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The effects of Rhes, a striatal specific protein, on the expression of behavioral and neuropathological symptoms in a transgenic mouse model of Huntington's disease

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The effects of Rhes, a striatal specific protein, on the expression of behavioral and neuropathological symptoms in a transgenic mouse model of Huntington's disease

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Abstract

Huntington's disease (HD) is a neuropsychiatric disorder characterized by choreiform movement of the limbs, cognitive disability, psychosis and dementia. It is untreatable, incurable, and ultimately fatal. HD is invariably associated with an abnormally long CAG expansion within the IT15 gene on human chromosome 4. Although the mutant huntingtin protein (mHtt) is ubiquitously expressed in HD patients, cellular degeneration occurs only in neurons within the striatum and cerebral cortex. The Ras homolog Rhes is expressed very selectively in the precise brain areas affected by HD. Recent work using cultured cells suggests that Rhes may be a co-factor with mHtt in cell death. However, there is controversy as to whether cell death underlies the symptoms of HD. We used a validated transgenic mouse model of HD crossed with Rhes knockout mice to show that the behavioral symptoms of HD are regulated by Rhes. HD/Rhes$^{-/-}$ mice showed greatly delayed expression of HD-like symptoms in this *in vivo* model. Drugs that block or inhibit the actions of Rhes may be useful as the first treatments for HD.

Huntington’s disease, Rhes, sumoylation, motor deficits, neurodegeneration

Introduction

Huntington's disease (HD) is a complex, progressive neurodegenerative disorder that results in deficits of motor function, cognition, and mood. HD is a result of a genetic defect that is characterized by an unstable expansion of a trinucleotide repeat (CAG, which codes for glutamate) on chromosome 4 in the huntingtin gene (Huntington's Disease Research Group, 1993). It has been shown that an unstable CAG repeat sequence results in neuronal intranuclear inclusions of the striatum and cerebral cortex (Davies, Turmaine, Cozens, DiFiglia, Sharp, Ross et al., 1997; Vonsattal, Meyers, Stevens, Ferrante, Bird, & Richardson, 1985; Hedreen, Peyser, Folstein, & Ross, 1991), which leads to the degeneration of selectively targeted medium spiny neurons in the striatum (Graveland, Williams, & DiFiglia, 1985; Albin, Reiner, Anderson, Dure, Handelin, Balfour et al., 1992). The striatum is a subcortical structure located at the base of the forebrain and is part of the basal ganglia, a larger set of nuclei that are involved in the behavioral activation system of the brain (Kandel, Schwartz, & Jessell, 2000). The striatum is part of two overarching and distinct circuits. The first circuit is comprised of the ventral tegmental area and nucleus accumbens, forming the ventral striatum (Groenewegen, Wright, Beijer, & Voorn, 2006), which is concerned with assigning salience to the environment (Cardinal, Parkinson, Hall, & Everitt, 2002; Di Chiara, Bassareo, Fenu, De Luca, Spina, Cadoni, et al., 2004). The latter circuit is comprised of the dorsal striatum, pallidum, substantia nigra pars compacta, and subthalamic nuclei (Kandel et al., 2000), which is responsible for the downstream regulation of the motor cortex and motor learning. However, the striatum is not the only anatomical region deteriorated by HD. The cortex is also severely affected, whereas

the hippocampus and cerebellum are spared until late in the disease (Cudkowicz & Kowall, 1990; Hedreen et al., 1991; Vonsattal & DiFiglia, 1998).

Affected individuals usually begin to experience symptoms in mid-adulthood and gradually progress over the next 10-15 years to more severe deficits followed by death (Craufurd, Thompson, & Snowden, 2001). The hallmark motor abnormality is chorea, which is characterized by quick movements of the hands and feet similar to dancing (Carter, Lione, Humbly, Mangiarini, Mahal, Bates et al., 1999). Although chorea is the clinical hallmark of the disease, akinesia (slowed initiation of movement), bradykinesia (slowness in the execution of movement), and dystonia (abnormal posture) can also be prevalent (Rosas, Salat, Lee, Zaleta, Pappu, Fischl et al., 2008). As far as cognitive deficits, problems with attention, visuospatial abilities, emotional processing, and memory can be seen in the early stages of the disease (Butters, Wolfe, Granholm, & Martone, 1986; Claus & Mohr, 1996; Sprengelmeyer, Young, Calder, Carnat, Lange, Homberg, et al., 1996; Brandt, Bylsma, Gross, Stine, Ranen, & Ross, 1996). Eventually, the cognitive impairments deteriorate to dementia, which can lead to numerous cognitive and emotional problems (Montoya, Price, Menear, & Lepage, 2006). Changes in personality and psychiatric disturbances are also cardinal features of HD, but these symptoms are often heterogeneous and lack a clear course of progression (The Huntington Study Group, 1996). Studies have provided support for the variability of psychiatric symptoms by reporting the presence and absence of depression, apathy, irritability, impulsiveness, antisocial behavior, and suicidal behavior (Kirkwood, Su, Conneally, & Foroud, 2001). Although researchers are in disagreement over which psychiatric symptoms are present in HD individuals, it has become clear that some specific psychiatric disturbance is present in HD with frequencies

between 35-75% (Dewhurst, Oliver, Trick, & McKnight, 1969; Folstein, 1989). The prevalence rates of specific psychiatric symptoms have been diverse, with some symptoms occurring more often than others. The most common symptoms are lack of energy and apathy, which has been reported to occur in more than 70% of HD patients (Craufurd et al., 2001; Caine & Shoulson, 1983; Folstein & Folstein, 1983). Irritability, which is also a common feature, has prevalence rates between 31-50% for HD patients (Crauford et al., 2001; Folstein, Chase, Wahl, McDonnell, & Folstein, 1987). Besides the typical mood disorders that are present in HD individuals, schizophrenia-like psychosis has also been reported to be present in 5-16% of HD patients, which is much higher than the 1% prevalence rate in the general population (Faraone & Tsuang, 1995; Tsuang, Almqvist, Lipe, Strgar, DiGiacomo, Hoff et al., 2000).

Huntingtin vs. Mutant Huntingtin

Huntingtin (Htt), the protein product of the HD gene, is composed of 3,144 amino acids with a molecular mass of ~349 kDa and is expressed ubiquitously, with the highest levels in neurons of the CNS and the testes (Cattaneo, Zuccato, & Tartari, 2005; Trottier, Lutz, Stevanin, Imbert, Devys, Cancel et al., 1995; Ferrante, Gutekunst, Persichetti, McNeil, Kowall, Gusella, et al., 1997; Fusco, Chen, Lamoreaux, Figueredo-Cardenas, Jiao, Coffman et al., 1999; Gil & Rego, 2008). At the intracellular level, Htt is associated with the nucleus, endoplasmic reticulum, and the Golgi complex (DiFiglia, Sapp, Chase, Schwarz, Meloni, Young et al., 1995; Velier, Kim, Schwarz, Kim, Sapp, Chase et al., 1998; Hilditch-Maguire, Trettel, Passani, Auerbach, Persichetti, & MacDonald, 2000; Kegel, Meloni, Yi, Kim, Doyle, Cuiffo et al., 2002). This widely expressed protein has also been found in synaptic vesicles and mitochondria (Sharp, Loev, Schilling, Li, Li,

Bao et al., 1995; Li, Plomann, & Brundin, 2003). Studies have indicated that Htt may bind to various proteins that may play a role in intracellular functions such as protein trafficking, vesicle transport and anchoring to the cytoskeleton, clathrin-mediated endocytosis, postsynaptic signaling, transcriptional regulation, and anti-apoptotic function (Gil & Rego, 2008).

Mutant Huntingtin (mHtt) has been characterized by the expansion of the polyglutamine tract beyond 35 glutamine residues, which begins at the 18^{th} amino acid position (Li & Li, 2004). The presence of an expanded polyglutamine tail, which is a key feature of HD, can lead to disruptions of the interactions between the normally functioning protein, Htt, and its effector proteins, ultimately leading to a modification in intracellular functioning.

While it is clear that the mHtt associated with HD leads to abnormalities in a variety of intracellular functions, controversy has developed over whether HD neurodegeneration is a result of a gain of function of mHtt or a loss of function of Htt. Evidence was provided that HD results from gained activity from the extended CAG and the interactions with other proteins producing neurological symptoms (Mangiarini, Sathasivam, Seller, Cozens, Harper, Hetherington et al., 1996). In contrast, there is also support for the loss of normal Htt function contributing to a decrease in survival and HD symptoms (Rigamonti, Bauer, De-Fraja, Conti, Sipione, Sciorati et al., 2000; Dragatsis, Levine, & Zeitlin, 2000). Recently researchers have come to the conclusion that neural degeneration in HD is a result of the combined effects of a gain of function of mHtt and a loss of function of Htt (Landles &Bates, 2004). The combined effects of mHtt and Htt underlie the mechanisms responsible for neurodegeneration in HD, such as activation of proteases, protein misfolding, inhibition of protein degradation,

transcription dysregulation, synaptic dysfunction, mitochondrial dysfunction, and oxidative stress (Gil & Rego, 2008).

Huntington's Disease Aggregates

In HD, the expansion of the CAG repeat into the pathological range results in the polyglutamine sequence undergoing a conformational change (Scherzinger, Lurz, Turmaine, Mangiarini, Hollenbach, Hasenbank et al., 1997). This conformational change, to be discussed in later paragraphs, results in the formation of aggregates. Protein aggregates have been known to disrupt cytoskeletal structures associated with protein and mRNA trafficking to synapses and reduce the availability of the proteasome for removal of other toxic proteins, resulting in cell dysfunction and death (Li, Li, Yu, Shelbourne, & Li, 2001; Bates, 2003). While the formation of aggregates is considered by most to be toxic to the cell, there are many studies suggesting that aggregates may actually serve a neuroprotective role in the cell. Evidence has shown that toxic effects of mHtt have been shown prior to the formation of large aggregates (Saudou, Finkbeiner, Devys, & Greenberg, 1998; Arrasate, Mitra, Schweitzer, Segal, & Finkbeiner, 2004). Kummerle and colleagues have suggested that mHtt aggregates may only represent a side-effect of progressive cell dysfunction and may even serve a protective role against cell death (Kummerle, Gutekunst, Klein, Li, Li, Beal et al., 1999). Regardless of the role of aggregates in the cell dysfunction and death in HD, the formation of these aggregates is the neuropathological hallmark of HD.

Both gain of function and loss of function can be indicated as a key component of aggregation formation (Gil & Rego, 2008). mHtt, in both soluble and insoluble aggregate form,

can disrupt intracellular pathways by abnormal protein interactions and sequestering key components of these pathways into these aggregates. On the other hand, loss of Htt functioning due to its sequestration into the aggregates can also result in neuronal dysfunction and death.

Activation of proteases. The cleavage of mHtt by caspases has been shown to precede the onset of neurodegeneration and cell dysfunction (Tarlac & Storey, 2003; Wellington, Ellerby, Gutekunst, Rogers, Warby, Graham et al., 2002). Caspase-3 and calpain have been shown to produce N-terminal fragments of mHtt after proteolysis, which are more toxic and more likely to aggregate (Kim, Yi, Sapp, Wang, Cuiffo, Kegal et al., 2001). Interestingly, the proteolysis actually increases in the presence of longer polyglutamine tails and N-terminal fragments from mHtt, which are smaller in size and can diffuse passively into the nucleus (Gafni & Ellerby, 2002; Sun, Fan, Balciunas, Cooper, Bitan, Steavenson et al., 2002). Once the fragments are in the nucleus, they can recruit more proteases into the aggregates, creating further aggregation and proteolytic cleavage which will contribute to cell death. It is clear that a mHtt gain of function is responsible for increased proteolysis and subsequent aggregate formation, but there is also a loss of function of Htt characterized by the reduction of Htt's ability to bind and inhibit caspase-3 (Zhang, Leavitt, van Raamsdonk, Dragatsis, Goldowitz, MacDonald et al., 2006). Not only is there a reduction in the Htt-mediated inhibition of capsase-3, but there is also a reduction in the ability of Htt to bind huntingtin-interacting protein 1 (HIP1), which will then bind to HIP1-protein interactor (HIPPI) (Gervais, Singaraja, Xanthoudakis, Gutekunst, Leavitt, Metzler et al., 2002). The activation of HIPPI activates caspase-8, which induces apoptotic cell death.

Abnormal protein folding. Abnormally long polyglutamine tracts have been associated with an abnormal folding of the protein. The folding of proteins into the correct conformation requires the actions of several molecular chaperones and the interaction of mHtt with these chaperones can be disrupted (Fink, 1999). Heat-shock protein 70 (Hsp70) and heat-shock protein 40 (Hsp40) are the chaperones responsible for correct protein folding and refolding (Hartl & Hayer-Hartl, 2002). If the abnormal proteins are not refolded by chaperones, they will be degraded by the ubiquitin-proteasome system (UPS) (Voges, Zwickl, & Baumeister, 1999). The chaperones and UPS normally prevent aggregation, but when the amount of misfolded proteins exceeds the degradative capacity, aggregation occurs. Evidence has suggested that chaperones are actually sequestered into the aggregates, decreasing the amount of chaperones available, which results in further increases in protein misfolding (Hay, Sathasivam, Tobaben, Stahl, Marber, Mestril et al., 2004). It has been shown that Htt actually interacts with Hsp70 and Hsp40 to prevent aggregation and protein misfolding. It is the Htt loss of function that may be responsible for abnormal protein folding and progressive aggregation formation that gives rise to HD pathology. In fact, Schaffar and colleagues have shown that Hsp70 and Hsp40 can actually interfere with mHtt aggregation (Schaffar, Breuer, Boteva, Behrends, Tzvetkov, Strippel et al., 2004). However, as mentioned earlier, the chaperones are sequestered into the aggregates of mHtt preventing their normal protective role, which leads to an enhancement in abnormally folded proteins (Ho, Carmichael, Swart, Wyttenbach, Rankin, & Rubinsztein, 2001).

Ubiquination process. If chaperones are unable to refold abnormal proteins, then the chaperones promote ubiquination, which directs proteins to the proteasome for degradation (Landles & Bates, 2004). Interestingly, researchers have found inclusions associated with HD to

be ubiquinated, implying that there is a malfunction in the degradation processes (Ciechanover & Brundin, 2003). Numerous researchers have provided support for the sequestering of ubiquitin into aggregates *in vitro* and *in vivo* (Wyttenbach, Carmichael, Swartz, Furlong, Narain, Rankin et al., 2000; Jana, Zemskov, Wang, & Nukina, 2001). These findings have influenced researchers to suggest the impairment of the UPS as the central feature of pathology in HD and other polyglutamine diseases (Landles & Bates, 2004). The fact that ubiquitin, which is responsible for tagging proteins for degradation, is sequestered into the aggregates suggests the existence of an impairment in the UPS, which has been found to lead to cell death via apoptosis (Jana et al., 2001). It can be argued that the presence of ubiquitin in mHtt aggregates may be a result of an unsuccessful attempt to mark the mHtt for the proteasome.

Mitochondrial Dysfunction in HD

Research over the years has demonstrated that mitochondrial dysfunction plays a role in many neurodegenerative diseases by disrupting energy metabolism, calcium homeostasis (Ca^{2+}), and activating apoptotic pathways (Chan, 2006; Green & Kroemer, 2004; Green & Reed, 1998; Newmeyer & Ferguson-Miller, 2003). In fact, studies conducted with transgenic HD mice and human patients with HD have discovered lower membrane potentials in mitochondria, as well as depolarization at lower Ca^{2+} loads (Panov, Gutekunst, Leavitt, Hayden, Burke, Strittmatter et al., 2002). This reduction in membrane potential has been shown to be correlated with CAG repeat expansion (Sawa, Wiegand, Cooper, Margolis, Sharp, Lawler et al., 1999). These findings are important because one of the major roles of mitochondria is to

control Ca^{2+} homeostasis without which increased cytoplasmic Ca^{2+} levels leads to neuronal toxicity (Bossy-Wetzel, Schwarzenbacher, & Lipton, 2004).

Aggregates Impair Mitochondrial Movement. Not only can mHtt interact with the outer mitochondrial membrane destabilizing the mitochondrial membrane potential, it can also lead to reduced mitochondrial mobility as well. Reduced mobility of mitochondria can disrupt the incorporation of certain proteins, which can ultimately lead to mitochondrial dysfunction (Orr, Li, Wang, Li, Wang, Rong et al., 2008). Studies have indicated that mitochondria may be embedded into aggregates formed by mHtt, which can eradicate mitochondrial movement resulting in reduced assimilation of proteins (Chang, Rintoul, Pandipati, & Reynolds, 2006). Interestingly, the cleaved N-terminal fragment of mHtt is less likely to impair mitochondrial mobility than the full-length mHtt (Chang et al., 2006). This finding seems counterintuitive when considering the tendency for N-terminal fragments to be more toxic and likely to aggregate. Further investigation into these findings is needed to better understand the relationship between mitochondrial mobility and aggregates.

Reactive Oxygen Species (ROS). ROS have been found to be increased in HD patients and transgenic HD mice, which can ultimately lead to mitochondrial dysfunction (Browne & Beal, 2006; Stack, Matson, & Ferrante, 2008). Inhibition of enzymes in the electron transport chain can lead to the leakage of electrons from the mitochondria to form ROS (Ramaswamy, Shannon, & Kordower, 2007). The increase in ROS can produce mitochondrial impairment by increasing the likelihood of induction of the mitochondrial permeability transition pore (mPTP) in the presence of Ca^{2+}. Researchers have shown that cells with mHtt undergo a permeability

transition, a result of mPTP induction, at lower Ca^{2+} concentrations (Milakovic, Quintanilla, & Johnson, 2006). Therefore, the presence of mHtt in HD increases ROS, which makes the mPTP sensitive to Ca^{2+}, leading to future cell death. This induction of mPTP can play a role in apoptosis by producing mitochondrial swelling and eventual cell death. This is a key step in glutamate-induced excitotoxicity in HD individuals and it will be discussed in a later section.

Transcription Factors Affecting Mitochondria. Alterations in transcription factors have been shown to play a role in mitochondrial dysfunction that leads to HD neurodegeneration. Researchers have been able to provide support for the detrimental effects of transcription factor modification on mitochondrial functioning. For instance, researchers were able to prevent the reduction in Ca^{2+} handling capacity in HD mice, which is characteristic of mitochondrial impairment, by applying histone deacetylase (HDAC) inhibitors (Oliveira, Chen, Almeida, Riley, Goncalves, Oliveira et al., 2006). Also, reductions in CREB-dependent transcription have been demonstrated in HD and CREB has been known to regulate respiratory chain proteins, such as cytochrome C, which is important for proper mitochondrial function (Gopalakrishnan & Scarpulla, 1994). Finally, mHtt has been associated with p53, which is a protein that plays a role in apoptosis and has been known to regulate various mitochondrial proteins (Bae, Xu, Igarashi, Fujimuro, Agrawal, Taya et al., 2005). The abundance of p53 resulting from the interaction with mHtt can actually induce p53-dependent transcription, resulting in further mitochondrial dysfunction. Based on this evidence, mitochondrial dysfunction may be secondary to transcriptional dysregulation. However, impairment in mitochondrial function is not the only effect of abnormal transcription and the interactions of transcription factors and mHtt will be discussed to greater lengths in a future section.

Glutamate-Induced Excitotoxicity. It has been shown that mitochondrial dysfunction can occur through anomalies produced by mHtt in mitochondrial membrane potential, ROS production, and protein expression which can all have detrimental effects on Ca^{2+} handling. However, mitochondrial Ca^{2+} handling can be reduced by the stimulation of glutamate receptors (NMDA receptors) as well. It is believed that chronic activation of NMDA receptors increases the intracellular Ca^{2+} concentrations, leading to mitochondrial dysfunction. Research has indicated that the reduction in ATP production that is characteristic of HD can lead to dysfunctional ionic pumps, which are needed to maintain electronic gradients across membranes (Ramaswamy et al., 2007). These deficient pumps do not allow the membrane to repolarize after action potentials, resulting in prolonged depolarization and the removal of the magnesium (Mg^{2+}) block in the NMDA receptor. This allows an influx of Ca^{2+}, which will result in radical production (Beal, 1992), which can increase mitochondrial dysfunction (see above sections).

Research has also shown an abnormal release of Ca^{2+} from the endoplasmic reticulum (ER) in neurons expressing mHtt (Damiano, Galvan, Deglon, & Brouillet, 2010. In fact, mHtt facilitates the activity of type 1 inositol 1,4,5-triphosphate receptors (IP_3R), which are located in the ER and promote an increase in Ca^{2+} release (Tang, Tu, Chan, Maximov, Wang, Wellington et al., 2003). In conclusion, the presence of impaired mitochondrial Ca^{2+} handling in HD (derived from abnormalities in membrane potential, mitochondrial immobility, protein expression, and ROS production) along with prolonged stimulation of NMDA receptors and increased Ca^{2+} release from the ER produces an excitotoxic level of Ca^{2+} leading to neuronal apoptosis.

Although the exact mechanism behind HD-induced mitochondrial dysfunction remains elusive, it is clear that anomalies in mitochondrial function play a vital role in HD pathogenesis.

Transcriptional Dysregulation

There are several transcription factors that bind to Htt, but CREB binding protein (CBP) and specificity protein 1 (SP1) have been two of the most studied in HD (Li & Li, 2004). Being that they are transcription factors, they are important for proper gene expression and neuronal function. Specifically, reductions in CBP have been shown to reduce CREB expression, leading to neurodegeneration in the hippocampus and striatum similar to HD pathology (Mantamadiotis, Lemberger, Bleckmann, Kern, Kretz, Villalba et al., 2002). Despite direct effects on neuronal function, transcription of CREB has also been linked to mitochondrial dysfunction, which has been associated with HD pathogenesis. Interaction between CBP and Htt has been shown to take place between the acetyltransferase domain of CBP and the polyglutamine domain of Htt (Chai, Wu, Griffin, & Paulson, 2001; Steffan, Bodal, Pallos, Poelman, McCampbell, Apostol et al., 2001). The effect of CBP is depleted due to the sequestration of CBP into the aggregates of mHtt, rendering CBP ineffective (Cong, Pepers, Evert, Rubinsztein, Roos, van Ommen et al., 2005; Jiang, Poirier, Liang, Pei, Weiskittel, Smith et al., 2006). More importantly, the interaction of the acetyltransferase domain of CBP with mHtt can reduce acetylation of histones, resulting in neurodegeneration (Steffan et al., 2001). However, the administration of HDAC inhibitors, which promotes histone acetylation, will attenuate neurodegeneration in animal models of HD (Steffan et al., 2001; Hockly, Richon,

Woodman, Smith, Zhou, Rose et al., 2003; Ferrante, Kubilus, Lee, Ryu, Beesen, Zucker et al., 2003).

In a similar fashion, SP1 is also sequestered into the aggregates of mHtt, which disrupts SP1-mediated transcription (Landles & Bates, 2004). Impairment of SP1-mediated transcription can disrupt the dopamine-D2-receptor gene expression, which has been shown to be a characteristic of HD (Dunah, Jeong, Griffin, Kim, Standaert, Hersch et al., 2002). The sequestering of SP1 into the aggregates can disrupt its interaction with the coactivator TATA-binding protein associated factor (TAFII130) and the reduction in the interaction between SP1 and TAFII130 was demonstrated in HD patients (Dunah et al., 2002). Interestingly, Dunah and colleagues (2002) demonstrated that the over-expression of both SP1 and TAFII130 ameliorated the inhibition of D2 receptor gene expression, but over-expression of only one did not restore normal transcription.

REST. Research has also provided support for the interaction of Htt with the repressor element-1 transcription factor (REST) in the cytoplasm (Zuccato, Tartari, Crotti, Goffredo, Valenza, Conti et al., 2003). REST is known to associate with the neuron restrictive silencer element (NRSE), which will affect the transcription of certain genes, such as brain-derived neurotrophic factor (BDNF). By interacting with NRSE, REST reduces the availability of NRSE-binding sites, which promotes transcription of BDNF to promote cell survival (Zuccato et al., 2003). When mHtt is present, there is an Htt loss of function leading to the inability of Htt to interact with REST. This will lead to an increase in NRSE-binding sites, which has been shown to

inhibit BDNF expression (Zuccato et al., 2003). The reduction in BDNF levels has detrimental effects on cell survival for HD individuals.

Rhes

Rhes, the Ras Homolog Enriched in Striatum, is an intermediate-size GTP binding protein (Falk, Vargiu, Foye, Usui, Perez, Danielson et al,, 1999). Although its full functions are not yet known, Rhes has been shown to be preferentially expressed in neurons of the striatum (Falk et al., 1999; Vargiu, Morte, Manzano, Perez, de Abajo, Sutcliffe et al., 2001; Vargiu, de Abajo, Garcia-Ranea, Valencia, Santisteban, Crespo et al., 2004; Harrison & LaHoste, 2006; Errico, Santini, Migliarini, Borgkvist, Centonze, Nasti et al., 2008), and evidence points to its role in dopaminergic signaling and behavior. For example, Harrison & LaHoste (2006) showed that lesions of the nigrostriatal pathway with 6-hydroxydopamine decreases Rhes mRNA in the striatum. As a protein mainly expressed in the striatum, Rhes function has mainly been investigated in the context of this structure. Interestingly, Htt is ubiquitously expressed with no differences in expression among various brain structures and it is present in all organs of the body (Subramaniam & Snyder, 2010). However, degeneration during HD progression occurs mainly in the striatum, with minimal effect on other brain structures. This would imply that a certain striatal phenomenon is responsible for the activation the mHtt. In fact, it has recently been shown *in vitro* that Rhes accounts for the striatal-specific cell death in HD (Subramaniam, Sixt, Barrow, & Snyder, 2009; Subramaniam & Snyder, 2010) by participating in sumoylation, a process by which the small ubiquitin-like modifier, SUMO, is attached to the protein, decreasing aggregation and inducing cell death (Steffan, Agrawal, Pallos, Rockabrand, Trotman, Slepko et

al., 2004). In a groundbreaking study, Subramaniam and colleagues (2009) were able to provide evidence of Rhes mediating mHtt cell death. In that study, overexpression of mHtt or Rhes alone *in vitro* did not increase cell survival but overexpression of Rhes with mHtt present reduced cell survival by 50%. Interestingly, there was normal cell survival when cells expressed Rhes and Htt. Also, the cell death in cells expressing mHtt was reversed when Rhes was depleted. The authors argued that Rhes was able to facilitate mHtt-induced cell death by increasing sumoylation. Sumoylation can modify the functional role of a protein leading to alterations in protein–protein interactions that affect cell functioning by masking existing binding sites or making conformational changes to the target protein that destroy existing binding sites (Geiss-Freidlander & Mechoir, 2007). By increasing sumoylation, Rhes is capable of decreasing aggregation and inducing cell death.

In summary, cells with a remarkable overlap of mHtt and Rhes show degeneration in HD, whereas mHtt cells lacking Rhes seem to survive. The simultaneous reduction in aggregation and increase in cell death brings about the question of whether aggregation is toxic or neuroprotective. In this case, it seems to be neuroprotective and the decrease in aggregation in the presence of Rhes allows cell death to occur. Therefore, the decrease in aggregation may be evidence of an absence of the cell's ability to protect against cell dysfunction, which ultimately leads to cell death.

Purpose and Hypothesis

It is evident that further investigation of the interaction between Rhes and mHtt is needed to elucidate the mechanisms behind HD selective neurodegeneration. Relationships

between Rhes and mHtt have only been shown *in vitro* and future studies should attempt to determine if the interaction of Rhes and mHtt can be responsible for the HD phenotype *in vivo*. The availability of transgenic HD mice and Rhes knock-out mice ($Rhes^{(-/-)}$) has allowed crossbreeding in our laboratory to test the interaction of Rhes and mHtt *in vivo*. Based on the findings of Subramaniam et al. (2009), it is hypothesized that mice carrying a HD/$Rhes^{(+/-)}$ or HD/$Rhes^{(-/-)}$ genotype will show a reduction or absence of the HD motor phenotype and neurodegeneration when compared to mice possessing the HD/$Rhes^{(+/+)}$ genotype. This will be a result of a significant decrease in Rhes expression, which leads to a reduction in sumoylation activity and delay or decline in HD symptoms. The current project tested the following hypothesis: *Deletion of the Rhes gene* in vivo *will render mutant HD mice less vulnerable to the motor deficits and neuropathological symptoms of HD.*

Methods

Although there is debate as to which type of symptoms occur first, the current project has focused on motor symptoms. Motor symptoms are far easier to assay than cognitive symptoms, and have been universally labeled as the indicator of disease onset.

Animals

Breeding. Spano, Branchi, Rosica, Pirro, Riccio, Mithbaokar et al. (2004) used homologous recombination to delete the mouse Rhes gene, thereby generating three genotypes: $Rhes^{+/+}$, $Rhes^{+/-}$ and $Rhes^{-/-}$ (WT, Het, and KO, respectively). Several years ago Dr. Spano kindly provided to us breeder mice from which we have generated a sizeable colony. Wild-type (WT) and knockout (KO) Rhes mutant mice from our colony were cross-bred with

transgenic mice (purchased from The Jackson Laboratory) which either carried (HD) or did not carry (NC) the human mutated Huntington's allele (115 CAG repeats). We chose the R6/1 strain of HD mice (Mangiarini et al., 1996) because of ease in breeding and the mass of data already gathered on these mice. Both male and female offspring for each group (n = 8) were used in testing procedures. The method of cross-breeding (see below) resulted in 6 genotypes, which are as follows:

1) HD/Rhes$^{(+/+)}$; 2) HD/Rhes$^{(+/-)}$; 3) HD/Rhes$^{(-/-)}$; 4) NC/Rhes$^{(+/+)}$; 5) NC/Rhes$^{(+/-)}$; 6) NC/Rhes$^{(-/-)}$

In order to produce these 6 genotypes, we crossbred HD^{+} mice from Jackson Laboratory with Rhes$^{(-/-)}$ mice, which did not carry the mHtt gene. This produced both the HD/Rhes$^{(+/-)}$ and NC/Rhes$^{(+/-)}$ mice. A second crossbreed of HD^{+} mice with Rhes$^{(+/+)}$ mice produced both HD/Rhes$^{(+/+)}$ and NC/Rhes$^{(+/+)}$ mice. Generation of HD/Rhes$^{(-/-)}$ and NC/Rhes$^{(-/-)}$ mice required two rounds of crossbreeding. The first round consisted of crossbreeding HD+ mice with Rhes$^{(-/-)}$ mice to produce animals that were either HD/Rhes$^{(+/-)}$ or NC/Rhes$^{(+/-)}$. The HD/Rhes$^{(+/-)}$ and NC/Rhes$^{(+/-)}$ mice were crossbred with Rhes$^{(-/-)}$ mice that did not carry the mHtt gene for the second round of crossbreeding. This produced HD/Rhes$^{(-/-)}$ and NC/Rhes$^{(-/-)}$ mice that were needed for the current project in approximately 50% of the offspring from this second round of breeding.

Material and Apparatus

Rotarod Apparatus. The rotarod (Med Associates Inc., Georgia, VT) consists of a horizontally oriented 30 mm diameter rotating rod that is used to measure coordination and

balance in rodents. This rod is suspended above a cage floor filled with bedding at a height (10.5”) that is not high enough to injure the animal, but high enough to induce avoidance of a fall. The machine contains a digital time counter to record time spent on the apparatus and the counter will run from the beginning of the test until the animal falls from the rod to the cage floor below. The apparatus also consists of a partition on each side of the rod to prevent any distractions to the animal while testing is taking place.

Suspended Bar. The suspended bar apparatus consists of a 3/8” (~1 cm) wooden dowel suspended 30 cm in the air. A cushioned plastic bin filled with mouse bedding was placed directly under the suspended rod to protect the animals from injury if they fell from the suspended bar.

Triple Beam Balance. A OHAUS® (Florham Park, NJ) triple beam balance was used to calculate the body weight of all animals in the study. The balance has a weight capacity of 2,610 g and contains a metal bin with a top to place the animal inside for proper weight calculations.

Analytic Balance. A Mettler Toledo AG64 balance (Mettler Toledo Inc., Columbus, OH) was used to calculate the weight of the animals’ brains in milligrams. This balance consists of a draft shield to allow for accurate measurements with minimal interference from environmental factors.

Digital Fractional Caliper. A 3” digital fractional caliper (General Tools & Instruments, New York, NY) was used to measure the brain size of all the animals. This instrument calculates brain size to the hundredth of a millimeter.

Procedure

Genotyping.

To determine the genotypes, mice were anesthetized for tail biopsies using 100-150 mg/kg of a ketamine/xylazine solution (AVMA Guidelines on Anesthesia). 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 for a DNA sample using a clean straight razor, and the site was cauterized using a razor blade heated over a spirit lamp. Antibiotic ointment was applied to the wound and animals were monitored until they had fully recovered from anesthesia. The tail clipping was placed in a solution containing 300µl of DirectPCR™ (Viagen Biotech Inc., Los Angeles, CA) lysis reagents and 11.5µl of proteinase K. Samples were incubated 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 1 hour to deactivate the proteinase K, an enzyme that can inactivate the PCR reaction enzymes. DNA was amplified using three separate polymerase chain reactions (PCR) for each DNA sample, one using primers for the mHtt allele, one for the wild type Rhes allele, and one using primers for the Rhes EGFP allele which uniquely recognizes the Rhes null mutation. PCR Reactions for $HD^{+/+}$ were performed in a solution containing 12.5µl GoTaq Green Master Mix™ (400 µM dNTPs, 3mM $MgCl_2$, Taq Polymerase, and proprietary buffer), 3µl each of sense and antisense primers, 9.5µl of nuclease free water, and 2µl of DNA sample. PCR Reactions for Rhes were prepared as above except for 11.5µl of nuclease free water and 0.5µl of DNA sample.

PCR Temperature Parameters

	Melting		*Annealing*		*Extension*		
	^{o}C	*Time*	*^{o}C*	*Time*	*^{o}C*	*Time*	*Cycles*
HD+	94°	0:30	55°	1:00	72°	1:00	31
Rhes+	94°	0:30	60°	1:00	72°	1:00	35
Rhes-	94°	0:30	55°	0:30	72°	1:00	35

Gel Electrophoresis. Gel electrophoresis was carried out using a 3% agarose gel to allow proper separation of PCR products. 10 µl of each PCR reaction sample was placed in each well. A 100 bp 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 0.5 µg/ml solution of ethidium bromide. The gel was then imaged using a Biorad™ gel imager.

Motor Performance on Rotarod.

The rotarod apparatus was used to assess motor performance in all animals from the six conditions over a 6 month period. 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 6 days of testing. Testing was similar to the procedure of Stack et al. (2005) and was conducted once a month for 6 months, with animals evaluated at 1mo old, 2 mos old, 3 mos old, 4 mos old, 5 mos old, and 6 mos old. 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.

Motor Performance on Suspended Bar.

Motor performance on the suspended bar was assessed after the completion of the rotarod evaluation for each monthly test day. Each animal was placed on the end of the dowel facing outward and the latency to turn 180 degrees was recorded. Based on previous experience in the lab, when mice are placed on a ledge they have a natural tendency to turn away from the ledge. In the current apparatus, when the animal is placed on the edge of the dowel, the latency to turn around or fall off the dowel in an attempt to turn around was recorded. Previous research has shown that HD mice consistently lose balance when turning (Mangiarini et al., 1996), which can cause significant differences among the genotypes in the amount of falls from the dowel. Therefore, this test assessed the animal's balance and ability to turn. The animal was removed from the dowel as soon as the 180 degree turn was performed. If the animal did not turn or fall off after 60 seconds had elapsed, the animal was removed from the dowel and given a score of 60. Similar to the rotarod apparatus, each animal was habituated for one day, tested on 6 different days, and each testing day consisted of 3-60sec trials. Each trial was separated by a 60 sec resting period and the cumulative score of all 3 trials per day was recorded.

Limb Movements.

Assessment of limb movements was similar to procedures performed by Stack et al. (2005). Evaluation of limb movements was conducted immediately after testing of motor performance on the suspended bar for each month. Assessment of limb movements entailed 3-10sec trials of suspending the animal in the air by the tail for 10 seconds and individual limb movements were recorded. Whereas normal mice splay their limbs outward when suspended, animals suffering from HD have the tendency to clasp their limbs inward towards their body (Mangiarini et al., 1996; Rubinsztein, 2002). 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 out away from the body. Therefore, 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 and tested once a month for 6 months.

Body Weight Assessment.

Previous research has shown that the body weight of HD mice will plateau with the onset of symptoms and progressively decrease along with the increase in other behavioral symptoms (Mangiarini et al., 1996). In fact, Mangiarini and colleagues (1996) have observed HD mice to weigh as little as 60%-70% of their control counterparts in the end stages of the disease. For this reason, body weight was measured immediately following completion of the other behavioral testing procedures for each month. Therefore, animals of each genotype were weighed every month for 6 months, similar to the other behavioral testing.

Assessment of Brain Size

At the completion of behavioral testing (6 months old), mice of all genotypes were sacrificed by decapitation and the brains were quickly removed and placed in liquid isopentane (~-20^{O}C) to rapidly freeze the brain, which was then stored in a -80°C freezer.

Analysis of differences among genotypes in whole brain weight was estimated by measurement in milligrams. The frozen brains were removed one at a time and placed directly on the scale to measure the weight of each brain to the tenth of a milligram. Immediately after weighing, the brain area was estimated by measurement of the distance from the most caudal to the most rostral parts of the brain. This was done using the digital fractional caliper. The brain was also measured from the most dorsal to the most ventral parts of the brain as well as from the two most lateral parts of the brain. All measurements were conducted in millimeters and were rounded off to the nearest hundredth of a millimeter.

Results

All animals included in the study were produced by the breeding protocol described in earlier paragraphs. The data were examined for statistical outliers and none were found. Therefore, no data were excluded from the analyses. All analyses were performed using SPSS for Windows (version 16.0) with the probability of a Type I error set at 0.05. Data for rotarod performance, suspended bar performance, limb movements, and body weight were analyzed in separate 2 x 3 x 6 mixed factorial ANOVAs with HD gene (HD+, NC) and Rhes gene (WT, Het, KO) serving as between subjects variables and Testing Age (testing once every month) serving as a within-subjects variable. A 3-way interaction was followed by separate analyses of the HD gene

X Rhes gene (2x2) interaction for each day of testing. Significant 2-way interactions on any day were followed by analysis of 1) the simple effects of HD on rotarod performance, suspended bar performance, limb movements, and body weight, 2) the simple effects of Rhes on rotarod performance, suspended bar performance, limb movements, and body weight, and 3) interaction contrast to determine if the reduced amount of Rhes expression reversed or partially reversed the effects of HD on rotarod performance, suspended bar performance, limb movements, and body weight. Tukey's HSD test was used for all post-hoc tests to control the family-wise error rate.

Analysis of Rotarod Performance

Rotarod performance data is summarized in Figure 1. The 3-way interaction indicates that there is a significant difference among the genotypes in rotarod performance [$F(25, 210) = 28.67$, $p < .0001$]. Tukey's HSD post-tests subsequently revealed that HD/Rhes$^{(-/-)}$ animals performed significantly better on the rotarod task than HD/Rhes$^{(+/+)}$ and HD/Rhes$^{(+/-)}$ at 4, 5, and 6 months of age. However, the HD/Rhes$^{(-/-)}$ animals performed significantly worse on the rotarod task than all NC animals at 5 and 6 months of age. Both HD/Rhes$^{(+/+)}$ and HD/Rhes$^{(+/-)}$ animals show significantly more impairment on the rotarod task than all NC animals at 4, 5, and 6 months of age. When comparing HD/Rhes$^{(+/+)}$ animals to HD/Rhes$^{(+/-)}$ animals, the HD/Rhes$^{(+/-)}$ animals performed significantly better than HD/Rhes$^{(+/+)}$ animals at 4, 5, and 6 months of age. The performance of HD/Rhes$^{(+/-)}$ mice was roughly mid-way between HD/Rhes$^{(-/-)}$ and HD/Rhes$^{(+/+)}$ mice (Fig. 1). There were no differences in rotarod performance among the NC groups.

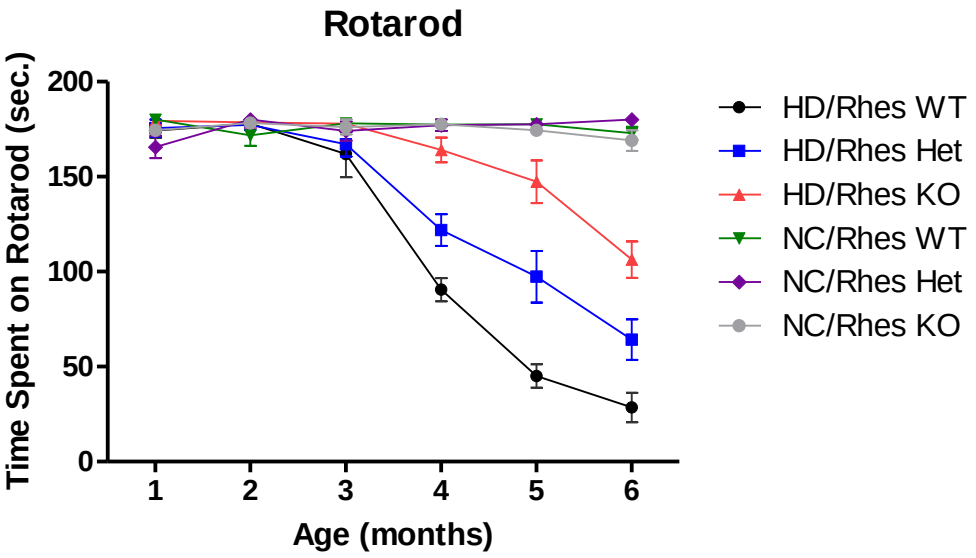


Figure 1. Mean (+/- SEM) time spent on the rotarod for each month of age

Analysis of Suspended Bar Performance

A 3-way interaction was conducted and revealed no significant differences among genotypes in suspended bar performance. Without a significant 3-way interaction, post-hoc tests were unnecessary.

Analysis of Limb Movements

Limb movement testing data are summarized in Figure 2. A significant 3-way interaction [$F(25, 210) = 22.12$, $p < .0001$] was observed and subsequent analysis revealed similar results to rotarod performance. As expected, HD+ mice showed clasping of the limbs when suspended by the tail. There was a significant reduction of clasping in HD/Rhes$^{(-/-)}$ animals when compared to the other HD+ animals. HD/Rhes$^{(-/-)}$ animals had significantly less clasping than HD/Rhes$^{(+/+)}$

animals and HD/Rhes$^{(+/-)}$ animals at 3-6 months of age and 5-6 months of age respectively. However, HD/Rhes$^{(-/-)}$ animals had significantly more clasping than all NC animals at 5 and 6 months of age. The HD/Rhes$^{(+/+)}$ animals showed significantly more clasping than all NC and other HD+ animals at 3-6 months of age. The HD/Rhes$^{(+/-)}$ animals had significantly more clasping than all NC animals at 4-6 months of age. As in the Rotarod test, the performance of HD/Rhes$^{(+/-)}$ mice was roughly mid-way between HD/Rhes$^{(-/-)}$ and HD/Rhes$^{(+/+)}$ mice (Fig. 2). There were no differences in clasping among the NC animals.

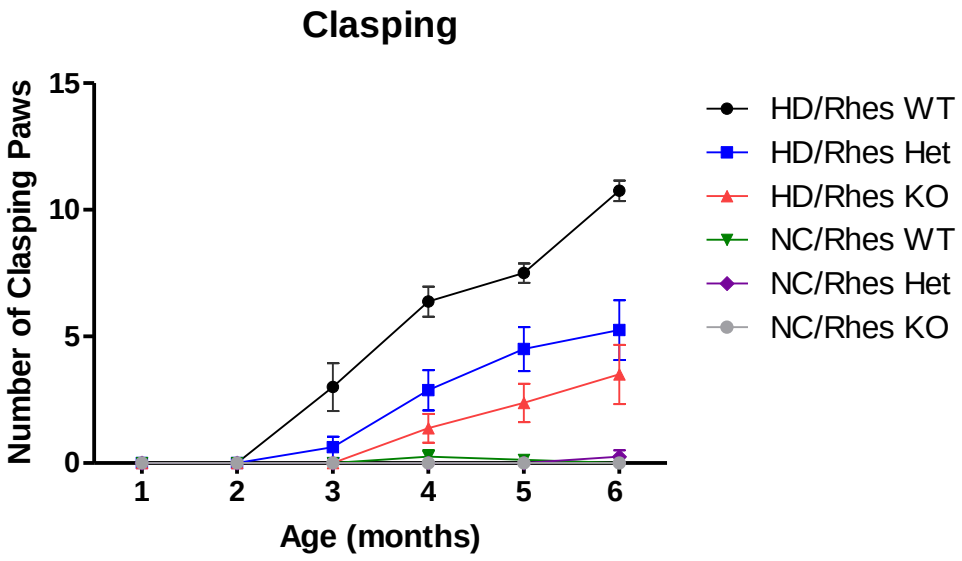


Figure 2. Mean (+/- SEM) number of limb clasps in all genotypes for each month of age.

Analysis of Body Weight

Data for body weight were separated by gender and analyzed in two separate 2 X 3 X 6 mixed factorial ANOVAs. This was done to correct for the anatomical differences between males and females regardless of genotype.

Male Body Weight. The data for male body weight is summarized in Figure 3. A significant 3-way interaction [F (25, 100) = 21.56, p < .0001] reveals a disparity among the different genotypes of male mice. Subsequent post-hoc tests show HD/Rhes$^{(-/-)}$ and HD/Rhes$^{(+/-)}$ mice to weigh significantly less than all NC mice at the ages of 4, 5, and 6 months. There is also a significant reduction in body weight at 5 and 6 months of age for HD/Rhes$^{(+/+)}$ mice when compared to all NC mice, in addition to a significant reduction at 4 months of age when compared to NC/Rhes$^{(-/-)}$ only. The NC/Rhes$^{(+/-)}$ mice had a significantly reduced body weight when compared to all other genotypes at 1 month of age. There were no differences among HD+ animals in body weight.

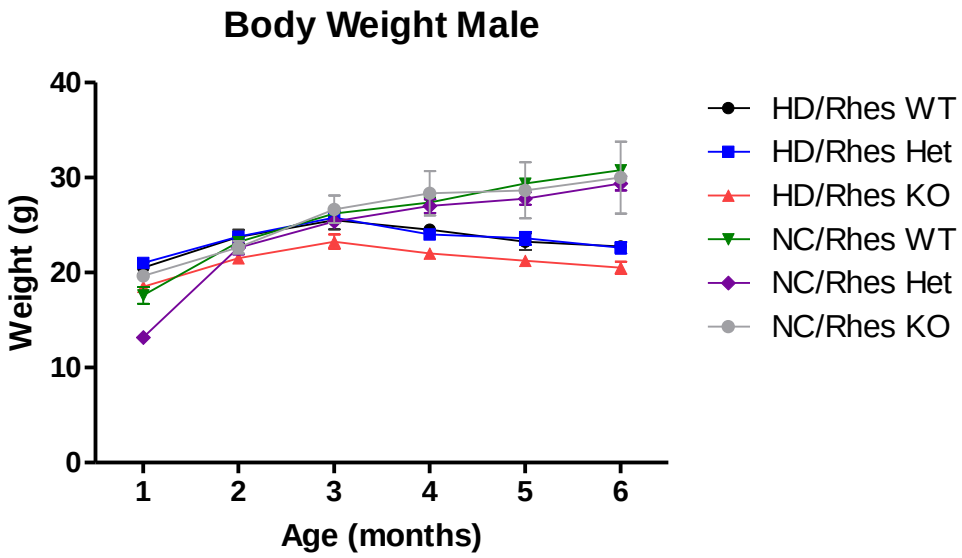


Figure 3. Mean (+/- SEM) body weight of male mice for each genotype.

Female Body Weight. NC/Rhes$^{(+/-)}$ mice weighed significantly less than all genotypes, excluding NC/Rhes$^{(+/+)}$ animals, at 1 month of age. However, there were no differences in body weight between female HD+ animals regardless of Rhes expression.

Analysis of Brain Size

Two separate measures were taken to determine brain size and whether significant atrophy had taken place. After the brain was extracted from each animal, the brain was weighed in milligrams and was then measured with a digital fractional caliper. Therefore, separate analyses were conducted for each one of these measurements.

Brain Weight. Data for brain weights are summarized in Figure 4. A one-way ANOVA was conducted to determine if the mean brain weight of each genotype was significantly different from other brain weights. It was determined that there was a significant difference [$F(5, 37) = 21.38$, $p < .0001$] and subsequent Tukey's HSD post-hoc tests were conducted to determine where these differences take place among the different genotypes. The HD/Rhes$^{(+/+)}$ animals have significantly less brain weight than all NC animals. Both HD/Rhes$^{(+/-)}$ and HD/Rhes$^{(-/-)}$ animals also have significantly less brain weight than NC/Rhes$^{(+/+)}$ and NC/Rhes$^{(+/-)}$ animals, but there is no significant difference between these two HD+ groups and NC/Rhes$^{(-/-)}$ animals. Surprisingly, the brain weight of the NC/Rhes$^{(-/-)}$ mice was significantly less than all other NC brains.

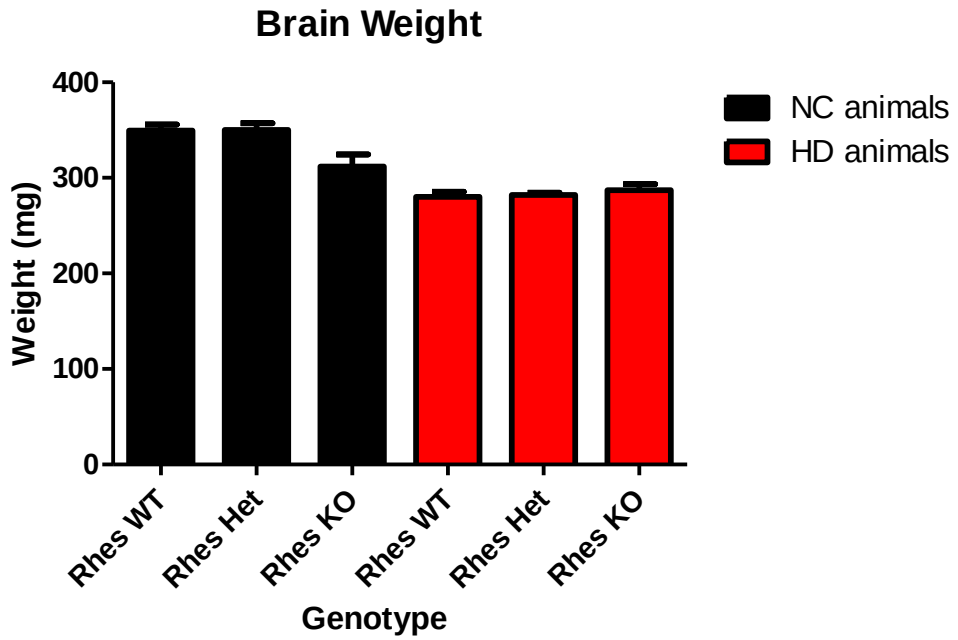


Figure 4. Mean (+/- SEM) brain weight of each genotype.

Brain Surface Area. Data for brain surface area are summarized in Figure 5. For each mouse, the caudal to rostral measurements from both brain hemispheres were averaged to attain an accurate measurement of brain length. This number was then multiplied by the width of the brain (distance from the two most lateral parts of the brain) to produce a value for brain surface area. A one-way ANOVA was conducted to determine if the mean brain surface area of each genotype was significantly different from other brain surface areas. It was determined that there was a significant difference [$F(5, 36) = 15.80$, $p < .0001$] and subsequent Tukey's HSD post-hoc tests were conducted to determine where these differences take place among the different genotypes. In slight contrast to the brain weight measurement, the HD/Rhes$^{(+/+)}$ animals have significantly less brain surface area than both NC/Rhes$^{(+/+)}$ and NC/Rhes$^{(+/-)}$

animals, but no significant difference when compared to NC/Rhes$^{(-/-)}$ animals. However, similar to the brain weight measurement, both HD/Rhes$^{(+/-)}$ and HD/Rhes$^{(-/-)}$ animals also have significantly less brain surface area than NC/Rhes$^{(+/+)}$ and NC/Rhes$^{(+/-)}$ animals, but there is no significant difference between these two HD+ groups and NC/Rhes$^{(-/-)}$ animals. The NC/Rhes$^{(-/-)}$ mice had significantly less brain surface area than all other NC brains.

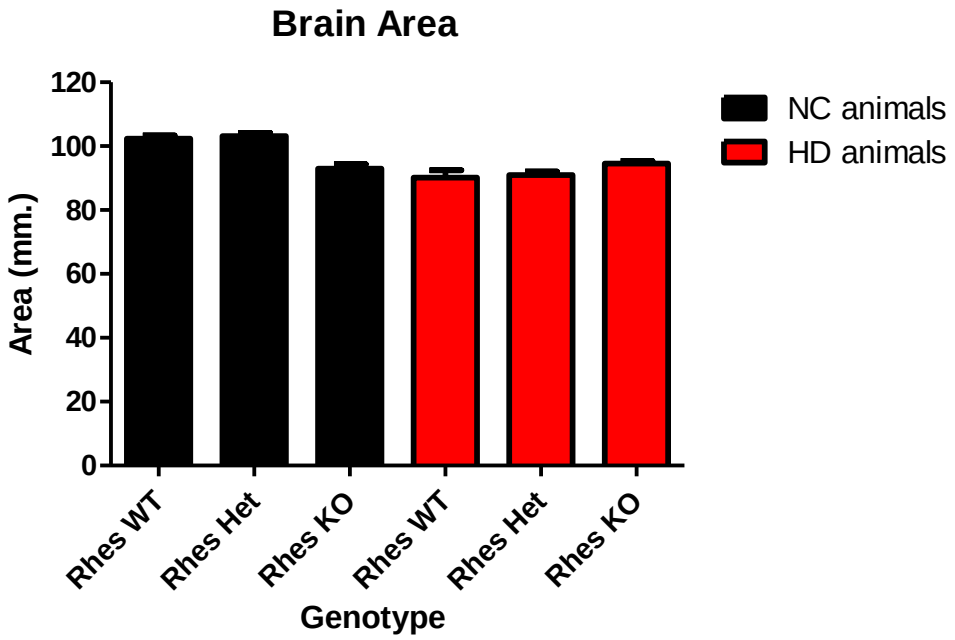


Figure 5. Mean (+/- SEM) brain surface area of each genotype.

Discussion

In agreement with the stated hypothesis, the deletion of Rhes *in vivo* rendered mutant HD mice less vulnerable to motor deficits. While the full deletion of Rhes from HD+ mice did not eliminate motor deficits completely, the deletion resulted in a two-month delay in the onset of symptoms. HD/Rhes$^{(+/+)}$ mice had significant deficits in the rotarod and clasping

measures by 3 months of age. The HD/Rhes$^{(-/-)}$ mice had onset of significant motor deficits at 5 months of age. It is unclear how this translates to human populations, but two months of a C57 mouse's life represents a proportion equivalent to >6 years of the *Homo sapiens* average life expectancy. It is therefore likely that the delay in HD symptoms observed in the present experiment is clinically significant.

There was concern in regards to expectations for those HD+ mice that were heterozygous for the Rhes gene. The level of Rhes expression in Rhes$^{(+/-)}$ has yet to be determined but it is assumed that these mice have less Rhes expression than the Rhes$^{(+/+)}$ but higher Rhes levels than Rhes$^{(-/-)}$. If this is the case, then one would expect the HD/Rhes$^{(+/-)}$ animals to perform better than HD/Rhes$^{(+/+)}$ mice. At the same time, these animals would be expected to perform worse than HD/Rhes$^{(-/-)}$ mice. The findings of the current study revealed this precise scenario, which indicates differences in Rhes expression among the WT, Het, and KO mice. In fact, a study conducted by Dr. Laura Harrison using real-time quantitative PCR has provided concrete evidence of reduced Rhes expression in Rhes$^{(+/-)}$ mice when compared to Rhes$^{(+/+)}$ mice (personal communication).

Despite significant findings of reduced motor deficits in HD+ animals with lower levels of Rhes expression, there were no differences across HD+ animals in regards to suspended bar performance, body weight, and brain size. All the HD+ animals had significant weight and brain size loss when compared to NC animals, but differences in Rhes expression among HD+ animals did not have an effect on these measures.

It is clear that Rhes plays a role in HD pathology and evidence from other laboratories has demonstrated a link between Rhes and sumoylation, a post-translational modification

process. At the same time, researchers have also provided evidence of the importance of sumoylation in HD pathology. The discovery of sumoylation processes within HD pathology (Steffan et al., 2004) and the ability of Rhes to influence sumoylation (Subramaniam et al., 2009; Subramaniam et al., 2010) has led researchers on a chase to understand HD pathology in light of this new evidence. The current study provides far-reaching evidence that Rhes plays a pivotal role in the progression of HD. Previous work on this topic has only been conducted *in vitro* and the current project provides additional and concise evidence in an *in vivo* model. The deletion of Rhes in HD+ mice via crossbreeding has shown a significant decline in typical motor deficits. Furthermore, there is a gene-dose effect that is shown across various HD+ mice with different levels of Rhes expression. The data clearly show that HD/Rhes$^{(+/+)}$ mice have a significant decline in motor deficits, while HD/Rhes$^{(+/-)}$ mice show a less severe decline in motor function than their HD/Rhes$^{(+/+)}$ counterparts. Finally, those HD/Rhes$^{(-/-)}$ animals show significant resistance to decline in motor performance when compared to their HD/Rhes$^{(+/+)}$ and HD/Rhes$^{(+/-)}$ counterparts. This gene-dose curve provides evidence of the importance of Rhes levels in the pathology of HD.

It is believed that Rhes plays a key role in HD by regulating the sumoylation of mHtt, which results in subsequent decreases in Huntingtin aggregation leading to cell death. This decrease in Huntingtin aggregation is an important feature of the sumoylation process that needs to be addressed. As mentioned in earlier paragraphs, it is still unclear whether these aggregates are neuroprotective or neurotoxic, but the general consensus is that their presence is either the result of or the cause of cellular dysfunction. However, recent findings have shown aggregates not to be correlated with neuronal death. In fact, numerous studies have shown

aggregates to decrease during neuronal loss (Arrasate et al., 2004; Slow, Graham, Osmand, Devon, Lu et al., 2005; Bowman, Yoo, Dantuma, & Zoghbi, 2005) and the highest percentage of aggregate formation has been shown to take place in non-degenerating areas of an HD+ brain (Kuemmerle et al., 1999). Therefore, cells containing aggregates are less likely to die than those cells that do not, which is an indication of the protective nature of aggregates. So the sumoylation of mHtt results in decreased aggregation, which results in an increase in cell death.

In order to better understand the mechanisms behind HD, it is imperative to fully explain how Rhes levels influence sumoylation, which in turn affects aggregate formation and cellular death. The following paragraph will summarize the sumoylation process and explain how Rhes can regulate sumoylation to influence HD pathology.

Sumoylation

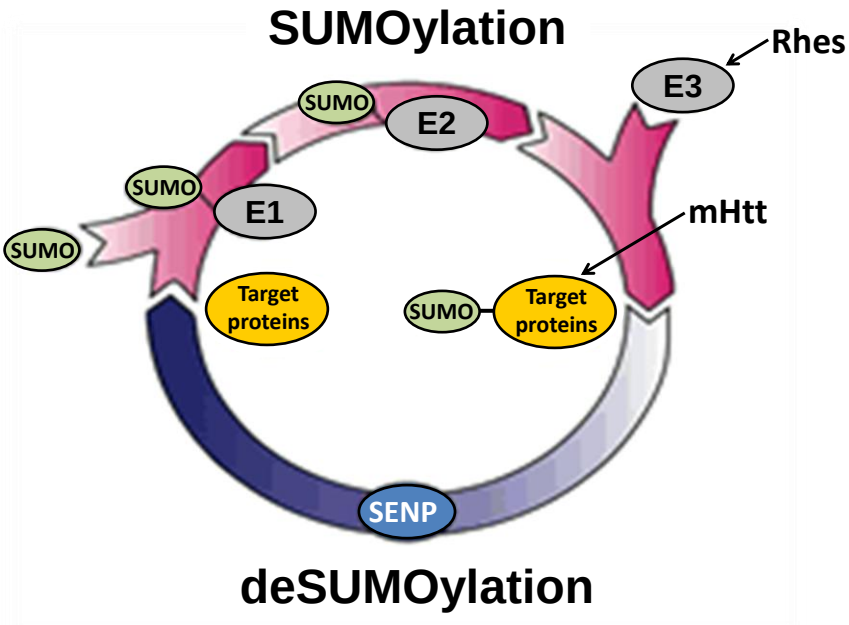
Post-translational modification processes have been shown to be a proficient way of regulating the stability of a protein (Kersher, Felberbaum, & Hochstrasser, 2006). The sumoylation process will be the modification process of focus here, but other processes include but are not limited to phosphorylation, acetylation, ubiquination, palmitoylation, transglutamination, and proteolytic cleavage (Johnson, 2004; Lieberman, 2004; Dorval & Fraser, 2007; Pennuto, Palazzolo, & Poletti, 2009). These modifications regulate a myriad of cellular functions such as targeting of proteins to the membrane, cellular localization of proteins, activation and deactivation of protein functions, and marking of proteins for degradation (Pennuto et al., 2009). Research has shown these post-translational modifications to interact with the polyglutamine proteins, which are responsible for a host of neurodegenerative diseases including HD (Pennuto et al., 2009). More specifically, any disturbances within these

post-translational processes have been shown to contribute not only to neurodegenerative diseases, but to numerous inflammatory diseases and various cancers as well (Dorval & Fraser, 2007).

The sumoylation pathway consists of multiple enzymes that are responsible for activating the SUMO protein and allowing it to conjugate to the target protein to alter the localization, activity, and stability of the target. (For review of this, see Geiss-Friedlander & Melchior, 2004; Lieberman, 2004; Johnson, 2004; Dorval & Fraser, 2007). The enzyme responsible for activating the SUMO protein is called E1. E1 activates the SUMO protein and transfers it to Ubc9 (E2), which is the enzyme responsible for conjugating SUMO to its target protein. Then Ubc9 transfers SUMO to the target protein with the help of E3 ligases, which contribute to the target specificity. These E3 ligases have been shown to enhance specificity of the SUMO protein by interacting with the target protein to make it more likely for the SUMO to bind to the target. However, there are a large number of SUMO protein targets that have been identified and a limited number of E3 ligases. It is this small number of known E3 ligases that suggest that there are additional ligases to be discovered that are responsible for key sumoylation processes. It was not until the groundbreaking study of Subramaniam et al. (2010) that researchers realized the role of Rhes as an E3 ligase in the sumoylation of mHtt.

Rhes, the E3 Ligase. As mentioned earlier, there are a few E3 ligases that have been identified, such as the E3 ligase family PIAS which is responsible for the sumoylation of the transcription factor p53 (Kahyo, Nishida, & Yasuda, 2001; Schmidt & Muller, 2002). However, a study conducted by Subramaniam et al. (2009) found Rhes to act as an E3 ligase for the sumoylation of mHtt. Next, Subramaniam et al. (2010) were able to show evidence of Rhes as

the principal determinant of sumoylation in the striatum. This was discovered by comparing the presence of sumoylated proteins in the striatum for normal mice and Rhes$^{(-/-)}$ mice. Results indicated multiple sumoylated proteins in the normal mice and a greatly reduced amount of sumoylated proteins in the Rhes$^{(-/-)}$ mice. Even more interesting, there was no difference in the amount of sumoylation in the cerebellum (where Rhes is not expressed) when comparing normal mice to Rhes$^{(-/-)}$ mice. This finding is paramount to explaining the mechanism by which Rhes affects HD pathology. As mentioned in earlier paragraphs, neurodegeneration resulting from HD takes place primarily in the striatum, which is the neuroanatomical location of Rhes-induced sumoylation. Therefore, Rhes, a striatal-specific protein, serves as an E3 ligase to induce sumoylation of mHtt in a striatally specific manner leading to the motor disturbances seen in HD.



Possible Explanation for Sumoylation Causing HD Pathology

Ubiquitin vs. SUMO. There are quite a few proteins that are conjugated by both SUMO and ubiquitin, which is not surprising when considering the similarities between sumoylation and ubiquitination (Dorval & Fraser, 2007). Ubiquitin and SUMO proteins share the same mechanisms of conjugation, but the SUMO pathway only contains one conjugation E2 enzyme while the ubiquitin pathway contains multiple E2 enzymes (Johnson, 2004). Since there are so many commonalities between SUMO and ubiquitin, many researchers believe sumoylation and ubiquination are in competition since they act on the same lysine residues of the target protein (Hoege, Pfander, Moldovan, Pyrowolakis, & Jentsch; 2002; Lin, Liang, Liang, Brunicardi, & Feng, 2003; Lee, Chang, Liu, & Derynck, 2003; Steffan et al., 2004;). SUMO can prevent the ubiquination of a protein, specifically mHtt, by blocking the lysine where ubiquitin would normally attach. As explained in earlier paragraphs, the ubiquination process is meant to degrade unused, obsolete, or abnormal proteins, which is a means of maintaining cellular health. In the case of HD, it can be suggested that marking of mHtt by ubiquitin would lead to degradation of the protein therefore allowing the cell to survive. The presence of Rhes in the striatum promotes sumoylation of mHtt in the striatum, resulting in the blockade of ubiquitin from performing the degradative process of the abnormal mHtt protein. At the same time, Rhes is not expressed in other brain regions allowing ubiquitin to conjugate to mHtt and eliminate the toxic proteins. This phenomenon can explain the selective neurodegeneration in the striatum as a result of HD despite the ubiquitous expression of mHtt throughout the brain.

Evidence of this explanation was provided by a study in *Drosophila* models of HD. Researchers found a significant reduction in progressive degeneration in flies heterozygous for

SUMO (Steffan et al., 2004). Even more convincing, the degeneration worsened in flies heterozygous for the ubiquitin gene (Ubi63E) which caused the flies to have genetically reduced levels of ubiquination. Furthermore, the mutation of lysine residues at the conjugation site of mHtt significantly reduced degeneration, indicating the importance of ubiquitin binding motifs for degeneration. When the lysine residues at the ubiquitin binding motif are available, sumoylation at these sites results in increased degeneration, while ubiquination at the same sites results in slight amelioration. These results indicate the role of sumoylation in mHtt toxicity and the promotion of cell survival by ubiquination.

Putative Explanations for Non-significant Findings

Suspended Bar. As described in the results section, the genotype of the mice had no effect on the suspended bar performance. In fact, the presence of HD did not increase or decrease time spent on the suspended bar when compared to NC mice. There was a high level of variability among all groups on this specific task, which leads to the assumption that there was a confounding factor in the task at hand. From observations, all mice, regardless of genotype, were not negatively affected by the ledge of the suspended bar. Based on previous data in the lab, an animal suspended in the air near a ledge would experience some anxiety-like behaviors. This would lead to a tendency to turn away from the ledge to escape this anxiety-provoking situation. However, none of the animals in the study displayed these behaviors at the ledge and spent most of their time exploring rather than evading. Two possible explanations for this phenomenon are the height of the suspended bar and/desensitization. The bar was 30 cm high and this may not have been high enough to provoke any anxiety-induced body rotation on the suspended bar. In fact, some animals were tempted to jump

from the apparatus to more stable ground rather than turning around to avoid the ledge. The second, more plausible explanation could be desensitization. These animals were placed on this apparatus a total of 18 times (6 days of testing with 3 trials per day) during the course of testing. After numerous tests where the animals have failed to fall from the bar, it is possible that the animals have learned not to experience any anxiety when placed on the bar. Therefore, after numerous attempts without any negative consequences, the suspended bar no longer provoked anxiety or fear resulting in no motivation to turn away from the ledge.

Weight Loss. All HD+ mice lost a significant amount of weight when compared to NC mice, as previously found (Mangiarini et al., 1996). However, when comparing the different HD+ mice to each other, differences in Rhes expression did not cause disparities in the amount of weight lost. One plausible explanation for these findings is the specificity of the sumoylation process. As mentioned in previous paragraphs, Rhes acts as an E3 ligase to determine specificity of sumoylation targets. It could be that Rhes acts as an E3 ligase for cells responsible for motor functioning only. If this is the case, some other E3 ligase not yet discovered could allow sumoylation to occur in cells responsible for weight loss. Therefore, the alteration of Rhes expression has no effect on weight loss, but a significant effect on motor performance. This could also be the case for neurons responsible for cognitive processing. This scenario is explained in detail below.

Brain Size. Similar to weight loss data, all HD+ mice had significant decreases in brain size when compared to NC mice, but there were no differences among HD+ mice with different levels of Rhes expression. This is surprising given the amelioration of motor symptoms in HD+ animals with no Rhes expression. With the attenuation of motor symptoms, resistance to

reduction in brain shrinkage would be expected due to the ability of striatal neurons to survive as a result of a decrease in sumoylation processes. It is believed that maybe there is still significant loss of neurons responsible for cognitive processes, while the neurons responsible for motor function are partially spared. This selective neurodegeneration could explain how HD/Rhes$^{(-/-)}$ mice have larger brain sizes than other HD+ mice, but not a large enough increase to create a significant difference. There is still enough neural degeneration in cognitive cells to compensate for the neural survival of motor cells. Future testing on cognitive symptoms would help elucidate these findings. A second possibility is that the gross measure of whole brain dimensions is not sensitive enough to detect the proportionally small loss of neurons relative to the entire brain. Nevertheless, such differences are detectable in the later stages of human HD postmortem (Rosas, Koroshetz, Chen, Skeuse, Vangel et al., 2003).

An even more surprising and unexpected finding was the reduction in brain size for NC/Rhes$^{(-/-)}$ mice. This may have important consequences on the measurements of brain size in HD+ animals. In HD+ animals, a negative correlation was observed with Rhes expression and brain size. On the other hand, the NC animals presented a positive correlation with Rhes expression and brain size. This discrepancy can possibly explain the non-significant results of the brain size measurements among HD+ animals with differential Rhes expression. The absence of Rhes prevented the death of striatal neurons in HD+ animals. However, this absence of Rhes causes reductions in brain size regardless of mHtt presence. In conclusion, the absence of Rhes may rescue some of the neurons typically lost in HD animals. However, this attenuation of volume loss seen in HD/Rhes$^{(-/-)}$ is reversed by the natural loss of neural volume

seen in animals that lack Rhes expression, thus counteracting the cellular rescue from reduced sumoylation.. The end result is a decrease in brain size that is similar to normal HD animals.

Shortcomings

The major drawback to the current project is the uncertainty in the motor performance measurements. The rotarod and clasping task are universally used to measure motor deficits in HD mice. However, it is still unclear whether these tasks are tapping into specific HD-like symptoms (chorea, akinesia, etc.) or whether it is just a measurement of global motor function. As mentioned in the introduction, HD involves a host of motor deficits including chorea, akinesia, bradykinesia, and dystonia. The motor measurements used in the current project and in the majority of HD research literature do not distinguish between the different motor deficits that can be experienced with HD. Therefore, it is possible that assessment of motor performance on these tasks involves a measurement of gross motor behavior that involves several different neural functions. This becomes problematic because some of these neural functions may involve Rhes neurons, whereas other functions may not. This can help explain why there is only a delay in the onset of motor symptoms rather than a complete impediment of symptoms.

Another drawback of the current project is the inability to assess Rhes' effect on all HD symptomology. HD not only includes motor deficits, but also includes cognitive deficiencies and psychiatric disturbances. Therefore, positive results in the current paradigm can only allow assumptions of similar results in other areas of functioning for HD animals. Future tests are needed to fully understand Rhes' role in the whole HD symptomology spectrum. Furthermore, it is possible that Rhes does not alter cognitive or psychiatric disturbances, which can possibly

disrupt performance on motor tasks. For example, an inability to concentrate on the rotarod task can result in the animal falling of the apparatus prematurely despite normal motor functioning. In fact, from a qualitative standpoint, the HD/Rhes$^{(-/-)}$ mice appeared to lose concentration on the task for months 5 and 6. The animals appeared to look around the room with curiosity rather than the rotating beam. This resulted in an early fall from the apparatus despite no visible difficulties in motor movements, which is in contrast to HD/Rhes$^{(+/+)}$ and HD/Rhes$^{(+/-)}$ mice. This confound can possibly explain why HD/Rhes$^{(-/-)}$ mice eventually developed motor deficits later on in the course of the disease.

Another drawback to the current study that requires further investigation is the possibility of the presence of other E3 ligases that are specific for mHtt. As mentioned in a previous paragraph, evidence suggests there are numerous E3 ligases that have not been discovered yet. The delay in onset and eventual progression of HD indicates that Rhes is not the only factor that influences mHtt toxicity. While Rhes plays a significant role, it is evident that there are other factors that can also influence the progression of HD symptoms. The involvement of undiscovered E3 ligases in HD pathology is a strong possibility and the addition of other E3 ligases can compensate for the absence of Rhes, resulting in the eventual manifestation of HD symptoms. Future studies should focus on the discovery of other possible means by which mHtt results in HD pathology.

Future Studies and Therapeutic Consequences

The significant findings of the current study have opened the door for future studies and additional questions that require further investigation. More specifically, does the modification of the Rhes protein attenuate cognitive and psychiatric symptoms of HD in a similar fashion or

is Rhes' influence specific to motor pathways. Future studies will involve an in depth look at the influence of Rhes on HD-induced cognitive deficits. Studies of this nature in animal models will be a complicated endeavor due to the possibility of confounding motor deficits. The majority of cognitive measurements for animal models involve motor movements, which could be negatively affected by HD resulting in misleading cognitive data. Procedures conducted by Giralt et al. (2009) may be considered in future studies as a possible means of measuring cognitive performance in HD animals [see Giralt, Rodrigo, Martin, Gonzalez, Mila et al. (2009) for review of memory tasks].

Another enticing question that requires answers is the applicability of these findings to the clinical setting. It is obvious that methods conducted in animal research that entail DNA alteration via gene deletion are not ethically acceptable in a clinical context. Therefore, another means of repressing Rhes expression is needed to apply these findings to humans with HD. Farnesyl transferase inhibitors (FTIs) seem to be the most plausible way of reducing levels of Rhes activity without altering the DNA sequence. Farnesyl transferase (FTase) is an enzyme that is involved in targeting of proteins to the cell membrane, which is critical for cellular signaling (Appels, Beijnen, & Schellens, 2005). More importantly farnesylation is the predominant method of targeting members of the Ras superfamily to the membrane, including Rhes. For many years FTI's have been used in cancer chemotherapy due their ability to block the signaling properties of Ras superfamily members which play crucial roles in cancer pathogenesis (Appels et al., 2005). Administration of FTIs could potentially block the ability of Rhes to bind to the membrane therefore reducing Rhes' ability to act as an E3 ligase for sumoylation. If this is the case, FTIs could provide a clinically significant chemotherapy, of

which none exist for HD. Studies in animal models to evaluate the potential benefit of FTIs are desperately needed.

Concluding Remarks

In conclusion, these findings have profound therapeutic implications for the treatment of HD. The deletion of Rhes expression has proven to be an effective method of delaying the onset of HD motor symptoms, thus resulting in a longer life. This is a significant finding when one considers the genetic nature of this disease. Genetic testing at a young age for those individuals with a known risk for developing HD allows for early detection and the beginning of disease management before the onset of symptoms occur. While the current findings provide a means of decelerating the progression of HD, Rhes expression is not the only determinant of mHtt toxicity due to the eventual development of symptoms. Until future investigations lead to the discovery of other factors involved with HD, Rhes seems to be a favorable target to treat this fatal disease.

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Institutional Animal Care and Use Committee

UNIVERSITY OF NEW ORLEANS

DATE: November 11, 2009

TO: Dr. Gerald LaHoste

FROM: Steven G. Johnson, Ph.D.
Chairman

RE: *IACUC Protocol* # **UNO-09-011**
Entitled: Breeding and Cross-breeding of Huntington's Disease Mice

Your application for the use of animals in research (referenced above) has been approved beginning November 11, 2009 and expiring November 10, 2012. Please note that an annual/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.

Institutional Animal Care and Use Committee

UNIVERSITY OF NEW ORLEANS

DATE: November 11, 2009

TO: Dr. Gerald LaHoste

FROM: Steven G. Johnson, Ph.D.
Chairman

RE: *IACUC Protocol* # **UNO-09-012**
Entitled: Rhes and Huntington's Disease

Your application for the use of animals in research (referenced above) has been approved beginning November 11, 2009 and expiring November 10, 2012. Please note that an annual/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

Brandon Baiamonte was born in Chalmette, Louisiana and earned his B.S. in Psychology and a minor in Sociology from Louisiana State University in 2005. He went on to earn his M.A. in General Psychology from Southeastern Louisiana University in 2007. In 2008, Brandon enrolled into the University of New Orleans to earn his M.S. and Ph.D. in Applied Biopsychology. Under the tutelage of both Dr. Gerald LaHoste and Dr. Rodney Denis Soignier, he successfully completed all the requirements necessary to earn his doctorate in Applied Biopsychology.