology.

2. Glial dysfunction in polyglutamine diseases

Glial cells, once only considered as supporting cells, are now recognized as vital components of the CNS and carry on many functions that support neuronal function and survival. Glial cells compose 90% of cells in the brain and consist of three major types: astrocytes, microglia, and oligodendrocytes (Kim and de Vellis, 2005; Lobsiger and Cleveland, 2007; Shin et al., 2005); and each has a unique role in the CNS. Signaling occurs between neurons and glia and involves neurotransmitters, ion fluxes and other signaling molecules. Although glia lack the membrane potential to fire action potentials like neurons, they nevertheless have ion channels and membrane transporters that allow

them to sense surrounding neuronal activity (Fields and Stevens-Graham, 2002). In particular, neurotransmitters and other extracellular signaling molecules that are released from neurons need to be taken up by astrocytes in order to maintain normal neuronal excitability and synaptic transmission.

2.1 Types of glia in the central nervous system

Astrocytes are the most abundant of the glial cells, and are vital to maintaining synaptic function and plasticity, and they accomplish this task through catabolism and synthesis of specific amino acids, like glutamate (Maragakis and Rothstein, 2006). For example, astrocytes have a very important role in preventing neuronal excitotoxicity by clearing the synapse of extracellular glutamate. Microglia function as immune cells in the CNS, and normally exist in a quiescent state until they are exposed to an insult or injury (Tai et al., 2007). Following injury, these cells become activated and will transform into phagocytes, which are able to remove dying cells and will release proinflammatory cytokines, proteases and oxygen radicals to combat the insult (Kim and de Vellis, 2005; Sapp et al., 2001). Oligodendrocytes are the third major type of glia in the CNS and are responsible for myelinating neuronal axons. Myelination of axons is critical in promoting neuronal survival and also in maintaining rapid conduction of action potentials (Howng et al., 2010). Astrocytes are the major type of glia responsible for maintaining normal neuronal function and survival and are important in preventing excitotoxicity. These cells have also been implicated in other neurodegenerative diseases like amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD), and Alzheimer's disease (AD). Based on this knowledge, we have chosen to study the role of astrocytes in HD pathology, which has not been extensively explored before.

2.2 Astrocyte glutamate transporters

Glutamate is the most important excitatory neurotransmitter in the CNS and is involved in synaptic plasticity, learning, and development (Su et al., 2003). Extracellular glutamate levels are controlled by sodium dependent transport systems that are primarily located on astrocyte membranes. The two main glutamate transporters located in astrocytes are glutamate transporter-1 (GLT-1) and the glutamate/aspartate transporter (GLAST), whose human homologs are EAAT2 and EAAT1, respectively (Li et al., 2006; Yang et al., 2009) (**Fig 1.2**). Astrocytic GLT-1 is responsible for the majority of glutamate uptake in the mature brain, as mice depleted of this transporter display only ~5% of normal transport (Tanaka et al., 1997). Astrocytes take up extracellular glutamate and metabolize it via the glutamine synthetase pathway to convert it to glutamine (Maragakis and Rothstein, 2001). The glutamine is then transported back to presynaptic terminals and converted into the neurotransmitter glutamate (Maragakis and Rothstein, 2006). Excess glutamate, due to glutamate transporter dysfunction, has been shown to result in neurotoxic effects and may contribute to CNS injury seen in diseases like ALS, AD, stroke and HD (Tanaka et al., 1997). In fact, mice that are GLT-1 deficient show excitotoxic phenotypes like lethal spontaneous seizures, hyperexcitability, and increased susceptibility to acute cortical injury and early death, when compared to WT littermates (Tanaka et al., 1997). Medium spiny neurons, which are specifically targeted in HD, seem to be particularly vulnerable to excitotoxicity because they are innervated by glutamatergic axons from the neocortex, and their viability is largely dependent on removal of excess extracellular glutamate by astrocytes. It is widely known that GLT-1 expression is decreased in HD mouse models (Behrens et al., 2002; Lievens et al., 2001)

and in human patients (Arzberger et al., 1997; Hassel et al., 2008). Decreased glutamate uptake could lead to excess glutamate in the extracellular space, and eventually neuronal excitotoxicity.

2.3 Reactive gliosis

Central nervous system injury or disease can lead to reactive gliosis, a process that produces reactive astrocytes and microglia (Buffo et al., 2008). Reactive astrocytes are characterized by up-regulation of glial fibrillary acidic protein (GFAP), cell proliferation, morphology changes (Ridet et al., 1997), and an increase in cytokine and inflammatory responses (Streit et al., 2004). GFAP is an intermediate filament protein that is expressed in astrocytes and is commonly used as a mature astrocytic marker (Brenner and Messing, 1996). Reactive astrocytes have been implicated in many neurodegenerative diseases like AD, ALS and HD (Simpson et al., 2008; Tanaka et al., 1997; Yamanaka et al., 2008), and are often present prior to cell death. Microglial activation also occurs during CNS insult or injury, and often precedes neuronal degeneration in many neurodegenerative diseases such as AD and PD (McGeer et al., 1988; von Bernhardi, 2007). HD patient brain, and in particular, the striatum and cortex, have been shown to contain activated microglia, with activation increasing as the severity of disease increases (Sapp et al., 2001).

Glial responses to injury can be harmful to neuronal tissue and probably promote pathogenic phenotypes seen in many CNS diseases like HD, AD and PD (Griffin et al., 1989; Streit et al., 2004). In fact, reactive astrocytes occur in the striatum and increase in number during HD progression (Sapp et al., 2001; Vonsattel et al., 1985; Yu et al., 2003). Reactive gliosis is also an early event seen in many HD mouse models (Lin et al., 2001;

Reddy et al., 1998; Yu et al., 2003), but it is not obvious in mouse models where mutant htt expression is restricted to cortical neurons (Gu et al., 2005). Along those lines, astrocytosis, which is observed by an increase in GFAP, even occurs in HD mouse models that do not express mutant htt in neurons (Bradford et al., 2009), indicating that mutant htt expression in astrocytes alone is sufficient to cause this glial response.

These findings, along with the knowledge that mutant htt is expressed in glia, suggests that glial-neuron interactions may play a very important role in HD pathogenesis (Shin et al., 2005). The link between glial dysfunction due to expression of mutant htt and neuronal excitotoxicity is an attractive hypothesis for explaining HD progression, but further studies are needed to confirm and identify the mechanism behind this interaction.

2.4 Glia and Huntington's disease

Evidence that glial cells express mutant htt and develop htt aggregates comes from several different groups. Examination of HD human patient brains reveals the presence of mutant htt in glial cells (Shin et al., 2005; Singhrao et al., 1998), and patients have also been found to have microstructural changes in the white matter of the corpus callosum (Rosas et al., 2009). In the R6/2 HD mouse model, exon1 htt with 115-150 CAG repeats is expressed in both neurons and glia (Chou et al., 2008; Shin et al., 2005). The *Hdh* KI mouse model with 150 CAG repeats (Lin et al., 2001) also expresses mutant htt in glia, with the frequency of aggregates increasing with age (Bradford et al., 2010). However, fewer glia than neurons contain aggregates in mouse models and human patients, and glial aggregates are usually smaller than neuronal ones (Shin et al., 2005; Wang et al., 2008). There are several explanations that would account for fewer glia containing smaller htt aggregates than neurons. One possibility is that glial cells could

intrinsically express htt at a lower level than neuronal cells, though this possibility remains to be verified. Another possibility is that, unlike neurons, glial cells are capable of dividing, which could deplete the amount of mutant protein in the cell. Our recent studies have found that UPS activity is lower in neurons than in glia and that UPS activity levels decrease with age (Tydlacka et al., 2008). The UPS is responsible for clearing misfolded protein, and its lower activity in neuronal cells could result in increased accumulation of N-terminal mutant protein, and subsequently, htt aggregation in neurons. The age-related decline in UPS activity in neurons and glial cells could explain the age-dependent formation of polyQ aggregates and disease progression in HD. The more abundant accumulation of mutant polyQ proteins in neurons than in glial cells could also in part explain for the selective neuronal loss common to these polyQ diseases.

The presence of toxic proteins in glial cells could affect their function, and because astrocytes play important roles in maintaining synaptic plasticity and neuronal communications (Lobsiger and Cleveland, 2007; Maragakis and Rothstein, 2006), astrocyte dysfunction could be deleterious to surrounding neurons. As mentioned before, one of the most important roles of astrocytes is to remove excess synaptic excitatory neurotransmitter glutamate (Maragakis and Rothstein, 2006), which protects surrounding neurons from death by excitotoxicity (Lobsiger and Cleveland, 2007; Maragakis and Rothstein, 2006). Mutant htt is known to decrease expression of the primary glutamate transporter (GLT-1) in both mouse models and HD patient brains (Arzberger et al., 1997; Behrens et al., 2002; Lievens et al., 2001). Specifically, the R6/2 mouse shows a significant decrease in GLT-1 transcript and protein levels in the striatum and cortex at 12 weeks of age, but not at 4 weeks, while a decrease in GLAST levels have not been

indicated in HD (Behrens et al., 2002; Lievens et al., 2001). Through microdialysis in freely moving R6/2 mice, it was found that the R6/2 mice had a progressive reduction in GLT-1 function (Behrens et al., 2002). Importantly, the striatum region of HD patients has decreased GLT-1 mRNA levels and decreased glutamate uptake (Arzberger et al., 1997; Hassel et al., 2008).

A *Drosophila* model of HD was also created to examine the potential roles of glial dysfunction in HD pathology (Lievens et al., 2005). In this study, the UAS-GAL4 system was used to express exon1 of human htt with 20 or 93 CAG repeats in *dEAAT1* expressing glia. dEAAT1 is the sole glutamate transporter in the fly and is expressed in a subset of glia. Although the transgenic flies died, degeneration was not seen in the glial cells. This implies that glial dysfunction due to mutant htt expression but not glial degeneration probably contributed to the phenotypes seen. Since MSNs are innervated by glutamatergic axons from the neocortex (Calabresi et al., 1998), a decrease in astrocyte glutamate uptake might make the MSNs more susceptible than other types of neurons to excitotoxicity, which at least, can result in neuronal dysfunction.

The involvement of glia has also been implicated in SCA7 disease pathology. A mouse model that expresses mutant ataxin-7 in Bergmann glia was developed to study this role (Custer et al., 2006). Bergmann glia are a type of astrocyte that are located in the cerebellum and are closely associated with Purkinje neurons, the cells that are selectively targeted in SCA7. Like other astrocytes, they are responsible for removing extracellular glutamate from the synapse. This mouse model revealed that overexpression of ataxin-7 in Bergmann glia alone was sufficient to cause Purkinje cell pathology and to impair motor coordination, as measured by the rotarod test (Custer et al., 2006). These findings

from the SCA7 model also imply an important role for glial cells in polyQ disease progression.

Recently, drug studies that target glia have been shown to improve or delay HD symptoms, which indicate that treatment of non-neuronal cells might be a more useful method than treating degenerating neurons. Ceftriaxone and other β-lactam antibiotics that increase GLT-1 levels have been used to improve glial function in HD mouse models. Ceftriaxone, in particular, has been found to effectively increase glial GLT-1 transcription levels and also delayed onset of phenotypes and extended the life span of the R6/2 HD mouse model (Miller et al., 2008), and also of an ALS mouse model (Rothstein et al., 2005). The mechanism by which ceftriaxone increases GLT-1 expression is through activation of the promoter of this gene, but the pathway has not yet been identified (Rothstein et al., 2005). Drugs aimed at targeting peripheral effects of polyQ diseases may be easier to administer or may have a more pronounced effect than current drugs aimed at treating neurons.

Given the importance of glia in the CNS, and their involvement in other neurodegenerative diseases, it is important to know if glial function contributes to HD pathology. To this end, we have generated novel mouse models that express human mutant htt in astrocytes. Characterization of behavioral phenotypes and the molecular mechanisms contributing to the observed phenotypes will allow us to better understand the impact mutant htt has in astrocytes and how glial htt contributes to HD.

Figure 1.1

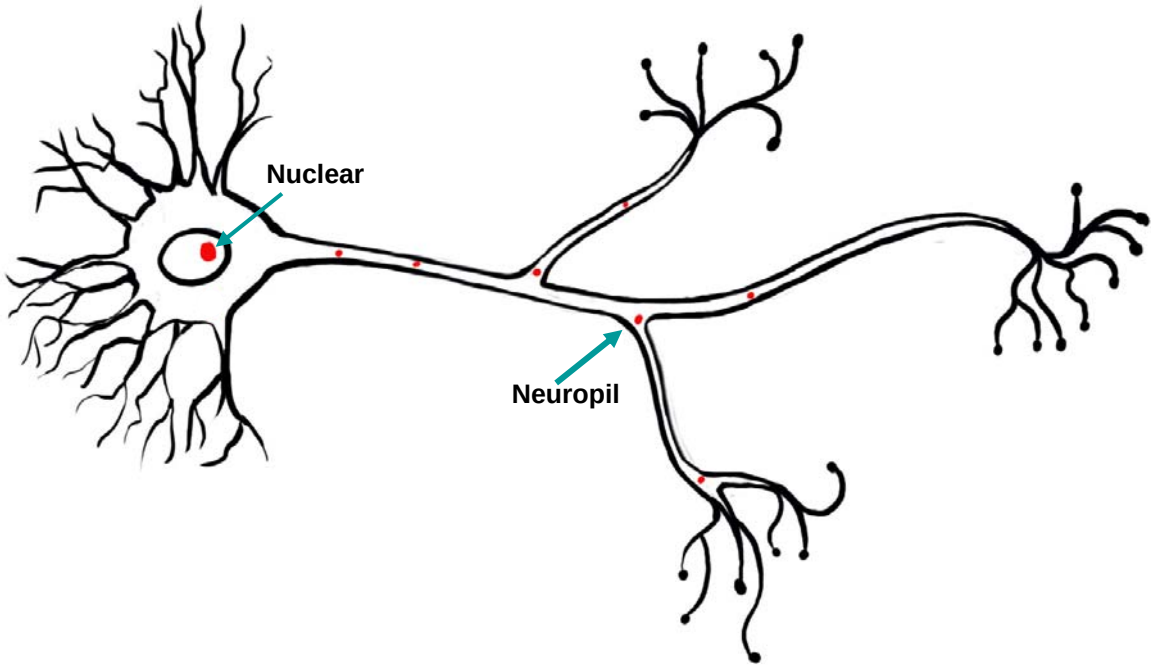


Figure 1.1

Aggregates can form throughout the neuron. Cartoon depicts a neuron with nuclear and neuropil (axon and dendrites) aggregates. Huntingtin is normally a cytoplasmic protein, but the N-terminal fragments produced from the expanded mutant protein can migrate to the nucleus and neuropil and form aggregates.

Figure 1.2

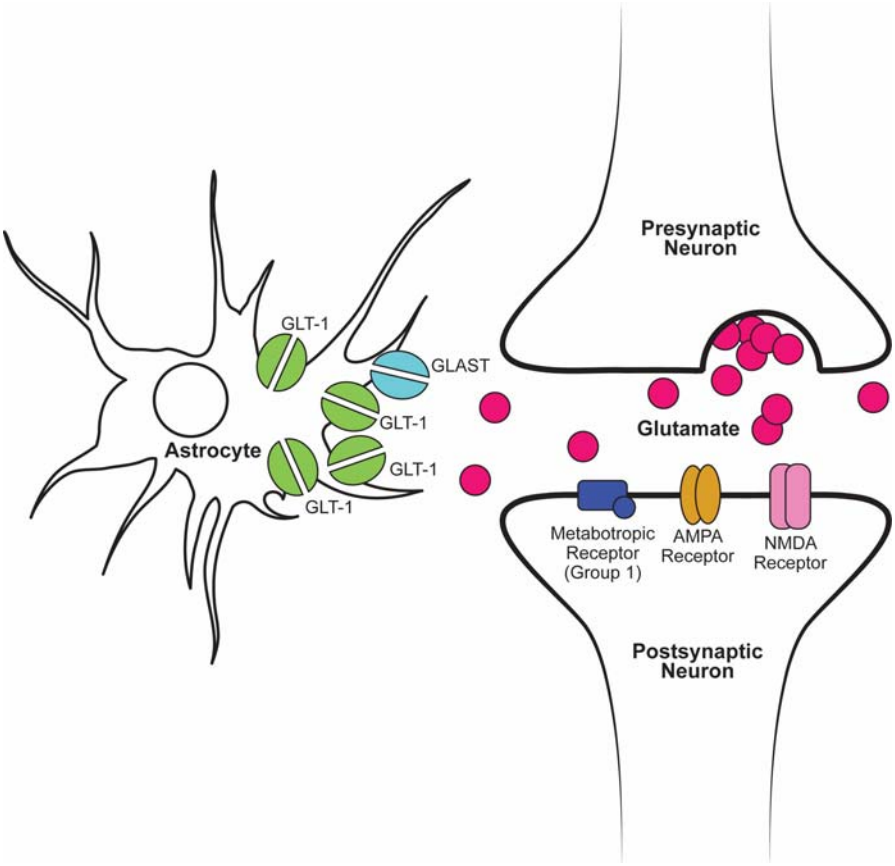

Presynaptic Neuron
GLT-1
GLAST
GLT-1
GLT-1
GLT-1
Astrocyte
Glutamate
Metabotropic Receptor (Group 1)
AMPA Receptor
NMDA Receptor
Postsynaptic Neuron

Figure 1.2

Astrocytes remove excess synaptic glutamate via glutamate transporters. Cartoon shows a presynaptic neuron releasing excitatory neurotransmitter glutamate into the extracellular space. The glutamate will then stimulate glutamate receptors on the postsynaptic neuron. Excess glutamate will be taken up through glutamate transporters which are primarily located on astrocytes (GLT-1 or GLAST).

Chapter 2

Characterization of a novel HD mouse model that expresses mutant huntingtin in astrocytes

This chapter includes findings and discussions previously published as Bradford, J., Shin, J.Y., Roberts, M., Wang, C.E., Li, X.J., and Li, S. 2009. Expression of mutant huntingtin in mouse brain astrocytes causes age-dependent neurological symptoms. *Proc Natl Acad Sci U S A* 106(52):22480-22485, and Bradford J, Shin JY, Roberts M, Wang CE, Sheng G, Li S, Li XJ. 2010. Mutant huntingtin in glial cells exacerbates neurological symptoms of Huntington disease mice. *J Biol Chem*. 285(14):10653-10661.

Design and construction of the GFAP-23Q/98Q/160Q transgenic mouse models was performed by Ji-Yeon Shin, Shihua Li and Xiao-Jiang Li. Guoqing Sheng performed experiments characterizing the 98Q/N171-82Q double transgenic mouse. Chaun-En Wang assisted with the RT-PCR comparing transgenes of different HD mouse models. All other experiments were performed by Jennifer Webster Bradford. Xiao-Jiang Li helped in writing and editing the manuscripts.

2.1 Abstract

Huntington's disease (HD) is an inherited neurological disorder caused by a polyglutamine expansion in huntingtin (htt) and is characterized by selective neurodegeneration that preferentially occurs in striatal medium spiny neurons (MSNs). Since MSNs are innervated abundantly by glutamatergic axons from cortical neurons, the preferential degeneration in the striatal neurons supports the glutamate excitotoxicity theory for HD pathogenesis. Thus, glutamate uptake by glia may be particularly important for preventing glutamate excitotoxicity in HD. Although mutant htt is expressed ubiquitously in various types of cells, it accumulates and forms aggregates in fewer glial cells than in neuronal cells. It remains largely unknown whether and how mutant htt in glia can contribute to the neurological symptoms of HD. We generated transgenic mice that express N-terminal mutant htt in astrocytes, a major type of glial cell that remove extracellular glutamate in the brain. Although transgenic mutant htt in astrocytes is expressed below the endogenous level, it can cause age-dependent neurological phenotypes in transgenic mice. Mice expressing mutant htt show body weight loss, have motor function deficits, and die earlier than wild type or control transgenic mice. Mice expressing mutant htt in astrocytes were mated with N171-82Q mice that express mutant htt primarily in neuronal cells. Double transgenic mice expressing mutant htt in both neuronal and glial cells display more severe neurological symptoms and earlier death than N171-82Q mice. These findings indicate a role for glial mutant htt in exacerbating HD neuropathology, and underscore the importance of improving glial function in treating HD.

2.2 Introduction

In HD, selective neuronal loss occurs preferentially in the MSNs of the striatum and then extends to other brain regions as the disease progresses (Vonsattel and DiFiglia, 1998). Because of the selective neuronal loss seen in HD, most studies to date have been focused on neurons. However, the majority of cells in the brain are glia that support the survival of neuronal cells. Astrocytes are the major type of glia and express glutamate transporters that uptake extracellular glutamate to prevent glutamate neurotoxicity (Lobsiger and Cleveland, 2007; Maragakis and Rothstein, 2001; Nagai et al., 2007). Although mutant htt is expressed in glial cells in the brains of HD mice and patients (Hebb et al., 1999; Shin et al., 2005), whether and how mutant htt in glia contributes to neuropathology in vivo remains unknown. Since glial cells can be therapeutic targets, establishing a transgenic mouse model expressing mutant htt specifically in glia can help develop treatment for HD.

Current HD mouse models have limitations for studying glial htt contribution because transgenic htt in these HD mice is either overexpressed in neurons or widely expressed in neuronal and non-neuronal cells. While overexpression of polyQ proteins in the astrocytes of flies (Lievens et al., 2005) or in mice (Custer et al., 2006) can induce neuropathology, it remains to be investigated whether mutant htt in glia at the endogenous level can induce neurological phenotypes. This is particularly important for validating the pathogenic role of mutant htt in glial cells, as less mutant htt accumulates and forms aggregates in glial cells than in neurons in HD mouse brains (Shin et al., 2005; Wang et al., 2008). Another important issue is how mutant htt affects glial function if glial mutant htt does contribute to HD neuropathology. Although our early study has

shown that mutant htt can affect glutamate uptake in cultured glial cells (Shin et al., 2005), the mechanism underlying this defect remains to be understood.

To examine the in vivo contribution of glial mutant htt to HD neuropathology and to investigate the mechanism by which mutant htt affects glial function, we generated transgenic mice that express human mutant N-terminal htt with 160Q in astrocytes (GFAP-160Q). Although transgenic mutant htt is expressed at a lower level than endogenous normal htt, it can induce age-dependent neurological phenotypes that include body weight loss, decreased motor performance, and early death when compared with 23Q mice and WT littermates. To see if the addition of mutant htt expressed in astrocytes would exacerbate phenotypes of a neuronal mouse model, we crossed our GFAP-HD mice with the N171-82Q HD model that expresses truncated mutant htt in neurons under the control of the prion promoter. These double transgenic mice had accelerated phenotypes when compared to the N171-82Q parental line, indicating that the additional expression of mutant htt in astrocytes can worsen HD neurological phenotypes.

2.3 Materials and Methods

Antibodies: Rabbit polyclonal antibody (EM48) and mouse monoclonal antibodies (mEM48) against the N-terminal region (amino acids 1-256) of human htt were described in our previous study (Li et al., 2000; Shin et al., 2005; Yu et al., 2003). Rabbit antibodies against GLT-1 (cGLT T88) were provided by Dr. Jeffrey Rothstein and purchased from Millipore Inc. Other antibodies used included mouse monoclonal antibodies against polyglutamine (1C2) or GFAP (Millipore Inc), rabbit anti-GFAP (Gene Tex, Inc), mouse anti-gamma tubulin (Sigma), -GLAST (Chemicon), and goat anti-mouse or -rabbit antibodies conjugated with Alexa Fluor 488 (Molecular Probes).

HD Mice: N171-82Q mice and *Hdh* CAG(150) knock-in mice (KI), which express a 150Q repeat, were bred and maintained in the animal facility at Emory University under specific pathogen-free conditions in accordance with institutional guidelines of The Animal Care and Use Committee at Emory University. To generate GFAP-HD mice, cDNA encoding N-terminal human htt (208 amino acids) containing 23Q, 98Q, or 160Q was subcloned into the eukaryotic expression vector pGfa2 at the *Bam*HI restriction site. This vector uses the 2.2-kb fragment of astrocyte-specific human glial fibrillary acidic protein (GFAP) promoter (Brenner et al., 1994; Brenner and Messing, 1996). Microinjection of GFAP-HD vectors into the pronucleus of fertilized oocytes from FVB mice was conducted by the Emory University transgenic mouse core facility. Genomic DNA was isolated from mouse tails, and a PCR genotyping method was employed for screening transgenic mice. Primers with sequences flanking the polyglutamine repeat were used for PCR. Sequences of the primers are: the forward primer (5'-ATGAAGGCCTTCGAGTCCCTCAAGTCCTTC-3') and reverse primer (5'-AAACTCACGGTCGGTGCAGCGGCTCCTCAG-3') were used for PCR. All the positive founders and their corresponding lines carry the expected length (23Q, 98Q or 160Q) of the polyQ repeat in transgenic htt.

Western Blot Analysis: For western blots, cultured cells or brain tissues were homogenized in RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA pH 8.0, 1mM EGTA pH 8.0, 0.1% SDS, 0.5% DOC and 1% Triton X-100) with 1X protease inhibitor from Sigma (P8340). The cell or tissue lysates were diluted in 1X SDS sample buffer (62.6mM Tris-HCl pH6.8, 2% SDS, 10% glycerol and 0.01% bromophenol blue) and sonicated for 10 s after incubation at 100°C for 5 min. The total lysates were resolved

in a 4-12% Tris-glycine gel (Invitrogen) and blotted to a nitrocellulose membrane. The western blots were developed using the ECL-Plus Kit (GE Health Care/Amersham).

Immunohistochemistry: For immunohistochemistry, brains of transgenic and littermate WT control mice were rapidly isolated and cut to sections (8-10, or 40 μm) with a cryostat (Leica) at –18°C. Mouse brain sections were examined with immunofluorescence labeling as described (Li et al., 2000). Light micrographs were taken using a Zeiss microscope (Axiovert 200 MOT) and a 63X lens (LD-Achroplan 63X/0.75) with a digital camera (Hamamatsu Orca-100) and Openlab software. Electron microscopy was performed as described previously (Friedman et al., 2007; Li et al., 2000).

Behavioral Analysis: Mouse body weight, survival, and growth were measured. The motor function of mice was assessed using the accelerating rotarod test (AccuScan Instruments, Inc.), as described previously (Friedman et al., 2007). In the accelerating rotarod test, each mouse was placed on a rotating cylinder that gradually accelerated to 40 RPM over a 5-min period. Latency to fall from the rotarod was recorded in 3 trials per day over a 3-day period. At least 5 minutes of recovery time was allowed between trials (Friedman et al., 2007).

Glial Cultures: Enriched astrocyte cultures were prepared from 1-2 day postnatal mouse pups (Shin et al., 2005). The cultures were enriched for astrocytes by shaking the 15-day culture plates for 15 minutes to dissociate any oligodendrocytes. Immunostaining with antibodies to specific cellular markers (GFAP for astrocytes, F4/80 for microglia, and myelin basic protein for oligodendrocytes) was used to identify different types of glial cells.

RT-PCR: Total RNA from mouse cortex was isolated using the RNeasy Lipid Tissue Mini Kit (QIAGEN #74804). First strand cDNA was obtained using the Invitrogen SuperScript™ First Strand Synthesis System for RT-PCR (11904-018). RT-PCR of mouse brain htt and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers has been previously described by our lab (Wang et al., 2008).

Four- to six-week-old cultured astrocytes were treated overnight with 0.25 mM dBcAMP in serum-free media to increase glutamate transporter expression for RT-PCR. Total astrocyte RNA was collected using the Qiagen RNeasy Mini Kit (74104), and cDNA was produced using the same method as the brain lysate. Primers for GLT-1, GLAST (Shin et al., 2005), GAPDH, and htt (Wang et al., 2008) have been previously described in our early studies.

Statistical Analysis: All values were expressed as means ± SE. Statistical significance was assessed by the use of Student's t test or ANOVA with Newman-Keuls Multiple Comparison Test. A probability level of $p < 0.05$ was considered to be statistically significant for all statistical tests.

2.4 Results

Our lab had previously found that mutant htt is expressed in glial cells in HD mouse brains and that fewer glial cells than neurons display nuclear htt aggregates (Shin et al., 2005; Tydlacka et al., 2008). We chose to study the role of mutant htt in astrocytes because they are critical in preventing neuronal death via excitotoxicity, and mutant htt is known to decrease expression of astrocyte glutamate transporters. To generate a mouse model that selectively expresses mutant htt in astrocytes, we constructed a vector that

expresses human htt under the control of the human glial fibrillary acidic protein (GFAP) promoter (**Fig. 2.1 A**), an astrocytic promoter that has been widely used to express a variety of genes in astrocytes (Brenner et al., 1994; Brenner and Messing, 1996). We verified that this promoter can specifically drive the expression of transfected htt in cultured astrocytes, but not in non-glial HEK293 cells (Bradford et al., 2009). Furthermore, transfected mutant htt formed small aggregates in a cultured glioma cell line (Bradford et al., 2009), which confirms the expression and accumulation of mutant htt in glial cells.

We then began pronuclear injection of GFAP-htt vectors to generate transgenic mice. Based on the findings that N-terminal htt fragments are more toxic than full-length mutant htt (Hackam et al., 1998; Wang et al., 2008), we used cDNAs encoding the first 208 amino acids with 23, 98 or 160 glutamines (Q) in the polyQ domain. We were able to generate three founders for GFAP-23Q, 2 founders for GFAP-98Q, and five founders for GFAP-160Q mice. Using western blotting or immunocytochemistry with the 1C2 antibody, we identified three GFAP-160Q mouse lines (line-1, -11, and -31) that showed detectable mutant htt in their brains. Western blotting revealed that htt-160Q is expressed in various brain regions including the cerebellum, brain stem, striatum, and cortex (**Fig. 2.1 B**). Transfected HEK293 cells were used as a control. To verify the expression of transgenic htt in astrocytes, we cultured primary astrocytes from GFAP-160Q mouse brains. Western blots with 1C2 clearly detected the expression of transgenic mutant htt in GFAP-160Q pups, but no band was seen in WT cultured astrocytes (**Fig. 2.1 C**). Similarly, our control GFAP-23Q model also expressed mutant htt protein in various brain regions (**Fig. 2.2**).

Since transgenic htt protein levels were low in the GFAP-160Q mice, we next analyzed transgene expression levels of our GFAP-HD mice and compared them to other commonly used HD mouse models. Using primers that specifically amplify human htt (i.e., will amplify transgenic mutant htt only), we found that expression levels of htt-160Q in brain lysate are lower than htt-23Q and N171-82Q mRNA levels, as well as lower than in HD KI mouse brains, which express mutant htt at the endogenous level (**Fig. 2.3 A**). To compare the expression levels of transgenic htt and endogenous mouse htt in the same astrocytes, we isolated astrocytes from WT and GFAP-160Q transgenic mice for RT-PCR. The cultured astrocytes allowed us to perform RT-PCR with primers that amplify the repeat region, so that the transgenic human htt and endogenous mouse htt could be distinguished in the same cells, under the same PCR conditions. The results also showed that the levels of transgenic human htt are lower than endogenous mouse htt in cultured astrocytes (**Fig. 2.3 B**). We also used different primers that amplify mouse and human htt transcripts together, which will produce one PCR product for both mouse and human htt. We observed that the level of total htt in GFAP-160Q astrocytes is slightly higher than in WT astrocytes, also suggesting that the additional expression of transgenic mutant htt is at a low level (**Fig. 2.3 C**). Furthermore, we performed real-time PCR to analyze the expression of transgenic htt transcripts in cultured astrocytes from WT, GFAP-23Q, GFAP-98Q and GFAP-160Q mice. This real time PCR experiment employed two different sets of primers that amplify either endogenous mouse htt or transgenic human htt. The relative levels of htt were then quantified and normalized to the level of endogenous GAPDH. The results showed that all transgenic htt transcripts are expressed at a lower level than the endogenous mouse htt (**Fig. 2.3 D**).

Immunostaining of brain sections of the GFAP-160Q transgenic mice shows an obviously low density of mutant htt-positive cells (**Fig. 2.4 A**). Htt-160Q is expressed in glial cells in various brain regions, including the brain stem and striatum, but accumulates and forms more nuclear aggregates in glial cells in the brain stem and spinal cord. This is consistent with the finding that the GFAP promoter drives the expression of transgenes in astrocytes in the spinal cord of adult mice (Yamanaka et al., 2008). The low density of mutant htt labeling, which was not seen in WT mouse brain under the same staining condition, reflects the restricted expression of mutant htt in glial cells. At higher magnification, htt aggregates can also be seen in cells that have astrocyte morphology (**Fig. 2.4 B**). We were also able to detect mutant htt aggregates in the white matter of the corpus callosum and the brain stem of GFAP-98Q mice at 19 months of age, but not in WT littermates (**Figure 2.5**).

The immunostaining data also suggests low expression of transgenic mutant htt in the GFAP-160Q model. To further confirm this, we compared htt staining in the same brain cortex regions from GFAP-160Q mice and N171-82Q mice that express N-terminal mutant htt under the control of the neuronal prion promoter (Schilling et al., 1999a). It is clear that htt-160Q is expressed in few glial cells in the GFAP-160Q mouse brain whereas N171-82Q htt is much more abundant in neuronal cells (**Fig. 2.6**). The low density of mutant htt staining in GFAP-HD mice also suggests that the expression of transgenic htt is restricted to glial cells.

Double immunostaining clearly demonstrates that GFAP-positive astrocytes express htt-160Q in large astrocytes (**Fig. 2.7**). Such double labeling was not seen in the glial cells that had a similar morphology in WT mouse brain, and NeuN-positive neurons

do not display mutant htt staining. Although the GFAP promoter can also drive transgene expression in a subset of neurons during embryogenesis (Casper and McCarthy, 2006), we did not detect the expression of mutant htt in neurons or microglial cells in our GFAP-HD mice, which is consistent with the fact that the GFAP promoter primarily expresses transgenes in astrocytes (Brenner et al., 1994). By electron microscopy of a 26 month old GFAP-160Q mouse, we were able to detect the presence of mutant htt in astrocytes but not in neurons or other glia (**Fig. 2.8**). Astrocytes can be distinguished from neurons because they have a condensed, irregular shaped nuclear membrane, a small cell body, and they are overall much smaller than neurons. EM data confirmed that transgene expression was seen only in astrocytes, but did not reveal any obvious evidence of cellular degeneration (**Fig. 2.8**).

Because we found no obvious degeneration of neurons and glia in GFAP-HD mouse brain, we believe that the expression of mutant htt in glial cells is more likely to cause cellular dysfunction than degeneration. This possibility is also consistent with the fact that neuronal dysfunction rather than overt neuronal loss mediates severe neurological phenotypes and early death in some HD mouse models (Davies et al., 1997; Schilling et al., 1999a), and that neuronal dysfunction can be induced by defective astrocytes even in the absence of neurodegeneration (Yamanaka et al., 2008).

GFAP-160Q mice developed late-onset neurological symptoms that are common with phenotypes of other well characterized HD mouse models. Three lines of GFAP-160Q mice all showed similar neurological phenotypes, which include body weight loss, clasping, and hunchback appearance (**Fig. 2.9**). These phenotypes also occurred in the transgenic offspring of GFAP-160Q mice that had been crossed with WT mice of the

B6C3 genetic background, indicating the phenotypes are not the result of an FVB background effect (**Fig. 2.9 B**). Clasping is an indication of neurological defects in mice, and was seen in our GFAP-160Q mice (**Fig 2.9 C**). Body weight, rotarod ability and survival were measured in the GFAP-160Q mice (**Fig. 2.10**). Body weight began to decrease in all three lines beginning around 16-17 months of age and progressively decreased as the mice aged (**Fig 2.10 A**). The accelerating rotarod test was used to measure body strength and coordination of the GFAP-160Q mice. Results showed that the GFAP-160Q mice had a decreased latency to fall beginning at around 12 months, and became significant at 13 months of age (**Fig 2.10 B**). It is known that the rotarod test can more sensitively measure neurological disease than even body weight measurement, so the GFAP-160Q mice appear to have phenotype onset at around 1 year of age. Once GFAP-160Q mice show obvious body weight loss, their behavioral phenotypes progressively worsen, and they often die 1-2 weeks after the onset of severe symptoms. Thus, there is clearly an age-dependent neurological phenotype in GFAP-160Q mice. Earlier death occurs in all three GFAP-160Q mouse lines (**Fig. 2.10 C**). These results indicate that expression of mutant htt in astrocytes alone is sufficient for producing HD symptoms in mice.

It is important to understand if glial mutant htt can contribute to HD phenotypes in the context of neuronal htt expression. Thus, we crossed GFAP-HD mice with N171-82Q mice, which express the first 171 amino acids of mutant htt with 82 CAG repeats under the control of the neuronal prion promoter (Schilling et al., 1999a). We chose N171-82Q mice because they do not express mutant htt in astrocytes but do show well-characterized neurological phenotypes during early ages (2-5 months), such that any

exacerbating effects that arise from glial htt can be readily observed. We crossed both GFAP-160Q and GFAP-98Q mice with N171-82Q mice in order to produce double transgenic mice. Even though the GFAP-98Q mice do not show obvious HD phenotypes (Bradford et al., 2010), they allowed us to see if expression of htt-98Q in glia can exacerbate neuronal phenotypes in the double transgenic mouse. Starting at 11-12 weeks of age, the double transgenic mice began to show a trend of decreased rotarod performance when compared to the N171-82Q parental line, and this became significant at 18-19 weeks of age (**Fig 2.11 A**). The double transgenic mice carrying GFAP-160Q showed more severe deficits in rotarod performance than those carrying htt-98Q, indicating a CAG repeat length effect. We also examined body weight and found that double transgenic mice had lower body weight than their littermates with N171-82Q or other genotypes, which is significant ($P<0.05$) after the age of 14 weeks (**Fig. 2.11 B**). Moreover, double transgenic mice carrying N171-82Q and GFAP-98Q or N171-82Q and GFAP-160Q die earlier than those carrying only N171-82Q or only htt-98Q transgenes (**Fig. 2.11 C**). All of these findings provide strong evidence that the expression of mutant htt in astrocytes can worsen the neurological symptoms of HD mice, and this exacerbating effect is dependent on the length of the polyQ repeat in mutant htt.

2.5 Discussion

Huntingtin is expressed in various types of cells, including neurons and non-neuronal cells. While our recent studies and others have demonstrated the presence of mutant htt in glial cells (Chou et al., 2008; Hebb et al., 1999; Shin et al., 2005; Singhrao et al., 1998; Wang et al., 2008), the in vivo role of glial htt in the HD pathology of mice

remains unknown. In this study, we show for the first time that mutant htt in astrocytes can promote HD neurological symptoms in mice.

Our findings support the idea that cell-cell interaction plays an important role in HD pathogenesis (Gu et al., 2005; Shin et al., 2005). In the brain, glia-neuron interactions are important for maintaining the normal function and survival of neurons. In other neurological disorders that are also characterized by selective neurodegeneration, such as Alzheimer's disease and amyotrophic lateral sclerosis, non-cell-autonomous effects of mutant proteins and involvement of glial dysfunction are well documented (Choi et al., 2008; Maragakis and Rothstein, 2006; Yamanaka et al., 2008). Importantly, our study reveals that mutant htt expression in glia contributes to HD neurological symptoms even when it is not overexpressed, providing strong evidence for the critical and pathogenic role of mutant htt in glial cells.

Expression levels of transgenic htt are controlled by the promoters, the polyQ repeat number, and the length of N-terminal mutant htt. All of these factors also determine the severity of neuropathology observed in various HD mouse models (Wang et al., 2008). For example, R6/2 mice, in which exon1 htt is abundant in both glia and neurons, show very severe phenotypes (Davies et al., 1997), whereas mice expressing the same exon1 protein only in neurons live normally and show much milder neurological phenotypes (Gu et al., 2005). Such differences raised the interesting issue of whether glial mutant htt could contribute to HD pathology. Overexpression of N-terminal htt in neuronal cells by the neuronal prion promoter also elicits severe neurological phenotypes in N171-82Q mice (Schilling et al., 1999a). Thus, different expression levels of transgenes in various types of cells in HD mice make it difficult to define the role of

mutant htt in glial cells. To solve this problem, we produced a mouse model that expresses truncated mutant htt below the endogenous level in astrocytes. Since mutant htt naturally accumulates more slowly in glial cells than in neurons when it is expressed at the endogenous level in HD knock-in (KI) mice, our GFAP-160Q model should reveal specific pathology that is not due to overexpression of mutant htt in glia.

The low level of transgenic mutant htt in mouse astrocytes could be due to low htt transgene expression or the greater capacity of glial cells to clear misfolded proteins, or both. Previous work in our lab has found that there is higher UPS activity in glial cells than in neurons (Tydlacka et al., 2008). The age-dependent accumulation of mutant htt in astrocytes in our transgenic mice further supports the idea that age-related decreases in UPS activity can promote htt accumulation and cytotoxicity. Because glia can also divide, their division could also help deplete mutant protein levels.

Smaller htt fragments with larger polyQ repeats are known to be more prone to aggregation and misfolding. Notably, our transgenic mice expressing N-terminal htt (208 aa) with 160Q show early death, a phenotype not seen in HD mice that express full-length htt in both neurons and glial cells. Because we see neurological phenotypes consisting of body weight loss, rotarod deficit and decreased lifespan in GFAP-160Q, but not GFAP-23Q or GFAP-98Q transgenic mice, it is clear that these phenotypes are dependent on the length of the polyQ repeat. The combination of the small htt fragment, long polyQ repeat, and promoter all influence htt expression and phenotypes in the GFAP-160Q mice.

An important issue is whether glial htt can contribute to HD pathology in the context of neuronal mutant htt. HD patients express mutant htt in both glia and neurons

so it is important to know if the addition of glial htt can exacerbate phenotypes observed in a neuronal HD model. The other issue is whether mutant htt that carries a smaller polyQ repeat (98Q as compared to 160Q) can cause glial dysfunction in the brain or contribute to HD neuropathology when neurons also express mutant htt. Addressing these issues is important in establishing the deleterious role of mutant htt in glial cells and would help validate the importance of improving glial function in the treatment of HD. By crossing transgenic mice expressing mutant htt in astrocytes with N171-82Q mice that express mutant htt primarily in neurons, we demonstrated the exacerbating effect of glial htt in HD transgenic mice. These effects were seen by decreased body weight and rotarod ability, and reduced lifespan in double transgenic mice expressing mutant htt in both neurons and glia. These results provide evidence that glia are clearly contributing to the pathology of HD. Establishing HD models that express mutant htt in different types of glial cells will allow one to further test these possibilities. Moreover, establishment of the role of htt in glial cells will help us to find therapeutic targets for treating HD by improving glial function.

Figure 2.1

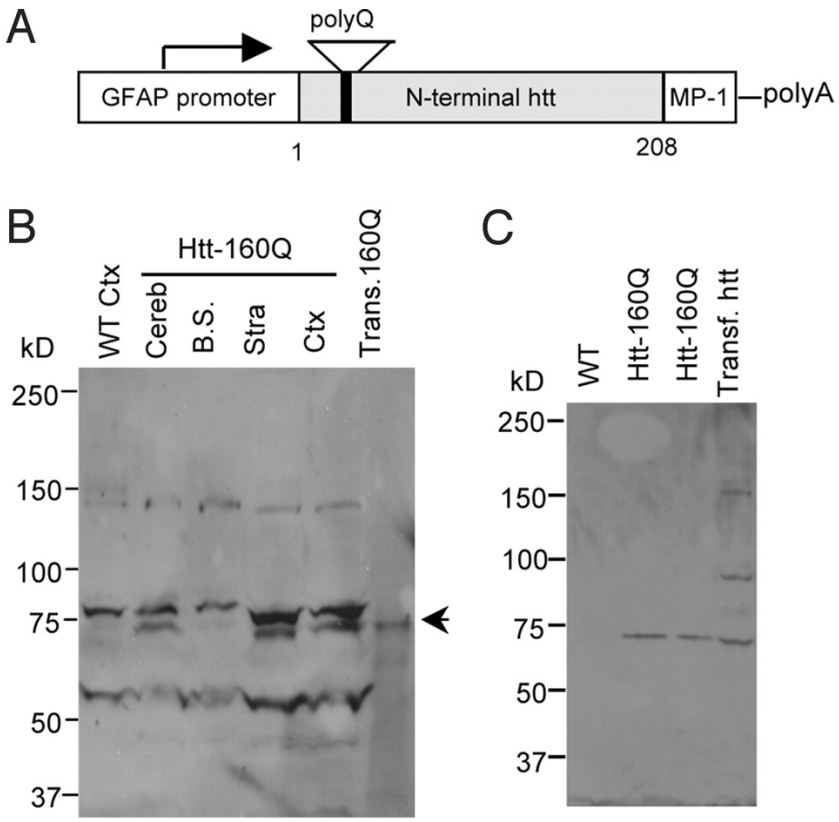

A
polyQ
GFAP promoter
N-terminal htt
MP-1
polyA
1
208
B
Htt-160Q
WT Ctx
Cereb
B.S.
Stra
Ctx
Trans.160Q
kD
250
150
100
75
50
37
C
WT
Htt-160Q
Htt-160Q
Transf. htt
kD
250
150
100
75
50
37

Figure 2.1

GFAP-160Q mutant htt protein expression in glial cells by the GFAP promoter. (A) DNA vector for expressing N-terminal htt (1-208) with either 23Q, 98Q or 160Q under the control of the human GFAP (*gfa*2) promoter. An intron and polyadenylation signal are provided by a fragment of the mouse protamine-1 gene (MP-1). (**B**) EM48 western blot analysis reveals the specific expression of transgenic htt (arrow) in different brain regions of GFAP-160Q mice. Cereb: cerebellum, B.S.: brainstem, Stra: striatum, Ctx: cortex. Arrow indicates transgenic htt. (**C**) 1C2 western blot analysis of cultured astrocytes from WT and GFAP-160Q mice. Transfected htt in HEK293 cells served as a control.

Figure 2.2

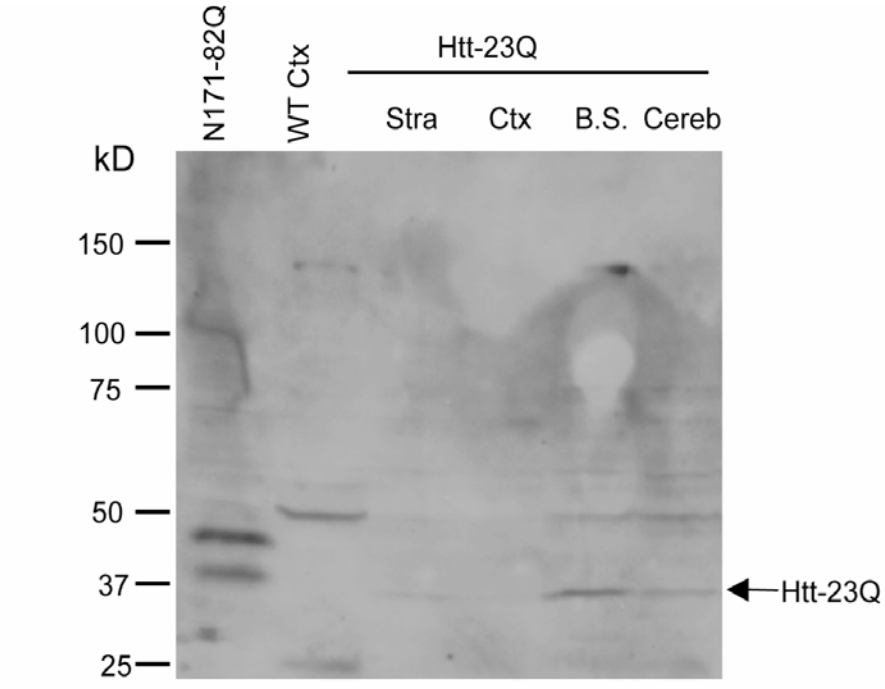
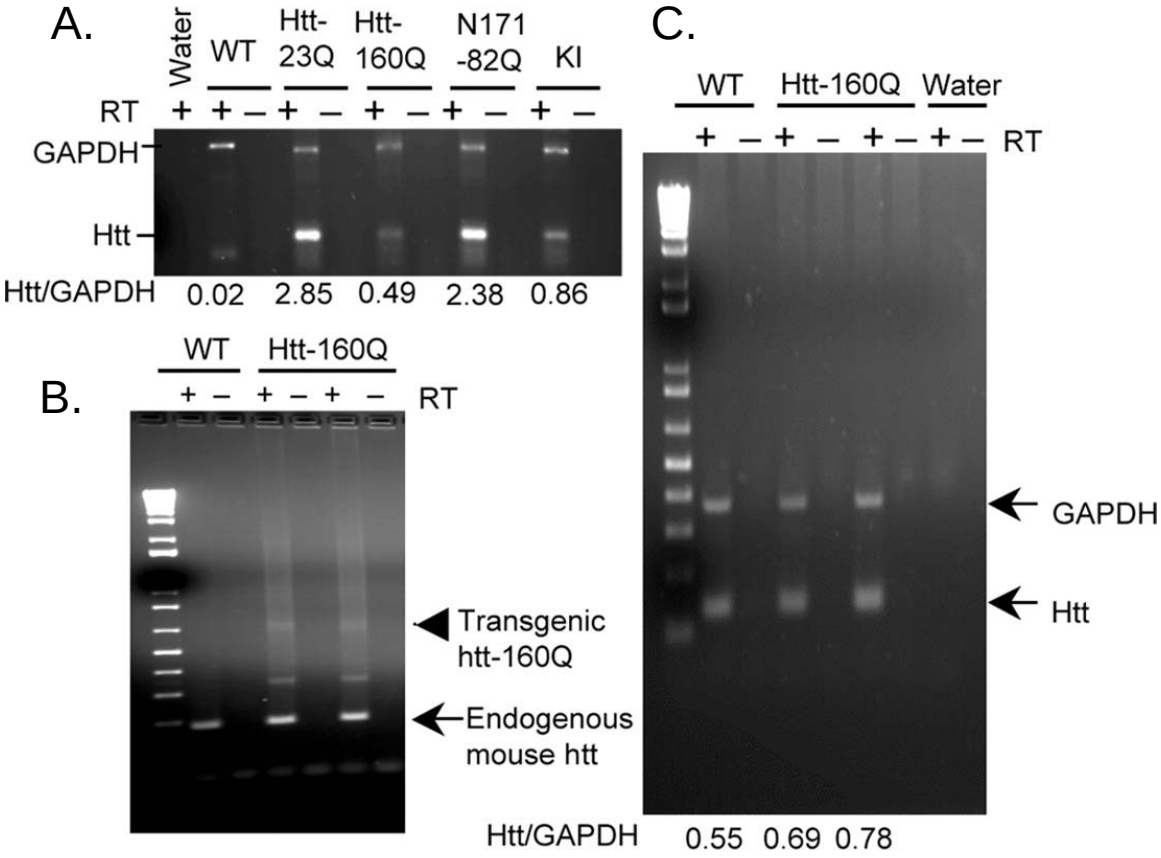

N171-82Q
WT Ctx
Htt-23Q
Stra
Ctx
B.S.
Cereb
kD
150
100
75
50
37
25
Htt-23Q

Figure 2.2

Protein expression of transgenic GFAP-23Q in the mouse brain. EM48 western blot analysis reveals the expression of transgenic htt-23Q (arrow) in the striatum (Stra), cortex (Ctx), brainstem (B.S) and cerebellum (Cereb). The cortical tissues from WT and N171-82Q mice serve as negative and positive control, respectively. Note that the level of transgenic htt in GFAP-23Q mouse brain is lower than transgenic htt in N171-82Q mouse brain.

Figure 2.3


A.
Water
WT
Htt-23Q
Htt-160Q
N171-82Q
KI
RT
GAPDH
Htt
Htt/GAPDH
0.02
2.85
0.49
2.38
0.86
B.
WT
Htt-160Q
RT
Transgenic htt-160Q
Endogenous mouse htt
C.
WT
Htt-160Q
Water
RT
GAPDH
Htt
Htt/GAPDH
0.55
0.69
0.78

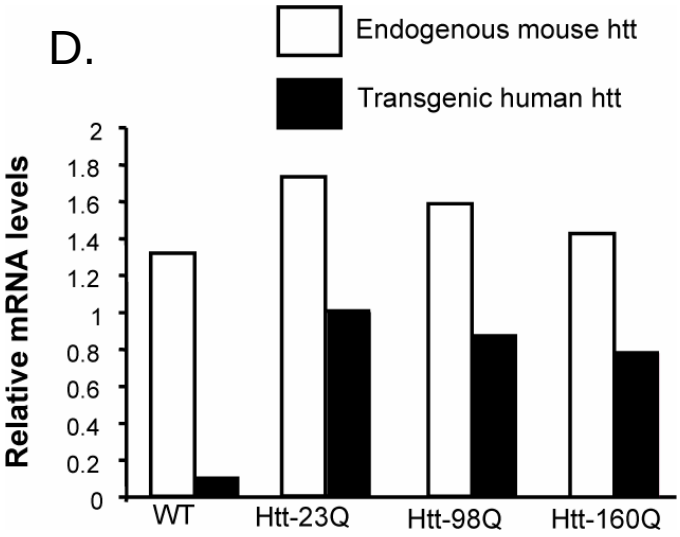
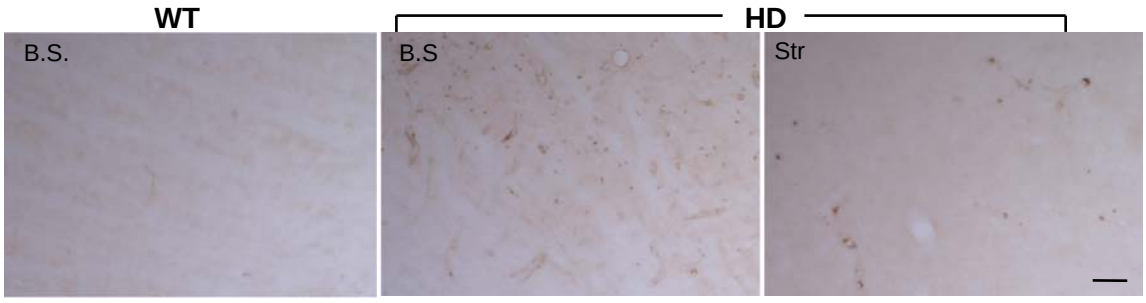

D.
Endogenous mouse htt
Transgenic human htt
Relative mRNA levels
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
WT
Htt-23Q
Htt-98Q
Htt-160Q

Figure 2.3

Low transgene expression levels in GFAP-160Q mice. (A) RT-PCR analysis of transgenic htt transcript levels in mouse brain cortex from various HD models. Primers for transgenic human htt were used for PCR. GAPDH was also amplified and served as an internal control in the same PCR reaction to generate the ratios of transgenic htt to GAPDH. RT: reverse transcriptase. **(B)** RT-PCR analysis of cultured astrocytes from wild-type (WT) and GFAP-160Q mouse cortex tissues were analyzed using the primers crossing over the CAG repeat for distinguishing htt-160Q (arrowhead) and endogenous mouse (arrow) htt transcripts. **(C)** RT-PCR analysis of cultured astrocytes using the primers that are common to both mouse and human htt sequences. Note that the transcription levels of htt in two cultures of GFAP-160Q astrocytes are slightly higher than in WT astrocytes, suggesting that additional transgenic htt expression is at a low level. **(D)** Real time RT-PCR of cultured astrocytes from WT and transgenic mice (GFAP-23Q, GFAP-98Q, GFAP-160Q) that express mutant htt in astrocytes. The relative expression levels of transgenic htt were normalized to endogenous GAPDH levels and were obtained from 3 independent real-time PCR assays.

Figure 2.4

A.



B.

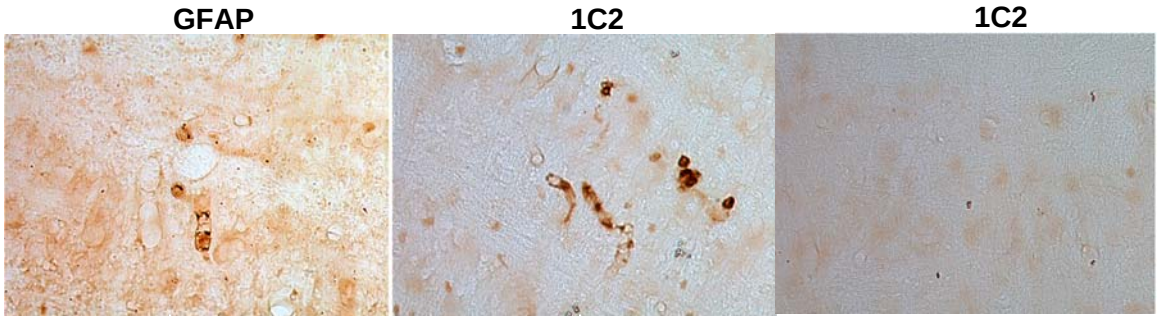


Figure 2.4

Immunostaining shows low levels of transgenic htt in GFAP-160Q mouse brain.

(A) Low magnification micrographs showing brain sections of WT and GFAP-160Q transgenic mice at the age of 18 months. B.S.: brainstem; Str: striatum. Scale bar: 50 m.

(B) Bottom panels indicate higher magnification of htt aggregates in glia of GFAP-160Q mice.

Figure 2.5

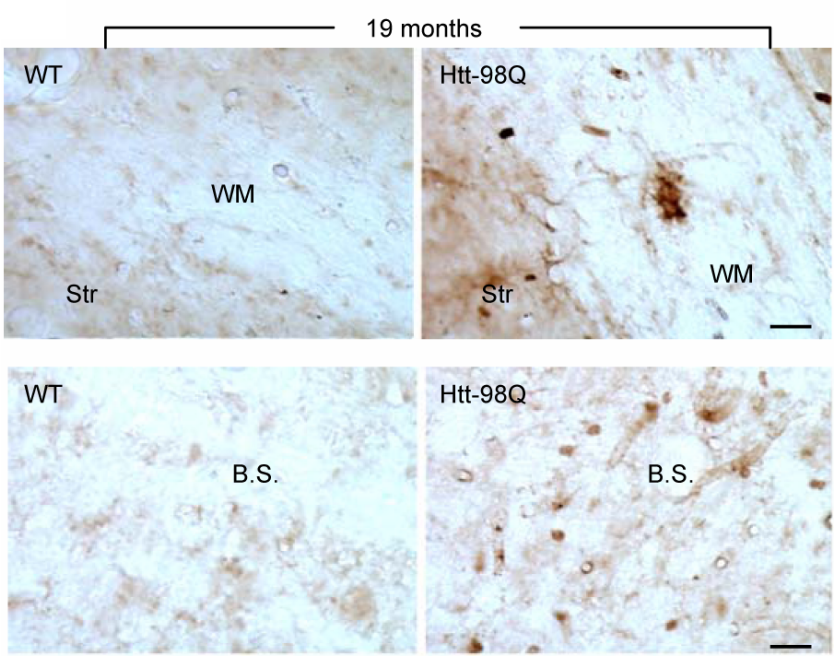


Figure 2.5

Accumulation of mutant htt in glial cells in GFAP-98Q transgenic mice. Wild type and GFAP-98Q mice at 19 months of age were examined using 1C2 immunocytochemical staining. Note that mutant htt is expressed in glial cells in the white matter (WM) of the corpus callosum and brain stem (B.S.) in GFAP-98Q mice. Str: striatum. Scale bars: 10 μm.

Figure 2.6

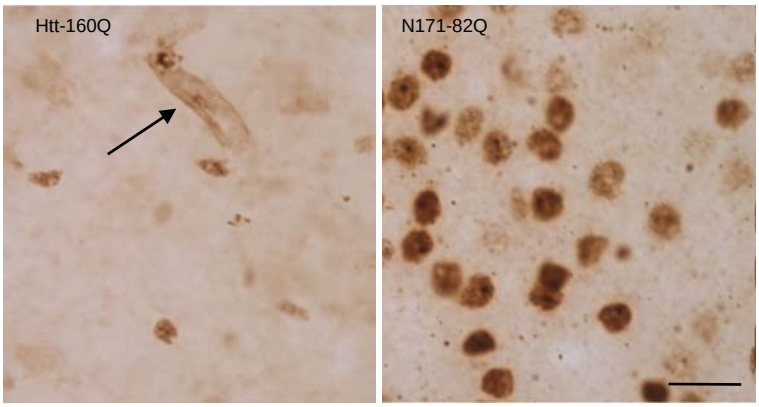


Figure 2.6

Comparison of mutant htt staining density between GFAP-160Q and N171-82Q mouse brain cortex. The frontal cortex region was stained with 1C2 at a 1:3000 dilution. Smaller nuclear size with htt labeling in glial cells and reactive astrocytes (arrow) are seen in GFAP-160Q mouse brain, whereas N171-82Q mouse brain shows more abundant and larger neuronal nuclear staining as well as small neuropil aggregates. Scale bar: 20 μm.

Figure 2.7

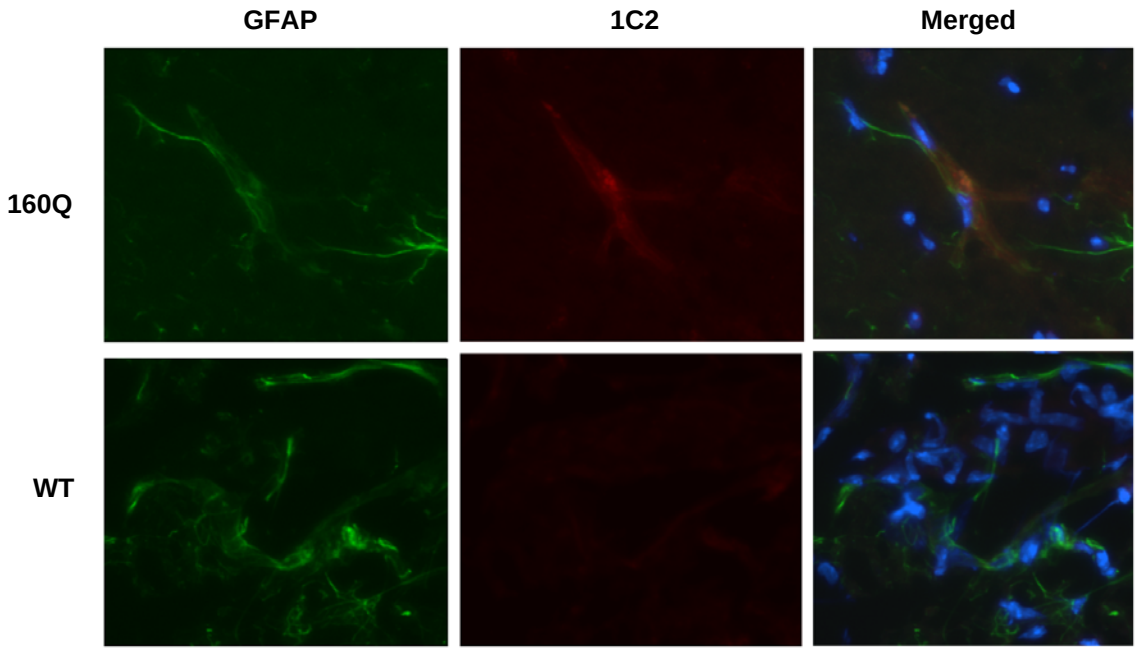


Figure 2.7

Mutant htt is localized to astrocytes in GFAP-160Q mouse brain. Double immunostaining of GFAP-160Q mouse brain with mouse antibody 1C2 for mutant htt (red) and rabbit antibody for GFAP (green). The merged image also shows nuclear staining by Hoechst dye (blue). WT brain does not show mutant htt staining. Scale bar: 10 μm.

Figure 2.8

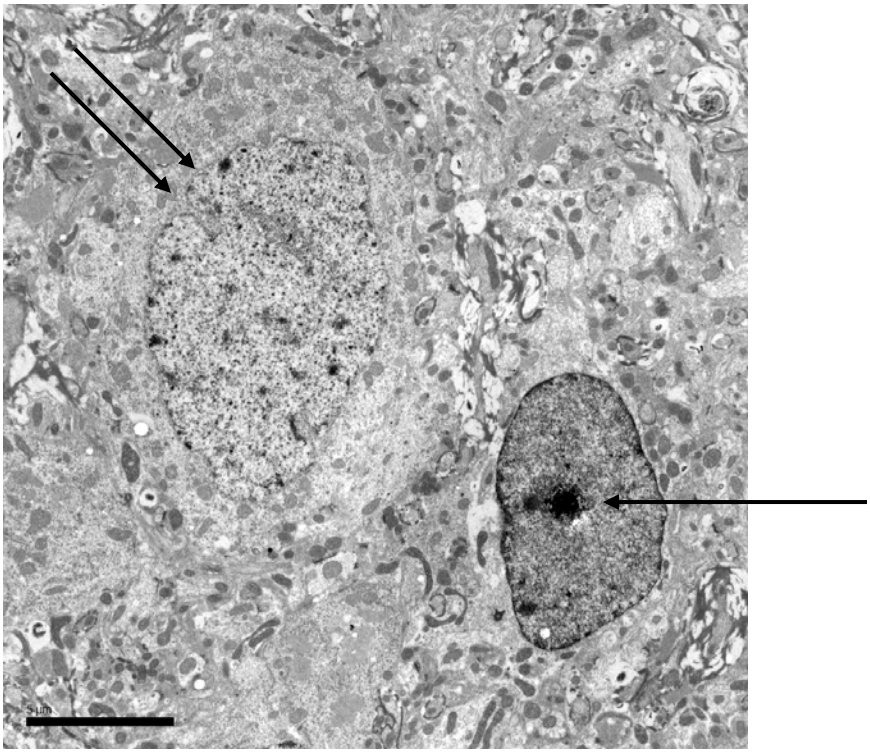


Figure 2.8

Electron microscopy of GFAP-160Q mouse brain. Electron micrograph of the brain stem of GFAP-160Q-1 mouse at 26 months of age. Intranuclear htt aggregates (arrow) in glial cells were detected by 1C2 and 3,3'-Diaminobenzidine (DAB) staining. No evidence of degeneration was found in either astrocytes (arrow) or adjacent neurons (double arrows) in this transgenic HD mouse brain. Scale bar: 5 m.

Figure 2.9

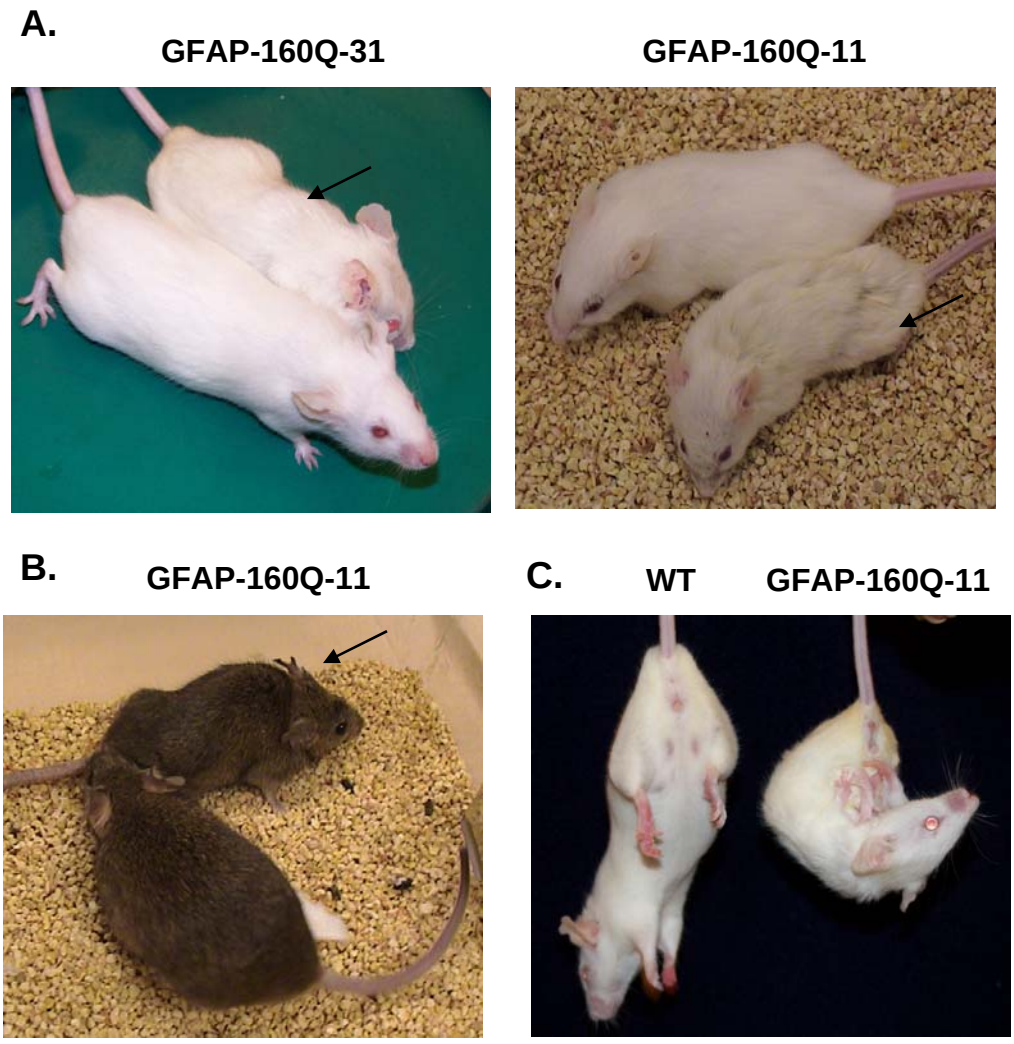


Figure 2.9

Progressive neurological phenotypes in GFAP-160Q mice. (A) Photos of GFAP-160Q mice**.** GFAP-160Q-31 (20 months old) and GFAP-160Q-11 mice (18 months old) on FVB background and their littermate controls are shown. **(B)** GFAP-160Q-11 mouse and its littermate control (11 months old) that also carried the B6C3 genetic background are shown. Arrows indicate mutant mice. **(C)** Old GFAP-160Q mouse (18 months old) showing the clasping phenotype.

Figure 2.10

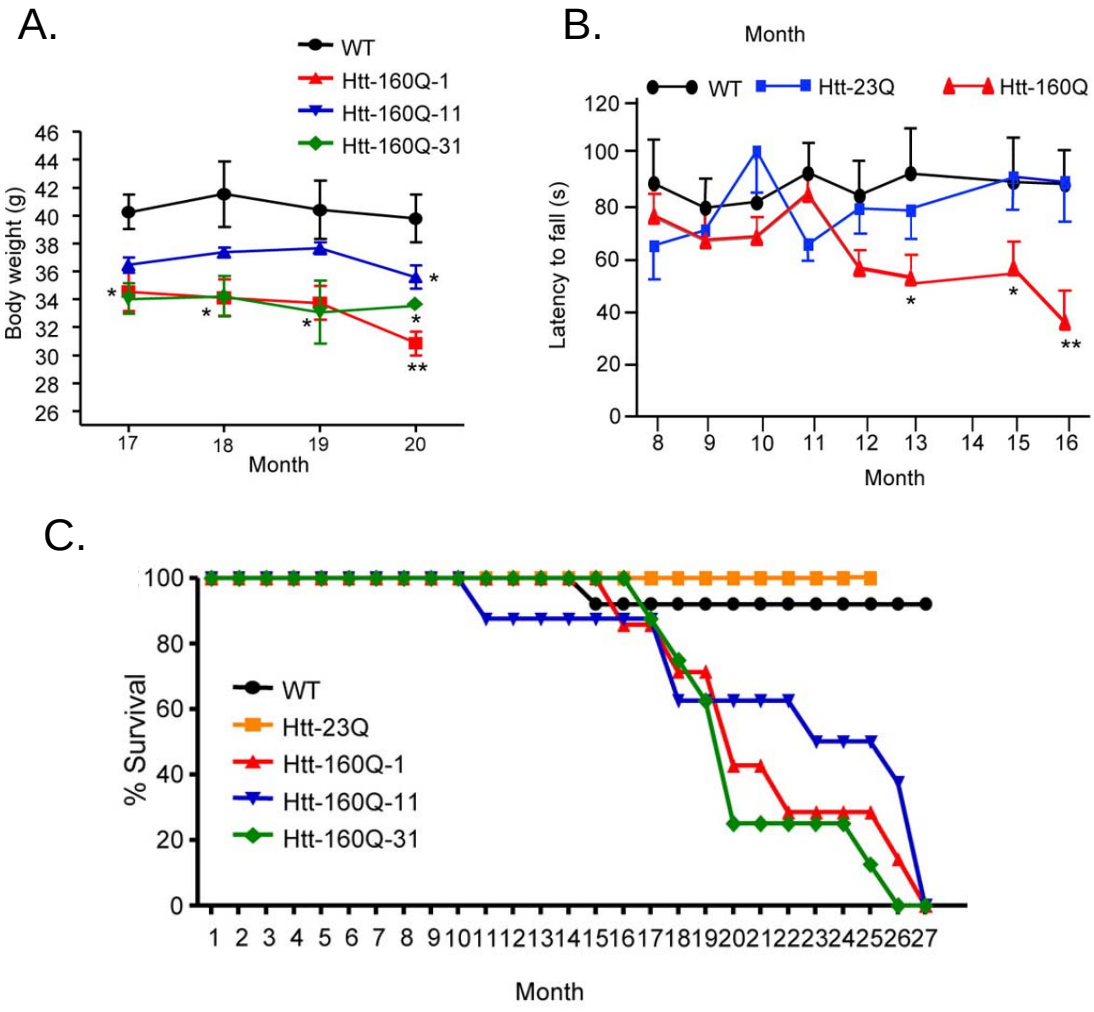

A.
WT
Htt-160Q-1
Htt-160Q-11
Htt-160Q-31
Body weight (g)
Month
B.
Month
WT
Htt-23Q
Htt-160Q
Latency to fall (s)
Month
C.
% Survival
WT
Htt-23Q
Htt-160Q-1
Htt-160Q-11
Htt-160Q-31
Month

Figure 2.10

Characteristic age related phenotypes in GFAP-160Q mice. (**A**) Body weight loss of male GFAP-160Q mice is observed after 16 months. (**B**) Rotarod performance of WT, GFAP-23Q, and GFAP-160Q mice. * $p < 0.05$; ** $p < 0.01$. (**C**) Surviving plots for WT, GFAP-23Q, and three GFAP-160Q mouse lines.

Figure 2.11

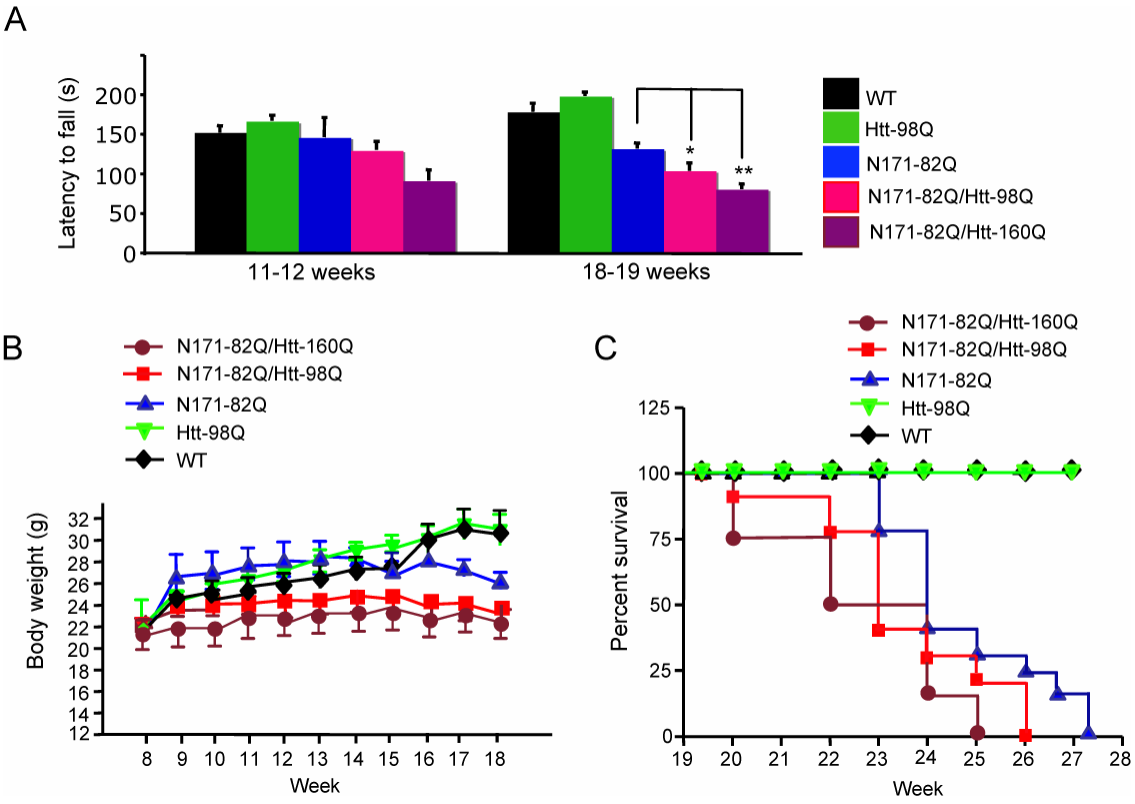

A
Latency to fall (s)
200
150
100
50
0
11-12 weeks
18-19 weeks
*
**
WT
Htt-98Q
N171-82Q
N171-82Q/Htt-98Q
N171-82Q/Htt-160Q
B
N171-82Q/Htt-160Q
N171-82Q/Htt-98Q
N171-82Q
Htt-98Q
WT
Body weight (g)
32
30
28
26
24
22
20
18
16
14
12
8 9 10 11 12 13 14 15 16 17 18
Week
C
N171-82Q/Htt-160Q
N171-82Q/Htt-98Q
N171-82Q
Htt-98Q
WT
Percent survival
125
100
75
50
25
0
19 20 21 22 23 24 25 26 27 28
Week

Figure 2.11

Exacerbation of neurological phenotypes in double transgenic mice expressing mutant htt in neuronal and glial cells. (A) Comparison of latency to fall of mice of different genotypes, including double transgenic mice expressing htt-98Q or htt-160Q. Mice at the age of 11-12 or 18-19 weeks (n = 8-12 each group) were compared. * $p < 0.05$; ** $p < 0.01$. **(B-C)** Body weight **(B)** and survival plot **(C)** of mice of different genotypes showing that double transgenic mice lose more body weight and die earlier than N171-82Q mice.

Chapter 3

Mutant huntingtin expression in mouse brain astrocytes results in glutamate transporter dysfunction

This chapter includes findings and discussions previously published as Bradford, J., Shin, J.Y., Roberts, M., Wang, C.E., Li, X.J., and Li, S. (2009). Expression of mutant huntingtin in mouse brain astrocytes causes age-dependent neurological symptoms. *Proc Natl Acad Sci U S A* 106(52):22480-22485. Plasmids used in the luciferase assay were cloned by Jonathan Cornett. The EAAT2-luciferase construct was provided by Dr. Jeffrey Rothstein at Johns Hopkins University. All other experiments were performed by Jennifer Webster Bradford. Xiao-Jiang Li helped in writing and editing the manuscript.

3.1 Abstract

The GFAP-160Q HD mouse model that expresses mutant htt in astrocytes exhibited progressive, late onset neurological phenotypes, including body weight loss, decreased rotarod ability and reduced lifespan, when compared to WT littermates or the GFAP-23Q control model. When crossed with the neuronal N171-82Q HD mouse model, the double transgenic mouse that expressed mutant htt in both glia and neurons developed accelerated neurological phenotypes. These results indicate that expression of mutant htt in astrocytes clearly is contributing to HD phenotypes in mice. In order to understand how mutant htt in astrocytes is contributing to HD, it is important to identify the molecular mechanisms driving the observed phenotypes. Since glia-neuron interactions are critical for maintaining normal neuronal function and survival, and since glutamate transport is known to be affected in various HD models and patients, we examined the major glutamate transporter (GLT-1) in the astrocytes of our GFAP-160Q mice. We found that GLT-1 transcript and protein levels were decreased in the GFAP-160Q mice, which resulted in decreased GLT-1 glutamate uptake in their brains. It was found that decreased Sp1 binding at the GLT-1 promoter, due to aberrant interaction with mutant htt, was leading to lower promoter activity, and consequently, reduced GLT-1 expression. These results indicate that mutant htt expression in astrocytes is leading to their dysfunction, which can contribute to neuronal dysfunction and HD pathology.

3.2 Introduction

The in vivo contribution of mutant htt in astrocytes to HD pathogenesis is poorly understood. To better understand the role these cells play in HD, we produced the GFAP-160Q HD mouse model. This model expresses truncated human mutant htt in astrocytes under the control of the GFAP promoter. Expression in this model was found to be below the endogenous htt level and was seen only in astrocytes. Even with low expression levels restricted to astrocytes, this model produced a late onset phenotype including body weight loss, decreased rotarod ability and reduced lifespan. When crossed to the N171-82Q mouse model that expresses mutant htt in neurons, the double transgenic mouse expressing mutant htt in both astrocytes and neurons exhibited an exacerbated phenotype. These results indicate that mutant htt in astrocytes is clearly contributing to HD pathogenesis.

Discovering the molecular mechanisms behind these observed phenotypes in the GFAP-160Q model is critical to understanding how mutant htt expression in astrocytes is contributing to HD pathology. Since medium spiny neurons (MSNs) are innervated by glutamatergic axons from cortical neurons (Calabresi et al., 1998), the MSNs are particularly vulnerable to glutamate excitotoxicity because they are naturally exposed to higher levels of glutamate than other cell types. This unique environment makes MSN death by excitotoxicity a possible pathogenic mechanism that could explain the preferential neurodegeneration seen in the striatum of HD patients (Beal, 1994). In support of this theory, excitotoxicity of the NMDA receptor, an ionotropic receptor for glutamate, is now associated with HD in various animal models (Cepeda et al., 2001; Zeron et al., 2002). As astrocyte glutamate transporter dysfunction has been indicated in

several HD models (Behrens et al., 2002; Lievens et al., 2001) and also in human HD patients (Arzberger et al., 1997; Hassel et al., 2008), we examined GLT-1 function in our GFAP-160Q mice. Here, we show that mutant htt in astrocytes causes these cells to become reactive, as indicated by an increase in GFAP production. We also show that GLT-1 transcript and protein levels are decreased in the GFAP-160Q mice, which results in decreased GLT-1 glutamate uptake. By co-immunoprecipitation (co-IP) we show that mutant htt binds strongly to the transcription factor Sp1 in primary astrocytes and that this abnormal binding causes a reduction in the association of Sp1 with the promoter of GLT-1. In primary astrocyte culture we find that decreased GLT-1 protein levels can be rescued in GFAP-160Q astrocytes by treatment with the β-lactam antibiotic ceftriaxone. Our findings demonstrate that glial mutant htt can contribute to neurological phenotypes and suggest that improving glial function could be an effective route in the development of new HD therapies.

3.3 Materials and Methods

Antibodies and Plasmids: Rabbit polyclonal antibody (EM48) and mouse monoclonal antibodies (mEM48) against the N-terminal region (amino acids 1-256) of human htt were described in our previous study (1-3). Rabbit antibodies against GLT-1 (cGLT T88) were provided by Dr. Jeffrey Rothstein and purchased from Millipore Inc. Other antibodies used included mouse monoclonal antibodies against polyglutamine (1C2) or GFAP (Millipore Inc), rabbit anti-GFAP (Gene Tex, Inc), mouse anti-gamma tubulin (Sigma), -GLAST (Chemicon), and goat anti-mouse or -rabbit antibodies conjugated with Alexa Fluor 488 (Molecular Probes).

Plasmids encoding different N-terminal htt (67-150Q, 208-120Q, 508-120Q) and NLS-tagged htt were generated in our previous studies (Shin et al., 2005; Yu et al., 2003). All transfections were performed with the Amaxa Biosystems Nucleofector II electroporator and the corresponding Amaxa Biosystems Mouse Astrocyte Nucleofector kit.

HD Mice: GFAP-160Q mice were bred and maintained in the animal facility at Emory University under specific pathogen-free conditions in accordance with institutional guidelines of The Animal Care and Use Committee at Emory University. Primers with sequences flanking the polyglutamine repeat were used for PCR genotyping. Sequences of the primers are: the forward primer (5'-ATGAAGGCCTTCGAGTCCCTCAAGTCCTTC-3') and reverse primer (5'-AAACTCACGGTCGGTGCAGCGGCTCCTCAG-3') were used for PCR. All the positive founders and their corresponding lines carry the expected length (23Q and 160Q) of the polyQ repeat in transgenic htt.

Western Blot Analysis: For western blots, cultured cells or brain tissues were homogenized in RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA pH 8.0, 1mM EGTA pH 8.0, 0.1% SDS, 0.5% DOC and 1% Triton X-100) with 1X protease inhibitor from Sigma (P8340). The cell or tissue lysates were diluted in 1X SDS sample buffer (62.6mM Tris-HCl pH6.8, 2% SDS, 10% glycerol and 0.01% bromophenol blue) and sonicated for 10 s after incubation at 100°C for 5 min. The total lysates were resolved in a 4-12% Tris-glycine gel (Invitrogen) and blotted to a nitrocellulose membrane. The western blots were developed using the ECL-Plus Kit (GE Health Care/Amersham).

Immunohistochemistry: For immunohistochemistry, brains of transgenic and littermate WT control mice were rapidly isolated and cut to sections (8-10, or 40 μm) with a cryostat (Leica) at –18°C. Mouse brain sections were examined with immunofluorescence labeling as described (Li et al., 2000). Light micrographs were taken using a Zeiss microscope (Axiovert 200 MOT) and a 63X lens (LD-Achroplan 63X/0.75) with a digital camera (Hamamatsu Orca-100) and Openlab software.

Reporter Assays: The GLT-1 (E2-Luci) reporter was provided by Dr. Jeffrey Rothstein (Rothstein et al., 2005). Ten-day-old cultured astrocytes from P2 mouse brains were transfected with this reporter vector and N-terminal htt fragments containing 67 (67), 208 (208), 508 (508) amino acids plus 20, 120, or 150 glutamines (Q). Transfections were conducted using electroporation and the corresponding astrocyte kit (Amaxa Biosystems). ONE-Glo Luciferase Assay System reagent (Promega) was used to detect reporter activity with a FLUOstar Galaxy luminescence plate reader (BMG Labtechnologies).

Glutamate Uptake Assay: Coronal brain slices were prepared from 20 month old GFAP-160Q or WT littermates (600μm slices, 3 slices per mouse). A vibratome was used to cut the slices in ice-cold artificial cerebral spinal fluid (aCSF) ([mM] 118 NaCl, 4.8 KCl, 2.6 $CaCl_2$, 1.2 $MgSO_4$, 25 $NaHCO_3$, 1.2 KH_2PO_4, 11 glucose, 0.6 ascorbic acid) with aeration of 95% O_2 and 5% CO_2. The coronal slices were cut in half down the midline and one half was treated with 1mM dihydrokainic acid (DHK, Sigma) in aCSF and the other half was preincubated in aCSF without DHK treatment, for 1 h at 37°C, with aeration. Subtracting DHK treated values from non-treated values will give GLT-1 specific uptake. After preincubation, L-[3,4-^{3}H]-glutamic acid was added at a final concentration of 25 nM and

incubated for 15 minutes. The glutamic acid treatment was terminated by quickly removing the solution and washing with 4ml ice-cold aCSF 3 times, 2 minutes each. Each half slice was sonicated for 10 seconds in aCSF and 0.1N NaOH. Radioactivity was determined using a liquid scintillation counter (Beckman LS6500). Each sample was performed in triplicate and protein concentration and total L-[3,4-^{3}H]-glutamic acid levels was determined to normalize the radiation results. Values are given as pmol/μg protein/15min. T-test was used to determine statistical significance from 4 GFAP-160Q and 4 WT littermate mice.

RT-PCR: Total RNA from mouse cortex was isolated using the RNeasy Lipid Tissue Mini Kit (QIAGEN #74804). First strand cDNA was obtained using the Invitrogen SuperScript™ First Strand Synthesis System for RT-PCR (11904-018). RT-PCR of mouse brain htt and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers has been previously described by our lab (Wang et al., 2008).

Four- to six-week-old cultured astrocytes were treated overnight with 0.25 mM dBcAMP in serum-free media to increase glutamate transporter expression for RT-PCR. Total astrocyte RNA was collected using the Qiagen RNeasy Mini Kit (74104), and cDNA was produced using the same method as the brain lysate. Primers for GLT-1, GLAST (Shin et al., 2005), GAPDH, and htt (Wang et al., 2008) have been previously described in our early studies.

Chromatin Immunoprecipitation (ChIP): ChIP assays with semi-quantitative PCR were performed as described previously (Friedman et al., 2007). Frontal brain lysate from GFAP-160Q and WT littermate control mice was used along with reagents from the

Millipore Chromatin Immunoprecipitation (ChIP) Assay Kit. After cross-linking, anti-Sp1 (Millipore) was used to precipitate the Sp1-DNA complex. Rabbit IgG (Jackson ImmunoResearch Laboratories, Inc) was used as a control antibody. PCR was conducted with primers to amplify the promoter region of the mouse GLT-1 gene (forward 5' CGAGGCGCTAAAGGGCTTAC 3'; reverse 5' GAGTGCGGCGGAGAGCGGTA 3') and mouse PCNA (forward 5' TCCTAAGGATGGAAACTGCAGCCT 3'; reverse 5'ATAGGCGAGGGGCATCACGG 3'). PCR conditions were as follows: 1 cycle of 96 C for 3 min and 37 cycles of 96 C for 30 s followed by 72 C for 1 min and 62ºC for 45 s. PCR with the precipitates by rabbit IgG or without template served as negative controls.

Ceftriaxone Drug Treatment: Primary astrocytes were cultured from P1-P3 GFAP-160Q or WT littermate pups for 6 weeks. Six week old cultured astrocytes were treated for 7 days with 50μM or 100μM ceftriaxone or 250μM dBcAMP in astrocyte media with 5% serum. Following treatment, cells were collected for western blotting with antibodies against GLT-1 or tubulin.

Statistical Analysis: All values are expressed as means ± SE. We assessed statistical significance using Student's t-test and considered a P value of < 0.05 significant.

3.4 Results

By studying the molecular mechanism underlying the phenotypes in our GFAP-160Q mice, we were able to better understand the effects of mutant htt expression in astrocytes. Although we did not detect overt cellular degeneration in older GFAP-160Q

mice, we were able to confirm the presence of reactive astrocytes, which is a glial response to CNS injury and occurs early in neurodegeneration. In older GFAP-160Q mice (>1.5 year), there is an increase in GFAP staining in the cortex, striatum, brain stem, and cerebellum (**Fig. 3.1 A**). Increased GFAP protein levels were also seen in cultured astrocytes from GFAP-160Q mice, compared to WT controls, by either immuno-fluorescence (**Fig 3.1 B**), or western blotting (**Fig. 3.1 C**).

Since astrocyte glutamate transporter (GLT-1 in mice or EAAT2 in humans) transcripts are decreased in other HD mouse models (Behrens et al., 2002; Lievens et al., 2001) and in cultured glial cells expressing mutant htt (Shin et al., 2005), we were interested in investigating whether GFAP-160Q transgenic mice also show decreased glutamate transporters in their brain. RT-PCR from primary cultured astrocytes shows a decrease in GLT-1 expression in GFAP-160Q mouse pups when compared to WT littermates, but there was no difference in glutamate/aspartate transporter (GLAST) transcript levels (**Fig. 3.2 A**). Western blotting with adult brain lysate also showed that GLT-1, but not GLAST, is reduced in GFAP-160Q mouse brains (**Fig. 3.2 B**). Since GLT-1 mRNA expression is reduced, and therefore protein levels are reduced in the GFAP-160Q mice, we wondered if there was a decrease in glutamate uptake in the brains of these mice. Using brain slices from either WT or GFAP-160Q mice, we found that there is a significant reduction of GLT-1 specific glutamate uptake in brain slices (cortex-striatum) from GFAP-160Q mice (**Fig. 3.3**).

Although the above findings and previous reports have found deficient GLT-1 expression in HD mice, we still lack mechanistic insight into this phenomenon. Based on the fact that the promoter of the GLT-1 gene has multiple Sp1 binding sites which are

required for full activity (Su et al., 2003), we asked whether mutant htt in astrocytes binds to Sp1 and affects the Sp1-dependent transcription of GLT-1. To better analyze the repeat-dependent effect in astrocyte nuclei, we tagged transfected htt proteins with a nuclear localization signal (NLS). Truncated WT htt has a tendency to enter the nucleus and then exit back into the cytoplasm, whereas truncated mutant htt will enter the nucleus and remain there. The addition of the NLS will help retain the WT htt in the nucleus so we would be able to compare the impact of WT and mutant htt nuclear localization in astrocytes. We electroporated either WT or mutant exon1 htt constructs tagged with an NLS into primary cultured astrocytes and performed fluorescent immunostaining with antibodies to htt. The immunostaining shows that mutant htt (150Q) forms nuclear aggregates, whereas the WT htt fragments are diffuse in the nucleus of cultured astrocytes (**Fig 3.4 A**). Higher magnification images are shown in (**Fig 3.4 B,C**). To see if mutant htt aberrantly binds to Sp1 in astrocytes, we performed Sp1 co-immunoprecipitation from WT astrocytes transfected with either WT or mutant NLS tagged exon1 htt. Although there was less htt-150Q than htt-20Q in the input, more htt-150Q than htt-20Q co-precipitated with endogenous Sp1 **(Fig. 3.5 A**). Quantification of the ratios of immunoreactive htt-20Q or htt-150Q to tubulin verified that the input of 150Q was much lower than the input of 20Q, while similar amounts of 20Q and 150Q were precipitated (**Fig. 3.5 B**). Thus, more htt-150Q than htt-20Q was precipitated by anti-Sp1, which was verified by measuring the ratio of the precipitated htt to its input (**Fig. 3.5 B**).

Next we assessed the influence of transfected htt on the promoter activity of the human GLT-1 gene in transfected astrocytes. To perform this luciferase activity assay,

we co-transfected primary astrocytes with the GLT-1 promoter fused to luciferase, as a readout of activity, and either WT or mutant htt of varying lengths. Small N-terminal mutant htt (67-150Q and 208-120Q) produced more inhibitory effects on GLT-1 promoter activity than a larger N-terminal htt fragment (508-120Q) (**Fig. 3.6**), which also supports the idea that protein context can modulate htt toxicity (Cornett et al., 2006; Yu et al., 2003). Although transfection of normal exon1 htt (N67-20Q) also elicited some inhibition of the GLT-1 promoter activity, mutant htt inhibited significantly more GLT-1 promoter activity, indicating a repeat-dependent inhibition (**Fig. 3.6**). These results indicate that in astrocytes, the presence of mutant htt is able to decrease the activity of the GLT-1 promoter.

Since mutant htt aberrantly binds to Sp1 in astrocytes, we next wanted to investigate whether mutant htt reduces Sp1 occupancy of the GLT-1 promoter. To do this, we performed a chromatin immunoprecipitation (ChIP) assay using brain tissues from GFAP-160Q transgenic mice and WT littermate controls. This assay revealed that the presence of transgenic htt-160Q reduces the association of Sp1 with the GLT-1 promoter (**Fig. 3.7 A**). Quantitative analysis of the ChIP results further confirmed that this reduction was specific to the GLT-1 promoter, but not to the Sp1-regulated PCNA promoter (**Fig. 3.7 B**). Taken together, these findings suggest that mutant htt binds Sp1 and reduces Sp1-mediated GLT-1 expression in astrocytes, which can lead to defective glial glutamate uptake and increased neuronal excitotoxicity.

Because reduced GLT-1 expression causes astrocyte dysfunction and eventually HD phenotypes in our mice and in other models, we tested to see if drugs aimed at increasing GLT-1 expression would work in primary astrocytes cultured from our GFAP-

160Q mice (**Fig. 3.8**). We chose to examine the effect of the drug ceftriaxone, a β-lactam antibiotic, in our model because it has been shown to increase the transcript expression of GLT-1 (Rothstein et al., 2005). Recently, this drug has been shown to improve behavioral phenotypes and reverse the glutamate uptake deficiency in the R6/2 HD mouse model (Miller et al., 2008). We treated 6 week old primary astrocytes from either WT or GFAP-160Q pups with either ceftriaxone or dibutyryl cyclic adenosine monophosphate (dBcAMP) for 1 week (**Fig 3.8**). We used dBcAMP as a positive control, as this compound is also known to increase glutamate transporter levels (Eng et al., 1997). Treatment of primary astrocytes with either 50μM or 100μM of ceftriaxone increased GLT-1 protein levels in both WT and GFAP-160Q cells, as seen by western blotting, when compared to untreated cells. However, the increase in GLT-1 levels was not as striking as what was seen in the dBcAMP treated cells. However, ceftriaxone is an FDA approved drug that could be used for human treatment, and may prove useful in treating patients in the future based on these results and the results seen in the R6/2 mouse (Miller et al., 2008).

3.5 Discussion

Although previous findings have shown that mutant htt reduces GLT-1 expression in transgenic HD mice (Behrens et al., 2002; Lievens et al., 2001; Shin et al., 2005), and decreases GLT-1 mRNA and glutamate uptake in the brains of HD patients (Hassel et al., 2008), a mechanism underlying this phenomenon had not been revealed. Our GFAP-160Q mice that express mutant htt in astrocytes allowed us to identify one of the possible molecular mechanisms leading to the observed HD phenotypes.

In this study, we have demonstrated that mutant htt can aberrantly bind to the

transcription factor Sp1 in astrocytes. This aberrant interaction has been shown before (Dunah et al., 2002; Li et al., 2002) and is thought to result in the decreased expression of genes specifically critical to neuronal function and survival (Chen-Plotkin et al., 2006). By performing a luciferase activity assay, we found that smaller fragments of mutant htt were able to significantly decrease the activity of the GLT-1 promoter in primary astrocytes. However, a larger mutant htt fragment of 508aa was not able to decrease GLT-1 promoter activity. This leads us to believe that mutant htt conformation contributes to toxicity in astrocytes in the same manner as in other cell types. Through ChIP analysis, we found that there is reduced Sp1 occupancy of the GLT-1 promoter in the brains of our GFAP-160Q mice, but there was no Sp1 binding difference between WT and GFAP-160Q mice at the PCNA promoter. Again, this indicates that mutant htt specifically affects certain promoters.

These findings provide some molecular insight into the phenomenon of GLT-1 expression being decreased in various HD mouse models expressing toxic N-terminal htt fragments (Davies et al., 1997; Shin et al., 2005). Miller et al. have recently shown that up-regulation of GLT-1 transporter by ceftriaxone treatment attenuates some of the behavioral alterations in the R6/2 transgenic model (Miller et al., 2008). Ceftriaxone treatment of primary astrocytes from our GFAP-160Q mice increased GLT-1 expression and may be a good candidate for further research for HD treatment. Another recent finding revealed that decortication of glutamatergic projections to the striatum significantly lowered striatal glutamate levels and reduced behavioral phenotypes of R6/2 mice (Stack et al., 2007). Defective glial glutamate uptake has also been found in ALS, and neuronal dysfunction can be induced by defective astrocytes even in the absence of

neurodegeneration (Lievens et al., 2001; Yamanaka et al., 2008). Although our study focused on the effect of astrocyte htt on GLT-1, we should point out that dysfunctional astrocytes can also be attributed to other cellular problems like the production of neurotrophic factors and cytokines, and recent studies suggest that astrocytes secrete a substance that kills motor neurons in ALS (Di Giorgio et al., 2007; Nagai et al., 2007). Establishing HD models that express mutant htt in different types of glial cells will allow these possibilities to be tested.

Figure 3.1

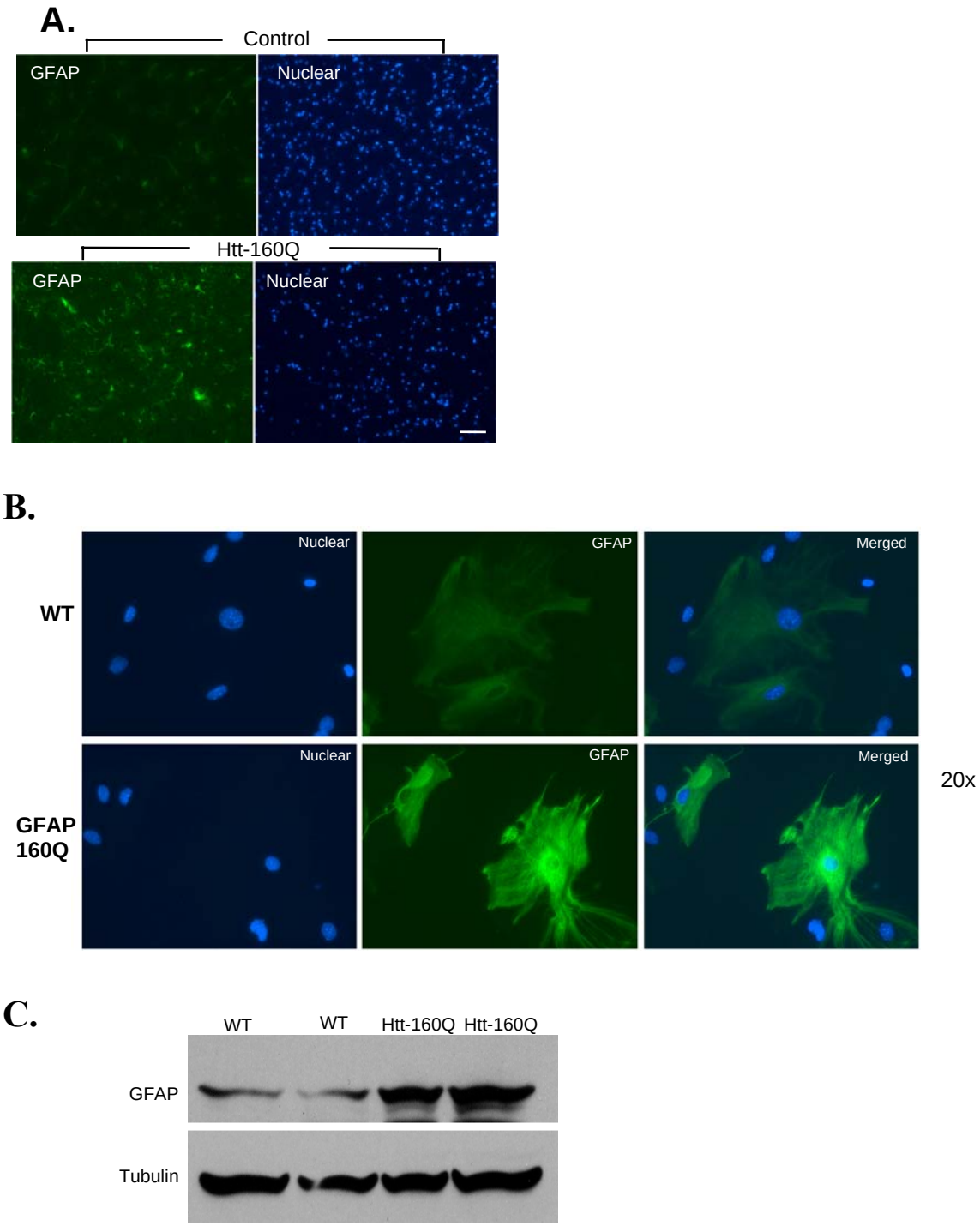
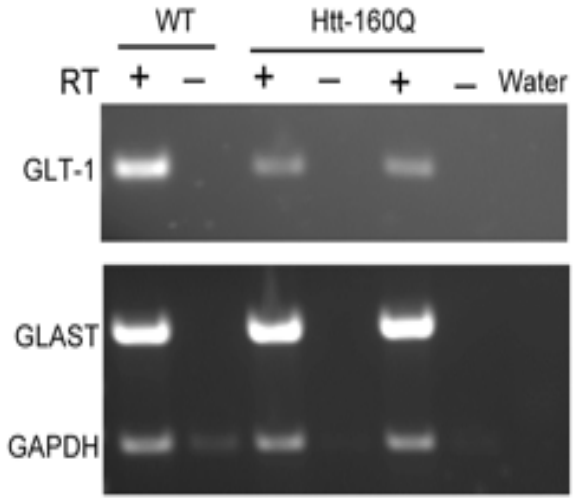

A.
Control
GFAP
Nuclear
Htt-160Q
GFAP
Nuclear
B.
WT
Nuclear
GFAP
Merged
GFAP 160Q
Nuclear
GFAP
Merged
20x
C.
WT
WT
Htt-160Q
Htt-160Q
GFAP
Tubulin

Figure 3.1

Increased activation of astrocytes in GFAP-160Q mice. (A) Comparison of GFAP staining in the brain stem of mice expressing normal htt (control) and htt-160Q (HD). Scale bar: 20 m. **(B)** Fluorescent staining of 6 week old primary cultured WT and GFAP-160Q astrocytes shows an increase in GFAP staining in GFAP-160Q astrocytes. **(C)** Western blot analysis of GFAP expression in astrocytes. Two independent 6-week old cultures from GFAP-160Q or WT littermate mice were examined.

Figure 3.2

A.


WT
Htt-160Q
RT + − + − + − Water
GLT-1
GLAST
GAPDH

B.

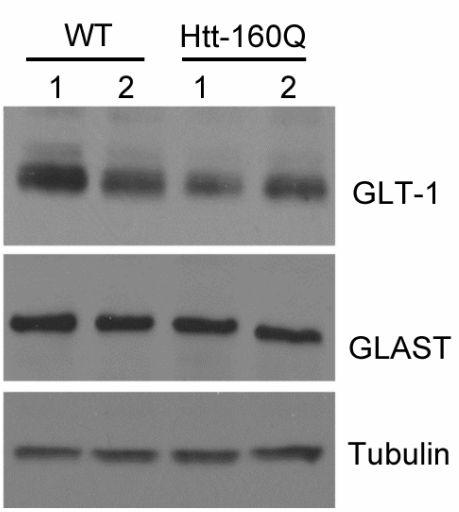

WT
Htt-160Q
1 2 1 2
GLT-1
GLAST
Tubulin

Figure 3.2

Decreased expression of GLT-1 in GFAP-160Q mice. (**A**) RT-PCR analysis of GLT-1 and GLAST transcripts in cultured astrocytes from two GFAP-160Q pups and one WT littermate control pup. RT: reverse transcriptase. (**B**) Representative western blot analysis of GLT-1 in the cortex of GFAP-160Q transgenic mice. Two mice (1 and 2) for each group are shown. The blots were also probed with antibodies to tubulin or GLAST.

Figure 3.3

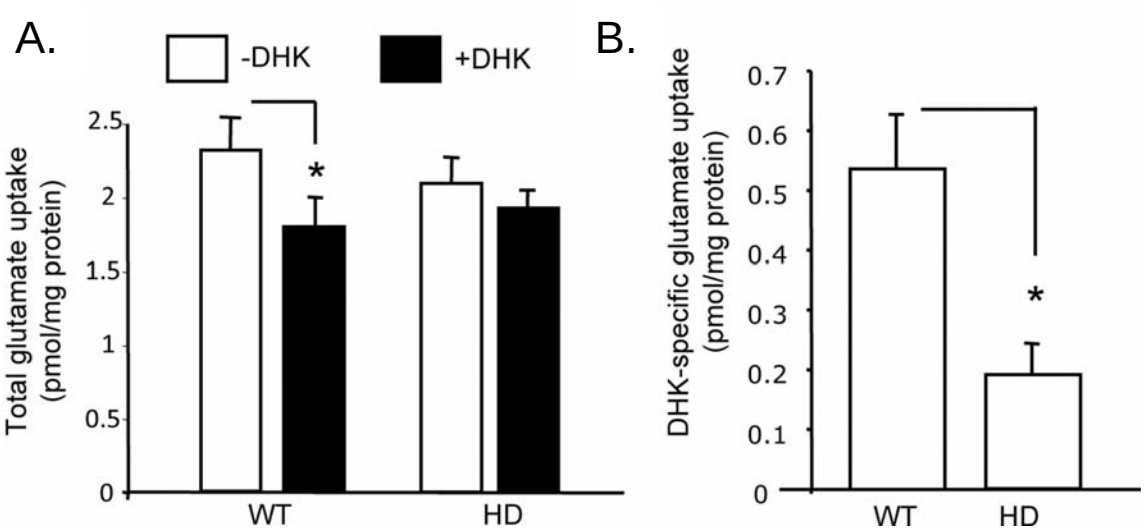

A.
-DHK
+DHK
Total glutamate uptake
(pmol/mg protein)
2.5
2
1.5
1
0.5
0
*
WT
HD
B.
DHK-specific glutamate uptake
(pmol/mg protein)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
WT
HD

Figure 3.3

Decreased glutamate uptake in brain slices of GFAP-160Q mice. (A) [^{3}H]-glutamate uptake assays of cortico-striatal brain slices from 20-month-old GFAP-160Q or WT littermate mice (n=4 each group). Glutamate uptake (pmol/mg protein/15min) was measured in the absence (-DHK) and presence (+DHK) of the GLT-1 specific blocker DHK (1 mM). **(B)** The differences between DHK treated and non-treated samples were obtained and show that DHK-specific glutamate uptake is significantly reduced in GFAP-160Q brains. * $P<0.05$.

Figure 3.4

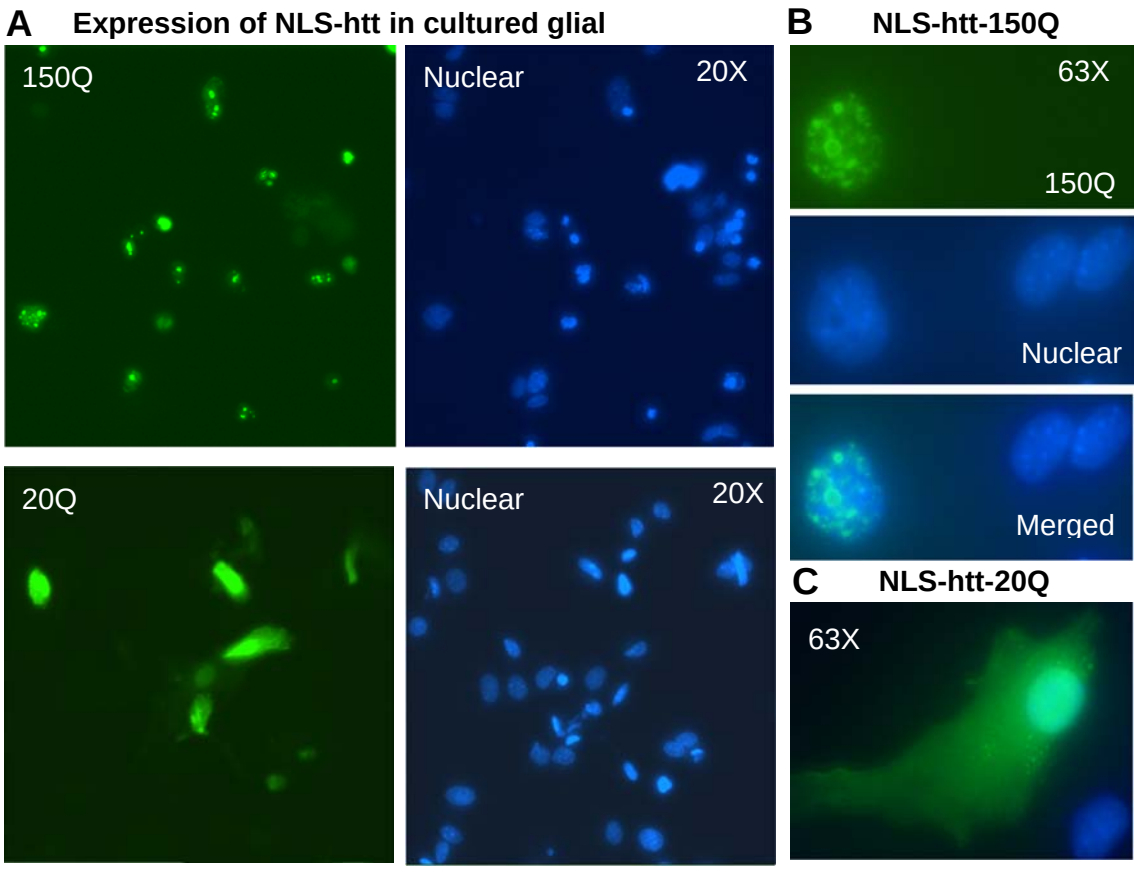

A
Expression of NLS-htt in cultured glial
150Q
Nuclear
20X
20Q
Nuclear
20X
B
NLS-htt-150Q
63X
150Q
Nuclear
Merged
C
NLS-htt-20Q
63X

Figure 3.4

Expression of htt in primary cultured astrocytes. (A-C) Images of EM48 immunostaining of cultured WT mouse astrocytes that were transfected with NLS-exon1 htt (green) containing 150Q or 20Q. The high-magnification graphs in **(B)** show that mutant htt (150Q) forms nuclear aggregates, whereas the normal htt fragment (20Q) in **(C)** is diffuse in the nucleus (blue).

Figure 3.5

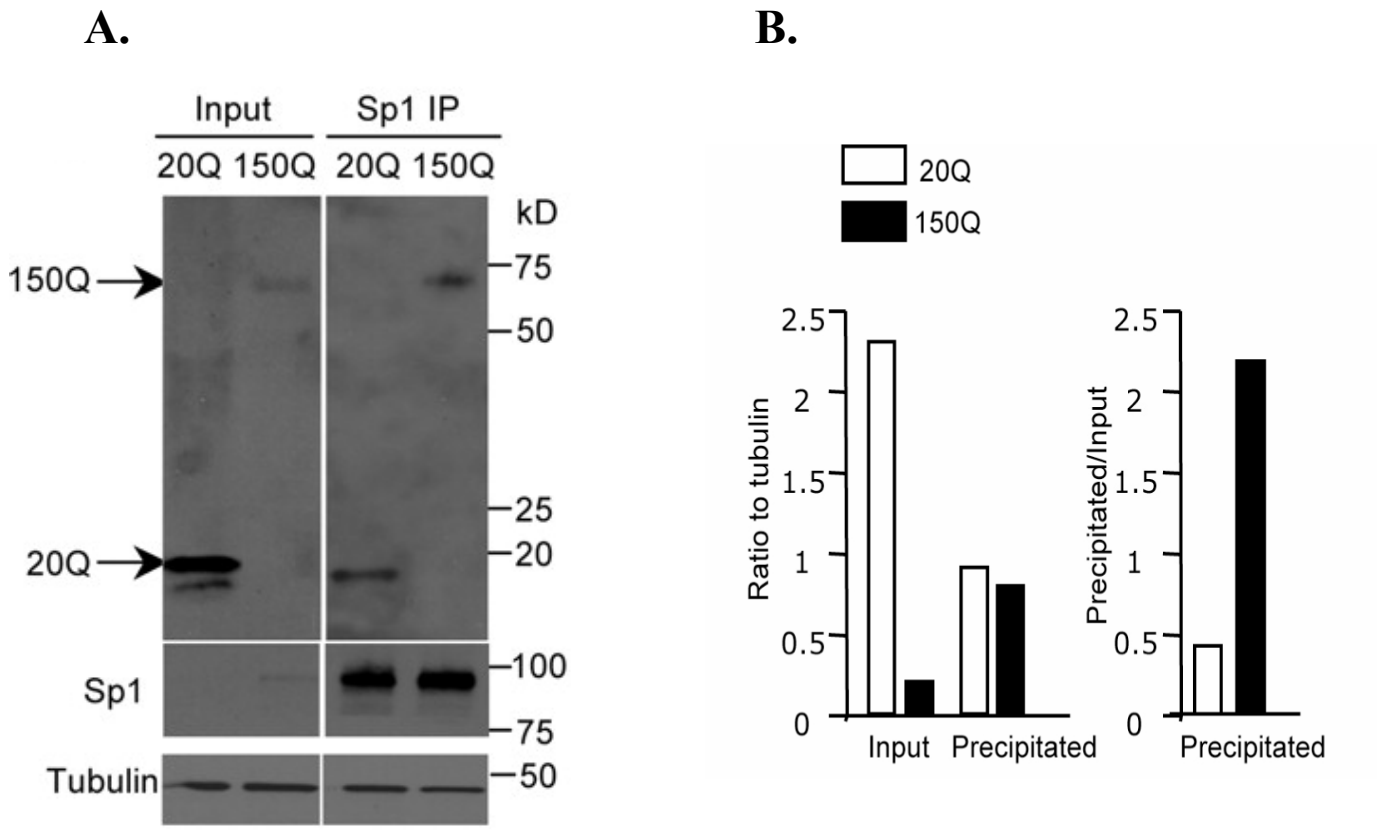

A.
B.
Input
Sp1 IP
20Q 150Q 20Q 150Q
kD
150Q
20Q
Sp1
Tubulin
75
50
25
20
100
75
50
20Q
150Q
Ratio to tubulin
Precipitated/Input
2.5
2
1.5
1
0.5
0
Input
Precipitated
Precipitated

Figure 3.5

Mutant htt pulls down more Sp1 transcription factor than WT htt in primary cultured astrocytes. (A) Immunoprecipitation of Sp1 in transfected astrocytes shows that more mutant htt (150Q) than normal htt (20Q) is co-precipitated with Sp1. **(B)** Quantification of the ratios of immunoreactive htt to tubulin (left panel) or of the precipitated htt to its input (right panel).

Figure 3.6

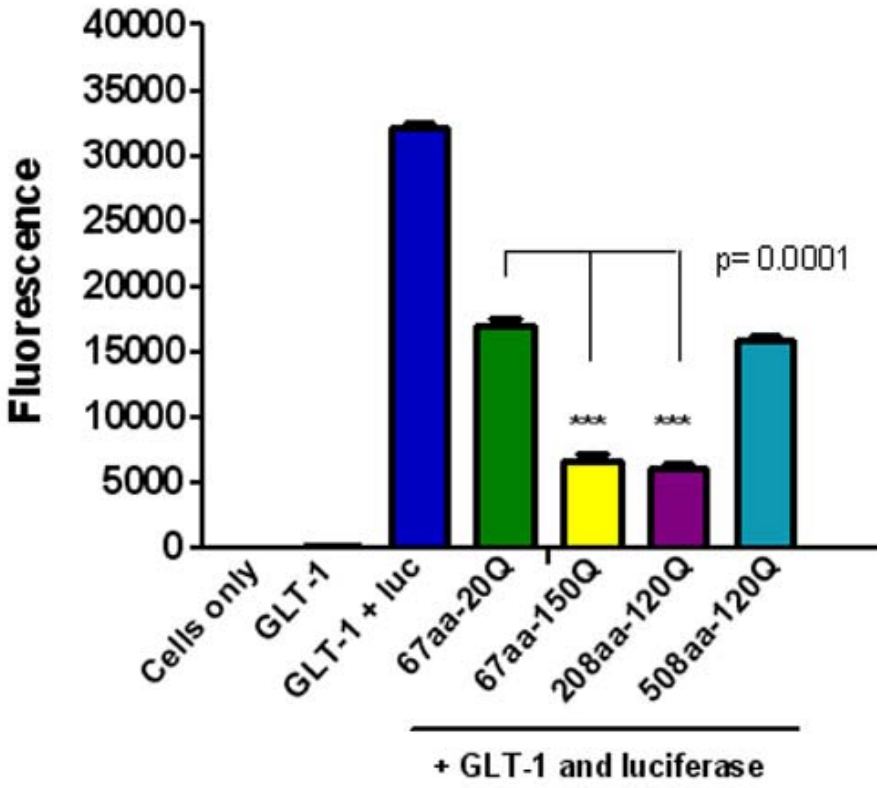

Fluorescence
40000
35000
30000
25000
20000
15000
10000
5000
0
p= 0.0001
***
***
Cells only
GLT-1
GLT-1 + luc
67aa-20Q
67aa-150Q
208aa-120Q
508aa-120Q
+ GLT-1 and luciferase

Figure 3.6

GLT-1 luciferase activity assay in primary astrocytes. Co-expression of the GLT-1 promoter reporter with N-terminal htt fragments containing different numbers of amino acids (67, 208, 508) plus either 20Q, 150Q, or 120Q. Note that mutant htt fragments (67-150Q and 208-120Q) significantly inhibit the reporter activity. *** $P < 0.001$.

Figure 3.7

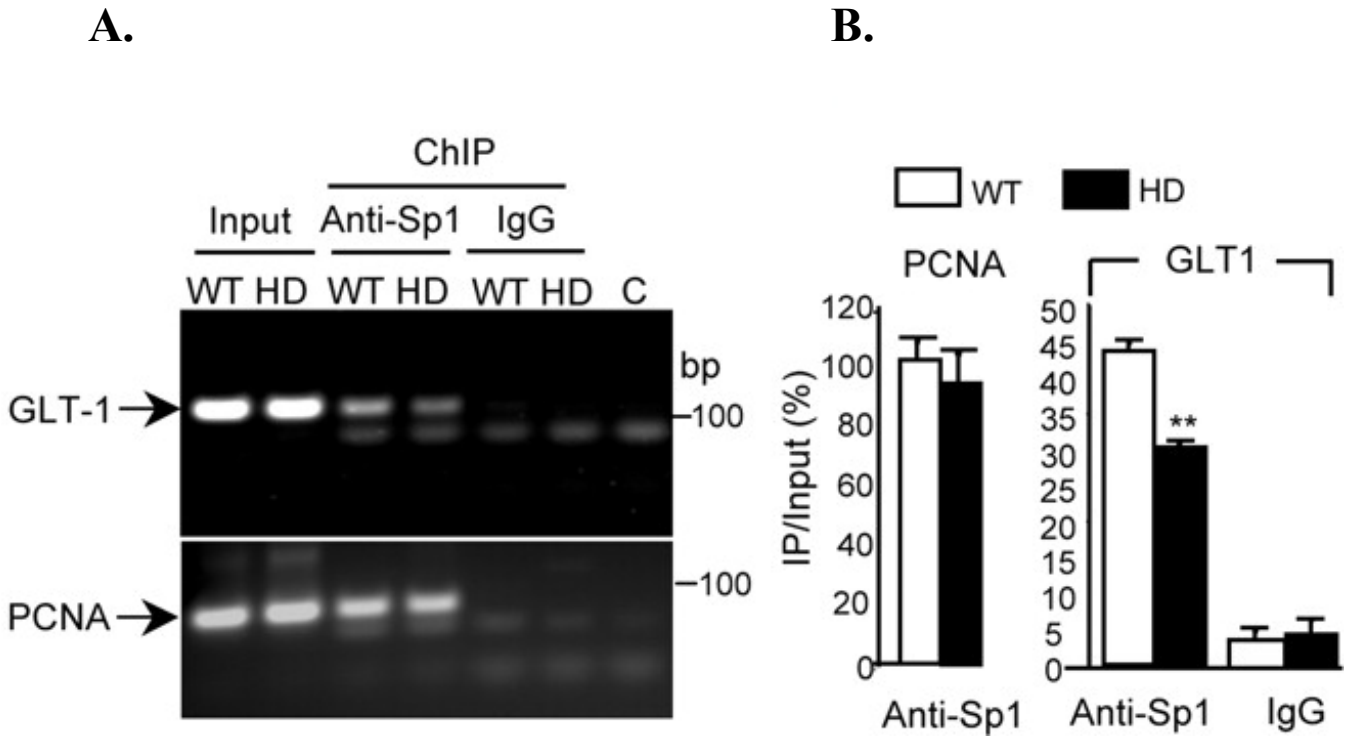

A.
B.
ChIP
Input
Anti-Sp1
IgG
WT HD WT HD WT HD C
bp
GLT-1
PCNA
100
100
WT
HD
PCNA
GLT1
IP/Input (%)
120
100
80
60
40
20
0
50
45
40
35
30
25
20
15
10
5
0
**
Anti-Sp1
Anti-Sp1
IgG

Figure 3.7

Chromatin immunoprecipitation (ChIP) assay examining Sp1 binding at the GLT-1 promoter. (A) PCR results show decreased association of Sp1 with the GLT-1 promoter in the frontal brain tissue of GFAP-160Q transgenic mice compared with WT littermate controls. Rabbit anti-Sp1 and IgG, which served as a control, were used for immunoprecipitation. C: no template. **(B)** Quantification of the ratios of PCR products from immunoprecipitated (IP) to input. ** $P < 0.01$ compared to WT controls (n= 4).

Figure 3.8

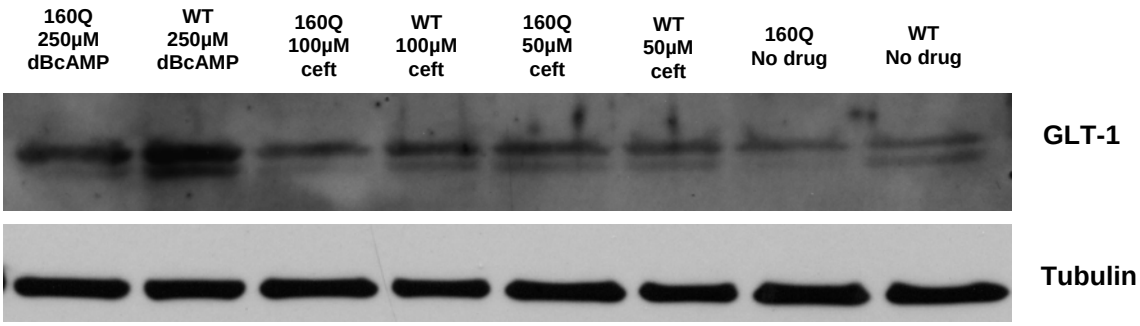

160Q 250μM dBcAMP
WT 250μM dBcAMP
160Q 100μM ceft
WT 100μM ceft
160Q 50μM ceft
WT 50μM ceft
160Q No drug
WT No drug
GLT-1
Tubulin

Figure 3.8

β-lactam antibiotic ceftriaxone improves GLT-1 expression levels in primary cultured astrocytes from GFAP-160Q mice. WT or GFAP-160Q 6-week old primary cultured astrocytes were treated for 7 days with various concentrations of ceftriaxone (50μM or 100μM) in reduced serum media. Western blots show ceftriaxone was able to increase GLT-1 in GFAP-160Q astrocytes to WT levels. Cells left untreated or treated with 250μM dBcAMP served as controls.

Chapter 4

Characterization of a BAC transgenic HD mouse model that expresses mutant htt in astrocytes

Design of the GFAP-BAC 23Q/141Q mouse model was performed by Shihua Li and Xiao-Jiang Li. Chuan-En Wang assisted with RT-PCR data. All other experiments in this chapter were performed by Jennifer Webster Bradford.

4.1 Abstract

The production of bacterial artificial chromosome (BAC) mouse models have been used to mimic endogenous expression levels of transgenes. Due to the large size of BAC vectors, they are able to contain all of the essential regulatory elements for transgene transcription. Because of this, expression levels of most transgenes are thought to be stable and close to that of endogenous genes. Thus, we produced a BAC transgenic HD mouse model that expresses truncated human mutant htt with either WT (23Q) or mutant (141Q) polyglutamine repeats. We placed the htt transgene under the control of the mouse GFAP promoter, which is flanked by the 3' and 5' GFAP regulatory regions, for expression of mutant htt in astrocytes. We were able to produce four mouse lines for both the 23Q and 141Q models. We confirmed production of mutant htt transcript in the brains of the mice, but were unable to detect the presence of mutant protein. These mice lived normally and did not show an obvious phenotype, even in late ages. The lack of transgenic htt expression at the protein level could be due to changes or lack of important regulatory elements in the BAC vector during the construction or cloning process. Despite this, the absence of neurological phenotypes in the BAC mice due to the lack of mutant htt protein expression supports the findings from our GFAP-HD transgenic mouse model that express mutant htt under a shorter GFAP promoter fragment.

4.2 Introduction

Chapters 2 and 3 discuss the characterization of the GFAP-160Q mouse model that expresses mutant htt in astrocytes. This model produced age-dependent HD phenotypes and has glutamate transporter dysfunction. This chapter discusses another HD mouse model that expresses mutant htt in astrocytes: the GFAP-BAC mouse model. The BAC transgenic approach is becoming popular because this technique has the advantage of more natural regulation of the promoter used to drive transgene expression. A BAC HD mouse expressing full length human htt with 97Q under the human htt promoter was recently produced (Gray et al., 2008). This HD BAC mouse exhibited progressive neurological phenotypes and late onset neuropathology (Gray et al., 2008).

We produced our BAC mice by replacing the endogenous mouse GFAP gene with a truncated human htt fragment (208aa) carrying either WT (23Q) or mutant (141Q) repeats. Thus, the htt transgene will be under the control of a large portion of the mouse GFAP promoter in the BAC vector. Instead of using an unstable CAG repeat like we did for the GFAP-160Q mouse model, we used a mixed CAA/CAG repeat for the BAC model because it is known to be more stable than a pure CAG repeat (Choudhry et al., 2001; Dorsman et al., 2002).

While we were able to obtain GFAP-BAC 23Q and 141Q mice that produced the mutant htt transcript, we were unable to detect mutant protein in the brains of these mice. Accordingly, the GFAP-141Q BAC mice did not show an obvious phenotype, as there was no difference in body weight, rotarod ability or survival when compared to the GFAP-23Q BAC mice or WT littermates. The only noticeable difference we saw was the

tendency for weight gain in the 141Q mouse, especially in males. We conclude that the BAC transgene expression levels may be too low to produce a phenotype.

4.3 Methods

Antibodies: Mouse monoclonal antibodies (mEM48) against the N-terminal region (amino acids 1-256) of human htt were described in our previous study (Li et al., 2000; Shin et al., 2005; Yu et al., 2003).

GFAP-BAC Mouse Production: An ~230 kD bacterial artificial chromosome (BAC) (Clone # RP23-117E15) was used to create a mouse model that expresses truncated human htt with either 23 or 141 glutamine repeats (23Q or 141Q) under the control of the endogenous mouse glial fibrillary acidic protein (GFAP) promoter. Mixed CAG and CAA glutamine repeats were used as they are more stable than CAG repeats alone. The cloning of the BAC construct was performed by the German company, Gene Bridges. The GFAP gene was replaced by a cDNA encoding the first 208 N-terminal amino acids of human htt gene in the BAC vector containing the mouse GFAP promoter. The GFAP-BAC 23Q or 141Q DNA was purified using a CsCl gradient preparation method, and 200μg of DNA was digested with ASC1 and separated on a Pulsed Field Electrophoresis Apparatus (Bio-Rad). Inserts containing human htt and GFAP regulatory elements and promoter were further purified with the QIAEX II DNA gel extraction kit. The Emory Transgenic Core Facility injected the DNA at 100μg/ml into FVB mouse embryos. Four 23Q and four 141Q founders were produced from the injections. The founders were confirmed positive for either the 23Q or 141Q transgene by PCR based genotyping. Forward (5'-CCTTCGAGTCCCTCAAGTCC TTC-3') and reverse

(5'TTCTTTCTTTGGTCGGTGCAGC-3') primers were designed to hybridize outside of the mixed glutamine repeats to detect for the presence of the transgene and to differentiate approximate repeat lengths as well. The primers are specific to human htt so that endogenous genomic mouse htt will not be amplified.

Western Blot Analysis: For western blots, cultured cells or brain tissues were homogenized in RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA pH 8.0, 1mM EGTA pH 8.0, 0.1% SDS, 0.5% DOC and 1% Triton X-100) with 1X protease inhibitor from Sigma (P8340). The cell or tissue lysates were diluted in 1X SDS sample buffer (62.6mM Tris-HCl pH6.8, 2% SDS, 10% glycerol and 0.01% bromophenol blue) and sonicated for 10 s after incubation at 100°C for 5 min. The total lysates were resolved in a 4-12% Tris-glycine gel (Invitrogen) and blotted to a nitrocellulose membrane. The western blots were developed using the ECL-Plus Kit (GE Health Care/Amersham).

Behavioral Analysis: Mouse body weight, rotarod ability and survival were measured. The motor function of mice was assessed using the accelerating rotarod test (AccuScan Instruments, Inc.) as described previously (Friedman et al., 2007). In the accelerating rotarod test, each mouse was placed on a rotating cylinder that gradually accelerated to 40 RPM over a 5-min period. Latency to fall from the rotarod was recorded in 3 trials per day over a 3-day period. At least 5 minutes of recovery time was allowed between trials (Friedman et al., 2007).

RT-PCR: Total RNA from mouse cortex was isolated using the RNeasy Lipid Tissue Mini Kit (QIAGEN #74804). First strand cDNA was obtained using the Invitrogen SuperScript™ First Strand Synthesis System for RT-PCR (11904-018). RT-PCR of mouse brain htt and

glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers has been previously described by our lab (Wang et al., 2008).

4.4 Results

We generated a BAC transgenic HD mouse model using BAC clone #RP23-117E15. The mouse GFAP gene was deleted in this clone and was replaced by the first 208aa of human htt with either 23 or 141 mixed polyQ repeats (**Fig. 4.1**). The htt transgene is driven by the mouse GFAP promoter and endogenous regulatory elements. Therefore, the htt transgene should be expressed under relatively normal mouse GFAP levels. We characterized 4 founding lines from both the GFAP-BAC 141Q and 23Q models. Genotyping with primers that flank the polyQ repeat give a clear distinction between the 141Q and 23Q transgene, and WT littermates do not give a PCR band, as mouse htt is not amplified by these primers (**Fig. 4.2**). To detect mutant htt protein, we performed western blotting with brain lysate using anti-htt antibodies (**Fig. 4.3**). No mutant protein was able to be detected via western blotting in any brain region using the mEM48 antibody (**Fig 4.3**) or other commonly used htt antibodies (data not shown). Immunostaining of brain sections also did not show the accumulation in htt aggregates in the brains of GFAP-BAC 141Q mice at the ages of 6 months or 20 months (data not shown).

Because we could not obtain evidence of protein expression via western blotting or by immunostaining of brain sections, we performed RT-PCR to examine whether the transgene was transcribed (**Fig. 4.4**). cDNA was produced from total brain lysate and primers against human htt were used to detect human transcripts in GFAP-BAC mice. GAPDH was amplified as an internal control. The RT-PCR results do show that the

GFAP-BAC transgene is being transcribed even though we can not detect mutant protein (**Fig. 4.4**). Expression levels from BAC-141Q mice are much lower than those from the BAC-23Q mice.

We also analyzed the GFAP-BAC mice for any common HD phenotypes such as body weight loss, decreased rotarod ability and early death. The 23Q and 141Q mice were analyzed for up to 25 months but showed no obvious phenotype. Some male BAC-141Q mice had a tendency to have increased body weight, especially at the ages of 8-12 months. However, there is no significant difference in body weight between BAC-141Q and WT littermates or 23Q controls (**Fig. 4.5**). The BAC-141Q mice showed a decreased ability to perform on the rotarod when compared to BAC-23Q controls (**Fig. 4.6**), which could be attributable to their increased weight. However, there was no difference between BAC-141Q mice and WT mice on the rotarod. BAC-141Q mice did not die earlier than the 23Q controls or WT littermates (data not shown).

4.5 Discussion

There are several possible reasons to explain why we could not detect mutant htt protein in the brains of the BAC mice. First, the expression levels might have been too low due to DNA mutations in the BAC vector that could have occurred during cloning, which may affect correct protein translation or transcript stability. Indeed, the RT-PCR data shows that the expression levels of transgenic mRNA are very low, especially in the BAC-141Q lines. Also, there could have been some sort of repressor element in the BAC construct that we are not aware of that could have inhibited expression. Because the BAC vector was constructed by other investigators at the German company, Gene Bridges, these possibilities remain to be verified.

The lack of overt phenotypes in these BAC mice supports the fact that we did not see mutant htt protein accumulation in their brains. Although these BAC-HD mouse models can not be used for further analysis, they provide another line of evidence to support the fact that the neurological phenotypes seen in our GFAP-160Q mice are due to the expression of mutant htt at the protein level in astrocytes.

Figure 4.1

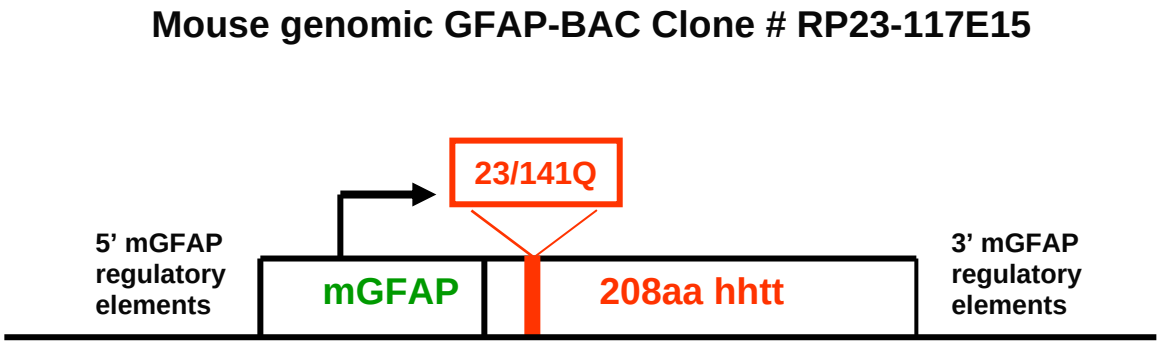

Mouse genomic GFAP-BAC Clone # RP23-117E15
23/141Q
5' mGFAP regulatory elements
mGFAP
208aa hhtt
3' mGFAP regulatory elements

Figure 4.1

GFAP-BAC human htt construct. BAC construct for expressing N-terminal htt (1-208) with either 23Q or 141Q under the control of the mouse GFAP promoter. The mouse GFAP gene was deleted and the human htt cDNA was inserted in its place. The flanking endogenous mouse regulatory elements are also included in the BAC vector.

Figure 4.2

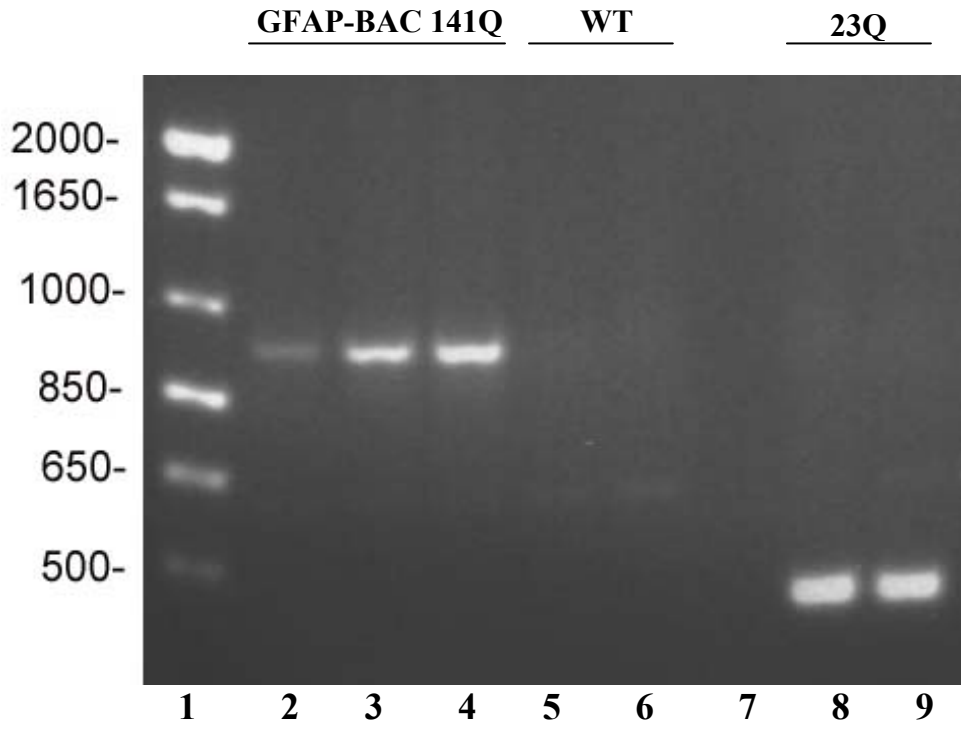


Figure 4.2

PCR genotyping result for GFAP-BAC mice. This is a representative gel showing PCR genotyping data from 3 GFAP-BAC 141Q founder mice (lanes 2-4) and 2 GFAP-BAC 23Q founder mice (lanes 8-9). Two WT mice did not produce a band with human htt primers (lanes 5-6). Lane 7 is empty.

Figure 4.3

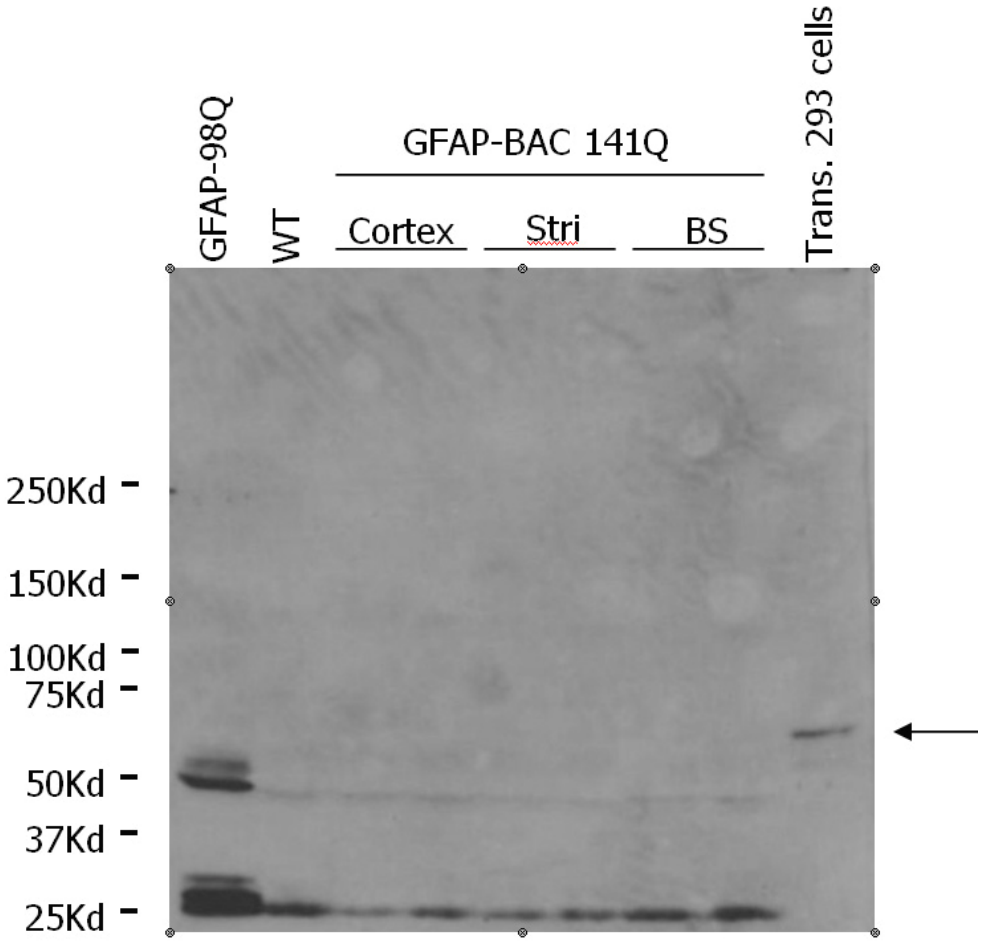

GFAP-98Q
WT
GFAP-BAC 141Q
Cortex
Stri
BS
Trans. 293 cells
250Kd
150Kd
100Kd
75Kd
50Kd
37Kd
25Kd

Figure 4.3

Western blot showing lack of mutant htt protein in BAC mice. mEM48 antibody was used to detect transgenic htt protein in this representative western blot using brain lysate from GFAP-BAC 141Q mice. The arrow indicates a mutant htt band in HEK293 cells transfected with the same mutant htt vector that the BAC-141Q mice were made with. Lane 1 shows GFAP-98Q mutant htt band and serves as a positive control for brain lysate. No mutant htt protein was detected in any region of the BAC-141Q brain. Stri: striatum, BS: brainstem.

Figure 4.4

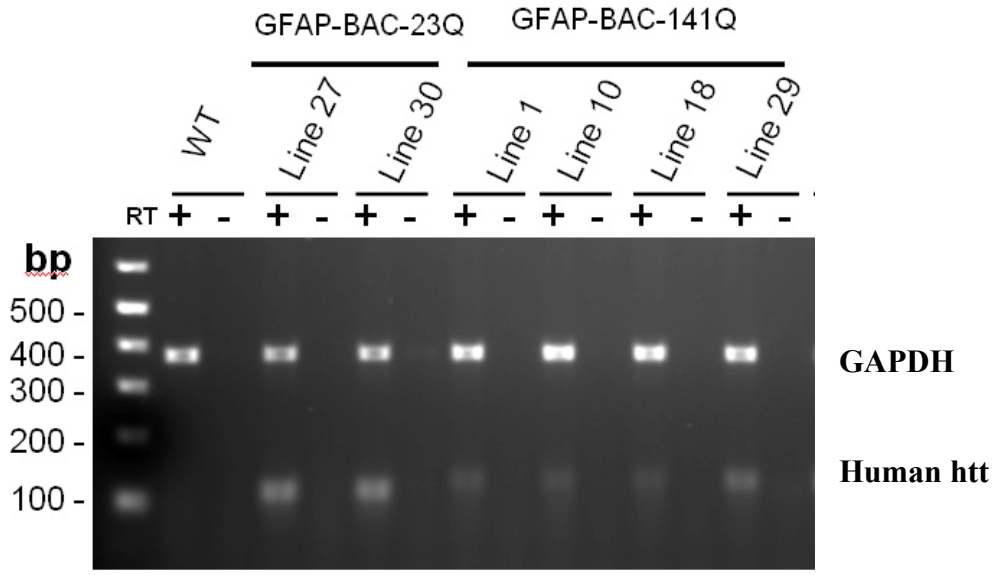

GFAP-BAC-23Q
GFAP-BAC-141Q
WT
Line 27
Line 30
Line 1
Line 10
Line 18
Line 29
RT + - + - + - + - + - + - + -
bp
500 -
400 -
300 -
200 -
100 -
GAPDH
Human htt

Figure 4.4

RT-PCR shows human mutant htt transcript is produced in the GFAP-BAC HD mouse model. Total brain mRNA was collected and reverse transcribed into cDNA for 4 BAC-141Q lines and 2 BAC-23Q lines. Primers to human htt were used to detect the transgene product. Lower expression of BAC-141Q was seen compared to BAC-23Q expression. No product was detected in WT mouse.

Figure 4.5

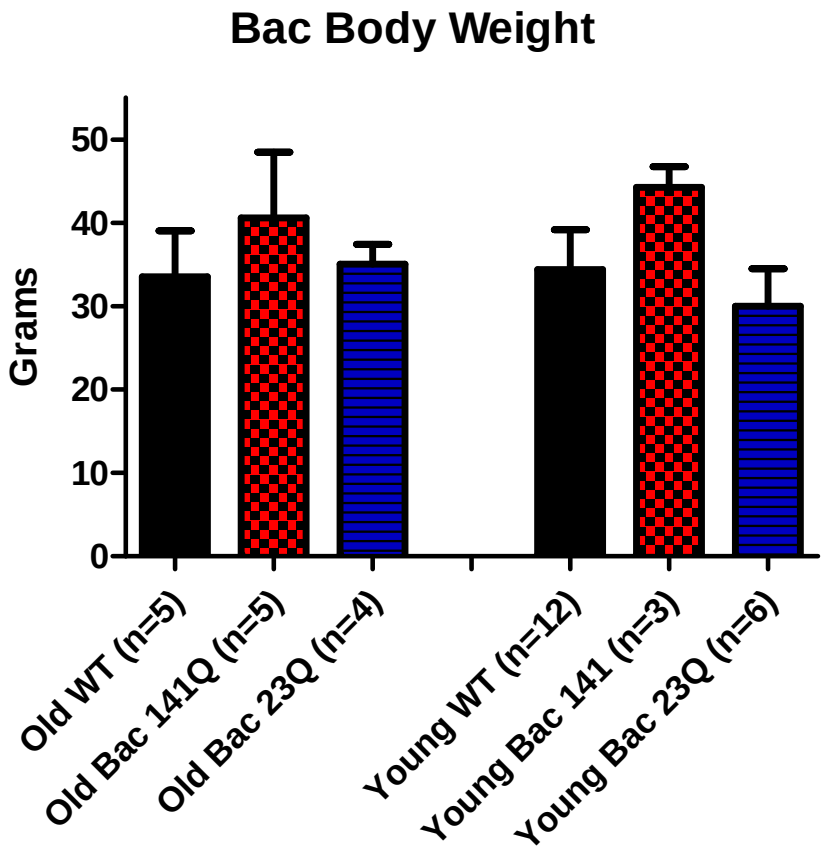

Bac Body Weight
Grams
50
40
30
20
10
0
Old WT (n=5)
Old Bac 141Q (n=5)
Old Bac 23Q (n=4)
Young WT (n=12)
Young Bac 141 (n=3)
Young Bac 23Q (n=6)

Figure 4.5

Body weight analysis of old and young GFAP-BAC mice. Young mice (8-12 months) and older mice (21-25 months) of different genotypes were weighed. We found no significant difference in body weight between GFAP-BAC 141Q mice and either GFAP-BAC 23Q controls or WT littermates at either time point, although BAC-141Q males tended to weigh more.

Figure 4.6

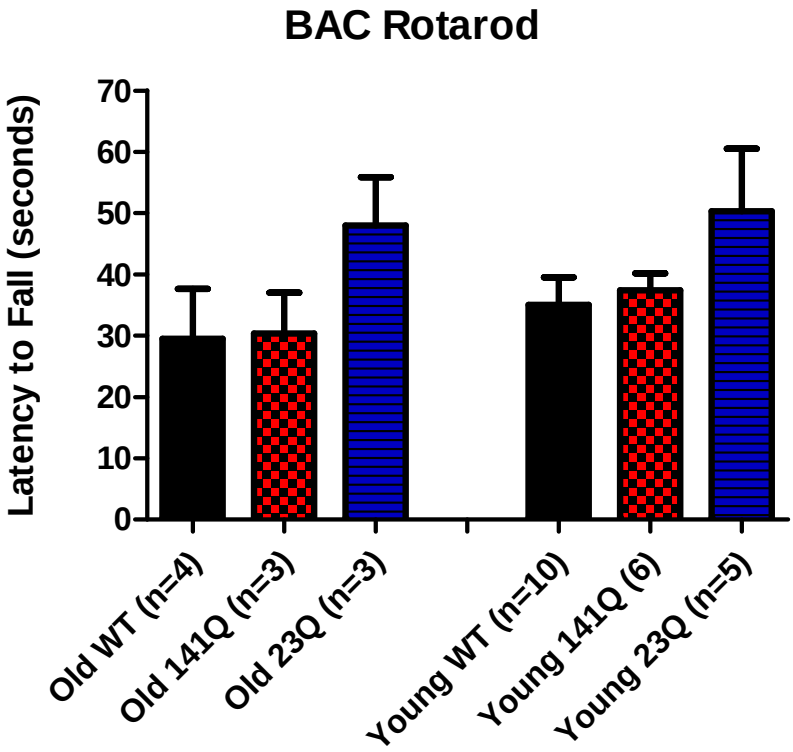

BAC Rotarod
Latency to Fall (seconds)
70
60
50
40
30
20
10
0
Old WT (n=4)
Old 141Q (n=3)
Old 23Q (n=3)
Young WT (n=10)
Young 141Q (6)
Young 23Q (n=5)

Figure 4.6

Rotarod analysis of old and young GFAP-BAC mice. Young mice (8-12 months) and older mice (21-25 months) of different genotypes were analyzed for differences in rotarod ability. We found no difference in the latency to fall between GFAP-BAC 141Q mice and either GFAP-BAC 23Q controls or WT littermates at either age.

Chapter 5

General conclusions and future directions

5.1 General conclusions

In this study, we set out to define the in vivo role that mutant htt plays in astrocytes. Previous in vitro studies in the lab had found that mutant htt is expressed in astrocytes in HD mouse models and HD patient brains. These studies also found that astrocytes that expressed mutant htt had a decreased ability to protect neurons in co-culture from excitotoxicity (Shin et al., 2005). The results from these in vitro experiments and the fact that glia are involved in other neurodegenerative diseases led us to make two different HD mouse models that express mutant htt in astrocytes: the GFAP transgenic model with 23, 98 or 160Q repeats and the GFAP-BAC model with 23 or 141Q repeats. These mice were characterized for expression of mutant htt protein and for neurological phenotypes.

While we were able to detect the production of transgene mRNA in the GFAP-BAC 141Q mouse, we were unable to detect the presence of mutant htt protein in brain by either western blotting or immunostaining. On the other hand, the GFAP-160Q transgenic mouse model proved to be very useful in studying the role mutant htt plays in astrocytes. These mice carried a 208aa fragment of human htt with 160 polyglutamine repeats and expression was driven by the human GFAP promoter (2.1 Kb) in astrocytes. This plasmid promoter has been widely used to drive gene expression in astrocytes in many previous studies (Brenner et al., 1994). The BAC vector, which was constructed by other investigators, remains to be verified for its intact structure after replacing the GFAP gene with human mutant htt cDNA. The lack of phenotypes in the BAC mice can be attributed to the lack of mutant htt protein expression. This supports the association of GFAP-160Q neurological phenotypes with the presence of mutant htt protein, which is

driven by a shorter GFAP promoter. Although the GFAP-160Q mice had low expression of the mutant htt transgene, they develop aggregates that were seen via immunostaining in various brain regions including the striatum, cortex and brain stem. By double immunofluorescence labeling and electron microscopy, we were able to detect the presence of mutant htt in astrocytes, but not other glia or neurons.

Even with low expression levels restricted to astrocytes, the GFAP-160Q mice show a late onset, progressive HD phenotype. Compared to WT and GFAP-23Q control mice, the GFAP-160Q mice have decreased body weight beginning around 17 months, reduced rotarod ability beginning around 12 months, and reduced survival. These phenotypes were seen in all three lines studied and in two different genetic backgrounds, indicating that the phenotypes were specifically caused by the expression of transgenic mutant htt. Moreover, the lack of mutant htt protein expression and neurological phenotypes in BAC-HD mice also clearly indicates that expression of mutant htt proteins in astrocytes is necessary to contribute to HD neurological phenotypes.

One interesting observation was that the GFAP-98Q mice did not have an obvious phenotype, suggesting that a very long repeat (very toxic repeat) is necessary to elicit an HD phenotype when expressed only in astrocytes. This could be due to several reasons. First, astrocytes may be able to clear mutant proteins with a smaller repeat more efficiently than neurons. Evidence for this comes from the fact that in other HD mouse models and human patients, mutant htt aggregates are smaller and less frequently seen in glia than in neurons (Shin et al., 2005). It is also known that astrocytes have higher UPS activity than neurons, and they are capable of dividing, both of which can decrease the amount of mutant protein load in a cell. Astrocytes may naturally express lower levels of

htt than neurons, but this has yet to be verified. Regardless, our studies show that even low levels of mutant htt can still produce late stage HD symptoms.

An important question that we wanted to address in this study was whether mutant htt expression in astrocytes can exacerbate HD phenotypes in a neuronal model of HD. To answer this question, we crossed our GFAP-98Q and 160Q mice to the N171-82Q HD mouse model, which expresses truncated mutant htt primarily in neurons (Schilling et al., 1999a). We found that the additive expression of mutant htt in astrocytes exacerbated the phenotypes in the N171-82Q neuronal model. The double transgenic mice had an earlier decline in body weight and rotarod ability and died earlier than the parental N171-82Q mice. Even though the htt-98Q transgene in astrocytes did not produce obvious phenotypes, expression of this mutant htt fragment can still enhance neurological phenotypes in double transgenic HD mice. These results clearly indicate that mutant htt in astrocytes can contribute to HD pathogenesis.

Electron microscopy showed no obvious cell death in the brains of the GFAP-160Q mice, suggesting that the observed phenotypes were most likely due to cellular dysfunction. However, reactive astrocytosis was observed in both brain tissue and in primary cultured astrocytes from the GFAP-160Q mice, which is an early indication of neurodegeneration, and also demonstrates that mutant htt is negatively affecting astrocytes in our HD mice.

The most important question that we had was to investigate the molecular mechanisms behind the phenotypes in the GFAP-160Q mice. The most logical starting point for our investigation was to determine if the astrocyte glutamate transporter, GLT-1, was dysfunctional in our mice. This transporter has been found to be down-

regulated in other HD mouse models (Behrens et al., 2002; Lievens et al., 2001), and in human HD patients (Arzberger et al., 1997). We first checked transcript levels of GLT-1, the major glutamate transporter in astrocytes, and GLAST, which is known not to be affected in HD. We found that GLT-1 transcript and protein levels were decreased in the brains and primary astrocytes cultured from our GFAP-160Q mice. We also found that GLT-1 specific glutamate uptake was decreased in brains of old GFAP-160Q mice compared to WT littermates. The promoter of the human GLT-1 gene has been previously characterized (Su et al., 2003), which revealed multiple Sp1 transcription factor binding sites that are required for full promoter activity. Our lab and others have reported previously that mutant htt aberrantly binds to Sp1 and sequesters it away from certain Sp1 dependent promoters that are primarily involved in neuron function and survival (Chen-Plotkin et al., 2006). Knowing this, we decided to see if mutant htt expressed in astrocytes could abnormally bind to Sp1 and affect GLT-1 activity. We confirmed an aberrant interaction of mutant htt with Sp1 in primary cultured astrocytes by co-IP. Using a luciferase activity assay, we also found that short mutant htt fragments decreased the activity of the GLT-1 promoter in astrocytes. We next performed a ChIP assay to determine if there was less Sp1 binding at the endogenous GLT-1 promoter in the brains of the GFAP-160Q mice. This experiment revealed that there was in fact less Sp1 binding at the GLT-1 promoter in GFAP-160Q mice than in WT littermates. Decreased Sp1 binding appears to be only at certain promoters, as there was no difference in binding at the PCNA promoter, which is also regulated by Sp1. This finding suggests that, although Sp1 mediates the transcription of a large number of genes, mutant htt may only selectively affect some of these genes. The selectivity is likely dependent on

the context of the promoters and other regulatory elements, as the expression of each gene is regulated by multiple transcription factors whose expression may be cell-type specific. Additionally, the accumulation of mutant htt in different types of cells also varies, which could contribute to the selective effects of mutant htt on the expression of certain genes.

By studying the phenotypes and molecular mechanisms behind the phenotypes in our GFAP-160Q mice, we are able to propose a model of how mutant htt may be affecting astrocytes in HD (**Fig. 5.1**). During the course of HD, mutant htt accumulates in astrocytes to a point where it begins to aberrantly bind to and sequester Sp1 away from specific promoters. Genes that are regulated by Sp1, like GLT-1, will have decreased promoter activity, which results in decreased gene expression. Decreased GLT-1 expression causes a reduction in glutamate transporter-1, which in turn, leads to a decrease in glutamate uptake by astrocytes. If this major glutamate transporter is dysfunctional, a build up of glutamate can occur in the synaptic space (Behrens et al., 2002; Tanaka et al., 1997), which could lead to over stimulation of glutamate receptors on postsynaptic neurons. Over stimulation of glutamate receptors can lead to their desensitization and dysfunction, and eventually death by excitotoxicity. As we did not see cell death in our mice, mutant htt is most likely leading to cellular dysfunction, which is sufficient to cause neurological phenotypes (Davies et al., 1997; Schilling et al., 1999a). Because mutant htt is also normally expressed in neurons, its expression in neurons and glia can additively promote neuron dysfunction and probably cell death.

Our results demonstrate that astrocytes play an important role in HD pathogenesis and could open new approaches for the advancement of therapies. These data clearly

underscore the importance of studying HD outside of neurons, and give support for the cell non-autonomous hypothesis for HD, which states that cells other than neurons contribute to HD progression. Mouse models that express mutant htt in other types of glia or in peripheral tissues will be important in showing that HD pathology is not only caused by neuronal mutant htt, but that HD pathology is also influenced by expression of mutant htt in other types of cells.

5.2 Future directions

The continuation of this project could go in many ways. One of the more interesting projects could be to see if treatment of our GFAP-160Q mice by β-lactam antibiotics, like ceftriaxone, could improve phenotypes. This drug has been shown to effectively improve HD phenotypes in the R6/2 mouse (Miller et al., 2008). The challenge of doing a drug treatment experiment would be figuring out when to treat symptomatic GFAP-160Q mice, because they begin to show phenotypes over a wide time frame. Initiations of phenotypes vary between mouse lines, and even within the same line. This obstacle could be overcome by treating pre-symptomatic mice of the same line and measuring phenotype latency between treated and untreated mice. Our very preliminary results of treating cultured astrocytes with ceftriaxone indicate that this treatment option might be useful.

Another interesting experiment would be to study how expression of mutant htt in astrocytes affects postsynaptic neuronal glutamate receptors. For example, given the reduced glutamate uptake by astrocytes in HD, we could see if there is increased activity of ionotropic and metabotropic receptors on postsynaptic neuronal membranes in the

GFAP-160Q mice. Also, calcium levels could be analyzed to see if the glutamate receptors are properly transmitting signals when a higher glutamate concentration is present in the synapse. As we see no cell death in the GFAP-160Q mice, neuronal dysfunction could be revealed by electrophysiology to determine whether neuronal membrane properties are altered in the HD mice. As MSNs are GABAergic neurons, we could also measure the release of GABA in brain slices of the GFAP-160Q mouse model to see if these neurons are functioning properly.

Based on our analysis of the molecular mechanisms underlying astrocyte dysfunction in the GFAP-160Q mice, we identified that increased binding of mutant htt to Sp1 results in decreased Sp1 association with the GLT-1 promoter, and thus, decreased expression of this transporter. Another study that could further explore this interaction would be to see if mutant htt is aberrantly binding to other transcription factors in astrocytes. The GLT-1 promoter contains other transcription factors binding sites such as NFkB (Su et al., 2003). It would be interesting to know if NFkB or any other transcription factors are sequestered by mutant htt. Such studies would also help us to understand why mutant htt selectively affects the expression of certain genes.

Although astrocytes are the major type of glia in the CNS, it is also important to understand the contribution of microglia and oligodendrocytes to HD. Microglia, in particular, are implicated in neurodegenerative diseases such as ALS, and may play important roles in HD pathogenesis as well. The first step to understanding their role in HD would be to see if human patients and HD mouse models express mutant htt and contain aggregates in these cell types. Our lab is interested in these questions and we have begun initial double fluorescent labeling experiments in the HD 150Q KI mouse

model with antibodies to microglia (F4/80) and oligodendrocytes (MBP) to see if these cells express mutant htt. Use of HD 150Q KI mice would allow us to define whether mutant htt also accumulates in other types of glial cells when full length mutant htt is expressed at the endogenous level. The results from these experiments will dictate whether future mouse models that express mutant htt in microglia and oligodendrocytes should be produced.

Figure 5.1

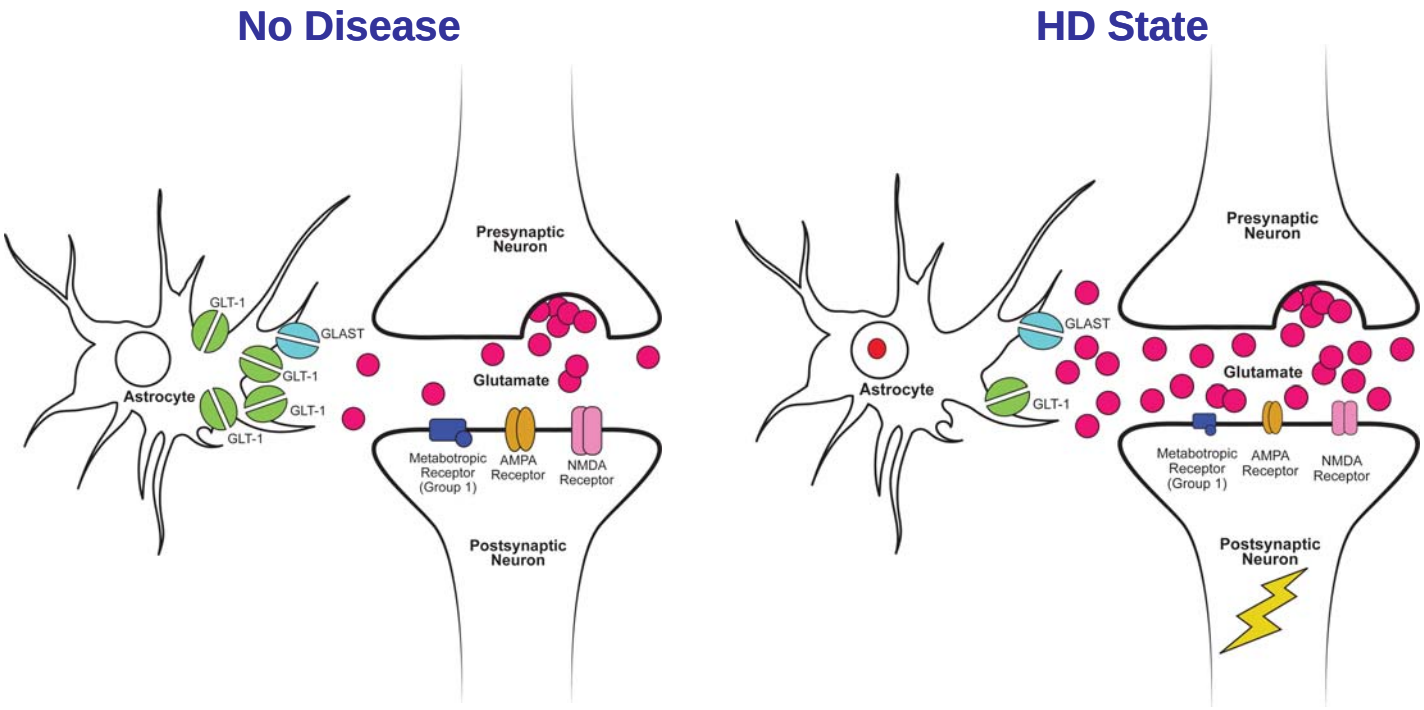

No Disease
HD State
Presynaptic Neuron
Glutamate
GLT-1
GLAST
GLT-1
GLT-1
GLT-1
Astrocyte
Metabotropic Receptor (Group 1)
AMPA Receptor
NMDA Receptor
Postsynaptic Neuron
Presynaptic Neuron
GLAST
Glutamate
Astrocyte
GLT-1
Metabotropic Receptor (Group 1)
AMPA Receptor
NMDA Receptor
Postsynaptic Neuron

Figure 5.1

Model of how mutant htt affects astrocyte function in the CNS. In a normal state, glutamate is released from presynaptic neurons and can bind to and stimulate glutamate receptors on postsynaptic neurons. Excess glutamate in the synaptic space is removed by glutamate transporters, primarily GLT-1, which are located on surrounding astrocytes. In the case of HD, the presence of mutant htt in the nuclei of astrocytes can aberrantly bind to the Sp1 transcription factor. This leads to reduced binding of Sp1 to specific promoters, including GLT-1, and results in decreased GLT-1 expression and protein production. This causes a reduction in GLT-1 glutamate uptake, which can lead to excitotoxicity of neurons and eventually neurological phenotypes.

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