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How the manipulation of the Ras homolog enriched in striatum alters the behavioral and molecular progression of Huntington's disease

A Dissertation

Submitted to the Graduate Faculty of the
University of New Orleans
in partial fulfillment of the
requirements for the degree of

Doctor of Philosophy
In
Applied Biopsychology

by

Franklin A. Lee

B.S. University of Texas at Tyler, 2006
M.S. University of New Orleans, 2010

December 2015

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Acknowledgement

This research project and dissertation has been a long and arduous road, and as such I could not have done it alone. I would like to give a special thanks to my mentor, my friend, and the most interesting man in the world Dr. Jerry LaHoste for helping me get through this. I would like to give special thanks to Dr. Laura Harrison as well who has always been a scientific role model to me, and showed me that even when things don't work out as planned you pick up the pipettes again and keep on rolling. To the rest of my committee members, Dr. Kevin Greve, Dr. Michelle Martel, and Dr. Carl Weems, thank you all for your critical analysis of this work. I would like to give special thanks to Dr. Brandon Baiamonte who spent countless hours learning techniques with me, collecting data, and writing papers. I would like to give a special thanks to all of the undergraduate students Khuong Tran, Mitzi Alfortish, and Peter brown, and one high school student, Alessandra LeDoux, who worked with me on this project. If pride is a sin so be it, but I am proud that each and every one of these students learned to do PCR, cryosection brain tissue, and do immunohistochemistry. This is all stuff I never even dreamed of learning till I got to graduate school, so I am confident that these guys will be awesome scientists one day.

Lastly, I would like to dedicate this dissertation to anyone who has ever and will ever suffer from Huntington's disease. We are working for you.

Table of Contents

| | |
|--|------|
| List of Figures..... | v |
| Terms..... | vi |
| Abstract..... | viii |
| Introduction..... | 1 |
| Background..... | 1 |
| Huntington’s Disease as a Public Health Concern..... | 3 |
| Costs..... | 4 |
| Genetics of Huntington’s Disease..... | 6 |
| Disease Onset..... | 6 |
| CAG Expansion..... | 8 |
| Paternal Transmission..... | 9 |
| Weakness of Diagnostic Criteria..... | 11 |
| Cellular Features of Huntington’s Disease..... | 12 |
| Role of Huntingtin..... | 12 |
| Transcriptional Dysregulation..... | 15 |
| Brain Derived Neurotrophic Factor..... | 19 |
| Mitochondrial Dysfunction..... | 20 |
| Excitotoxicity..... | 21 |
| Aggregates..... | 23 |
| Aggregates as Neuroprotective..... | 29 |
| Rhes..... | 32 |
| The Role of Rhes in Huntington’s Disease..... | 37 |
| Hypothesis..... | 41 |
| Design and Methods..... | 42 |
| Animals..... | 42 |
| Tail Biopsy..... | 42 |
| Polymerase Chain Reaction..... | 43 |
| Gel Electrophoresis..... | 44 |
| Preparation of Brain Tissue..... | 44 |
| Immunohistochemistry..... | 45 |
| Motor Performance..... | 46 |
| Clasping..... | 47 |
| Experimental Design..... | 48 |
| Results..... | 50 |
| Aggregate Formation..... | 50 |
| Motor Performance..... | 51 |
| Clasping..... | 52 |
| Discussion..... | 53 |
| Conclusion..... | 60 |
| Future Studies..... | 60 |
| References..... | 62 |
| Appendices..... | 78 |
| Vita..... | 79 |

List of Figures

| | |
|----------------|----|
| Figure 1 | 50 |
| Figure 2 | 51 |
| Figure 3 | 52 |
| Figure 4 | 53 |
| Figure 5 | 54 |
| Figure 6 | 54 |

Terms

AC – adenylyl cyclase

ADP – adenosine diphosphate

AMPA – α -amino-3-hydroxy-methyl-4-isoxazolepropionic acid

ATP – adenosine triphosphate

BDNF – brain-derived neurotrophic factor

CAAX – amino acid motif consisting of cysteine, any aliphatic amino acid, any aliphatic amino acid, any amino acid

cAMP – cyclic adenosine monophosphate

CREB – cAMP response element-binding protein

DXXD – amino acid motif consisting of aspartate, any amino acid, any amino acid, aspartate

ER – endoplasmic reticulum

GABA – gamma-amino butyric acid

GDP – guanosine diphosphate

GFP – green fluorescent protein

GEFs – guanidine exchange factors

GPCRs – G protein-coupled receptors

GTP – guanosine triphosphate

HD – Huntington's disease

Hdh – Huntington disease homolog

hDNs – huntingtin dystrophic neurites

Htt - Huntingtin protein

htt – huntingtin gene

IgG – Immunoglobulin G

mHtt – mutant Huntingtin protein

mhtt – mutant huntingtin gene

NIIs – neuronal intranuclear inclusions

NMDA – n-methyl-D-aspartate

mTOR – mammalian target of rapamycin

PBS – phosphate buffered saline

PKA – protein kinase A

PKC – protein kinase C

PLC – phospholipase C

polyQ – polyglutamine tract

Q – the one letter convention for the amino acid glutamine

Rhes^{+/+} - two normal (wild type, WT) alleles

Rhes^{+/-} - one WT and one knockout (KO) allele

Rhes^{-/-} - both alleles are knocked out

SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis

SUMO – small ubiquitin-like modifier

Abstract

Huntington's disease is an incurable, progressive neurological disorder characterized by loss of motor control, psychiatric dysfunction, and eventual dystonia leading to death. Despite the fact that this disorder is caused by a mutation in one single gene, there is no cure. The mutant Huntingtin (mHtt) protein is expressed ubiquitously throughout the brain but frank cell death is limited to the striatum. Recent work has suggested that Rhes, Ras homolog enriched in striatum, which is selectively expressed in the striatum, may play a role in Huntington's disease neuropathology. In vitro studies have shown Rhes to be an E3 ligase for the post-translational modification protein SUMO. Rhes increases binding of SUMO to mHtt which competes for the same binding site as Ubiquitin. SUMOylation of mHtt leads to disaggregation and cellular death, whereas ubiquitination leads to aggregation and cellular protection. In a previous study we showed that deletion of Rhes caused a decrease in the Huntington's disease phenotype in mice. We hypothesized that mice lacking Rhes would also show increased aggregation in the striatum and this increased aggregation would correlate in a rescue of behavioral symptoms. Despite the prior in vitro and in vivo evidence, deletion of Rhes in vivo did not alter the aggregation of mHtt in the striatum of mice however deletion of Rhes still showed a rescue from the diseased phenotype. This result would indicate that deletion of Rhes alters the neurobehavioral phenotype of Huntington's disease through a different pathway than promoting aggregation in striatal cells.

Keywords: Rhes, RasD2, Huntington's Disease, Huntingtin, mHtt, aggregate

Introduction

Background

George Huntington first characterized a novel disease in a landmark paper in which he described a 1xpression disorder with a specific genetic inheritance, manifest in mid-adulthood, and leading to insanity (1872). This far predated modern molecular genetics and we now know that Huntington's disease (HD) is an autosomal dominant disorder due to an unstable expansion of the trinucleotide repeat CAG coding for the amino acid glutamine located at 4p16.3 (Huntingtons Disease Collaborative Research Group, 1993). The CAG repeat is naturally occurring at a low frequency; however the threshold for showing symptoms in humans is in the range of 36-41 repeats (Andrew et al., 1993; Kremer et al., 1994; Rubinsztein et al., 1996). Studies have also shown that the length of the repeats is negatively correlated to the onset age of symptoms such that the disease is anticipated early (Duyao et al., 1993). The mean age of onset in HD is difficult to predict but typically is in middle age between 30 and 50 years of age (Langbehn, Brinkman, Falush, Paulsen, & Hayden, 2004) with a range of onset from 5 years of age to 85 years of age (Andrew et al., 1993; S. Chen, Ferrone, & Wetzel, 2002).

HD differentially affects the neurons of the striatum (Dunlap, 1927 ; Vonsattel et al., 1985), despite the fact that the allele is expressed ubiquitously throughout the body (Sharp et al., 1995). HD leads to eventual cell death as evidenced by studies showing severely decreased volume of basal ganglia structures (de la Monte, Vonsattel, & Richardson, 1988; Vonsattel et al., 1985). The cell death is further restricted to the medium spiny neurons (Sapp et al., 2004; Saudou, Finkbeiner, Devys, & Greenberg,

1998) which make up to 95% of the dorsal striatum (Graybiel, Ragsdale, & Edley, 1979), and especially that of the enkephalin and D2-receptor expressing medium spiny neurons (Saudou et al., 1998).

The exact cause of the cell death in HD has not been elucidated, but several factors have been identified that contribute to the process, including aggregation of mutant Huntingtin (mHtt) protein forming intranuclear inclusions (DiFiglia et al., 1997), impaired dopamine signaling (Filloux et al., 1990), decrease in Brain-Derived Neurotrophic Factor (BDNF) transcription (Zuccato et al., 2001), and oxidative stress leading to mitochondrial dysfunction (Browne, Ferrante, & Beal, 1999; Goswami et al., 2006; Polidori, Mecocci, Browne, Senin, & Beal, 1999). Reviewing the literature, it is evident that these processes are not mutually exclusive adding to the complexity of the disease process especially since they precede frank cell death.

The progression of the disease usually begins with the onset of either neurocognitive symptoms and/or psychological changes that precede actual motor dysfunction. Studies have shown early changes in visuospatial functioning, working memory, and set shifting, sometimes several years in advance of motor dysfunction (Foroud et al., 2004; Jason et al., 1988; Rupp et al., 2010). Numerous studies have also shown changes in psychological dimensions such as anxiety, depression, and loss of motivation (Craufurd, Thompson, & Snowden, 2001; Duff, Paulsen, Beglinger, Langbehn, & Stout, 2007; Thompson, Snowden, Craufurd, & Neary, 2002), which often give way to full blown psychosis (Rosenbaum, 1940) and often lead to suicide (Huntington, 1872; Shoenfeld et al., 1984).

Although the psychiatric and cognitive features of HD are very important, the hallmark symptoms for which the diagnosis is made involves motor disturbances. The main motor disturbances of HD are chorea, or involuntary movements, as well as dystonia, or rigidity of muscles. Generally the symptoms are due to loss of motor control beginning slowly with the inability to control minor movement, however; this leads to chorea which manifests as inability to control movement of the eyes, face, limbs, and trunk as well as erratic facial and oculomotor control, problems with ataxia and gait, and twisting or jerking of the limbs (Conneally, 1984; Huntington, 1872; Sturrock & Leavitt, 2010). Dystonia is the inability to move voluntarily and this is characteristic of patients with juvenile onset of HD (Vonsattel & DiFiglia, 1998), as well as in patients with advanced HD that had previously displayed expression movements (Sturrock & Leavitt, 2010). Aspiration pneumonia is the number one cause of death in HD due to eventual loss of the ability to control swallow and esophageal tone (Sorenson & Fenger, 1992).

Huntington's Disease as a Public Health Concern

Given its severe symptomology and prolonged time course, HD exacts a massive toll on the healthcare apparatus. Because the disease is incurable, the treatment options are limited. Treatment plans mostly focus on the management of symptoms through pharmacotherapy. According to two separate meta analysis of 2,620 subjects from 30 separate trials receiving various pharmacological treatments, only one drug, tetrabenazine, was shown to be effective in management of chorea (Mestre, Ferreira, Coelho, Rosa, & Sampaio, 2009a, 2009b). Given the ineffectiveness of

pharmacological treatment, managing the progression of the disease in such a manner as to provide for quality of life becomes the clear goal.

Costs

Utilization and costs are very difficult to estimate in HD. Prodromal symptomatology may go on for years and require treatment well before the patient is diagnosed with the disease. Another problem with estimating utilization and costs are detangling the direct costs of the disease from seemingly unrelated or peripheral treatments, and the problem of trying to estimate the long term care costs, especially when such care is provided at home and utilizes paid but unskilled workers. Family involvement may also indirectly account for costs in the form of lost productivity as a family member is pulled away from work and increasingly into the home to take care of the affected individual. One European study of 451 HD patients shows a large majority of patients utilizing both formal and informal healthcare (Busse et al., 2011). As of initial collection of data, the researchers found that 75% of the patients had used or were using hospital based services, and 89% were using community or outpatient services (Busse et al., 2011). They also showed a trend for an increase in utilization of in home care services (Busse et al., 2011), which is to be expected given the progressive nature of the disease. Even this well executed study does not provide a very clear picture of the progressive nature of the disease. Although utilization of services was measured at three time points, many of the participants were in different stages of the disease process. Given this information, inference from this study should be restricted to overall utilization, and only represents a limited population as the sample was from a registry of patients. No longitudinal studies could be identified that

directly measured patterns of utilization of healthcare services. Given the nature of the disease it is relatively easy to infer that the general course involves years of prodromal treatment, most likely in an outpatient setting. This would be followed by years of more intensive outpatient care with simultaneous home health care. This would again be followed by several years of intensive skilled nursing either in a facility or at the home eventually giving way to palliative or hospice care.

Costs can reflect the amount of services required over the course of the disease. One cross sectional study from 2002 estimated the direct costs of one year of treatment at an average of \$18,000 per patient (Murman et al., 2002). This study also lacks a well-defined approach to control for severity of disease at the time of sampling. It also has a low sample number (51) drawn from an Alzheimers disease registry which may not represent the population of HD patients (Murman et al., 2002). The Huntington's Disease Society of America estimates that some 30,000 people in the United States suffer from Huntington's disease and another 250,000 are at risk (Huntington's Disease Society of America, 2011). An extremely conservative estimate of total direct costs based on the average costs from the study previously discussed, adjusting for inflation and the estimated number of Americans affected, is \$650 million dollars annually. This does not take into account skyrocketing healthcare costs, the number of affected who are not identified, and indirect costs such as family loss of productivity. The Society for Neuroscience estimates the societal costs of Huntington's disease at a staggering \$2 billion dollars annually as of its publication in 2005 (Society for Neuroscience, 2005). Given these numbers, and the severity of the disease process, it is a clear public health concern that necessitates the need to for basic research.

Genetics of Huntington's Disease

The key feature of the huntingtin gene is an area located near the 5' end of the coding segment that is highly polymorphic for a triplet repeat codon of CAG (Huntingtons Disease Collaborative Research Group, 1993). As previously mentioned, CAG codes for the amino acid glutamine, and its repeating code results in a stretch of glutamine residues also known as a polyglutamine tract (polyQ). The presence of the CAG repeat in the huntingtin gene in humans is ubiquitous (Andrew et al., 1993); however there is much variation in the number of repeats occurring in individuals. One of the consistent findings in HD research is that an increase in the number of CAG repeats leads to a quicker disease progression and an earlier age of onset (Andrew et al., 1993; Brandt et al., 1996; Brinkman, Mezei, Theilmann, Almqvist, & Hayden, 1997; Penney, Vonsattel, MacDonald, Gusella, & Myers, 1997; Rosenblatt et al., 2006; Ruocco, Bonilha, Lopes-Cendes, & Cendes, 2008). Age of onset is an important discussion due to the clinical implications of the disease. Understanding the mean age of onset and progression of symptoms can help to provide information not only about the genetic aspects of the disease but also can help to guide timing with interventions that are developed in the future.

Disease Onset

It has been found that the CAG repeat length in the disease accounts for 50% of the variation in the age of onset of the disease (Andrew et al., 1993). Studies have identified cases in which individuals with repeats in the lower range, >35 and <42 repeats, may not ever express pathology during a normal lifetime (Andrew et al., 1993; Rubinsztein et al., 1996). Only one study has identified a case in which Huntington's

disease was contracted with ≤ 35 repeats in which 1 patient with 30 repeats and two patients with 31 repeats were identified (Snell et al., 1993). This may have been the result of very early genotyping of the huntingtin gene resulting in a primer design that did not take into account the flanking CCG repeats. No other cases of HD have been reported with a CAG repeat length of ≤ 35 repeats (Andrew et al., 1993; Kremer et al., 1994; Langbehn et al., 2004; Rubinsztein et al., 1996). This evidence suggests that, even though HD is highly genetically penetrant, it is not 100% certain that the disease will manifest in the lower range of pathological repeats.

Most patients will have an onset of Huntington's disease with a mean age range of 30-50 years (Andrew et al., 1993; Langbehn et al., 2004). Despite this clustering of onset around middle age the ranges of actual cases are quite extreme with one case exhibiting symptoms as young as 5 years of age (Andrew et al., 1993; S. Chen et al., 2002) and others not showing symptom onset until they are elderly (Andrew et al., 1993). Despite the mean, it is interesting that the juvenile onset cases do not always contain more repeats than normal mid-life onset cases (Andrew et al., 1993). Juvenile onset cases tend to present with a different phenotype than that of adult onset cases. Juvenile onset HD also has a different symptom profile as it is most often marked by immediate dystonia and less 7α expression activity as well as a much quicker neuropathological degeneration (Vonsattel & DiFiglia, 1998; Vonsattel, Keller, & Ramirez, 2011). One phenomenon that may be responsible for this exaggerated phenotype is that striatal tissue specifically induces large amounts of instability in the gene, such that there are increased numbers of CAG repeats, in some cases up to a thousand (Kennedy et al., 2003). The longer the original repeat, the more likely it is to

show large tissue specific instability (Kennedy et al., 2003). No evidence supports a current explanation as to why this might occur, but it may very well explain why juvenile cases occur even when the normal DNA sample collected from blood or buccal swab reveals a median CAG repeat length. Also, Kennedy et al. (2003) believe that this might also explain why juvenile onset cases present with immediate dystonia and a more widespread neuropathogenesis than adult onset cases. They propose that the extremely long CAG repeats in the brain impart a more global neurotoxic effect that essentially speeds up the neuropathological process, whereas with median length repeats seen in adults, the pathology is more limited to the striatum and temporally slower in degeneration (Kennedy et al., 2003).

CAG Expansion

Several theories of unstable expansion have been posed. In HD resulting from the expansion of intermediate sized CAG repeats (30-35), one of the key features for unstable expansion was a loss of two triplet sequences which fall in between the CAG_n and CCG_n repeats (Chong et al., 1997). The wild-type sequence normally looks like (CAG)_n CAA CAG CCG CCA (CCG)_n, but when the CAA and CCA are changed to match the CAG and CCG respectively, the net result is unstable expansion (Chong et al., 1997). Several studies utilizing yeast artificial chromosomes have repeatedly found that instability in both CAG and CTG repeats is the result of meiotic recombination (Cohen, Sears, Zenvirth, Hieter, & Simchen, 1999; Jankowski & Nag, 2002; Jankowski, Nasar, & Nag, 2000; Schweitzer, Reinke, & Livingston, 2001). Generally, the repeats form hairpin structures resulting in breaking of the DNA strand, which in turn results in a recombination event in which an expansion occurs (Jankowski et al., 2000).

Another set of studies have found evidence to implicate DNA replication slippage in CAG repeat expansion in which replication that occurs on the lagging strand leads to improper joining of Okazaki fragments that have formed hairpin structures (Freudenreich, Kantrow, & Zakian, 1998; Miret, Pessoa-Brandao, & Lahue, 1998; Schweitzer & Livingston, 1998). Another possibility for increases in length of repeats may result from defective repair of damaged DNA in somatic tissue that has generally been shown to increase with instability over the age range and can be highly varied based on the type of tissue (Fortune, Vassilopoulos, Coolbaugh, Siciliano, & Monckton, 2000; Kennedy & Shelbourne, 2000; Richard, Goellner, McMurray, & Haber, 2000; Telenius et al., 1994). The idea of differential expression based on tissue type is highly important and points to the possibility of epigenetic mechanisms.

Studies have also found that there may be a contribution by the chromosome regulatory factor transcriptional repressor CTCF. This regulator of chromatin structure and insulation has binding sites that flank the CAG repeats of huntingtin gene (Filippova et al., 2001). Another study has shown that binding of transcriptional factor CTCF at its sites adjacent to CAG repeats provides repeat stability through various methylation control abilities, whereas mutant binding sites leads to a much higher rate of CAG instability (Libby et al., 2008). Inability of this epigenetic regulator to properly protect the repeat site from instability may be a possible mechanism for differences in transmission between males and females.

Paternal Transmission

Many studies of HD have found significant correlations with paternal transmission and anticipation such that larger repeats are transmitted via the father to the offspring

and earlier ages of onset are expected via epigenetic imprinting (Farrer, Cupples, Kiely, Conneally, & Myers, 1992; Kremer et al., 1995; Norremolle, Sorenson, & Hasholt, 1995; Ranen et al., 1995; Rooij et al., 1993). In a study of the expansion of CAG repeats, it was found that fathers are much more likely to impart instability to their offspring, with a 21.9% chance of stability compared to mothers with a 36.3% chance of stability, and a much larger 25.2% chance of offspring contracting the CAG repeat (Kremer et al., 1995). The most interesting finding of this study is that in parent-child transmission in the normal population CAG expansion only happened with paternal transmission (Kremer et al., 1995). One study found that in sperm taken from the general population and from those with a family history of sporadic HD, the probability of passing on an expanded allele from an intermediate sized allele (30-35 CAG repeats) is two times greater in the families prone to sporadic HD (Chong et al., 1997). This evidence suggests that there is some epigenetic imprinting which makes males more prone to passing on an expanded repeat than females.

All of these ideas add intriguing support to the idea that males impart more instability to their offspring. Spermatogenesis in males is in an ongoing process throughout the lifespan, whereas in women, the formation of oocytes is a finite process that occurs before birth. These mechanisms all have a much higher probability of producing an expanded repeat due to successive damage in males whereas in females the DNA of the oocyte would be temporally unaffected. Most likely, all of these different mechanisms play a part in the potential for the CAG repeat to expand resulting in instability passed onto to successive generations.

Weakness of Diagnostic Criteria

One of the major weaknesses in the human studies on CAG repeat length and onset age is the lack of well-defined diagnostic criteria. Going over the methods of the major studies of CAG repeat length it is apparent that diagnostic criteria were not necessarily clear. In Andrew et al. (1993) age of onset was defined as “age at which the first clearly defined abnormality was apparent including involuntary movement, psychiatric or cognitive abnormalities, or inability to perform complex hand movements as defined by clumsiness.” This is a very broad interpretation of age of onset. Other studies use a more refined criteria such as Ranen et al. (1995) who defines age of onset as simply “motor disturbance defined as chorea (or, in the absence of chorea, as rigidity), and voluntary movement abnormalities.” Other studies failed to define age of onset altogether (Kremer et al., 1995; Rubinsztein et al., 1996). This lack of well-defined diagnostic criteria can lead to spurious interpretations of the results of these studies. It is now well accepted that cognitive symptoms often precede actual motor dysfunction in HD (Butters, Sax, Montgomery, & Tarlow, 1978; Ho et al., 2003; Lawrence et al., 1998). One approach may require that the genotypes be known prior to diagnosis. In this way it may be more relevant to look for onset of either cognitive or motor symptoms to mark onset as confidence in genetic testing is extremely high. When genotype cannot be inferred prior to diagnosis (e.g. Andrew et al., 1993) choosing a much more rigid diagnostic criteria provides a more useful operational definition and results in much better external validity.

Cellular Features of Huntington's Disease

Role of Huntingtin

As with many other genetic disorders, the classification and function of non-mutant Huntingtin protein (Htt) has largely been overshadowed by the necessity to understand the functions of the disease causing mutant Huntingtin (mHtt). As a result, much of what we know about the function of endogenous Htt is the result of the study of HD. However, one of the key questions that remain regarding HD is whether or not the pathogenesis of the disease is related to a loss of function of the normally functioning protein, or a gain of function of the mutant protein. For this reason it is important to understand the function of the normal protein to put into prospective findings regarding the pathogenesis related to the mutant protein.

The location of the gene was first found to be on chromosome 4 by a large linkage analysis study of individuals affected by HD (Gusella et al., 1983) and subsequently spurred the collaboration of a large group of investigators to identify the location of the responsible gene. This group, using a location cloning approach located the gene, IT15, at 4p16.3 (Huntingtons Disease Collaborative Research Group, 1993). Sequence analysis of the IT15 gene indicated that it was coding for 3,144 amino acids resulting in a \approx 348kD protein with a highly polymorphic area coding for a poly-L-glutamine tract (polyQ) starting at the 18th amino acid from the 5' terminus (Huntingtons Disease Collaborative Research Group, 1993).

The tertiary structure of Htt has yet to be resolved, but this is not surprising given the size of the protein. Much work has been done to understand the structure of the N-terminal region containing the polyQ expansion. X-ray crystallography has shown that

exon-1 of Htt resolves to form an alpha helix for the first 20 residues that gives way to a irregularly structured loop containing the remainder of the polyQ region that is then connected to a polyproline helix (M. W. Kim, Chelliah, Kim, Otwinowski, & Bezprozvanny, 2009). Proline is a special amino acid in that its side chain forms a loop with an unchanging dihedral angle of 60° making it the most conformationally restricted amino acid (Voet, Voet, & Pratt, 2008). This is interesting because it has been found that in expanded polyQ regions present in mHtt, the secondary structure resolves to a beta sheet conformation (Poirier, Jiang, & Ross, 2005). In fact one study has shown that manipulation of the number of prolines following a given number of glutamines can induce either a polyproline helix or beta sheet structure (Darnell, Orgel, Pahl, & Meredith, 2007). This work suggests that the polyproline tract that follows the polyQ region is responsible for preserving the polyQ secondary structure via steric hindrance. Evidence of this was also found by Scherzinger et al. (1997) who produced GST-huntingtin fusion proteins containing different polyQ expansions. Investigation of the cleavage products showed that insoluble aggregates were found for polyQ stretches containing 51 repeats or more, and further that the solubility of smaller products would have been increased by the GST tag suggesting that even smaller mHtt fragments can become insoluble (Scherzinger et al., 1997). Ultrastructural analysis of these insoluble products showed amyloid like plaques highly consistent with a β -sheet structure (Scherzinger et al., 1997). This is important as the difference between not having HD and having the full blown disease is simply 1 additional glutamine molecule from 35 to 36 as was discussed in the section regarding CAG repeats.

The exact function of normal Htt has not yet been identified, but several studies have provided evidence pointing to some potential functions of the protein. Certainly Htt is necessary for proper neurogenesis. Mice with a null mutation for the Huntington disease homolog (Hdh) gene are not viable and show an increase in apoptotic cell death in the ectoderm during differentiation and show abnormalities in development of the basal ganglia (Nasir et al., 1995; Zeitlin, Liu, Chapman, Papaioannou, & Efstratiadis, 1995). This is further supported by the fact that several knock-in and transgenic mouse models of HD are quite viable showing no gross anatomical abnormalities between unaffected controls (Duyao et al., 1995; Fortune et al., 2000; Gray et al., 2008 ; Hodgson et al., 1999; Mangiarini et al., 1996; Wheeler et al., 2002; Yamamoto, Lucas, & Hen, 2000). Mice heterozygous for a null mutation at the Hdh allele interestingly showed an increase in locomotor activity in an open field but a sharp decrease in time spent swimming in the platform quadrant of the Morris water maze suggesting an increase in locomotion and decrease in cognition (Nasir et al., 1995). Another study using a conditional knockout technique found that when Hdh was inactivated in mature mice they displayed deficits in locomotion, increased claspings, tremor, hypoactivity, and a decrease in lifespan of nearly half when compared with mutant controls (*cre/loxp* null/null) (Dragatsis, Levine, & Zeitlin, 2000). Taken together these findings certainly lead to the conclusion that Htt plays a crucial role in neurodevelopment as well as proper motor functioning and cognition.

Evidence regarding the cellular functions of Htt points strongly to a role in the regulation of neuronal vesicles. Htt has been found to be expressed ubiquitously throughout the brain (DiFiglia et al., 1995; Gutekunst et al., 1995). Further investigation

revealed that Htt is expressed in the cytosol and is co-precipitated with a number of proteins associated with vesicles including SV2, synaptophysin, and the 15xpression1515 receptor (DiFiglia et al., 1995). Immunohistochemical analysis revealed that Htt is closely associated with vesicle membranes and microtubules (DiFiglia et al., 1995; Gutekunst et al., 1995). Htt has also been shown to be closely associated with clathrin (Velier et al., 1998) as well as number of other proteins associated with vesicle transport (Block-Galarza et al., 1997), and endocytosis (Kalchman et al., 1997). Although the exact function of Htt has yet to be described, this evidence suggests that it does have a role in vesicle regulation and the secretory pathways. mHtt aggregates have also shown to be involved in the dysregulation in trafficking of both organelles and elements of the secretory system, namely vesicles. Ultrastructural analysis has shown that aggregates in neuronal process can immobilize mitochondria (Chang, Rintoul, Pandipati, & Reynolds, 2007; Orr et al., 2008), prevent dynamin activity by accumulation (Qin et al., 2004), and reduce the movement of vesicles in axons (Gunawardena et al., 2003). This evidence is supported by the fact that Htt has been shown to be associated with secretory machinery as previously mentioned. Loss of function of both the normal role of Htt in secretory pathways and possibility of gain of aberrant function in the same pathways could result in disruption of normal neural transmission.

Transcriptional Dysregulation

One of the contributors to the pathophysiology of HD is undoubtedly the dysregulation of transcription that occurs as a result of mHtt. Transcription is the activation of a series of proteins that are responsible for the coding of mRNA from DNA

and the subsequent translation of proteins in the cell. This process is paramount to the normal functioning of all other cellular processes. Essentially it is what determines which proteins will be expressed in a cell at any given time along the continuum of the organism's life. The complement of proteins present in a cell regulates every cellular function.

mHtt has been shown to interact with the transcriptional process at many levels. Evidence has been found that large groups of mRNAs are downregulated in HD. Downregulation of mRNA through investigation by in situ hybridization or microarray has been shown for a number of neuropeptides as well as neurotransmitters including brain-derived neurotrophic factor (BDNF) (Zuccato et al., 2001; Zuccato et al., 2008), oxytocin, vasopressin, CART, neuropeptide Y, prepro-thyroid-stimulating hormone-releasing hormone, prepro-somatostatin, enkephalin (Kotliarova et al., 2005). Receptors that have been shown to be downregulated include the BDNF receptor TrkB, (Zuccato et al., 2008) D1 and D2 receptors (Augood, Faull, & Emson, 1997; Cha et al., 1998), as well as N-methyl-d-aspartate (NMDA) and α -amino-3-hydroxy-methyl-4-isoxazolepropionic acid receptors (Giralt et al., 2009). Ion channels including Ca^{2+} , Na^{+} , and K^{+} have also been shown to have the same pattern of downregulation (Luthi-Carter et al., 2000). The mRNAs for cytoskeletal proteins protocadherin 20, neurobin 1, and actinin alpha 2 which are involved in regulation of synapses, synaptic plasticity, synapse formation, and dendrite integrity have also been shown to be downregulated (Becanovic et al., 2010). Clearly the presence of mHtt causes widespread perturbations in proper functioning of neural signaling via a net downregulation in the production of mRNA

related to normal neurotransmission at the level of the neurotransmitter, receptor, ion channel, and even the structure of the synapse.

One of the striking characteristics of this downregulation is the fact that it is not necessarily due to downstream regulation caused by inefficient or improperly functioning signaling cascades. mHtt has been shown to act as a regulator of transcription factors themselves (Benn et al., 2008; Hu, McCaw, Hebb, Gomez, & Denovan-Wright, 2004; Steffan et al., 2000) and in some cases act on the DNA directly to alter transcription (Benn et al., 2008; Sadri-Vakili et al., 2007; Steffan et al., 2001). Evidence points to several possible mechanisms for the ability of mHtt to interact with transcriptional regulation. One possibility is that aggregated mHtt acts as a molecular net to sequester transcription factors in the cytosol thus leading to decreased transcription (Huang et al., 1998; Nucifora et al., 2001; Shimohata et al., 2000). One of the properties of the polyQ expansion is the formation of the β -sheet structure as described earlier. This β -sheet conformation allows for a unique interaction between strands in the sheet such that hydrogen bonds are formed between them producing what has been termed the polar zipper (Perutz, 1995). This conformation, especially when formed into a large regular structure, would have the unique ability to hydrogen bond with other proteins that also possess a polyQ region. This also has the consequence of making mHtt fragments complimentary to the promoter regions that are bound by these polyQ containing transcription factors thus potentially blocking them or activating them inappropriately.

However, evidence has also been found that some of the more ubiquitously affected transcription factors including cAMP response element-binding protein (CREB)

and TATA-binding protein showed no co-immunoreactivity with mHtt aggregates suggesting other possible mechanisms (Yu, Li, Nguyen, & Li, 2002). Another more interesting mechanism for the transcriptional dysregulation is the idea that mHtt acts directly on DNA. Studies have found that mHtt can associate directly with DNA especially at the level of promoters thus effectively blocking transcription factors (Benn et al., 2008; Cui et al., 2006 ; Kegel et al., 2002). It has also been shown that mHtt may act at the level of histones to affect transcription. Studies have found that mHtt acts to block the site of histone acetyltransferases (Igarashi et al., 2003; Steffan et al., 2000), and also that mHtt increases the presence of monoubiquityl histone 2A which is responsible for methylation of histone H3 (M. Kim et al., 2008). Histones are proteins that are integral in the arrangement of DNA such that they function as substrates in which genetic material is wrapped around to form chromatin (Cheung, Allis, & Sassone-Corsi, 2000). Through modifications such as phosphorylation, acetylation, and methylation, histones act to regulate the transcriptional availability of genetic material by binding or unbinding it allowing for the availability of promoter regions to be bound by transcription factors (Cheung et al., 2000). By blocking the acetylation (Igarashi et al., 2003; Steffan et al., 2000) or increasing the methylation (M. Kim et al., 2008) of histones, mHtt can cause large areas of DNA to be unavailable to transcription factors thus downregulating transcription. One of the most promising treatments recently under review for use in HD is the use of histone deacetyltransferases. These drugs have shown a number of benefits in reduction of symptomology both at the behavioral and anatomical level in HD models (Bates, Victor, Jones, Shi, & Hart, 2006; Ferrante et al., 2003 ; Steffan et al., 2001).

Brain Derived Neurotrophic Factor

The most researched of the transcriptionally dysregulated proteins is that of the neuropeptide BDNF. BDNF is a trophic factor which is critically important in the differentiation and survivability of neurons (Barde, Edgar, & Thoenen, 1982; Jones, Farinas, Backus, & L.F., 1994; Korsching, 1993) and has been localized in both the nigrostriatal and corticostriatal pathways (Hyman et al., 1991; Ivkovic, Polonskaia, & Ehrlich, 1997; Yurek, Lu, & Hipkens, 1996 ; Zuccato et al., 2001). BDNF is produced in cortical neurons that project to the striatum providing trophic support and it has been shown that Htt upregulates the production of BDNF in cortical neurons projecting into the striatum (Zuccato et al., 2001). Htt has also been shown to be involved in the proper trafficking of BDNF containing vesicles along microtubules in cortical neurons that project into the striatum (Gauthier et al., 2004).

As Htt has been shown to affect regulation of BDNF so has mHtt been shown to disrupt regulation of BDNF. Levels of BDNF transcription are severely downregulated in animal models of HD and humans suffering from HD and (Ciammola et al., 2007; Ferrer, Goutan, Marin, Rey, & Ribalta, 2000; Zuccato et al., 2001; Zuccato et al., 2005; Zuccato et al., 2008). Post mortem examination of human HD brains has shown that there are reduced populations of the TrkB receptor in caudate and an upregulation in the low affinity neurotrophin receptors T-Shc and p75^{NTR} suggesting impaired BDNF signaling (Zuccato et al., 2008).

Another promising therapeutic intervention is the replacement of BDNF. Studies have found that in models of HD, upregulation of BDNF expression or replacement of BDNF through exogenous introduction causes a decrease in loss of striatal neurons, improved synaptic function, increased synaptic plasticity, and reversal of the HD phenotype (Bemelmans et al., 2004; Cepeda et al., 2004; Duan et al., 2008; Gharami, Xie, An, Tonegawa, & Xu, 2008; Kells et al., 2004; Peng et al., 2008; Simmons et al., 2009; Y. Xie, Hayden, & Xu, 2010). Interestingly, the selective serotonin reuptake inhibitor sertraline has been shown to accomplish this upregulation of BDNF (Duan et al., 2008; Peng et al., 2008). This research on BDNF is exemplary of the fact that Huntington's disease is both the interruption of normal Htt functioning and the gain of aberrant functioning of mHtt in that one normal transcript is not enough to overcome the gain of aberrant function of mHtt.

Mitochondrial Dysfunction

Mitochondria are organelles located in the cytoplasm that are responsible for cellular respiration which produces the main energy source of the organism; adenosine triphosphate (ATP). Mitochondria themselves most likely evolved in eukaryotes as a symbiotic prokaryotic organism as they have distinct features of prokaryotic cells including a membrane, inner and outer membrane environments, membrane potentials, DNA, and the ability to express proteins (McBride, Neuspiel, & Wasiak, 2006). Mitochondria not only function to produce the energy currency of the body, ATP, they also play a crucial role in intracellular Ca^{2+} regulation, and are intricately involved in intracellular signaling events (McBride et al., 2006; Rizzuto et al., 2009). The striatum is particularly vulnerable to perturbations of mitochondria as several studies have shown

that disruptions in mitochondrial function can lead to movement disorders and striatal degeneration (Martin et al., 1988; Sarzi et al., 2007; Spruijt et al., 2007).

Another important finding in HD research is that mHtt interacts with cellular mitochondria causing their dysfunction. mHtt N-terminal cleavage fragments have been shown to associate with the mitochondrial membrane and endoplasmic reticulum (ER) (Choo, Johnson, MacDonald, Detloff, & Lesort, 2004; Rockabrand et al., 2007).

Mitochondria are responsible for the proper storage of Ca^{2+} and regulate the intracellular Ca^{2+} concentration (McBride et al., 2006), but that has been shown to be disrupted with the presence of mHtt (Panov et al., 2002; Rockabrand et al., 2007; Seong et al., 2005). mHtt associates with the mitochondrial membrane destabilizing the membrane potential across the mitochondria causing poor Ca^{2+} permeability, and as a result of increased NMDA Ca^{2+} conduction, which is a separate effect of mHtt (Cepeda et al., 2001; Sun, Savanenin, Reddy, & Liu, 2001), causes excitotoxic cellular conditions (Gellerich et al., 2008; Milakovic, Quintanilla, & Johnson, 2006). A similar consequence of altered membrane potential is that it prevents the uptake of adenosine diphosphate (ADP) thus directly altering cellular respiration (Seong et al., 2005). Another mechanism of mitochondrial dysfunction is suggested by evidence that 2 subunits of the enzyme succinate dehydrogenase, which is critical to cellular respiration, are severely reduced in a striatal-specific manner in both humans suffering from HD and in HD models (Benchoua et al., 2006). Eukaryotic cells must contain properly functioning mitochondria in order to survive, and this evidence suggests that disruptions of mitochondrial function by mHtt may be a cause of neuronal loss in HD.

Excitotoxicity

Another possible cause for the neural degeneration seen in HD may be due to excitotoxicity. Much of the input to the striatum is the result of five parallel circuits that connect the frontal cortex to the striatum via glutamatergic efferents (Alexander, DeLong, & Strick, 1986; Roberts & Anderson, 1979; Tekin & Cummings, 2002). Early experiments showed that overstimulation of striatum with glutamate or the NMDA receptor agonist quinolic acid could indeed cause neurodegeneration (Foster, Collins, & Schwarcz, 1983; Mangano & Schwarcz, 1982; McBean & Roberts, 1984). In fact, quinolic acid has been used extensively to model HD in animals due to its ability to mimic the striatal neurodegeneration that existed in HD especially before genetic models of HD were designed (Beal et al., 1986).

As pointed out in the section above on mitochondrial dysfunction, mHtt has shown to be responsible for an increase in the magnitude of NMDA receptor-mediated calcium influx (Cepeda et al., 2001; Sun et al., 2001). Part of this increase in calcium conductance has been linked to an upregulation in the expression of the NR2B subunit of the NMDA receptor (Arzberger, Krampfl, Leimgruber, & Weindl, 1997; Li et al., 2003). Cells with an over-expression of the NR2B subtype of the NMDA receptor show much larger evoked potentials in cells expressing mHtt (N. Chen et al., 1999). In models of HD, it has also been shown that cells expressing larger numbers of NR2B type NMDA receptors show larger NMDA receptor-mediated currents than wild type animals (Shehadeh et al., 2006; Zeron et al., 2002). Studies in which intervention to down-regulate the expression of the NR2B subunit type of NMDA receptor or block it with NR2B antagonist prevents cell loss (Shehadeh et al., 2006; Zeron et al., 2002). This

net gain in calcium influx, along with calcium dysregulation by mitochondria, provides a sound hypothetical means for cellular death.

Aggregates

One of the earliest reported neuropathological findings in HD other than degeneration of the striatum was the presence of nuclear membrane indentations in striatal neurons observed through electron-microscopical ultrastructural analysis techniques (Bots & Bruyn, 1981; Roizin, Stellar, & Liu, 1979). It wasn't until 18 years later that the first studies found the cause of the indentations.

Not long after the discovery of the sequence for the huntingtin gene, researchers began trying to localize the Htt protein in the brain using immunohistochemical methods. In 1995, Marian DiFiglia and colleagues developed the first set of polyclonal antibodies to Htt. They generated, in rabbits, antibodies raised to amino acid sequence regions of the Htt protein at residues 1-17, 585-745, and 2911-3140 resulting in purified antibodies Ab1, Ab585, and Ab2911 respectively (DiFiglia et al., 1995). All three antibodies showed highly specific labeling for Htt as verified by SDS-PAGE and western blot analysis which confirmed specificity for a protein of 320kD (DiFiglia et al., 1995). Immunohistochemical techniques showed that Htt was expressed ubiquitously throughout the brain of both humans and rats (DiFiglia et al., 1995). Both ultrastructural

analysis as well as fractionation techniques revealed proximity of Htt in patches of the cytoplasm, membranes of vesicles, golgi, microtubules associated with vesicles, in the cell body, dendrites, axons, and axon terminals (DiFiglia et al., 1995). At the very same time another group of researchers were carrying out a nearly identical experiment. Sharp et al. (1995) raised polyclonal antibodies to the N-terminal region as well as to an internal area of the Htt protein from residues 650-663. Using the exact same methodology as DiFiglia et al. (1995), they were able to draw the same conclusions regarding the expression of Htt as well as its intracellular localization (Sharp et al., 1995). This was an important discovery. It showed that Htt is expressed throughout the brain which raised the ever more important question of why gross degeneration was largely confined to the striatum in HD.

Not long after this study was published a group of researchers, using a monoclonal antibody raised against 38 Q residues in the TATA-binding protein, was able to show specific binding to mHtt using western blot analysis (Trottier et al., 1995). However the mAB1c2 antibody bound polyQ stretches in other proteins rather indiscriminately, and because it was raised against a longer stretch of polyQ, had very poor return for repeats under the number of 40 and increasing signal with increasing repeat number (Trottier et al., 1995).

In 1996, Mangiarini and colleagues made the next big technical advance in the pathway to the discovery of mHtt aggregates. They created the R6/1 and R6/2 lines of HD transgenic mice using the human promoter and exon-1 of the HD gene (Mangiarini et al., 1996). cDNA was generated using E.Coli and this was microinjected into single cell CBAXC57B/L6 embryos (Mangiarini et al., 1996). Using this technique the

researchers created two viable lines, R6/1 and R6/2, with the former containing 116 CAG repeats and the latter containing 144 CAG repeats (Mangiarini et al., 1996). The R6/1 and R6/2 model both showed a robust behavioral, anatomical, and neuropathological phenotype beginning at 4 months and 2 months of age respectively (Mangiarini et al., 1996). Behaviorally the mice showed a progressive onset movement phenotype including limb dyskinesia and tremor (Mangiarini et al., 1996). They also showed a progressive onset of weight loss and increase in urinary incontinence (Mangiarini et al., 1996). Neuropathologically the model showed a decrease in brain weight, and a tendency to undergo handling induced epileptic seizures (Mangiarini et al., 1996). The R6/1 and R6/2 model of HD display the most comprehensive phenotype of any of the developed transgenic or knock-in models of HD produced thus far, and as such has been used most extensively of all the models in HD research. Development of this model would play a key role in not only the discovery of aggregates but also in the path of HD research.

In 1997 Davies and colleagues set out to determine the neuropathological characteristics of the R6/1 and R6/2 transgenic models created by Mangiarini (1996). Reverse transcription PCR showed ubiquitous expression of the transgene in both lines as consistent with previous findings regarding expression of Htt in humans (Davies et al., 1997). Using the both N-terminal antibodies described above and the mAB1c2 antibody, western blot analysis was able to detect the presence of large protein fragments consistent with the size of exon-1 of mHtt (Davies et al., 1997). Using immunohistochemical methods the researchers discovered densely stained circular “inclusions” in the nuclei of neurons of the striatum, cortex, cerebellum, and spinal cord

with a much lower incidence in the hippocampus, thalamus, globus pallidus, and substantia nigra of the R6/1 and R6/2 models (Davies et al., 1997). Ultrastructural analysis showed that the inclusions formed a distinct area within the nucleus separate from the nucleolus, and occasionally transited the nuclear membrane (Davies et al., 1997). For this reason the researchers coined these structures as neuronal intranuclear inclusions (NIIs) (Davies et al., 1997). Secondary staining with toluidine blue in the striatum shows the NIIs as a circular plate like granular structure that is larger than the nucleolus and occurs in 1 in 5 neurons (Davies et al., 1997). Ultrastructural analysis also revealed a sharp increase in the number of nuclear membrane indentations and increase in the number of nuclear membrane pores (Davies et al., 1997). Staining for ubiquitin also showed that NIIs contain a large amount of ubiquitin (Davies et al., 1997). Formation of NIIs happened within 3.5 weeks of age in the cortex and 4.5 weeks of age in the striatum of R6/2 mice, with a pattern of increasing size and density for neurons of the cortex, striatum, cerebellum, and spinal cord (Davies et al., 1997). In the striatum the researchers failed to find NIIs in large cholinergic interneurons or NADPH-diaphorase-containing interneurons (Davies et al., 1997). This study was the first to demonstrate in vivo aggregation of mHtt, and further describe its characteristic localization to the nucleus. The implication of this finding is that the aberrant aggregation of mHtt protein fragments participates in some gain of pathological function in the affected neurons. Transcriptional dysregulation seems to be a major pathological mechanism associated with NIIs.

One month later this group published a follow up study using similar methods in human HD brain tissue (DiFiglia et al., 1997). Using the Ab1 antibody which is specific

for the N-terminal segment of Htt and mHtt, the researchers found significant distribution of NIIIs in the striatum, and all layers of the cortex, but not in globus pallidus or cerebellum in patients with HD when compared with control brains (DiFiglia et al., 1997). NIIIs were again found to be prominent inside of the nuclear membrane and larger than the nucleolus (DiFiglia et al., 1997). Another important discovery was that processes were also labeled with aggregates assuming a spherical or ovoid shape in the neuropil of the cortex (DiFiglia et al., 1997). These areas were coined huntingtin dystrophic neurites (hDNs) (DiFiglia et al., 1997).

Attempts to stain these aggregates with the antibody Ab585 targeted at an internal segment of mHtt failed, suggesting that the aggregates are formed from a cleavage product of the full length mHtt protein containing only the N-terminal region (DiFiglia et al., 1997). Follow up western blot analysis found that in human HD cortical homogenates Ab1 detected a 350kD product consistent with polyQ mHtt, as well as a 40kD product that was not present in controls (DiFiglia et al., 1997). In an earlier study, researchers subjected mHtt expressing cells to cytosolic extracts of chicken hepatoma cells in apoptosis (Goldberg et al., 1996). Using a substrate analog inhibitor they were able to block mHtt cleavage by apopain (also known as caspase-3) proteases where others had no effect (Goldberg et al., 1996). Further using the same technique in cells expressing truncated mHtt cDNAs they determined that the protease cleaved mHtt in the N-terminal region, which contains multiple DXXD recognition sites for apopain leading to several different cleavage product sizes (Goldberg et al., 1996). One of these products was approximately 50kD, which is very similar to the product size found by DiFiglia et al., (1997). In mammalian cells expressing mHtt with variable polyQ

lengths, apoptosis was induced, and the researchers found that an increase in polyQ length increased dramatically the rate of apoptosis induced cleavage of mHtt (Goldberg et al., 1996) suggesting the idea that longer polyQ expansions lead to secondary structures in which the amino acid cleavage sites, DXXD, are more readily exposed to the protease. Treatment of R6/2 mice with the caspase-3 inhibitor minocyclin has been shown to delay the onset of rotarod performance deficits and increase life span by 14% when compared with controls (M. Chen et al., 2000). Apoptain has been shown to be necessary for proper apoptosis in mammalian cells through ubiquitin mediated lysis (Nicholson et al., 1995).

A more recent study has shown that apoptain (caspase-3) may not be necessary for improper neuronal degeneration. Lines of mice were generated expressing a full length human mHtt gene via the introduction of a yeast artificial chromosome that had either its caspase-3 or caspase-6 cleavage sites altered as not to be recognized by the proteases (Graham et al., 2006). Results show that caspase-3 resistant mice do not differ significantly from controls in neurodegeneration in the striatum, formation of NInIs, or performance in motor tasks (Graham et al., 2006). However, mice expressing mHtt resistant for caspase-6 cleavage site showed no degeneration in the striatum, motor performance on par with non-carrier HD mice, and morphologically different aggregate formation (Graham et al., 2006). This study was carried out to 10 months for all models involved including HD non-carriers and the YAC128 (HD53) model of HD. It should be noted that mortality times were not reported, however YAC128 (HD53) which were essentially used as controls in this study have a much longer life expectancy than R6/2 models. On the one hand this can also be considered a strength of this study. Given

the limited life-span of R6/2 models (Approximately 3-4 months), the experimenters were able to show that resistance to caspase-6 cleavage essentially produced a phenotype similar to non-carrier mice for an extended time period. This line of research implicates the importance of cleavage of mHtt. On the one hand mHtt can be cleaved by different proteases including apopain (caspase-3) (Goldberg et al., 1996), as well as calpain (Y. J. Kim et al., 2001), and most likely others that have yet to be identified. Each proteolytic event can cause different sized N-terminal fragments, and thus can regulate the behavior of the aggregate formation. As pointed out by Graham et al., (2006) other fragments not cleaved at the caspase-6 sites were still present in the nucleus, however the phenotype suggested no dysfunction. This finding may suggest that regulation of mHtt cleavage may be responsible for formation of toxic fragments versus products that may have no effect or are further targeted for degradation in the cell.

Aggregates as Neuroprotective

One of the biggest questions in HD research is the functional consequence of aggregate formation. On the one hand there are copious amounts of research to indicate that the formation of aggregates is pathological and one of the main reasons for the dysfunction and neurodegeneration seen in HD. However, there are lines of evidence that also suggest that formation of aggregates is a neuroprotective response.

One group of researchers transfected cultured striatal neurons with mouse N-terminal fragments containing either 17 or 68 Q repeats (Saudou et al., 1998). They found that there was an increase in NIIIs in cells that had been exposed to trophic factors but that this resulted in a much lower rate of cellular apoptosis compared with

cells that did not receive trophic support (Saudou et al., 1998). This line of evidence shows an increase in location of aggregates to the nucleus with no increase in cell death.

In another study, researchers created an automated microscopy procedure to follow striatal neurons in culture that had been transfected with GFP-tagged N-terminal mHtt fragments (Arrasate, Mitra, Schweitzer, Segal, & Finkbeiner, 2004). They found that cells transfected with mHtt N-terminal fragments die in a fashion that is dependent upon length of Q repeat, such that longer polyQ expansions lead to a quicker cell death, which was expected (Arrasate et al., 2004). However, they also found that neurons forming inclusion bodies (authors choice of terminology, analogous to NIIIs) were less likely to die, and that cells with a diffuse fluorescent return for N-terminal fragments were much more likely to die quickly (Arrasate et al., 2004). This would suggest that the aggregation and formation of NIIIs would play some part in potentially protecting the cell from mHtt.

The researchers responsible for creating the YAC128 mouse model of HD which expresses a full length human htt gene under the control of the human promoter, also inadvertently created a mouse expressing exon-1 and exon-2 of the same gene through accidental truncation of the yeast artificial chromosome during recombination (Slow et al., 2005). This ShortStop model was found to widely express the N-terminal fragment, and at 12 months of age showed NIIIs in 95% of neurons of the striatum (Slow et al., 2005). However, when compared to YAC128 mice, the ShortStop mice showed no neurodegeneration, no detriment to rotarod performance, and a much longer life span (Duan et al., 2008). Despite the very robust formation of NIIIs these mice did not display

the same phenotype of mice created in a similar fashion that express the full length mHtt gene. The authors suggest that maybe this is the inability of the ShortStop specific fragment to undergo caspase cleavage at a site that will in turn cause a toxic cycle (Slow et al., 2005). This is important because it shows that N11s do not necessarily signal cell death.

Clearly the biggest argument for a neuroprotective role of aggregates is quite obvious in that the expression of mHtt in the brain is ubiquitous, however many parts of the brain are spared whereas the striatum, and to a lesser degree the cortex, show the greatest amount of neurodegeneration. Why then are other areas of the brain not affected by HD? For the sake of current research in HD, it is beneficial at this point to assume that both possibilities are true. On the one hand the formation of aggregates is a cellular response that is an attempt of the cell to deal with toxic mHtt or toxic mHtt fragments. This would serve multiple useful purposes in the cell including removing mHtt from the cytosol in turn limiting its interference with mitochondria and secretory structures, and providing an aggregated target for degradation via proteolysis. On the other hand, it may be that the cell cannot properly regulate the lysing of the proteins, and by transporting the large aggregates into the nucleus, it begin to cause further disruptions to transcription. Support for why this may be the case might come by the investigation of the small GTPase Rhes which is selectively expressed in the striatum.

Rhes

Ras homolog enriched in striatum (Rhes) is a small GTP binding protein that is heavily expressed in the striatum and has a high degree of nucleotide conservation with the Ras superfamily of small GTPase proteins (Errico et al., 2008; J. Falk et al., 1999; Harrison & LaHoste, 2006a). Ras superfamily members act as cellular switching mechanisms that interact with other effector proteins to change specific cellular messaging systems within the intracellular cytoplasmic domain (Campbell, Khosravi-Far, Rossman, Clark, & Der, 1998). Ras superfamily members have been studied extensively for their role in the activation of the mitogen-activated protein kinase/extracellular signal-related kinase (MAPK/ERK) signaling pathway which is intimately involved in cell proliferation and implicated in the mechanics of many cancers (Bos, 1989; Campbell et al., 1998; Rodriguez-Viciana, Sabatier, & McCormick, 2004). However, despite the large body of research conducted on the relationship between the MAPK/ERK signaling pathways and small GTPases, Rhes has not been shown to affect MAPK/ERK signaling (Vargiu et al., 2004).

Rhes was originally characterized by Falk and colleagues after the discovery of its very closely related family member Dexras1/AGS1 (1999). In bacterial cells expressing Rhes, these researchers determined that Rhes did bind GTP likely giving it intrinsic GTPase activity, and that it was induced by thyroid hormone unlike its close

relative Dexras1, which is induced by dexamethasone. This group also made the observation that like Dexras1, Rhes also contained an unusually long c-terminus, the part of the protein that is known to associate with other proteins, making it likely that these proteins possess unique but similar signaling characteristics (J. Falk et al., 1999).

Vargiu and colleagues further characterized Rhes (2004). *In situ* hybridization was performed and it was determined that the Rhes mRNA was heavily expressed in the striatum, but to a lesser extent in the nucleus accumbens, olfactory bulb, olfactory tubercle, piriform cortex, hippocampus, anterior thalamic nuclei, inferior colliculus, cerebellum, cortex, and outside of the CNS in the thyroid. A later study provided evidence that expression of Rhes is determined by dopamine expression. By experimentally lesioning dopaminergic projections, a decrease in Rhes mRNA was measured in the caudate putamen and shell of Nac suggesting that Rhes expression is regulated by dopaminergic tone and also that supersensitivity of dopamine receptors correlates with Rhes mRNA downregulation (Harrison & LaHoste, 2006b).

By attaching a GFP (green fluorescent protein) to the CAAX terminal, an area of proteins susceptible to membrane binding, researchers were able to determine that Rhes is targeted to the plasma membrane *in vitro*. Through a series of pharmacological interventions it was also determined that farnesylation is the most likely candidate for its post translational targeting to the membrane (Vargiu et al., 2004).

In vitro immuno-precipitation showed evidence that Rhes did not stimulate the MAPK/ERK pathway, but that it did stimulate the phosphoinositide 3-kinase (PI3K) pathway (Vargiu et al., 2004). *In vitro* GTP binding found that 30% of Rhes is constitutively bound by GTP, meaning its effects remain active at basal conditions.

Evidence also suggest an inability of guanidine exchange factors (GEFs), small proteins that assist in exchanging GTP and GDP molecules on other proteins, to alter GTP binding. Most importantly, Rhes was found to inhibit cAMP concentration in cells expressing the metabotropic, G_{as} coupled, thyroid hormone receptor, suggesting that Rhes interferes with signaling of G_{as} coupled receptors (Vargiu et al., 2004). This was supported by Errico et al (2008), who found that protein kinase A-dependent phosphorylation of the GluR1 subunit of the AMPA receptor in medium spiny neurons, a direct result of $G_{as/olf}$ stimulation of AC/cAMP, was increased in $Rhes^{-/-}$ primary cultures (2008). Since this phosphorylation is a direct result of $G_{as/olf}$ stimulation of adenylyl cyclase (AC) and cAMP it suggests that Rhes provides negative modulation of the $G_{as/olf}$ stimulation of AC. A later study showed that Rhes interacts directly with D1 receptor signaling at the level of AC. In Chinese hamster ovary cells transfected with D1 receptors and Rhes, stimulation with the D1 receptor agonist SKF 83822 resulted in an attenuation of cAMP accumulation in a pertussis toxin sensitive manner, whereas stimulation of AC with forskolin failed to attenuate cAMP accumulation suggesting that Rhes interacts with the ability of $G_{as/olf}$ to be activated by the receptor (Harrison & He, 2011).

To determine if Rhes modifies $G_{ai/o}$ signaling like its closest relative Dexas1, researchers using PC12 cells transfected with Rhes and the M2-muscarinic receptor found no change in reporter activity suggesting no alteration of $G_{ai/o}$ (Vargiu et al., 2004). Errico et al. suggested that Rhes may have an effect on $G_{ai/o}$ signaling after finding that in $Rhes^{-/-}$ primary cultures of striatal medium spiny neurons, lack of Rhes significantly lowered the ability of GTP to bind $G_{ai/o}$ in agonist stimulated D2 receptors

(2008). A later study found that Rhes does influence signaling through $G_{ai/o}$. Cells stably expressing the M2-muscarinic receptor were shown to produce maximal inhibition of $Ca_{v2.2}$ channels under agonist stimulation when transfected with mutant Rhes, however agonist stimulation produced less inhibition in the presence of fully functioning Rhes, suggesting that Rhes attenuated $G_{ai/o}$ inhibitory signaling (Thapliyal, Bannister, Christopher, & Brett, 2008). Harrison and He (2011) also hypothesized that since Rhes acts to decrease cAMP accumulation in a pertussin toxin sensitive manner that it might be involved with activation of $G_{ai/o}$. Pull down assays showed that Rhes did in fact interact with $G_{ai/o}$, but only in its GTP bound form (Harrison & He, 2011). Rhes did not show any ability to affect cAMP accumulation through stimulation of D2 receptors with the agonist quinpirole (Harrison & He, 2011).

Another important finding of the previously mentioned study was the possible role of Rhes' effect on $G_{\beta\gamma}$ signaling. This group transfected M2-muscarinic receptor expressing cells with Rhes and showed tonic inhibition of $Ca_{v2.2}$ channels, a $G_{\beta\gamma}$ mediated effect, versus cells transfected with a Rhes CAAX mutated control (Thapliyal et al., 2008). In a recent study by Hill, Goddard, Ladds, and Davey (2009), cells expressing both Rhes and either $G_{\beta1}$, $G_{\beta2}$, $G_{\beta3}$, $G_{\beta4}$, or $G_{\beta5}$ were compared, and it was found that Rhes showed much more binding affinity for $G_{\beta1-3}$, but not $G_{\beta4,5}$. These results show evidence that Rhes could possibly interfere with $G_{\alpha s}$ binding due to its affinity to interact with $G_{\beta1-3}$, the units that preferentially bind $G_{\alpha s}$, and not $G_{\beta4,5}$, units that preferentially bind $G_{ai/o}$. Hill et al. suggests this might be possible due to the fact that $G_{\alpha s}$ interaction with the plasma membrane is much weaker than that of $G_{ai/o}$, therefore $G_{ai/o}$ subunits are able to overcome the interference of Rhes in binding G_{β}

subunits (2009). This would lend support to the assertion of Thalapiyal et al. (2008) that Rhes may interfere with $G_{\alpha i/o}$ signaling by allowing the $G_{\alpha i/o}$ subunit to disassociate from the heterotrimeric complex and signal but as this happens allowing Rhes to bind the $G_{\beta\gamma}$ subunits disrupting re-association of the $G_{\alpha i/o}$ subunit with the $G_{\beta\gamma}$ complex. They suggest that this may be due to the ability of Rhes to function as a guanidine exchange factor for $G_{\alpha i/o}$ (Thapliyal et al., 2008).

Rhes has also been shown to effect opioid signaling as well. We conducted an experiment to determine the effects of Rhes on opioid analgesia, tolerance, and withdrawal (Lee et al., 2011). Mice lacking Rhes showed a marked increase in opioid-mediated analgesia, no significant development of tolerance with repeated exposure to morphine, and a much milder withdrawal phenotype when compared with controls (Lee et al., 2011). These results were in stark contrast to the hypothesis that lack of Rhes would increase cAMP production through activation of G_s while simultaneously decreasing the ability of $G_{i/o}$ to decrease AC-mediated cAMP increases, the predominant mechanism by which μ -opioid receptors work to decrease neuronal excitability. An earlier study had shown that $Rhes^{-/-}$ mice show a decrease in D1 receptor-mediated grooming when compared with $Rhes^{+/+}$ mice which is a phospholipase C (PLC) –mediated behavior (Quintero, Spano, LaHoste, & Harrison, 2008). Likewise there is evidence that μ -opioid-mediated analgesia (Bonacci et al., 2006 ; Mathews, Smrcka, & Bidlack, 2008; Newton et al., 2007; W. Xie et al., 1999) as well as tolerance and withdrawal (Mathews et al., 2008; Smith, Lohmann, & Dewey, 1999; Zeitz, Malmberg, Gilbert, & Basbaum, 2001) are mediated by PLC-PKC (protein kinase C) dependent mechanisms. The evidence from this study points to the activation

of a pro-nociceptive pathway by which Rhes promotes the signaling of μ -opioid receptor associated $G_{\beta\gamma}$ subunits increasing activation of the PLC-PKC pathways. All of these results considered together show that Rhes has a highly important role in signal transduction within the striatum.

The Role of Rhes in HD

Rhes has also been found to play an important role in the pathogenesis of HD. In a series of experiments, Subramaniam and colleagues set out to determine if this striatally specific protein could in fact be responsible for much of the neurodegeneration that is striatally specific in HD. In cells expressing mHtt and Rhes, it was found that Rhes bound mHtt, and with a much higher affinity than Htt, while not binding a similar protein, ataxin, containing a polyQ repeat (Subramaniam, Sixt, Barrow, & Snyder, 2009). In cells expressing Rhes and mHtt together, it was shown that there was a 60% decline in cell survival when compared with cells expressing either Rhes or mHtt alone, or Rhes with Htt (Subramaniam et al., 2009). They were able to reduce apoptosis in PC12 cells, which contain endogenous Rhes, expressing mHtt by use of RNA-interference directed at Rhes expression (Subramaniam et al., 2009). They determined that overexpression of Rhes in vitro lead to an increase in soluble mHtt, an increase in SUMOylation of mHtt, and a decrease in ubiquitination of mHtt (Subramaniam et al., 2009). Targeted mutation of lysine residues in mHtt involved in SUMOylation resulted in a decrease in SUMOylation, a decrease in mHtt dis-aggregation, and a reversal in cytotoxicity (Subramaniam et al., 2009). They further determined that Rhes did directly bind the SUMO-conjugating enzyme/E2 ligase Ubc-9 and itself was SUMOylated (Subramaniam et al., 2009). Mutation of the CAAX prenylation/farnesylation site on

Rhes but not mutation of the GTPase catalytic core was sufficient to reverse the pattern of SUMOylation seen at mHtt suggesting that Rhes' association with the membrane was critical to the process whereas its GTPase activity was not required for SUMOylation of mHtt (Subramaniam et al., 2009).

The biochemical sequencing for the attachment of both ubiquitin and SUMO follow a very similar pathway in which 2 or 3 steps are involved. First, E1 SUMO activating enzyme complex is formed with SUMO (Hay, 2005). Next, SUMO is transferred to Ubc-9 which is known as the conjugating protein and from here can be directly ligated to the target protein (Hay, 2005). However, although SUMOylation can occur at the last step, in many cases an E3 ligase must be present (Hay, 2005). E3 ligases form an intermediate for the ligation reaction and can increase its efficiency (Hay, 2005). Evidence from this experiment suggests that Rhes acts in this last ligating step similar to other E3 ligases, and therefore provides a critical intermediate function in the SUMOylation of mHtt (Subramaniam et al., 2009). A follow up study by Subramaniam and colleagues also found that Rhes acts as an important striatal regulator of SUMOylation (Subramaniam et al., 2010). They found a major decrease in the SUMOylation of common SUMO targets Ubc-9, SP100, RanGAP1, and I κ B in the striatum of Rhes^{-/-} mice (Subramaniam et al., 2010). Importantly they found that Rhes increased the cross thioester transfer of SUMO between E1 and Ubc-9 up to 400% (Subramaniam et al., 2010).

Small ubiquitin-like modifier (SUMO) is a protein that shares a 20% sequence homology with ubiquitin and has been shown to be involved in many cellular processes by acting to direct the fate of proteins by modifying them and subsequently being

removed (Hay, 2005). SUMO requires a specialized binding domain in which it is covalently bonded to a lysine which is preceded by a large hydrophobic residue and followed by any amino acid, and finally followed by glutamic acid (Hay, 2005). Ubiquitin on the other hand can recognize a variety of binding domains (Hurley, Lee, & Prag, 2006), however they share this binding site in mHtt (Steffan et al., 2004). In vitro experiments in which either mHtt or SUMO were mutated to block binding sites showed a variety of interesting results (Steffan et al., 2004). First, it was shown that by permanently SUMOylating mHtt resulted in de-aggregation of mHtt and an increase in non-aggregated mHtt localization to the cytosol (Steffan et al., 2004). They also found that SUMOylated mHtt shows a greater suppressive effect on a number of promoters potentially giving mHtt a greater transcriptional suppressive effect (Steffan et al., 2004). Lastly, they showed that genetic mutation of SUMO in *drosophila* models of HD showed a much decreased neuropathological phenotype as compared to controls (Steffan et al., 2004). As Hay (2005) points out in his review of SUMO, one of its functions is as an antagonist of ubiquitin activity. It has already been mentioned that ubiquitin is highly colocalized with HD aggregates in vivo (Davies et al., 1997).

Recently in our laboratory we have found in vivo evidence to support the role of Rhes in SUMOylation induced pathogenesis. Rhes^{-/-} mice were successfully cross-bred with R6/1 mice. Behavioral and neuroanatomical phenotypes were observed for the mice throughout their lifespan. It was found that Rhes^{-/-}/HD⁺ mice show significant decreases in motor symptomology compared to Rhes^{+/+}/HD⁺ mice, and were identical to controls up to 5 months of age (Baiaomonte, Lee, Brewer, Spano, & LaHoste, 2013). Measures of brain weight and size were inconclusive, but this is most likely due to the

fact that Rhes^{-/-} show a diminished brain weight and size analogous to HD⁺ mice. No significant differences were found between brain weight and size of Rhes^{-/-}/HD⁺ when compared to Rhes^{+/+}/HD⁺ mice. Overall this behavioral evidence supports the assertion that Rhes acts as an E3 ligase for SUMOylation of mHtt, therefore deletion of Rhes in vivo results in a decrease in striatal neurodegeneration. Interestingly the Rhes^{-/-}/HD⁺ mice do begin to show a progressive onset of HD phenotype although not as severe as Rhes^{+/+}/HD⁺, but this is delayed until 5+ months. This may also lend support to the idea that progressive aggregate formation begins as a neuroprotective cellular event, and eventually causes problems that lead to eventual degeneration.

Hypothesis

Some researchers (e.g., Subramaniam et al., 2009; 2010) have argued that increased levels of soluble, cytosolic mHtt are toxic to neurons that express Rhes. In this scenario, the formation of intranuclear aggregates (Nlls) is neuro-protective by virtue of sequestering the mutant protein, thereby decreasing the levels of cytosolic, toxic mHtt.

We hypothesize that Rhes will significantly affect the time at which mHtt aggregates appear in striatal neurons such that Rhes deleted mice will have an earlier onset of aggregate formation when compared to their wild type counterparts suffering from HD. In addition, we propose that the timing of aggregate formation will be significantly correlated with the appearance of deficits in motor behavior. Specifically we predict that aggregate formation will not be a predictor of disease phenotype in Rhes deleted mice. This prediction is based on our previous research showing that the emergence of motor deficits is correlated with Rhes expression in a gene-dose manner (Baiamonte et. al., 2012).

Design and Methods

Animals

All procedures carried out in this study were done under the approval and supervision of the University of New Orleans Institutional Animal Care and Use Committee (Approval #UNO-12-007) and the United States Public Health Service.

All Rhes mice used in this study have from breeding pairs generously donated by Dr. Daniela Spano. Mice were created on a CD1 and C57BL/6 background. Using site-specific homologous recombination, a null mutation was created at the Rhes locus resulting in a strain of mice null for the Rhes gene. An EGFP cassette was inserted into the locus of the Rhes gene allowing access to a reporter gene for genotyping.

For this study we chose the R6/1 transgenic murine model of Huntington's disease. One male founder was shown to have integrated the transgene at one site allowing for ubiquitous expression of an N-terminal mHtt fragment containing a 116 CAG repeat sequence. Proven R6/1 breeders were purchased from The Jackson Laboratory (Jackson Laboratories, Bar Harbor, Maine, USA). These breeders were back crossed on a C57BL/6J background for at least 10 generations.

Tail Biopsy

Tail biopsies were performed on all mice to render a sample of DNA for genotyping. Mice were anesthetized using 100-150 mg/kg of a ketamine/xylazine solution. Depth of anesthesia was checked using toe pinch and corneal reflex. Once the animal displayed no reflex, the distal 2-5 mm of its tail was clipped using a straight razor, and the site was cauterized using a razor blade heated over a spirit lamp. Animals were monitored until they had fully recovered from anesthesia. The tail clip

was placed in a solution containing 300µl of DirectPCR™ lysis reagents and 11.6µl of proteinase K solution (Viagen BioTech, Los Angeles, California, USA). Samples were placed on rotation in an incubator at 55°C overnight to allow for complete cell lysis.

Polymerase Chain Reaction

After tail samples were incubated at 55°C overnight, they were placed in an oven at 85°C for approximately 1 hour to deactivate the proteinase K. For the rhes gene, reactions were prepared consisting of 12.5µl GoTaq Green Master Mix™ (Promega, Madison, Wisconsin, USA), 3µl of sense and antisense primers, 11.5µl of nuclease free water, and 0.5µl of DNA sample. Primers for the wild type gene include upstream 5'-TCCTAGCTCAGCGAGAGGAA-3', and downstream 5'-CTAGACAGGGCCCACAGAGA-3'. Primers for the EGFP reporter include 5'-CCTACGGCGTGCAGTGCTTCAGC-3', and 5'-GCGAGCTGCACGCTGCGTCCTC-3'. Reactions using WT primers were allowed to anneal at 60°C for 30 seconds with a 1 minute extension time, while EGFP reactions were allowed to anneal at 55°C for 30 seconds with a 1 minute extension time. Both reactions were allowed to complete 35 cycles and then held at 4°C to stop the reaction. Reactions for the mHtt allele were mixed using 12.5µl GoTaq Green Master Mix™, 3µl of sense and antisense primers, 9.5µl of nuclease free water, and 2.0µl of DNA sample. Primers for the HD transgene segment include 5'-CCGCTCAGGTTCTGCTTTT-3' and 5'-TGGAAGGACTTGAGGGAC-3'. The HD reaction was allowed to anneal at 55°C for 1 minute with an extension time of 1 minute for 31 cycles and subsequently held at 4°C.

Gel Electrophoresis

Gel electrophoresis was carried out using a 3% agarose gel. 5µl of each PCR reaction sample was placed in each well. A 100bp ladder was used at the distal wells of the gel providing a standard to judge the length of the alleles. Samples were run through the gel for 5 minutes at 35v to allow the DNA to move into the gel evenly. After the initial 5 minutes the voltage was increased to 95v, and the DNA was allowed to run until the marker dyes approached the edge of the gel. The gel was post-stained using a 0.5µg/ml solution of ethidium bromide. The gel was then imaged using a standard fluorescent light box. Expected size of the wild type gene was 400bp, while expected size of the EGFP reporter was 345bp. Expected size of the HD transgene was 305bp.

Preparation of Brain Tissue

At the specified experimental time points mice were taken from their cages live and promptly sacrificed by decapitation. Whole brains were harvested and flash frozen by immersion in -80°C isopentane for at least 5 minutes. Brains were then wrapped in aluminum foil and stored at -80°C. Prior to sectioning, the brains were placed in the cryostat at -20°C and allowed to equilibrate to that temperature for a minimum of 2 hours. Brains were sliced on the coronal plane at 20 microns and sections were mounted onto gelatin treated microscope slides. Slices were selected for mounting once the corpus collosum began to cross the midline and terminated with the upturning of the lateral edges of the anterior commissar. This allowed for reliable sectioning of the striatum.

Once slides were removed from the cryostat they were left at room temperature for 5 minutes. After five minutes the slides were incubated in 4% paraformaldehyde for

10 minutes. This was followed by a rinse in 0.1M phosphate buffered saline (PBS) for 5 minutes and a final rinse in deionized water for 5 minutes. Slides were air dried then placed in a slide mailer and stored at -80°C.

Immunohistochemistry

Day 1

All wash steps, unless otherwise specified, included 3 successive rinses for ten minutes each in 0.1M PBS. Slides were first placed in a solution of 0.1M PBS and 0.3% hydrogen peroxide for 30 minutes to allow for any endogenous peroxidase activity to be extinguished. Slides were then washed. Following this wash, the tissue was allowed to incubate for 60 minutes in a solution of 0.1M PBS, and 5% normal horse serum. This step served to block non-specific binding sites of the primary antibody. Following this blocking procedure the slides were again washed. The slides were then incubated for 48 hours at room temperature in a solution containing 0.1M PBS, 1% normal horse serum and mouse monoclonal anti-Huntingtin antibody (em48, 1:200) which preferentially recognizes the aggregated form of mHtt.

Day 2

After 48 hours of incubation in the primary antibody, the slides were again washed. They were then allowed to incubate for 1 hour in a solution containing 0.1M PBS, 1% normal horse serum, and biotinylated horse anti-mouse IgG secondary antibody at a dilution of 1:200. The slides were then washed. They were then incubated for 1 hour in a solution containing an avidin and biotinylated horseradish peroxidase macromolecular complex (Elite ABC reagent, Vectastain, Burlingame, CA, USA). Slides were washed again. After this wash the slides were stained for

approximately 6 minutes using the DAB staining kit (Vectastain, Burlingame, CA, USA) which consists of 3,3'-diaminobenzidine, hydrogen peroxide, and nickel chloride in distilled water. Immediately following staining the slides were washed in distilled water and allowed to air dry. After drying slides, glass cover slips were mounted on top of the tissue sections using a large drop of Histomount on each section (National Diagnostics, Atlanta, Georgia, USA). Slides were allowed to dry and harden for 24 hours.

Slides were analyzed using an Olympus BX 60 light microscope (Center Valley, Pennsylvania, USA). Images for each slide were captured and digitized at 400x objective representing a volume of 400um. The images were selected from the right and left caudate. Matlab (Natick, Massachusetts, USA) was used to analyze the images. Coding scripts were generated to account for image intensity variability. The gray scale intensity histogram of a representative slice was selected and analyzed. The values defining the histogram were then applied to all other slides using an automated script applying the 'imhistmatch' function to account for differences in exposure and lighting that occurred during image capturing. Once the images were standardized another script was generated to detect aggregates. This function was defined based on circular centers, radii of 1-8 pixels, and intensity. Aggregates were defined as intensity values $< X$ based on darkness against a light background Y .

Motor Performance

The Rotarod apparatus (Med Associates Inc., Georgia, Vermont, USA.) was used to assess motor performance in all animals. The rotating speed of the rod was set at 16 rpm (rotations per minute) throughout testing and each mouse was given one day of habituation and 1 day of testing. Testing was similar to the procedure outlined by

Stack et al. (2005) and was conducted once on each group before they were sacrificed at 2 months, 4 months, and 6 months respectively. During habituation and testing trials, the animals were placed on the rotarod and the latency to fall off the rod was recorded. If animals remained on the rod after 60 sec had elapsed, they were promptly removed and given a maximum score of 60. This procedure was conducted for 3 trials per day for each animal with a 60 sec resting period between each trial. The maximum rotarod score per day for each animal was 180.

Clasping

Whereas normal mice splay their limbs outward when suspended by their tails, animals suffering from HD have the tendency to clasp their limbs inward towards their body (Mangiarini et al., 1996; Rubinsztein, 2002). This behavior is considered to be a sign of muscle dystonia in HD models (Ferrante, 2009). Clasping was assessed according to Stack et al. (2005) immediately after testing of motor performance on the rotarod. Quantification of clasping entailed 3 trials of suspending the animal in the air by the tail for 10 seconds, during which the number of limbs withdrawn was recorded. Each limb was scored as a 1 if the mouse clasped the limb into the body and was scored a 0 if the limb remained extended away from the body. The maximum score per trial for each mouse was 4 and a total score of 12 for each day. Animals were allowed a 10 sec resting period between each trial. Similar to the other tasks in the study, each animal was given a habituation day.

Experimental Design

Experimental animals were housed with littermates on a 12hr light dark cycle and provided food and water ad libitum. A total of N = 29 mice were used. HD⁺ mice were cross-bred with Rhes^{+/-} mice to produce groups of HD⁺ mice that were either Rhes-normal (Rhes^{+/+}/HD⁺) or Rhes heterozygotes (Rhes^{+/-}/HD⁺). All subsequent crosses were made using Rhes^{+/-}/HD⁺ producing one of three genotypes (Rhes^{+/-}/HD⁺, Rhes^{+/+}/HD⁺, or Rhes^{-/-}/HD⁺). Limitations in the viability of offspring for Rhes x HD crosses resulted in a total n=9 Rhes^{-/-}/HD⁺ and n=17 Rhes^{+/+}/HD⁺ mice available for study. Given the previous research performed showing a gene dosing effect (Baiamonte, 2012) and lack of available Rhes^{-/-}/HD⁺ mice, an n=3 Rhes^{+/-}/HD⁺ were used at the 2 month time period. At this time point it was expected a priori that there would be no difference in genotypes. Each genotype was divided into 3 sub-groups according to the time of testing and sacrifice: 2, 4, and 6 months of age. These time points were chosen based on previous work that shows lack of symptomology between these genotypes at 2 months, with significant variation at 4 months, and symptomology in all groups at 6 months of age (Baiamonte et. al., 2012).

Differences in latency to fall from the rotarod were analyzed for each time point using a 2 (genotype) x 3 (time point) between-subjects factorial ANOVA. Claspings was also assessed using a 2 (genotype) x 3 (time point) between-subjects factorial ANOVA. Presence of aggregates in the striatum was statistically analyzed using a 2 (genotype) x 3 (time point) between subjects factorial ANOVA. Significant omnibus tests were further investigated using Tukey's HSD. The probability of making a type-1 error was set to $\alpha=0.05$. Pearson's r was calculated to determine if there was a correlation between the

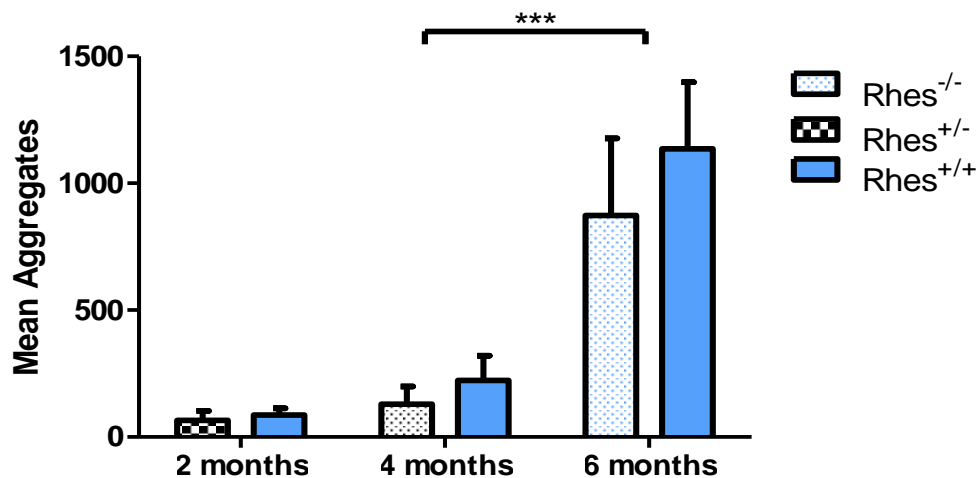
amount of aggregates that formed over time and behavioral variables for both genotypes.

Results

Aggregate Formation

A control experiment confirmed that no staining was present in non-HD carriers whereas HD carriers showed numerous intranuclear aggregates in the striatum. As expected aggregate formation increased with age for both genotypes [$F(2,28)=86.146$, $p < 0.0005$] (figure 1) and this result closely resembles the aggregation behavior observed in humans with HD (DiFiglia et.al., 1997). Follow up analysis using Tukey's HSD revealed that aggregate counts between 2 months of age ($M=80.6$ $SD=29.35$) did not significantly differ from aggregate counts at 4 months of age ($M=176.5$, $SD=93.95$). However, aggregate count increased dramatically at 6 months of age ($M=1019$, $SD=297.11$). The number of aggregates by Rhes genotype displayed a trend toward significance ($F(1,28)=4.008$, $p=0.057$). More importantly genotype had no effect on aggregate formation over time ($F(2, 28)=1.263$, $p=0.302$).

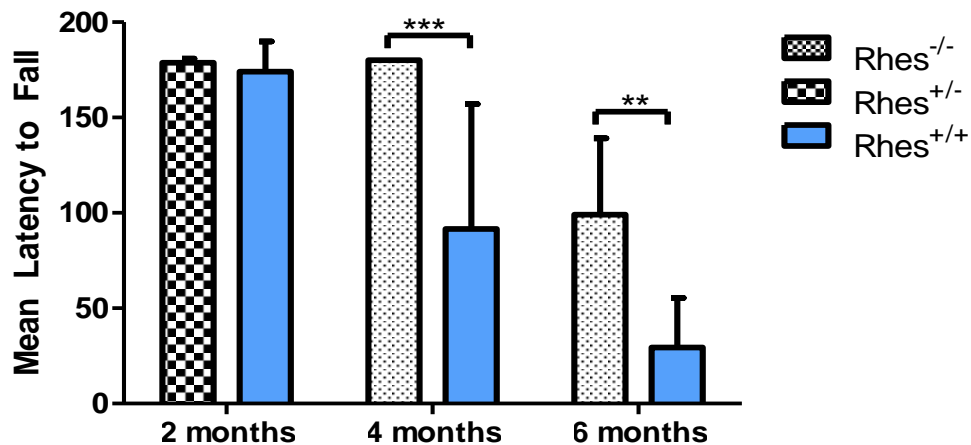
Figure 1.



Motor Performance

Rotarod results were consistent with the previous study (figure 2). A significant interaction of genotype by time point was found ($F(2,28)=3.759$, $p=0.039$). Follow up comparisons of the interactions using Tukey's HSD found that at the 2 month time period there was no difference between Rhes^{+/-}/HD⁺ mice ($M=178.67$, $SD=2.31$) and Rhes^{+/+}/HD⁺ mice ($M=174$, $SD=15.87$) on latency to fall from the rotarod. At the 4 month time period Rhes^{+/+}/HD⁺ mice ($M=91.6$, $SD=65.47$) performed significantly worse than Rhes^{-/-}/HD⁺ mice ($M=180$, $SD=0.0$). The same result was found at the 6 month time point with Rhes^{+/+}/HD⁺ mice ($M=29.4$, $SD=26.09$) performing much worse than Rhes^{-/-}/HD⁺ mice ($M=99$, $SD=40.84$). Rotarod performance was shown to steadily decrease as aggregate formation increased for both Rhes^{+/+}/HD⁺ mice ($r=-.073$, $n=0.001$) and Rhes^{-/-}/HD⁺ mice ($r=-0.82$, $p=0.001$).

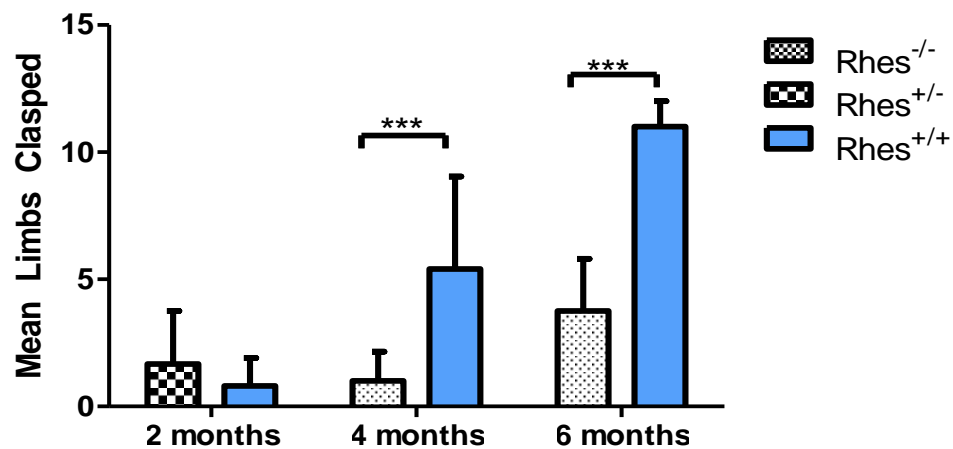
Figure 2.



Clasping

Clasping results were consistent with the previous study as well (figure 3). A significant interaction of genotype by time point was found ($F(2,28)=8.8$, $p=0.001$). Like performance on the rotarod, no difference in clasping was found between $Rhes^{+/-}/HD^{+}$ ($M=1.67$, $SD=2.08$) and $Rhes^{+/+}/HD^{+}$ mice ($M=1$, $SD=1.15$) at 2 months. $Rhes^{+/+}/HD^{+}$ mice performed significantly worse at 4 ($M=5.4$, $SD=3.65$) and 6 months ($M=11$, $SD=1$) when compared to $Rhes^{-}/HD^{+}$ mice at 4 ($M=0.8$, $SD=1.09$) and 6 months ($M=3.75$, $SD=2.06$). Interestingly aggregate formation did not correlate with clasping behavior in $Rhes^{-}/HD^{+}$ mice ($r=0.46$, $p=0.13$), whereas increasing aggregate count in $Rhes^{+/+}/HD^{+}$ mice strongly correlated with an increase in clasping behavior ($r=0.79$, $p<0.0001$).

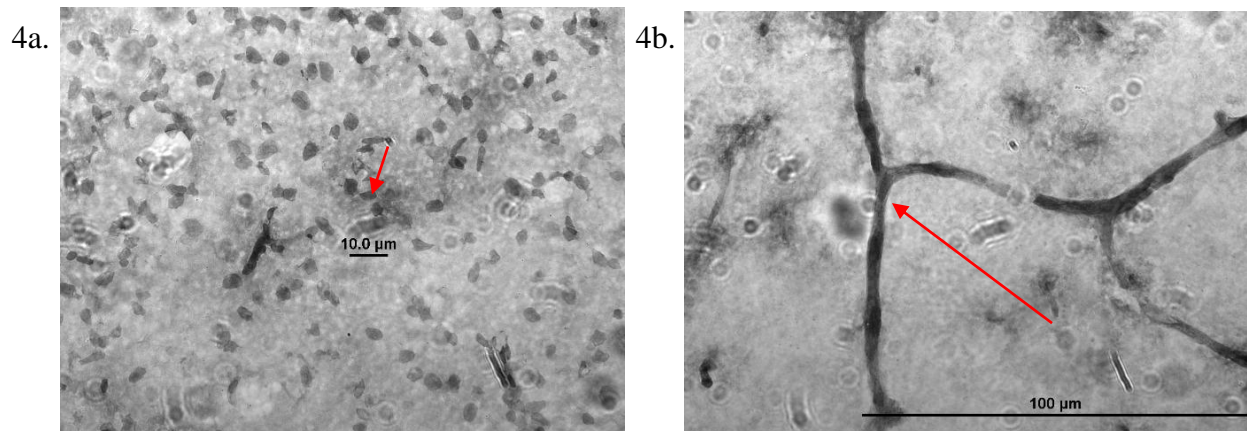
Figure 3.



Discussion

Aggregate formation in both genotypes was consistent with previously reported findings (Mangiarini et al., 1996). At two months of age both genotypes displayed a diffuse staining of cell bodies measuring approximately 5 μ m in length which corresponds to the size of MSNs (Kawaguchi, Wilson, & Emson, 1990). This diffuse staining is likely to be em48 tagging fragmented mHtt expressed in the cell body before aggregation (figure 4a). Heavier staining can be seen in some processes which is consistent with other reports (figure 4b) (Mangiarini et al., 1996; Wang et al., 2008).

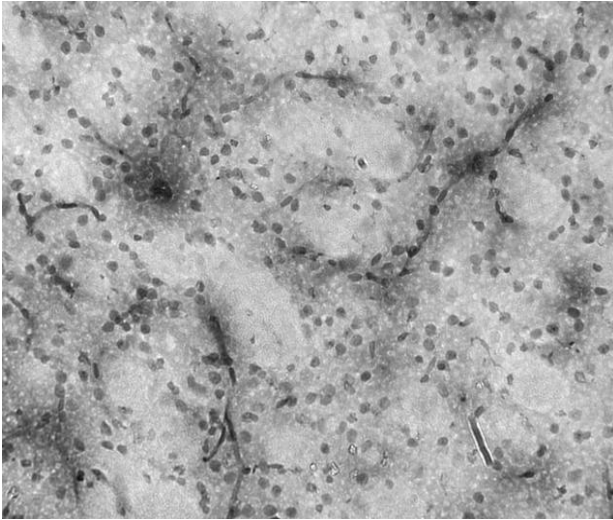
Figure 4.



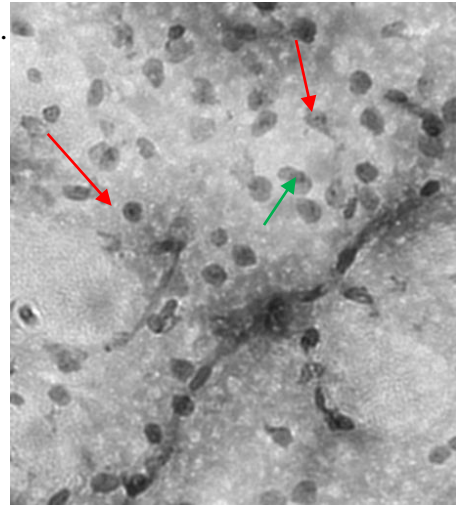
At four months, soma staining is more intense, as well as staining in processes (figure 5a). Small somatic aggregates (green arrow, figure 5b) are common and some aggregates can be seen localizing to the nucleus (red arrows, figure 5b).

Figure 5.

5a.

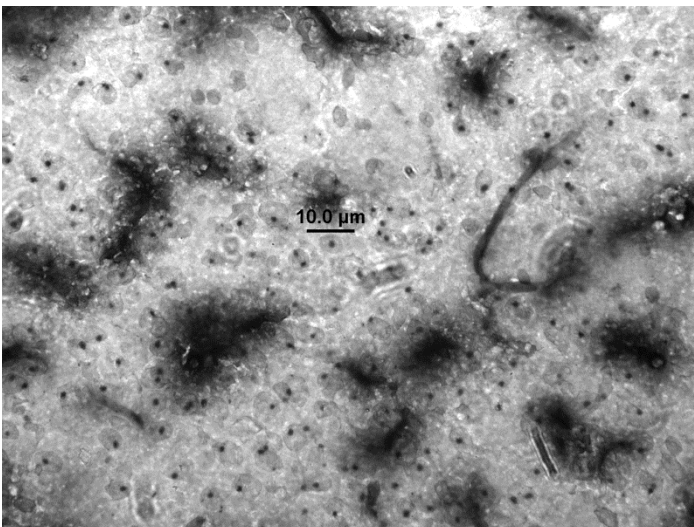


5b.



At six months of age there is massive intranuclear aggregation (red arrows, figure 6). Also, there are large areas of very intense staining that are assumed to be dystrophic neurites (green arrows, figure 6) which are similar to previous findings and occurred consistently in all 6 month old HD brains (DiFiglia et al., 1997).

Figure 6.



This study failed to find a significant difference in aggregate formation comparing Rhes deleted mice (Rhes^{-/-}/HD⁺, Rhes^{+/-}/HD⁺) with Rhes normal mice (Rhes^{+/+}/HD⁺). It was hypothesized that Rhes deleted mice would in fact show an increase in aggregation compared to Rhes normal mice due to evidence that Rhes acts as an E3 ligase for SUMOylation which hinders aggregation and promotes the stability of toxic soluble fragments of mHtt. Failing to reject the null hypothesis in this particular instance raises far more interesting questions than rejecting it as evidence of Rhes' role in neuroprotection and neuropathology has mounted.

The first evidence of the neuroprotective role of Rhes was provided by Subramaniam et al. (2009, 2010) showing that Rhes promotes SUMOylation of mHtt leading to disaggregation. They also found deletion of Rhes provided an in vitro increase in cell survivability of mHtt transfected cells (Subramaniam et al., 2010). Our lab, in response to this finding, followed up on this in vitro work with an in vivo behavioral study. We showed that Rhes deleted mice show a significant delay in the time course of the HD phenotype providing crucial in vivo evidence that Rhes does in fact play a major role in HD pathogenesis (Baiamonte et al., 2013). At the same time Mealer and colleagues (2013) published another report showing similar behavioral results in Rhes^{-/-} mice with induced 3-nitropropionic acid lesion, a long established model of striatal neurodegeneration. Subramaniam and colleagues have very recently published an in vivo report showing similar findings, this time using Rhes^{-/-} mice crossed with the N171-82Q murine model of HD (Swarnkar et al., 2015). Not all reports have been supportive of the in vivo results from the above combined studies. One other group has recently published findings that are in direct contrast to the previous studies.

Lee and colleagues (2014) have found that suppression of Rhes using RNAi in two murine models of HD, N171-82Q and BacHD, did not in fact cause a significant rescue of behavioral symptoms. In fact, they found that suppression of Rhes activity enhanced the disease phenotype (Lee et al., 2014). A follow up study by Lee and colleagues (Lee et al., 2015) found that over-expressing Rhes using a viral vector in N171-82Q mice actually diminished the disease phenotype. Conclusions drawn from comparisons of these studies should be made with caution as the methodologies used are very different from each other.

Along with these in vivo studies, new evidence of a role for Rhes' in the cellular functions in HD has come to light as well. One of the most interesting is the role of Rhes in mTOR (mammalian target of rapomycin) signaling. mTOR regulates many important cellular processes including ATP production, protein synthesis, lipid synthesis, and, most importantly for HD, autophagy (Laplante & Sabatini, 2013). Research shows that mTOR activation by mHtt may decrease autophagy thereby leading to an exacerbation of the HD phenotype (Pryor et al., 2014). Rhes has been shown to bind and activate mTOR (Subramaniam et al., 2012) which leads to the assumption that perhaps Rhes provides a means of inhibiting autophagy and clearance of mHtt. Rhes has also been implicated in Akt signaling. One study has found that Rhes increases Akt targeting to the membrane (Bang, Steenstra, Kim, 2012). Rhes deletion in vivo has also been shown to increase Akt phosphorylation (Harrison, Muller, & Spano, 2013). Akt is an upstream regulator of mTOR activity and presumably Rhes acts as a modulator of mTOR via Akt signaling. On the contrary one report has found that mTORC1 activation in HD striata promotes autophagy (Lee et al., 2015). However; evidence shows that

Rhes stimulates autophagy through a completely different mTOR-independent pathway that involves relieving the inhibition of Beclin-1 which itself is a potent stimulator of mTOR independent autophagy (Kang, Zeh, Lotze, & Tang, 2011; Mealer, Murray, Shahani, Subramaniam, & Snyder, 2014). Taken together, the suggestion by all of these reports is that Rhes may play a neuroprotective role, at least when it comes to inducing autophagy. Evidence from this study may help to answer the question of Rhes role in autophagy. Given the fact that aggregates did not differ between genotypes, and even trended toward being more frequent in Rhes normal mice, it might be assumed that Rhes is in fact not substantially affecting autophagy at the level of the mHtt aggregate.

Other recent evidence has also been found to support Rhes' role in neurotoxicity. It was shown that in a novel human embryonic stem cell model of HD loss of neurons was significantly decreased when Rhes was knocked down using RNAi (Lu & Palacino, 2013). Another study has found that Rhes complexes with mHtt and ACBD3, a scaffolding protein responsible for regulation of neurotoxicity (Sbodio, Paul, Machamer, & Snyder, 2013). Expression of Rhes with mHtt and ACBD3 in cultures resulted in a much higher cytotoxicity than expression of mHtt and ACBD3 together (Sbodio et al., 2013). Another study has found that Rhes expression in mHtt expressing cells results in a loss of mitochondrial potential leading to increased oxidative stress (Fabio, 2014).

All of this new evidence does not make it very easy to paint a uniform picture of Rhes' involvement in HD. On one hand we have an overwhelming line of evidence both in vivo and in vitro, suggesting that Rhes plays a neurotoxic role in the pathogenesis of Huntington's disease. On the other hand we have some evidence that Rhes actually

plays a neuroprotective role in Huntington's disease. Evidence from the current study would suggest that Rhes deletion does not diminish the effect of mHtt aggregation on striatal neurons. This is in contrast to the evidence suggesting that Rhes deletion should in fact cause an increase in aggregation.

One explanation for the results of this study may be that the methods used were not sensitive enough to properly distinguish a difference in aggregation between the genotypes. Rhes may indeed be promoting early onset of aggregation but the temporal spacing used to detect this was not close enough. The R6/1 mouse model of HD shows a relatively aggressive phenotype compared to other murine models of HD. BacHD mice, for example, containing a full length human HD gene display a much less aggressive and a much longer temporal phenotype. The methods used to stain and detect aggregates in this study would be more sensitive in a model with a longer temporal onset of aggregation. It could be that the critical times of aggregation difference in the R6/1 model are in between 4 and 6 months whereas in models such as BacHD the window of aggregation difference is 8-16 months. This also makes the important assumption that deletion of Rhes does not preclude the eventual cellular dysfunction and cell death, which is based on the previous evidence that Rhes deleted models of HD do eventually show a disease like phenotype but at a slower rate than Rhes normal mice.

Another possibility is that the R6/1 model changes aggregation behavior itself. A recent study has found evidence that SUMO shows a higher binding affinity for mHtt based on the length of the polyQ sequence (Bhat, Yan, Wang, Li, & Xio-Jiang, 2014). Interestingly the group found that ubiquitin serine residue K63 preferentially binds the n-

terminal of the expanded polyQ regions which promotes aggregation (Bhat et al., 2014). The suggestion is that there is a difference in the steric properties of the protein such that as the expanded polyQ changes in length, availability of the binding site for SUMO/Ubiquitin changes. The R6/1 model contains a 116Q repeat length HD transgene (Mangiarini et al., 1996). It is not out of the question that differential expression of repeat length size, size of expressed protein, or even different cleavage products based on either of these would result in differential effector protein interactions with mHtt. A follow up study that stains for co-localization of mHtt with either ubiquitin or SUMO could help to answer this question.

Another interesting explanation may lie with the nature of SUMO itself. SUMO exists in the 4 isoforms; SUMO-1, SUMO-2, SUMO-3, and SUMO-4 (O'Rourke et al., 2013; Saitoh & Hinchey, 2000). In a recent report it was shown that SUMO-1 and SUMO-2 differentially bind mHtt (O'Rourke et al., 2013). Most notably the study found that SUMO-1 modification of mHtt resulted in more soluble fragmentation whereas SUMO-2 modification resulted in less solubility and more aggregation (O'Rourke et al., 2013). Interestingly the authors also found that SUMO-2 binding to mHtt was not associated with Rhes whereas, as previously reported, SUMO-1 binding to mHtt was enhanced by Rhes (O'Rourke et al., 2013). This interesting result fits perfectly with the observed results from the current study. This suggests that Rhes deletion would downregulate the soluble fraction of toxic mHtt fragments via inability to act as an E3 ligase for SUMO-1. At the same time aggregation behavior would not be affected by Rhes as it does not promote SUMO-2 association with mHtt.

Conclusion

Rhes deletion does not significantly alter the aggregation of mHtt in a mouse model of Huntington's disease, but it does significantly reduce the phenotypic symptoms of the disease. Many important questions remain. Why does Rhes deletion not effect aggregation despite the overwhelming evidence that it should? As Subramaniam and colleagues (2009) pointed out in their first publication on the subject, some models of HD show a high correlation with aggregation and cell death and some do not. Model biochemical differences and methodologies used to study HD pathology perhaps underlie this discrepancy in the literature. Another important underpinning in regards to Rhes' contribution could lie in its ability to act as both a trophic entity and a neurotoxic species. Evidence is beginning to support the idea that Rhes presence is important as a positive regulator of autophagy and a negative regulator of proteolysis. Hypothetically Rhes involvement in a temporal fashion in proteolysis versus autophagy may help to explain why disease onset is later in life. Regardless, this is the first study of its kind to look at aggregation behavior as it relates to Rhes in an in vivo model of HD, and as a result careful follow up should be considered.

Future Studies

Rhes' role in Huntington's disease is an important avenue of research that should continue to be looked at very carefully. Following up on the findings in this particular study could shed important light on not only the functions of Rhes, but on how progressive neuropathological disorders work in general. One particularly interesting question is why did Rhes deletion not affect aggregation as expected? Studies using different models of HD may be considered to address the limitation of a short

neuropathological time course. A similar study using BacHD mice, which expresses the full HD transgene, and shows a much longer neuropathological and neurobehavioral phenotype, may be more sensitive for studying aggregate formation. Another consideration would be the possibility of using similar methodology but incorporating fluorescent-tagged secondary antibodies as well as staining for ubiquitin and SUMO in addition to mHtt. This would provide a much clearer picture of how these highly important regulators of HD neuropathology behave in relation to the formation of aggregates. Ultrastructural analysis of MSNs using electron microscopy techniques should also be performed on *Rhes*^{-/-}/HD⁺ mice. Aggregate formation could differ from expected wild type mice at the level of the soma that is not visible using light microscopy techniques. It would be interesting if the deletion of *Rhes* changes the quality of the aggregate on a structural or location level inside of the cell.

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Appendices

Institutional Animal Care and Use Committee

UNIVERSITY OF NEW ORLEANS

DATE: November 17, 2012

TO: Dr. Gerald LaHoste

FROM: Dr. Steven G. Johnson
Chairperson

RE: *IACUC Protocol # UNO-12-007*
Entitled: Rhes and Huntington's Disease: Genetic Studies

Your application for the use of animals in research (referenced above) has been approved beginning November 17, 2012 and expiring November 16, 2015. We are concerned about the use of personal funds to conduct such experiments and would like to see a major progress report at the end of the first year. To minimize the use of excess animals, we also need to see that scientific progress such as grants and publication is evident, especially given the dynamic nature of this area. We will require this at the end of the first year and make a determination about whether approval is given to extend the protocol beyond the first year. Please note that a final report must be provided to the UNO IACUC.

The University of New Orleans has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), National Institutes of Health. The assurance number is A3299-01.

Vita

The author was born in the sleepy East Texas town of Carthage. He graduated high school in 2000 from Subiaco Academy in Subiaco, Arkansas. He then went on to earn a Bachelors of Science in Psychology at the University of Texas at Tyler. He started in the Applied Biopsychology graduate program at the University of New Orleans in 2008 and earned his Masters in 2010.