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Effects of Pharmacological De-prenylation of Rhes on Motor Behavior in a Beta-Nitropropionic Acid Animal Model 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

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B.A. Elizabethtown College, 2008
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December, 2015

Acknowledgement

I sincerely thank my major professor, Gerald LaHoste, for his tireless efforts and guidance throughout my graduate studies. My fellow lab members, past and present, were also invaluable to my project, as they provided generous assistance and support. I would also like to thank my committee members, Dr. Leeroy Morgan, Dr. Monica Marsee, Dr. Connie Lamm, and Dr. Elliott Beaton for their helpful suggestions and commentary. Finally, I give my deepest appreciation to my family and friends for their continuous support for all of my endeavors.

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Terms

3-NP – 3-nitropropionic acid

AD – Alzheimer's disease

Akt – protein kinase B

ATP – adenosine triphosphate

BBB – blood brain barrier

BDNF – brain-derived neurotrophic factor

BOLD – blood oxygen level dependent signal

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

CAG – Cytosine Adenine Guanine nucleotide sequence

CNS – central nervous system

DTI – diffusion tensor Imaging

FA – fractional anisotropy

fMRI – functional magnetic resonance imaging

FPPS – farneysyldiphosphate synthase

FPP – farnesylpyrophosphate

FTI – farnesyltransferase inhibitor

GABA – gamma-amino butyric acid

GGTI – geranylgeranyltransferase inhibitor

GGPP – geranylgeranylpyrophosphate

HD – Huntington's disease

HEAT – Huntingtin, Elongation factor 3, the A subunit of protein phosphatase 2A, and TOR1 repeat

HMG-CoA – 3-hydroxy-3-methylglutaryl- Co enzyme A ; mevalonate pathway

HPLC – high performance liquid chromatography

HSP – heat shock protein

Htt – Huntingtin protein

JNK – c-Jun N-terminal kinases

mHtt – mutant Huntingtin protein

Miro – mitochondrial Rho

mTOR – mammalian target of rapamycin

NES – nuclear export signal

NMDA – n-methyl-D-aspartate

PBS – phosphate buffered saline

Rheb – Ras homologue enriched in the brain

Rhes – Ras homologue enriched in the striatum

ROS – reactive oxygen species

SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis

SUMO – small ubiquitin-like modifier

UPS – ubiquitin-proteasome system

Abstract

Huntington's disease (HD) is a heritable, neurodegenerative disorder characterized by motor, cognitive, and psychiatric disturbances. The progressive disease is caused by an unstable CAG expansion within the gene that normally encodes for the huntingtin protein (Htt). The expanded mutant form of Htt (mHtt) is expressed ubiquitously throughout patients' bodies; however, neuronal degeneration is prominent only in the corpus striatum and, to a lesser extent, the cortex. The Ras homolog Rhes is also preferentially localized to the striatum. The putative co-factor Rhes has been shown to act with mHtt to cause neuronal death. Simvastatin, a lipid lowering drug, and zoledronate, a nitrogen bisphosphonate, act on the mevalonate pathway, which gives both Rhes and its target cells, binding sites. The current study aimed to interrupt the mevalonate pathway and inactivate, via de-prenylation, Rhes in CD-1 mice exposed to 3-nitroproprionic acid, a neurotoxin that mimics HD mitochondrial dysfunction and striatal degeneration. Results suggest that drug treatment does not rescue motor impairments and may potentiate 3-NP damage. The persistent motor deficits are discussed in relation to possible Rhes de-prenylation.

Huntington's disease, neurodegeneration, 3-nitropropionic acid, animal model, motor deficits, Rhes, statin, bisphosphonate, prenylation

Introduction

Huntington's disease (HD) is a progressive neurodegenerative disease with no known cure. Affected individuals suffer disturbances across motor, cognitive, and psychiatric domains of functioning. The disease has an autosomally dominant inheritance pattern, leaving a biological child of a HD carrier with a 50% risk of carrying the responsible gene, regardless of sex. The disease is caused by a short arm mutation on chromosome 4 that results in an expansion of the Cytosine (C) Adenine (A) Guanine (G) repeat area (MacDonald et al., 1993). Healthy people with no risk of developing HD may have up to 25 repeats while individuals with 27 to 35 are said to have an intermediate repeat length, the prognosis of which is undetermined (Killoran et al., 2013). Those with at least 36 CAG repeats are labeled as HD carriers and are presumed to eventually experience symptom onset. In non-pathological contexts, the CAG repeat encodes for a polyglutamine section of the Huntingtin (Htt) protein that plays a role in healthy neuronal functioning. With pathological CAG expansion, the mutant Huntingtin (mHtt) protein undergoes conformational and functional changes, and develops hallmark intracellular protein aggregates. HD researchers remain divided over the neurotoxic, or neuroprotective role of mHtt aggregates. The mHtt protein simultaneously exerts emergent toxic effects while preventing the normal Htt functions, the mechanism of which remains largely unknown (Cattaneo, Zuccato, & Tartari, 2005).

The brain pathology of HD is curious, and has provided researchers with a basis for numerous lines of research. The hallmark of gross brain abnormalities in HD is an extreme loss of neurons within the subcortical striatum, despite the ubiquitous expression

of huntingtin throughout the entire brain and in peripheral tissues (Reiner et al., 1988). In particular, the GABAergic medium spiny neurons are lost in proportions up to 95% of the total population (Albin et al., 1992; Graveland, Williams, & DiFiglia, 1985). This finding suggests that a striatal-specific co-factor may be responsible for the frank cell death. One such putative contributor is Ras homologue enriched in the striatum (Rhes), a protein expressed preferentially in the striatum (Falk et al., 1999). Although the exact mechanisms of HD degeneration are yet to be fully understood, Rhes remains a promising lead for the few research groups worldwide who have pursued it.

The gross cell death seen in HD brains is indicative of the highly pervasive clinical deficits associated with the disease. The first formal description of what is now known as HD was penned by George Huntington and accurately describes the experience of HD patients (1895). He noted the involuntary, choreic movements, as well as other behavioral and psychiatric disturbances that could manifest into a dementia. Importantly, he also wrote that the condition affected families, rather than random individuals. HD has a lifetime prevalence rate of 4-8 per 100,000 individuals of European descent, with symptom onset typically between the ages of 30 and 50 (Craufurd, Thompson, & Snowden, 2001; Harper, 1992). Barring non-HD associated mortality, the symptoms progress for the next 10-15 years until death. Thus, from the time a HD carrier experiences the earliest symptoms until the time of death, they are subjected to a psychologically and physically painful deterioration. The most vivid example of this distress comes from the high rate of suicide within HD populations that was evident even as George Huntington wrote the first medical account (Schoenfeld et al., 1984).

Clinical Features

Despite inconsistent reports of specific symptom profiles, it is well known that some psychiatric, behavioral, and cognitive changes emerge before the telltale motor deficits (de Boo et al., 1997; Paulsen et al., 2014a). Furthermore, performance on certain cognitive tasks, such as those incorporating motor planning or sensory functioning, can predict motor symptom onset (Harrington, Smith, Zhang, Carlozzi, & Paulsen, 2012). Cognitive changes experienced by preclinical HD carriers are most evident on timed tasks and include problems with emotion perception (Novak et al., 2012), executive functioning (Papp et al., 2013), attention and working memory (Lemiere, Decruyenaere, Evers-Kiebooms, Vandenbussche, & Dom, 2004), and visuomotor control (Say et al., 2011). The onset of symptoms is significantly based upon the number of CAG repeats one has, and rate of clinical decline accelerates as time goes on (Andrew et al., 1993; Rosenblatt et al., 2012).

Estimates of lifetime prevalence rate among HD patients for psychiatric symptoms, varies between 33% and 76%, based on specific assessment methodologies (van Duijn, Kingma, & van der Mast, 2007). Such symptoms are often more distressing, over motor symptoms, and are an important point of consideration when caregivers and patients are deciding on hospitalization options (Hamilton et al., 2003). Three clusters of psychiatric features, each with its own longitudinal trajectory, have emerged in the research: apathy, irritability, and depression. Apathy may continually progress over time and throughout the disease. Irritability increases throughout the disease, but more so in early stages,

perhaps due to lifestyle adjustments and tiresome coping processes. Lastly, depression, the most common psychiatric symptom, is a point of contention. Many hold that depression is most common in the middle stages of the disease, while others point out increased rates as early as pre-clinical years (Julien et al., 2007; Paulsen et al., 2005). Demonstrated declines in depression may be due to anti-depressant medication relief (Thompson et al., 2012). In later stages, psychiatric functioning can deteriorate until psychosis emerges. In 5%-16% of HD patients, schizophrenia-like psychosis occurs, complete with paranoid delusions and auditory hallucinations (Tsuang et al., 2000).

Striatal degeneration is directly responsible for the prototypical symptom of HD, chorea (from the modern Greek, *"dance"), as* well as many other motor features. Chorea refers to unwanted, involuntary movements of extremities that can be described as dance-like. Beginning usually in distal extremities, e.g. digits and small facial muscles, these twitches develop from mild to moderate classification (Roos, 2010). Early and pre-clinical indications are abnormal saccadic eye movements, brisk muscle stretches, and decreased ability to quickly alternate movements (Penney et al., 1990). Slower reaction times are sometimes also cited, but like other early indicators of imminent disease, the literature is inconsistent on this point (de Boo et al., 1997; de Boo, Tibben, Hermans, Maat, & Roos, 1998; Kirkwood et al., 2000). Descriptions of the early symptoms are continuously revised, as researchers search for the most sensitive behavioral markers. In addition, akinesia, bradykinesia, slowness in performing movement, dystonia, and abnormal and contorted posturing, are well agreed upon symptoms displayed at various time points in the disease course (Rosas et al., 2008). Motor impersistance, or the

inability to maintain any voluntary muscle contraction may also be a unique and sensitive measure of disease severity (Gordon, Quinn, Reilmann, & Marder, 2000).

Gross Brain Pathology

As mentioned previously, the striatum experiences the most severe degradation in HD, but recent evidence shows that several other brain regions are also affected. In addition to detecting global volume changes, structural brain imaging techniques illustrate HD pathology in the human cortex, cerebellum, hippocampus, basal ganglia output structures, and brainstem nuclei (Aylward et al., 2011; Rees et al., 2014; van den Bogaard et al., 2011; Hobbs et al., 2010). Like striatal loss, some such alterations are apparent even before clinical diagnosis or onset of identifiable symptoms (Paulsen et al., 2010; Paulsen et al., 2014b). Within the cortex, projection neurons in layers III, V, and VI are particularly vulnerable in HD (Hedreen, Peyser, Folstein, & Ross, 1991; Jackson et al., 1995). Smaller interneurons are largely preserved in the cortex, as they are in the striatum (Cudkowicz & Kowall, 1990). Progressive cortical loss presents across all lobes, encompassing sensory and motor cortices. However, like phenotypes at any given point in disease progression, cortical changes are heterogeneous across individuals (Nana et al., 2014). Cortical thinning patterns can be tied to specific impairments, even within a class of symptoms. Rosas et al. show (2008) that individuals with greater bradykinesia and dystonia, compared to choreic movements, demonstrate greater cortical thinning in areas that are affected later in the thinning progression. Specifically, greater reductions are seen in anterior frontal cortices, including the pre-motor and supplementary motor areas, without observed striatal differences. Furthermore, cognitive impairments are also

correlated to extrastriatal atrophy (Peinemann et al., 2005). The finding of variable brain loss across individuals, regardless of CAG repeat length or disease severity, pushes researchers to more closely examine extra-striatal regions and attempt to map obscure inter-related functioning (Waldvogel, Kim, Thu, Tippett, & Faull, 2012).

Throughout the brain, connective white matter displays evidence of compromise, also (Poudel et al., 2014). The corpus callosum, composed principally of input fibers from cortical areas, shows early signs of deterioration which progress in a temporally predictable manner, many years prior to the onset of behavioral symptoms and even before measurable atrophy. Diffusion tensor imaging (DTI) and fractional anisotropy (FA) analyses reveal microstructural changes indicating possible dysmyelination, demyelination, degradation away from source projection neurons, reduced density of white matter projections, and directional disruption within axonal pathways (Rosas et al., 2010). Pre-clinical white matter disturbances are also shown within the same cortical areas associated with the earliest callosal loss, i.e. the sensorimotor cortices (Dumas et al., 2012). In fact, white matter loss within the sensorimotor cortex can be used to predict the probability of near symptom onset (Paulsen et al., 2010). Importantly, HD researchers are now able to tie motor, cognitive and psychiatric impairments to regional white matter disturbances depicting specific cortico-basal ganglia circuits (Delmaire et al., 2013).

Striatal Brain Pathology

Despite emerging literature on extrastriatal contributions to disease profiles, the striatum remains the most severely affected brain region in HD. This subcortical region is

the main input structure of the basal ganglia, a set of structures responsible for behavioral activation. In humans, the striatum is composed of the caudate nucleus and putamen. Both receive input from the cortex, the thalamus, and the substantia nigra pars compacta. Striatal output projects to the cortex via connections in the thalamus, substantia nigra pars reticulata, and the globus pallidus (Kandel, Schwartz, & Jessell, 2000). The striatum connects to the cortex via topographically organized thalamocortical circuits specializing in motor and cognitive functions, posing vast targets for HD dysfunction (Bohanna, Georgiou-Karistianis, & Egan, 2011). The motor loop, which governs the planning and execution of movement, connects the putamen with pre-motor, primary sensorimotor, and supplementary motor cortices. Cortical centers for cognition including the frontal and posterior parietal lobes also connect reciprocally to the caudate (Alexander, DeLong, & Strick, 1986). Using diffusion weighted MRI data, anatomical connections throughout the basal ganglia, thalamus, and cortex are apparent and show significant overlap in the prefrontal, premotor, and motor cortices (Draganski et al., 2008). In summary, striatal degeneration has far-reaching effects on other brain areas.

The motor loop appears to be predominantly affected in HD. Using DTI, Bohanna and colleagues (2011) show that in HD patients, the most marked differences to healthy controls are found in striatal areas connected to the primary motor cortex and somatosensory cortices. In this study, HD patients show volume reduction and microstructure abnormalities in both the caudate and the putamen. HD motor symptoms are most closely correlated with increased mean diffusivity of water in grey and white matter, signaling reduction in cell density or compromised membrane integrity. Another

study reports mean diffusivity increases in grey matter of the putamen, the pallidum and the thalamus, though not in the caudate (Douaud et al., 2009). FA data further suggests alterations in fiber organization in HD. Increases in white matter FA, compared to control subjects, signals increase in coherent fiber organization, whereas in grey matter, increases are related to cell body loss and efferent pathways, indirectly increasing organization. Increased FA in the putamen is consistently related to HD status in HD patients (Bohanna, et al., 2011; Douaud et al., 2009) and in pre-symptomatic or pre-clinical gene carriers (Kloppel et al., 2008). Increases are seen also in the caudate, though less consistently. The different FA patterns in the striatum could be because afferent and efferent pathways are, normally, less organized in the caudate than in the putamen (Douaud et al., 2009). Importantly, several studies demonstrate associations between striatal diffusivity and FA measures and performance on motor and neuropsychological tests, as well as disease duration. Even in pre-clinical HD, FA data in the putamen is positively correlated with the probability of diagnosis in the next five years (Magnotta et al., 2009), while multimodal approaches, using volumetric and diffusion methods, have successfully characterized pre-clinical carriers as early as 15 years before symptom onset (Georgiou-Karistianis et al., 2013). In early HD, increased diffusivity in the putamen and the caudate are positively correlated with global functional impairment and to the CAG repeat length, which largely determines age of onset of symptoms (Seppi et al., 2006).

While DTI data provide structural clues as to the pattern of degeneration within HD, fMRI data indicates impaired cortico-striatal functional communication in pre-clinical stages, as well. Unschuld et al. (2012) uses fMRI during a resting state to investigate

functional connectivity in pre-clinical HD carriers. Synchrony of blood oxygen level dependent (BOLD) signals between the caudate and twelve cortical seeds serves as a measure of functional communication (van de Ven, Formisano, Prvulovic, Roeder, & Linden, 2004). First, carriers show significantly decreased caudate and putaminal volumes compared to healthy age matched controls, corroborating numerous reports of reduced striatal volume and increased rate of atrophy in carriers (Aylward et al., 2004; Aylward, 2007; Paulsen et al., 2010). In addition, carriers have significantly decreased BOLD synchrony between the caudate and the premotor cortex, while synchrony with adjacent areas in the motor cortex are at the trend level (Unschuld et al., 2012). A moderate degree of variance in connectivity change can be explained by caudate atrophy, however unexplained variance suggests possible signaling dysfunction stemming from deficient inter-cellular and intra-cellular processes.

Huntingtin Protein

Despite the massive literature on HD, the wild-type or normal huntingtin protein's function remains elusive. Its complete structure and numerous functions are yet to be fully characterized, slowing the discovery of a proven clinical therapy or cure. No other known protein is homologous to huntingtin. Unlike other proteins with a high molecular weight (~348 kDa, depending on individualized structure), it is completely soluble and is expressed throughout the human and rodent body, and ubiquitously in the brain (Cattaneo et al., 2005). The central nervous system and the testes show the highest concentrations (Gil & Rego, 2008). Huntingtin is made up of 3,144 amino acids, of which only a few motifs have been identified. Included in the known structural components are the

following: a polyglutamine tract, an adjacent region rich in the non-essential amino acid proline, and 4 clusters of HEAT repeats (named for the first 4 proteins they were found in: Huntingtin, Elongation factor 3, the A subunit of protein phosphatase 2A, and TOR1), which are sequences of ~40 amino acids duplicated at various points along the protein (Andrade, Petosa, O'Donoghue, Muller, & Bork, 2001; Li & Li, 2004).

At the subcellular level, huntingtin is widely distributed throughout several compartments, serving as a complicating factor for researchers focused on functional identification. The protein is localized to both nuclear and cytoplasmic structures, such as the endoplasmic reticulum, the Golgi apparatus, synaptic vesicles, and mitochondria, as well as neurites (DiFiglia, Sapp, Chase, Schwarz, Meloni, Young et al., 1995; DiFiglia et al., 1995; Hilditch-Maguire et al., 2000; Kegel et al., 2002; Li, Plomann, & Brundin, 2003). Interestingly, evolutionary focused research proposes that the neural localization of Htt is a relatively new occurrence (Kauffman, Zinovyeva, Yagi, Makabe, & Raff, 2003).

Original insights into possible functions stem from huntingtin's structure. The polyglutamine tail, starting at the 18th amino acid position, elongates to no more than 25 repeats, under healthy circumstances (MacDonald et al., 1993). Early work suggests that the multiple glutamines formed a "polar zipper", facilitating binding between huntingtin and other polyglutamine-containing transcription factors, like brain-derived neurotrophic factor (BDNF) (Perutz, 1994). In fact, Htt interacts with multiple partners (Harjes & Wanker, 2003; Zuccato, Valenza, & Cattaneo, 2010).

Htt is thought to be involved in a host of important neural functions, such as, cytoskeletal anchoring, postsynaptic signaling, transcriptional regulation, and intracellular regulation of apoptosis (Caviston & Holzbaur, 2009; Gil & Rego, 2008; Kegel et al., 2002; Rigamonti et al., 2000). Not surprisingly, Htt is necessary for developing embryos. Reductions or elimination of Htt results in serious or fatal defects in the endodermal and neural plate tissue, respectively (Dragatsis, Efstratiadis, & Zeitlin, 1998; Henshall et al., 2009).

Because HEAT repeats are present in many other large eukaryotic proteins involved in nuclear and cytoplasmic transport processes, as well as chromosome separation, the same may be true for Htt (Neuwald & Hirano, 2000). The poly-proline portion is thought necessary for the formation of mHtt inclusion bodies or aggregates, perhaps by affecting solubility (Steffan et al., 2004). Htt also has a Carboxyl-terminal nuclear export signal (NES) and a less active, non-conventional nuclear localization signal, indicating a protein and vesicle transport role. These elements allow for the movement of Htt in and away from the nucleus, likely as part of a larger nuclear-cytoplasmic shuttling complex. Importantly, the NES is cleaved away in mHtt, leaving the protein able to enter the nucleus, but not exit (Xia, Lee, Taylor, Vandelft, & Truant, 2003).

Mutant Huntingtin (mHtt)

Evidence suggests that HD pathology may not only be the gain of aberrant mHtt function but also the loss of wild-type Htt function (Landles & Bates, 2004). In fact, the

many proposed HD mechanisms are all said to be driven by the decreased expression of functional Htt, the increased expression of mHtt, or by both. The hallmark histopathological finding in HD is the presence of intra- and extra-nuclear inclusion bodies within neurons of all cortical layers and medium sized striatal neurons (Davies et al., 1997; DiFiglia et al., 1997). HD aggregates differ in composition, with nuclear bodies consisting mostly of N-terminal mHtt fragments and extra-nuclear bodies consisting of both fragments and full length mHtt (Cooper et al., 1998; Martindale et al., 1998). Aggregation is caused by the expanded polyglutamine portion of mHtt, which induces a conformational change and subsequently initiates downstream pathogenesis. In healthy cases, exon 1 of Htt has an N-terminal α helical structure (Kim, Chelliah, Kim, Otwinowski, & Bezprozvanny, 2009). The shape is flexible and transforms into a well-defined α helix, a random coil, or a loosely-bound and extended loop conformation at various, randomly determined points along the glutamine chain. This flexibility creates a low threshold for interference from outside influences, such as length of polyglutamine tract and neighboring protein motifs (Kim, Chelliah, Kim, Otwinowski, & Bezprozvanny, 2009). In mHtt's first exon, the flexibility in shape is a disadvantage. In this case, the random coil portion of the polyglutamine expansion misfolds into a hairpin structure, described as two non-parallel β strands and a sharp turn (de Mezer, Wojciechowska, Napierala, Sobczak, & Krzyzosiak, 2011).

Two, non-mutually exclusive pathways to aggregation are proposed. The first involves proteolysis, the process by which proteins are either partially degraded into peptides, or completely broken down into amino acids. Specifically, N-terminal mHtt is cleaved by

two classes of protease enzymes, caspases (notably caspase-3 and caspase-6) and calpains, which leave behind mHtt fragments with a particular propensity for diffusing into the nucleus and forming inclusion bodies (Gafni & Ellerby, 2002; Goldberg et al., 1996; Graham et al., 2006). mHtt fragments recruit further proteases, thereby creating a positive feedback loop resulting in amplified aggregation until, possibly, the cell ultimately dies (Gil & Rego, 2008). Secondly, aggregation can stem from mHtt protein misfolding, a phenomenon which Htt protects against (Fink, 1999). Normally, chaperones such as heat-shock protein 70 (Hsp70) and heat-shock protein 40 (Hsp40) coordinate to correct misfoldings and maintain the solubility of Htt (Hartl & Hayer-Hartl, 2002). If misfoldings remain after these chaperones are active, the ubiquitin-proteasome system (UPS) acts as a secondary degradation mechanism for target proteins (Voges, Zwickl, & Baumeister, 1999). Working alongside the UPS is the even more capable autophagic machinery. Autophagy, sometimes called macroautophagy, can degrade even large structures, such as entire organelles. It is not known how the two systems coordinate, but there is evidence of a bidirectional influence (Korolchuk, Menzies, & Rubinsztein, 2010). Despite these multiple protections, if the misfolded mHtt protein generates faster than the degradation buffers can act, aggregation will begin. Exacerbation occurs while the important chaperones and proteasomes are further sequestered within the aggregates (Hay et al., 2004; Martin-Aparicio et al., 2001).

Controversy remains over the role of mHtt aggregates in HD neurodegeneration. Traditionally, researchers have viewed mHtt aggregates as cytotoxic. This argument cites evidence of intracellular aggregates in several neurodegenerative diseases and the

correlation between aggregation and cell death (Yang, Dunlap, Andrews, & Wetzel, 2002). Aggregates, generally, do seize important proteins that also contain a polyglutamine tract and that normally facilitate or regulate transcription, as well as parts of the essential proteasome safeguards (Cha, 2007). Overexpression of proteasome components reduces aggregation and prolongs cell life in transgenic and cell models of HD (Carmichael et al., 2000; Vacher, Garcia-Oroz, & Rubinsztein, 2005). Others contend that aggregation is noncausal and incidental to cellular dysfunction (Kuemmerle et al., 1999). Post-mortem human HD brains demonstrate the highest number of aggregates in the cortex, rather than the most severely affected striatum, suggesting such an incidental role (Gutekunst et al., 1999). Examination also found that neuropil aggregates in dendrites and dendritic spines are much more common than nuclear aggregates. Furthermore, non-CNS tissue also displays a number of mHtt aggregates (Moffitt, McPhail, Woodman, Hobbs, & Bates, 2009). Importantly, a mouse model containing only a fragment of the full length HD gene indicates it is not the aggregates that drive neuronal loss. The model displays early and widespread neuronal nuclear inclusions, without signs of neuronal dysfunction or cell death (Slow et al., 2005).

Still a third argument describes the neuroprotective capacity of aggregates. As mHtt continues to be sequestered by aggregates, the amount of diffuse or soluble form of mHtt in other cell locations theoretically decreases. Alterations in the length, and thus aggregation, of mHtt have shown related decreases in the number of neuronal aggregates and death (Ratovitski et al., 2009). Survival analysis shows that inclusions actually predict cell survival, indicating the presence of aggregation could be a coping mechanism

of the cell (Arrasate, Mitra, Schweitzer, Segal, & Finkbeiner, 2004). Interestingly it has been found that aggregates also sequester mTOR, a protein responsible for suppressing autophagy (Ravikumar et al., 2004). This kinase suppresses autophagy which alleviates toxicity and behavioral symptoms of HD (Jia, Hart, & Levine, 2007).

Rhes Protein

Present work builds from important findings from the Snyder lab at Johns Hopkins University showing the neuroprotective nature of mHtt aggregation and the protein Rhes' contribution to HD neuropathology. As evident in its name, Rhes or the Ras homolog enriched in the striatum, is preferentially located in the neural regions most vulnerable to degeneration. Rhes is an intermediate sized guanine-nucleotide binding protein (Falk et al., 1999). Similar to prototypical Ras family proteins, Rhes has guanosine triphosphate (GTP) binding and effector domains and a CAAX box (composed of a Cysteine which attaches to membranes, two aliphatic amino Acids, and one of several amino acids, X). It also has an extended C terminal, adding to its molecular weight (Falk et al., 1999; Harrison, 2012). The full functions of Rhes have yet to be elucidated but clear evidence has been found implicating Rhes in striatal GPCR signaling at the level of intracellular messaging cascades (Harrison & LaHoste, 2006; Spano et al., 2004 (Lee et al., 2011).

Rhes binds to mHtt to a greater extent than the protein binds with Htt. Subramaniam and colleagues (2009) show that Rhes overexpression in cells containing mHtt decreases cell survival by 50%. Such a decrease does not occur when either Rhes or mHtt is

overexpressed individually, nor is it shown in cells expressing both Rhes and Htt. Furthermore, Rhes depletion, through RNA interference, increases cell survival. Likewise, they find that, in mHtt, but not Htt, knock-in striatal cells, overexpression of Rhes further decreases cell survival by 60% overall (Subramaniam, Sixt, Barrow, & Snyder, 2009).

Rhes is argued to influence neuronal death by influencing sumoylation. Sumoylation is a post-translational modification process in which a small ubiquitin-like modifier (SUMO) is covalently attached to various proteins causing a change in functioning. When mHtt undergoes sumoylation, aggregation is reduced and neurotoxicity is increased. This finding lends support to the notion that non-aggregated, soluble mHtt is toxic (Steffan et al., 2004). Rhes enhances sumoylation of mHtt in a time- and concentration-dependent manner; thus, the potential cofactor is responsible for the disaggregation of mHtt bodies and consequential cytotoxicity (Subramaniam et al., 2009). Novel in vivo work demonstrates that HD mice without the Rhes gene are less susceptible to the motor deficits and neuropathological markers of HD, corroborating a cytotoxic role for Rhes (Baiamonte, 2012). However, contradicting evidence using RNA interference against Rhes mRNA shows the opposite effect behaviorally (Lee et al., 2014).

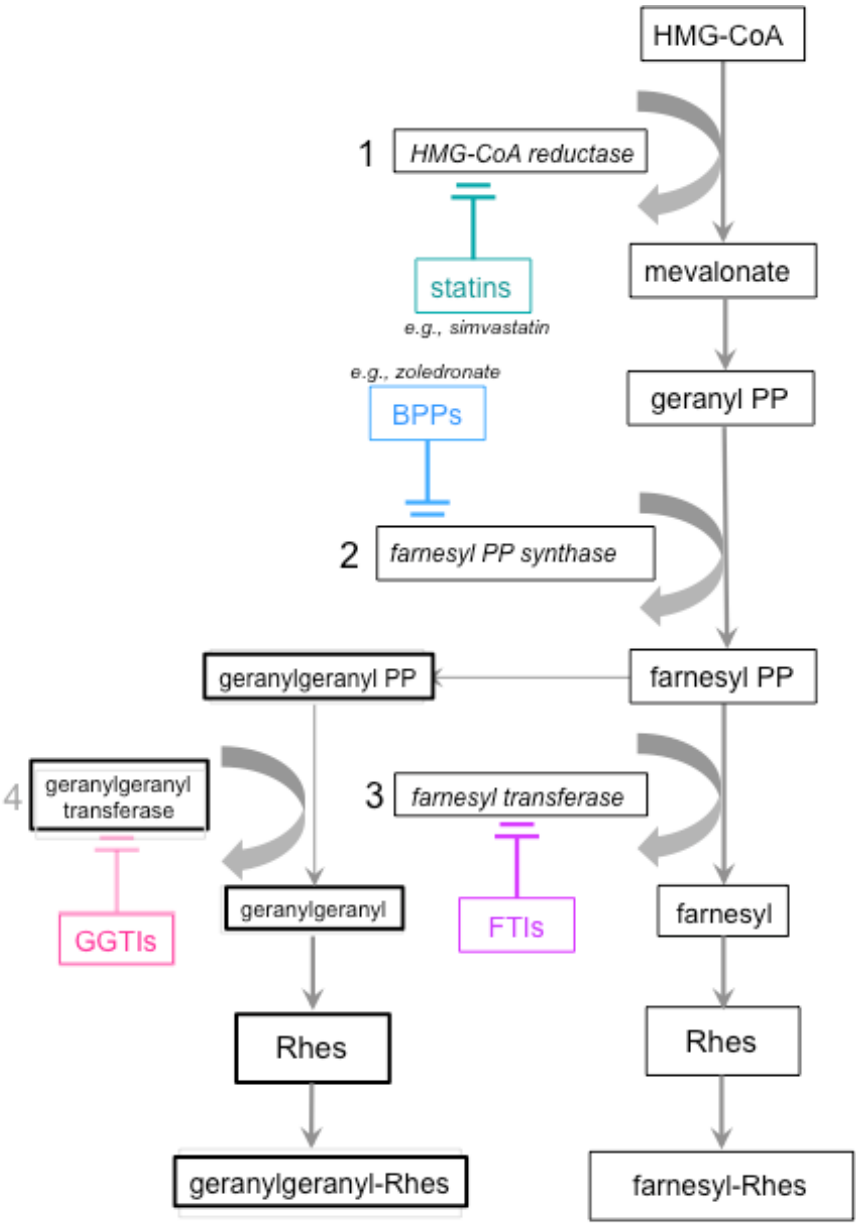
Furthermore, Rhes has independent influence on autophagy, a process that cells utilize to clear not only soluble mHtt but also aggregates. Findings paint a complicated picture where Rhes can have both anti- and pro-autophagic effects. Subramaniam et al. (2012) show that Rhes has a high affinity to binding autophagic factors likely a result of Rhes'

uniquely extended C-terminal which promotes binding in general (Subramaniam et al., 2012). Rhes bound to and activated both mTOR complexes 1 and 2. These complexes are known to inhibit autophagy. The Rhes-mTOR interaction was evident in striatal culture and tissue. However, the same group later found that in culture, Rhes can induce autophagy by circumventing mTOR and activating a regulator, Beclin-1, resulting in increased autophagy. Interestingly, co-expression with mHtt blocked Rhes' pro-autophagy influence (Mealer, Murray, Shahani, Subramaniam, & Snyder, 2014).

Mevalonate Pathway

In searching for therapeutic strategies focused on Rhes intervention, the current study looks toward the structure of Rhes as a potential target. Like other small GTP-binding proteins and Ras family proteins, Rhes shares a prenylated chemical structure (Hancock, Magee, Childs, & Marshall, 1989; Takai, Sasaki, & Matozaki, 2001; Vargiu et al., 2004). Prenylation, or isoprenylation, is the process by which at least one prenyl group, farnesylpyrophosphate (FPP) or geranylgeranylpyrophosphate (GGPP), is added to the C-terminal cysteine(s) of peptides and proteins by farnesyltransferase or geranylgeranyltransferase, respectively. The hydrophobic prenyl groups serve several purposes, including anchoring to cell membranes or to other proteins. After prenylation, Ras proteins undergo proteolysis and C-terminal methylation (Backlund, Simonds, & Spiegel, 1990; Zhang & Casey, 1996). Proteolysis and methylation facilitate proper membrane anchoring, but the initial prenylation is essential. Prenylation occurs as a downstream mevalonate pathway change, which is one of the most studied biological synthesis pathways in the human body. Also known as the 3-hydroxy-3-methylglutaryl-

CoA (HMG-CoA) pathway, the cascade most notably produces cholesterol and assists a multitude of functions, such as cell respiration, hormone production and maintaining membrane integrity (See Figure 1; Fritz, 2009).



The mevalonate pathway, with Rhes depicted, and the interfering classes of drugs. HMG-CoA: 3-hydroxy-3-methylglutaryl- CoA; BPPs: nitrogen bisphosphonates; PP: pyrophosphate; FTIs: farnesyltransferase inhibitors; GGTIs: geranylgeranyltransferase inhibitors

Given that the numerous mevalonate pathway metabolites influence such a diverse group of proteins, therapeutic interference is explored in various disease models. Pharmaceutical techniques can inhibit CAAX box post-translational modification by acting at intermediate stages or inhibit the pathway's key enzyme, HMG-CoA reductase, at the start of the pathway (Goldstein & Brown, 1990). Farnesyltransferase inhibitors (FTIs) and geranylgeranyltransferase inhibitors (GGTIs) prevent the protein prenylation. By preventing the transfer of farnesyl, geranygeranyl groups, or both, to CAAX box motifs, these drugs stop prenylated proteins from attaching to cell membranes, thereby interfering with signal transduction (Goldstein & Brown, 1990). Similarly, nitrogen bisphosphonates prevent prenylation by acting above FTIs and GGTIs in the pathway. This class of drugs targets farneysyldiphosphate or farnesylpyrophosphate synthase (FPPS), which is necessary for downstream prenylation (Goffinet et al., 2006). Since small GTP proteins, like H-, K-, and N-Ras, are the most common culprits of oncogenic mutations, there has been an impressive exploration of oncogenic therapies utilizing FTIs, GGTIs, and bisphosphonates (Drake & Cremers, 2010; Gao, Liao, & Yang, 2009). Animal models show benefits to Ras prenylation inhibition in colon, pancreatic, lung, prostate, bladder, and breast cancer (Ayral-Kaloustian & Salaski, 2002).

While nitrogen bisphosphonates take action toward the end of the mevalonate pathway, statins act at the beginning, inhibiting HMG-CoA reductase, which serves as the rate-limiting enzyme, producing mevalonate (Kuzuyama & Seto, 2012). Statins are best known for their clinical application in cholesterol management but they may prove useful in treating Alzheimer's disease (AD) and other dementia states. Epidemiological studies

show that chronic statin use is associated with decreased risk for AD (Jick, Zornberg, Jick, Seshadri, & Drachman, 2000). In vitro and in vivo work shows that statins reduce amyloid beta protein production that cause neural plaques in AD (Fassbender et al., 2001; Simons et al., 1998). Statins likely have therapeutic effects based on the regulation of isoprenoids, necessary for prenylation. Mevalonate derived compounds, FPP and GGPP are dramatically elevated in human AD brains. In vivo treatment with simvastatin, a highly potent statin that is able to cross the blood-brain barrier, significantly reduces both isoprenoids in mice (Eckert et al., 2009). Clinical trials using statins have shown mixed results, though the cholesterol pathway hypothesis of AD remains prominent (Hoglund & Blennow, 2007).

Because HD and AD are both neurodegenerative diseases involving intranuclear inclusion bodies and possible Ras contribution, HD researchers have taken a cue and are beginning to explore what the mevalonate pathway contributes to the disease. Valenza and colleagues (2005) found altered cholesterol biosynthesis resulting in lower overall cholesterol mass in cultured mHtt cells, as well as cortical and striatal tissue of HD mice. Importantly, by adding exogenous cholesterol into striatal mHtt neurons, they were able to rescue the cells in a dose dependent manner.

Relatedly, Del Toro and colleagues argue that augmented cholesterol homeostasis in HD takes the form of cholesterol accumulation and altered cellular distribution that contributes to excitotoxicity (2010). Not only is accumulation documented in striatal neurons and tissue of mHtt mice, but mHtt cells show an increased level of cholesterol

and altered distribution in the plasma membrane and intracellular deposits (Trushina et al., 2006). Not surprisingly, cultured striatal cells and neurons that contained mHtt also demonstrate an increase in cholesterol-rich, highly ordered portions of the plasma membrane and those in the cytosol (Valencia et al., 2010). Healthy Htt cells have only sporadic places of highly ordered domains, with more fluid membranes in the cytosol. Because NMDA receptors are located within such high cholesterol areas, known as lipid rafts, the extent of membrane order influences how susceptible neurons are to NMDA mediated excitotoxicity. Simvastatin causes decreases in plasma membrane order, reduces lipid rafts, and protects against NMDA excitotoxicity in both Htt and mHtt cells (Ponce et al., 2008). Interestingly, simvastatin treatment does not reduce overall cholesterol levels, supporting earlier work which suggested simvastatin redistributes plasma cholesterol content in other brain areas (Burns, Igbavboa, Wang, Wood, & Duff, 2006; Paolisso et al., 1991).

Statins' blocking of downstream protein prenylation affects more than membrane configuration. Statins, like nitrogen bisphosphonates, prevent the synthesis of isoprenoids FPP and GGPP, which as described above, are necessary for the anchoring of prenylated proteins to membranes or other proteins (Jasinska, Owczarek, & Orszulak-Michalak, 2007). Since Rhes is prenylated, de-prenylation techniques have been used previously, showing prevention of the normal sumoylation and subsequent disaggregation of mHtt, as well as Rhes-mediated cytotoxicity (Subramaniam et al., 2009). Thus, statins and nitrogen bisphosphonates offer the potential for clinically relevant means to inhibition of Rhes prenylation, an important step toward unlocking a treatment for HD.

In summary, statins and nitrogen bisphosphonates hold promising potential for HD pathology on two fronts. First, statins' cholesterol lowering effects could attenuate cholesterol accumulations found in striatal neurons of HD patients and mice. A decrease in such accumulations is protective against NMDA-mediated excitotoxicity, one proposed mechanism of HD degeneration. More relevant to the proposed research, statins and nitrogen bisphosphonates may pharmacologically interfere with the putative cofactor, Rhes. By compromising plasma membrane integrity and blocking prenyl group synthesis, the drugs could prevent the prenylation of Rhes, thus, rendering the striatally expressed protein largely inactive. Since the drugs act at different steps in the mevalonate pathway, the size and number of both target and any pleiotropic effects also likely differs, offering a potentiating effect of treatment with both compounds (Vincenzi et al., 2003). Combination therapies are beginning to be explored in some of the various disease models involving prenylated proteins (Blondel et al., 2014; Issat et al., 2007; Varela et al., 2008).

3-Nitropropionic Acid

To examine the mevalonate pathway mechanisms as they apply to HD, a disease model must be chosen carefully. Despite the usefulness of transgenic animal models of HD, in rodents and flies, the majority of animal HD research is conducted with chemical models which induce cell death and dysfunction (Tunez, Tasset, Perez-De La Cruz, & Santamaria, 2010). Such models offer a faster onset and, possibly, a faster physical decline than transgenic models, whether they employ quinolinic, malonic, kainic, or 3-

nitropropionic acid (3-NP). Derived from various fungi and plants, 3-NP was first described after infected American cattle demonstrated poor motor coordination and eventual paralysis. In fact, bodily uptake can mimic HD motor impairments (Ludolph, He, Spencer, Hammerstad, & Sabri, 1991).

3-NP is a toxin that disrupts the energy metabolism within brain cells. Exposure to 3-NP irreversibly inhibits succinate dehydrogenase, or complex II of the electron transport chain and Krebs cycle, which triggers a cascade of events culminating in neuronal death within the striatum (Alston, Mela, & Bright, 1977). Complex II inhibition causes multiple pathogenic mechanisms to take action, each contributing to apoptotic or necrotic cell death. By disrupting the mitochondrial membrane potential, adenosine triphosphate (ATP) depletion occurs, preventing Na^+ and Ca^{2+} from exiting the cell, leaving a state of depolarization. The NMDA receptors are left susceptible to excitotoxicity by normal levels of glutamate. In addition, a flood of Ca^{2+} enters the cell, activating calcium dependent enzymes, like calpains and nitric oxide synthase. Eventually, 3-NP causes brain lesions localized specifically to the striatum (Blum, Gall, Cuvelier, & Schiffmann, 2001; {Gould and Gustine, 1982, #78719}). The lesion affects medium sized spiny GABAergic neurons, while sparing interneurons, reproducing the well-known HD pathology (Beal et al., 1993). This model also shows motor impairment similar to that seen in human populations (Kraft, Osterhaus, Ortiz, Garris, & Johnson, 2009; Ubhi et al., 2009). Thus, 3-NP treatment mimics both the mitochondrial dysfunction, the preferential striatal degradation, and observable motor deficits displayed in HD, along with greater logistical control than transgenic models during testing.

The 3-NP HD model has been used to show a protective effect of Rhes depletion using transgenic mice designed to have varying levels of endogenous Rhes. Mealer et al. demonstrate that Rhes knockout mice are protected against 3-NP driven neurotoxicity and motor impairment (2013). Compared to wild-type controls, Rhes knockout mice could cross an elevated beam more quickly and with more coordination. The striatal lesions in mice without endogenous Rhes were mostly non-existent, apparent only slightly in two of ten mice. Interestingly, Rhes knockout mice did not live significantly longer, speaking to the toxicity of 3-NP. While these results are impressive, a more clinically applicable Rhes-focused strategy is still required. The use of post-transcriptional gene interference targeting brain cells has inherent complications preventing accessibility in human populations. Rather, pharmacological strategies, such as statin or bisphosphonate treatment, are the ideal method of treatment. To that end, both classes of drugs are already clinically available for use in other disorders.

In addition, statin treatment has previously been shown to attenuate striatal neurodegeneration caused by 3-NP. Lee et al.describe an anti-apoptotic effect of atorvastatin in rats (2008). After 5 days of simultaneous administration of both 3-NP and atorvastatin, experimental rats showed fewer behavioral sign of neurological dysfunction, as well as smaller striatal lesions and fewer apoptotic cells (Lee et al., 2008). Importantly, this study establishes the desired therapeutic effect of a statin-class drug. However, analysis did not include a measure of HMG-CoA pathway activity or associated proteins, e.g. Rhes.

Hypotheses

Current evidence implicates Rhes in promoting solubility of mHtt, potentially leading to HD neurodegeneration. The current study sought to inactivate Rhes via de-prenylation in a pharmacological model of HD, exploring the potential benefit of already clinically available drugs. Injection with 3-NP was hypothesized to mimic HD striatal lesions seen in humans, causing specific motor impairments seen in transgenic HD mouse models, compared to control mice receiving no 3-NP. Additionally, we predicted that statin and nitrogen bisphosphonate treatments would cause de-prenylation of Rhes, measured by western blot analysis, and protect against 3-NP neurotoxicity and motor impairments, measured by rotarod and clasping behavior, compared to animals receiving no drug treatment.

Methods

Animals

All procedures were carried out in this study under the approval and supervision of the University of New Orleans Institutional Animal Care and Use Committee (IACUC; Protocol 15-009). All animals in this study were purchased from Harlan Laboratories (Indianapolis, IN). The particular strain of mice, CD1 mice, were chosen for their widespread use in toxicology and behavioral experiments. Animals were housed with litter mates on a 12hr light dark cycle and provided food and water without restriction. A total of 60 animals were used, equally divided and randomly assigned to 5 experimental groups. The first group is a control group receiving no 3-NP or drug treatment. The remaining four groups received 3-NP treatment along with either no drug treatment,

statin treatment alone, bisphosphonate treatment alone, or both statin and bisphosphonate treatment.

Material and Apparatus

Rotarod apparatus. The rotarod device (Med Associates, Inc., Georgia, VT) is designed to assess animal motor coordination and balance. It is made of a suspended, rotating horizontal bar attached on both sides to the center of a 38 cm-wide metal wall. The walls, and thus, the bar, rotate at a rate of 16 revolutions per minute (rpm). The bar is 30 mm in diameter and positioned 27 cm above a floor of soft bedding material. The height of the bar is intended to encourage the animal to continue walking as to avoid a fall; but in the event of falling, the bedding acts as a protective padding.

Triple beam balance. Animals' body weights were measured using an OHAUS® (Parsippany, NJ) triple beam balance. Animals were placed in an attached, covered metal container which allows for proper measurement.

Analytical balance. A Mettler Toledo (Columbus, OH) AG64 scale was used to calculate all drug quantities. The scale has the capacity to measure masses within 1 mg. It is further equipped with a glass shield which protects against drafts, minimizing environmentally caused fluctuations in reading.

Simvastatin treatment. Simvastatin (Zocor® Merck & Co., Inc. Whitehouse Station, NJ) was generously provided by Dr. Lee Roy Morgan.

Zoledronate treatment. Zoledronate was purchased from Sigma-Aldrich (St. Louis, MO).

3-Nitropropionic acid. 3-NP was also purchased from Sigma-Aldrich.

Procedure

Drug and 3-NP administration. All drugs or compounds, including simvastatin, zoledronate, and 3-NP, were delivered to the animals via intraperitoneal (i.p) injection over the course of 3 days. Simvastatin was chosen over several other available statins based on its higher potency, greater capacity to cross the blood brain barrier, and more widespread use clinically (Saheki, Terasaki, Tamai, & Tsuji, 1994; Shepardson, Shankar, & Selkoe, 2011). Pills were crushed using a pestle and mortar, and then sifted to filter out the pill coating. Adjustments to measurements were made to account for filler in each pill. Zoledronate was chosen based on its relatively higher potency than other bisphosphonates and its greater prevalence among in vivo designs (Russell, 2011). Although zoledronate does not cross the blood brain barrier (BBB), 3-NP severely disrupts the barrier (Duran-Vilaregut et al., 2009). For this reason, 3-NP delivery occurred first on treatment days. Drug doses were measured and then diluted with sterile phosphate-buffered saline. The Ph level of 3-NP was also balanced to 7.4 using sodium hydroxide. Animals were treated with a clinically relevant dose of 40 mg of simvastatin/kg of body weight for 3 days. Literature shows simvastatin effects in daily doses ranging from .125 mg/kg to 100 mg/kg (Aprahamian et al., 2006; Zhang et al., 2011). Animals were treated with .4 mg of zoledronate/kg of body weight for 3 days. The dose of zoledronate was chosen after pilot work ruled out doses of 1mg/kg (92% mortality, n = 12) and, then, .5mg/kg (20% mortality, n = 6). For 3-NP, animals were treated with a dose of 105 mg/kg/day. This dose was based on one of the only studies to date using CD-1 mice in a 3-NP regimen (Kim & Chan, 2001) and was confirmed by a

pilot study comparing 4 doses ranging from 95 mg/kg/day to 125 mg/kg per day. The pilot study showed no mortality associated with the current dose when mice were treated for 7 days.

Motor assessment.

Rotarod performance. Using the rotarod apparatus at a fixed speed of 16 rpm, motor coordination and balance was measured after the seventh and last day of 3-NP injection and drug treatment. Latency to fall was recorded over three 60 second trials for each animal, with a 60 second rest period between each trial. If an animal remained on the rod after 60 seconds, it was removed and the time was recorded as 60 seconds. Based on established protocol and considering habituation effects shown in unpublished data of our laboratory, each animal underwent one day of habituation prior to behavioral testing and then a 60 second habituation trial prior to first testing trial. The best score over three trials was used for each animal in analyses.

Clasping behavior. Each animal's limb movements during midair, ground-facing suspension was assessed. Healthy wild-type animals normally splay their limbs outward, while transgenic and 3-NP HD model animals tend to clasp their front and back limbs inward (Fernagut et al., 2002; Mangiarini et al., 1996; Rubinsztein, 2002). Every animal was assessed during a 10 second trial, with 10 second rest periods in between, after the last of injections. For each limb that is pulled in toward the body, the animal obtained a score of 1. Thus, every trial offered a maximum score of 4. Animals were habituated on the day prior to testing. The total clasping score was used for analysis.

Body weight assessment. On each experimental day, animals' body weights were measured prior to handling. The weight was used as one of several measures of overall health and to calculate the proper amount of 3-NP, simvastatin, and zoledronate.

Health checklist. On each day of the experiment, all animals' were observed closely for indications of poor health. Animals received a score for hunching behavior, bradykinesia, pstosis (drooping of the upper eyelid) and recumbency, in addition to their recorded weight. If an animal received a score indicative of extremely poor health, the animal was sacrificed immediately, regardless of the experimental time point in order to ameliorate pain and suffering.

Preparation of Brain Tissue. Mice were taken from their cages live and promptly sacrificed by decapitation. Brains were removed from the skull, and a 2 mm medial coronal slice was removed containing the striatum. The hemispheres were divided and flash frozen by immersion in -80˚C isopentane. Brains were stored in thin, protective plastic tubes at -80˚C. The tissue was then used for prenylated Rhes quantification.

Prenylated Rhes Quantification. Western Blot techniques were employed in order to quantify the amount of prenylated Rhes in all experimental groups (Harrison, Muller, & Spano, 2013). Cytosol and membrane fractions were separated using the Sub-cellular Fractionation Kit (Thermo Scientific; Rockford, IL). Protein concentration was measured by a Bradford assay (Bio-Rad; Hercules, CA). Protein concentration was obtained for each sample with a spectrophotometer. Proteins were then separated using

SDS-PAGE methods with polyacrylamide TGX gels (Bio-Rad) and transferred to PVDF membranes. Membranes were first probed for MEK protein to ensure successful fractionation. Afterwards membranes were stripped with OneMinute Stripping buffer (GM Biosciences; Rockville, MD) and then probed for Rhes. Membranes were blocked with a nonfat dry milk and TBS-T (20 mM Tris Buffered Saline and .1% Tween) mixture for 60 minutes at room temperature and then incubated at 4° overnight in TBS-T with 5% BSA and anti-Rhes antibody (Fabgennix; Frisco, TX) at a dilution of 1:500. Anti-rabbit secondary antibody containing horseradish peroxidase was used at a dilution of 1:2000 in 5% milk for 60 minutes at room temperature (Cell Signaling; Danver, MA). After washing in TBS-T, blots incubated in Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific) for 15 minutes. Bands were visualized and analyzed using QuantityOne software (Bio-rad).

Results

Analyses were performed using SPSS for Windows version 23.0 with the probability of a Type I error set at 0.05. For rotarod, clasping, weight, and Rhes expression a one-way Analysis of Variance (ANOVA) between the 5 experimental groups was conducted. Bonferroni's follow up tests were conducted in the presence of a significant main effect of group differences, unless homogeneity of variance was violated, in which case a Games-Howell correction was used.

Rotarod Performance

Rotarod performance data are depicted in Figure 2. Animals' best performance across three trials on the spinning rotarod was used for group comparisons. The best performance was defined as the longest time spent on the rotarod before falling, or the longest latency to fall. Levene's test of homogeneity of variance showed significantly different variance across groups, $p < .05$. To correct for this violation, a Welch's analysis of rotarod performance was conducted and revealed a significant difference between groups [$F(4, 16.97) = 21.99$, $p < .01$]. Post-hoc comparisons, using the Games-Howell method, show that the healthy control group (M = 152.80, SD = 33.32) that received only vehicle injections did significantly better than all groups treated with 3-NP (see Table 1). This difference confirms that there was a deleterious effect of the toxin on rotarod performance. In contrast to hypotheses, no significant differences between the groups receiving 3-NP were found, regardless of drug treatment.

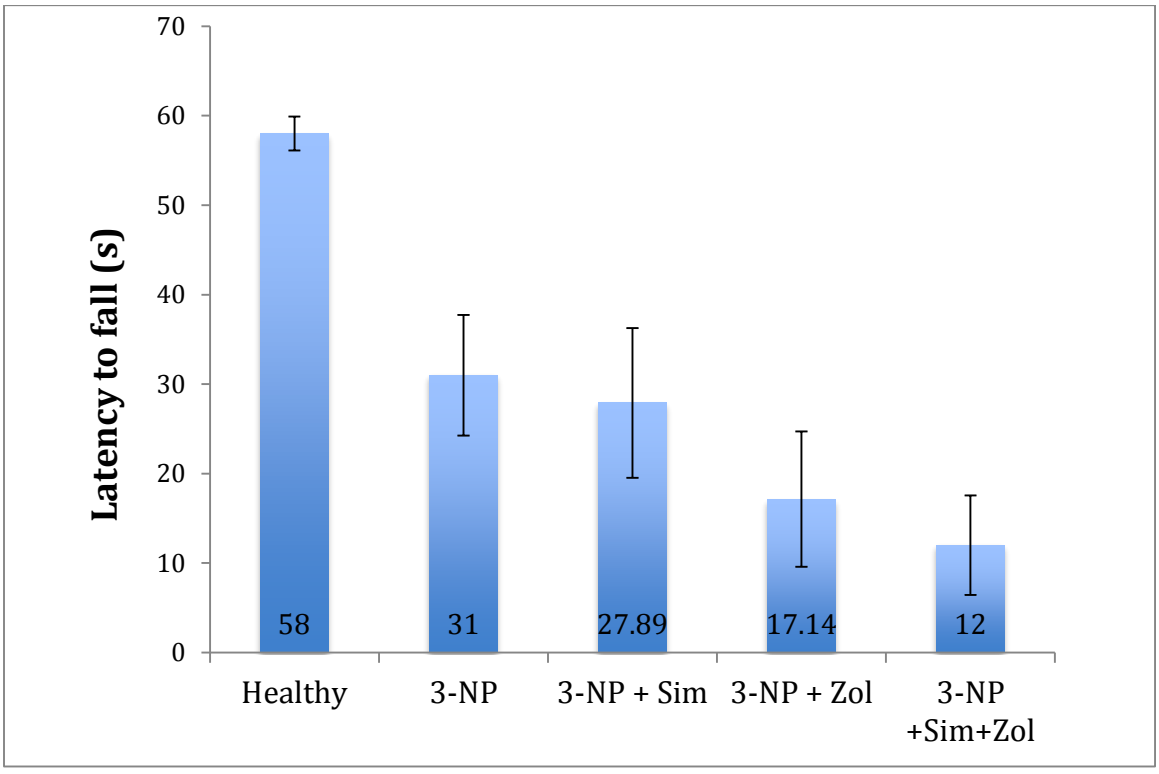


Figure 2. Rotarod performance is displayed as latency to fall from rod in seconds. Group means are shown with standard error. Sim = simvastatin. Zol = zoledronate.

Rotarod Performance

Group	Mean (s)	Standard Deviation
Healthy	58	6.01
3-NP	31	23.39
3-NP + Sim	27.89	25.07
3-NP + Zol	17.14	20
3-NP + Sim + Zol	12	15.75

Table 1. Mean rotarod performances are presented as latency to fall off rotarod in seconds. Sim = simvastatin. Zol = zoledronate.

Clasping

Clasping behavior is summarized in Table 2. Analysis failed to reveal any significant difference between groups [$F(4, 39) = .50$, $p > .05$]. Therefore, no post-hoc comparisons were computed. Overall, only 5 of 38 3-NP mice that were alive at time of behavioral testing showed any clasping behavior.

Clasping Behavior

Group	Mean	Standard Deviation
Healthy	0	0
3-NP	1	2.34
3-NP + Sim	.33	1
3-NP + Zol	1.14	3.02
3-NP + Sim + Zol	.63	1.77

Table 2. Mean clasping behaviors are totals of limbs clasped over three trials. Sim = simvastatin. Zol = zoledronate.

Weight

Weight observances are summarized in Table 3. Analysis of the animals' weight after injections showed a significant between group effect [$F(4,53) = 3.54$, $p < .05$]. Similar to rotarod performance, post-hoc comparisons showed only differences between healthy control animals ($M = 41.67$, $SD = 2.47$) and all 3-NP groups.

Weight

Group	Mean	Standard Deviation
Healthy	40.86	1.75
3-NP	37.29	2.77
3-NP + Sim	38.11	2.42
3-NP + Zol	37.64	4.55
3-NP + Sim + Zol	39.69	3.49

Table 3. Weights were measured on last day of life. Sim = simvastatin. Zol = zoledronate.

Mortality

Although pilot studies conducted separately for 3-NP and zoledronate guided the current doses, mortality did occur. In the 3-NP alone group, 1 mouse died after the third injection (8%). Comparatively, the simvastatin (33%) and combination simvastatin/zoledronate group contained 4 mice that died or were sacrificed according to IACUC guidelines, after the third injection (36%). In the zoledronate group, 5 mice died after the third injection (45%).

Rhes Expression

For Western blot data (Table 4), a ratio of cytosolic:membrane bound protein optical density was calculated for each animal represented. The drug groups were then normalized to account for the ratio observed in the 3-NP alone group and then compared to each-other. Levene's test of homogeneity of variance was violated, $p < .05$. Welch's analysis did not show any significant differences between groups [$F (3, 1.80) = .31$, $p > .05$]. The pattern of expression in the protein bands makes this assay difficult to interpret. The control animal which received only vehicle injections displayed greater protein in the cytosolic portion, in contrast to previous findings showing Rhes localization to the plasma membrane.

Rhes expression

Group	Mean	Standard Deviation
Healthy	0	0
3-NP	1	2.34
3-NP + Sim	.34	1
3-NP + Zol	1.14	3.02
3-NP + Sim + Zol	.63	1.77

Table 4. Rhes expression was measured by western blot and the ratio of cytosolic: membrane optical density of protein bands is shown. Sim = simvastatin. Zol = zoledronate.

Discussion

Interpretation of Findings

Corroborating pilot data, the results show that treatment with simvastatin, zoledronate, and a combination of both failed to improve motor deficits associated with 3-NP. Several possible explanations for this finding exist. First, the lack of behavioral improvement may stem from failure of the drugs to de-prenylated the target protein, Rhes, which is thought to contribute to HD neuropathology. Here, behavioral data is interpreted with regard to Western data of Rhes expression. Unfortunately, Western blots probed for Rhes neither support nor refute this possibility. The obtained protein bands show more Rhes in the cytosolic fractions, compared to membrane fractions, consistently across groups, which aligns with the predicted pattern one would see after de-prenylation. However, this pattern is problematic because it is also appeared in a control animal that received only vehicle injections. Vargiu et al. (2004) originally showed Rhes localization primarily at the plasma membrane using PC12 cells, derived from peripheral tissue. Thus, unaltered endogenous Rhes should show the same expression in control animals. Notably, the current study differs from Vargiu's, as it measures Rhes in vivo and via Western blot methods. Although differentiated PC12 cells have been commonly used to

model neurons, it is possible that endogenous Rhes in tissue displays more diffuse expression across the cell. Given the limited literature on Rhes, no other group has examined subcellular localization by Western, precluding more direct comparisons.

If the drugs failed to de-prenylate Rhes, it could be due to lack of sufficient presence in the brain. Simvastatin crosses the BBB, but zoledronate does not (Saheki, Terasaki, Tamai, & Tsuji, 1994; Sierra et al., 2011; Weiss et al., 2008). The 3-NP administration served as a theoretical means for the bisphosphonate transport to neural tissue. The neurotoxin disrupts the BBB after 3 days of i.p. injections with a dose over 5 times lower than the current dose (Duran-Vilaregut et al., 2009; Kim et al., 2003). Therefore, it is unlikely that the BBB was not disrupted in all 3-NP animals. However, to our knowledge, in vivo unassisted zoledronate transport to the brain with impaired BBB functioning has not been studied previously. Given that zoledronate is quickly taken up by bone, it is possible that no measurable amount successfully reached the brain. Conversely, the treatment timecourse may have been too short to have a measurable effect on the structure of Rhes, while still circulating in the brain. The novelty of pharmacological means to Rhes de-prenylation limits guidance in timecourse design.

Secondly, an off-target de-prenylation of a mitochondrial associated Ras family GTPase has the potential to worsen 3-NP dysfunction or nullify overcoming beneficial actions of the drugs. Mitochondrial Rho (Miro) anchors to the mitochondrial outer membrane, allowing the organelle to attach to its motor proteins, kinesin and dynein, which actively transport the structure to where it is needed within the cell, namely the axon and dendrites

(Abe, Kato, Miki, Takenawa, & Endo, 2003). Miro contains N-terminal and C-terminal GTPase domains that promote membrane attachment; thus, Miro is likely prenylated. Transgenic Drosophila lacking Miro's N-terminal not only show severely fragmented mitochondria that accumulate in the cell body, but die prematurely (Babic et al., 2015). In contrast, Drosophila lacking Miro's C-terminal show less severe reduced motility only.

Neuronal mitochondrial motility is greatly suppressed without Miro, resulting in a motor neuron disease phenotype in mice that shares many behaviors with HD or 3-NP mice (Nguyen et al., 2014). Miro also acts an intermediate substrate between mitochondria and Milton, which directly recruits kinesin (Glater, Megeath, Stowers, & Schwarz, 2006). Glater et al. (2006) postulate that Milton may also have a structural attachment to the mitochondrial membrane, which would possibly make it another target of de-prenylation. Importantly, a transgenic mouse that will not express Miro in adulthood may be under development to better study this potential complex in relation to neurodegenerative diseases.

Third, pleiotropic drug effects independent of the mevalonate pathway may hinder functioning. Though treatment with mevalonate pathway disrupters obviously does not impair physiologic functioning enough to pull them from the commercial market, there is a growing awareness of the negative off-target effects. For example, statin use has been linked to the development of diabetes; meta-analyses describe a 9% increase in the risk for incidental diabetes (Sattar et al., 2010). Many clinicians and researchers have concluded that the risk is negligible in individuals without pre-existing risk factors or

overinflated statistically, but this finding should lead neuroscientists to look closer at lesser known statin actions in the brain (Blackburn, Chow, & Smith, 2015). Simvastatin reduces insulin secretion and promotes insulin resistance (Yada, Nakata, Shiraishi, & Kakei, 1999). The mechanism by which simvastatin exerts this influence is not completely clear, but a recent study claims that simvastatin affects insulin pathways independent of the mevalonate pathway interference (Kain, Kapadia, Misra, & Saxena, 2015). The brain is an insulin sensitive organ; thus, alteration of insulin activity leads to alterations in neural activity (Heni, Kullmann, Preissl, Fritsche, & Haring, 2015). The literature on insulin signaling and neurodegenerative disease is often contradictory (White, 2014). Insulin resistance in the brain may actually exacerbate degenerative conditions, like AD (Sims-Robinson, Kim, Rosko, & Feldman, 2010). Moreover, mitochondrial dysfunction is observed in mice with a brain specific insulin receptor knockout mutation (Kleinridders et al., 2015). These mice show increased levels of pro-apoptotic ROS and behavioral deficits, such as cognitive impairment. Therefore, statins could potentially cause further mitochondrial dysfunction, beyond 3-NP's effects, that is mediated by insulin signaling changes.

Conversely, targets of insulin, insulin receptor substrate 1 (Irs1) and substrate 2 (Irs2) have also been linked, inconsistently, to degenerative conditions (Taguchi, Wartschow, & White, 2007; Selman et al., 2008). Sadagurski et al. (2011) crossbred HD mice with transgenic mice exhibiting brain Irs2 overexpression. They found that Irs2 overexpressing HD mice performed significantly worse on motor tasks, died sooner, and displayed greater oxidative stress and mitochondrial dysfunction. Whether

neurodegeneration is a direct result of dysregulated insulin cascades or an indirect result of peripheral effects is unclear (White, 2014). Though statins' cardiovascular actions outweigh any risk for diabetes in clinical settings, how this class of drugs affects insulin regulation demands attention in the presence of deleterious physiological effects and absence of behavioral rescue.

Other pleiotropic drug effects related to complicated Rhes interactions impart several other options for why drug treatment could fail to improve behavioral deficits in the current study. The drugs and the hypothesized target, Rhes, have multiple, sometimes paradoxical, effects on the brain's ability to clear damaged components. Simvastatin can increase apoptosis through suppression of protein kinase B (Akt) (Hwang et al., 2011) or through c-Jun N-terminal kinases (JNK) signaling (Gopalan, Yu, Sanders, & Kline, 2013). However, simvastatin's reactive oxygen species (ROS) scavenging activity can reduce apoptosis (Moon et al., 2011). Zoledronate can also induce apoptosis through via ROS upregulation (Ge et al., 2014). Relatedly, ROS has proven neurotoxic in another study using FTI-277 to interfere with the mevalonate pathway{Kim et al., 2010, #60084}. Apoptosis contributes to the striatally specific degeneration caused by 3-NP, so influences on the signaling pathway resulting in apoptosis can similarly influence the effects of 3-NP on physiological and motor functioning.

Furthermore, simvastatin, zoledronate, and Rhes affect autophagy. As mentioned previously, autophagy, sometimes called macroautophagy, is a complex intra-cellular degradation process that targets damaged cytosolic structures, like misfolded mHtt or

dysfunctional mitochondria (Qin et al., 2003). Complete molecular mechanisms have yet to be characterized; however, it is clear that the process includes double-membrane cellular machinery, called an autophagosome, which engulfs targets and traffics the complexes away for degradation. These regularly occurring events support homeostasis until the cell incurs a level of stress that surpasses the autophagic capacity. At that time, autophagy becomes, along with apoptosis and necrosis, a form of programmed cell death (Tsujimoto & Shimizu, 2005). Cellular stress can manifest because of lack of nutrients or an increased amount of dysmorphic or dysfunctional components, such as aggregated proteins found in numerous neurodegenerative diseases (Nixon, 2013). Researchers now generally agree that the process is majoritively neuroprotective, often prolonging cell survival. Notably however, a large proportion of mammalian autophagy research has been conducted in cancer paradigms that obviously seek to suppress such pro-survival activities in cancer cells (Bhutia et al., 2013). Zoledronate (Khandelwal et al., 2014) and simvastatin can both increase autophagy (Fukui, Ferris, & Kahn, 2015). Importantly, if either proves to be a capable de-prenylation agent for Rhes, indirect anti-autophagic effects are possible. Rhes' effects on autophagy are complex. Rhes binds to and activates mTOR, an autophagy suppressor (Subramaniam et al., 2012). This activity could potentially leave cells without the required degradation tools to clear dysfunctional mitochondria, in the present model, or mHtt, in transgenic models. If Rhes downregulates autophagy, it serves as one possible mechanism through which Rhes potentiates HD pathology and why blocking Rhes expression, either through genetic manipulation (Baiamonte, Lee, Brewer, Spano, & LaHoste, 2013; Mealer, Subramaniam, & Snyder, 2013) or de-prenylation (Subramaniam, Sixt, Barrow, & Snyder, 2009) have shown

protective effects. There is, in fact, limited contrasting evidence arguing Rhes is neuroprotective. Lee et al. (2014) used RNA interference to silence Rhes in HD mice. These animals show greater striatal atrophy and persistent HD motor deficits. This study directly contrasts previous work from our lab finding a protective effect of Rhes depletion on motor behavior (Baiamonte et al., 2013). This increased cytotoxicity may be explained by pro-autophagic effects of Rhes. Rhes has been argued to increase autophagy via indirect de-phosphorylation of Akt, which leads to decreases in mTOR activity (Harrison, Muller, & Spano, 2013). The same research group that has showed toxic Rhes influences also discovered that Rhes can circumvent the mTOR pathway to autophagy by activating Beclin-1, which has pro-autophagic activity (Mealer et al., 2014). If future work proves that the presently used drug regimen does de-prenylate Rhes, then the behavioral data support a neuroprotective Rhes theory. Furthermore, since the current study uses a mitochondrial model of HD, effects of the drugs on autophagy, which degrades dysfunctional mitochondria, could potentiate or diminish the induced 3-NP damage.

Differentially inefficient autophagy across brain areas is one proposed force behind striatally specific degeneration in HD. Indeed, increasing autophagy has proven beneficial in a number of HD models, while reducing autophagic responses tends to exacerbate pathology. By inhibiting the activity of calpains, one of the negative regulators of autophagy, several markers of cellular health are affected. Autophagy promotion in Drosophila and mouse models results in reduced mHtt aggregation and soluble mHtt levels while survival is increased and behavior deficits are tempered

(Berger et al., 2006; Menzies et al., 2015). Similar effects appear when caspase activity is disrupted (Wellington et al., 2000). Caspases, like calpains, negatively regulate autophagic clearance. In the presence of mHtt, striatal neurons may have lower clearance capacity than in other parts of the brain, e .g. cortex, whether due to deficits of the UPS or autophagy (Tsvetkov et al., 2013). This is a contested idea, as another study found no significant differences between the striatum and the cortex of transgenic HD mice (Baldo, Soylu, & Petersen, 2013).

Another possible explanation for lack of behavioral amelioration by drug treatment lies in peripheral toxicity of the drugs. Indeed, there was increased mortality among treatment groups compared to the 3-NP alone group. Zoledronate, and bisphosphonates in general, have long been associated with increased chances for renal failure, especially in those with already lowered kidney function (Bounameaux, Schifferli, Montani, Jung, & Chatelanat, 1983). Even in rats with normal kidney functioning, zoledronate can lead to renal failure, though using a higher dose (Bergner, Siegrist, Gretz, Pohlmeyer-Esch, & Kranzlin, 2015). Simvastatin has been implicated in sporadic hepatic failure of clinical patients (Bjornsson, 2014). Animal research also finds simvastatin to be especially toxic, compared to other statins, to liver cells. Higher levels of ROS and increased cytotoxicity were observed after simvastatin exposure in liver cells in vitro (Abdoli, Azarmi, & Eghbal, 2015). Statin use is also associated with muscle toxicity in patients. Statin-induce myopathy is possibly caused by multiple subcellular actions of statins, such as reducing cholesterol through HMG-CoA reductase inhibition, but also by direct effects on the mitochondrial respiration (Apostolopoulou, Corsini, & Roden, 2015).

Although peripheral toxicity could have affected behavior, unequal mortality rates across 3-NP groups point toward a third explanation for lack of behavioral rescue, a contributory negative effect of the drugs, which may or may not be independent of de-prenylation. Pilot data gathered on toxicity speak to an adverse interaction of mevalonate pathway inhibitors and 3-NP. The incidence of mortality in the zoledronate treated 3-NP group was higher than observed in zoledronate pilot mice that received no 3-NP. Furthermore, mortality was increased in the simvastatin and combination treatment groups, as well. Because simvastatin has such an established literature indicating it is largely well tolerated, a dose pilot with no additional 3-NP was forgone to reduce the number of animals used. However, the current mortality pattern for simvastatin groups does concur with findings from a previous study that paired the same dose of simvastatin with an escalating dose of 3-NP. In the first study, simvastatin, and zoledronate groups had higher mortality than 3-NP alone. The breadth of pleiotropic effects for simvastatin and zoledronate treatment certainly present numerous options for increased cellular dysfunction. Since the current drug groups did not perform significantly worse than the 3-NP group on the rotarod task, rather than adding dysfunction, treatments could have simply not been able to overcome the extent to which 3-NP caused cellular and neural damage.

Clasping behavior was not affected by drug treatment either. However, there were very few incidences of any clasping behavior observed across 3-NP groups. This is in contrast to transgenic mouse models, in which the majority of mice exhibiting any motor

disturbances also display clasping behavior. The lack of clasping is also in contrast to evidence that the 3-NP HD model is associated with clasping (Fernagut et al., 2002). Previous work was conducted in C57 mice, however, and with a higher cumulative dose. Notably, our pilot studies with CD-1 mice showed similarly absent clasping. It is possible that 3-NP may not cause clasping behavior in the CD-1 murine strain. A very limited number of studies have been published using 3-NP in CD1 mice and these studies did not assess clasping behavior (Kim & Chan, 2001; Kim et al., 2003). There is ample documentation of strain differences in susceptibility to 3-NP toxicity across species (Alexi, Hughes, Knusel, & Tobin, 1998) and across strains of the same species (Gabrielson et al., 2001), thus the extent to which 3-NP would cause clasping in a particular strain likely also displays strain differences. Our data informs future work using CD-1 mice with a 3-NP model of HD.

Limitations

There are prominent flaws in the current research that stem from the lack of specificity in the pharmacological interventions. While Rhes inactivation remains a promising target for HD therapies, the methods utilized leave room for improvement. First, pharmacological de-prenylation techniques do not offer any selectivity to Rhes. Rather, all proteins in the body which contain isoprenoids may be diminished. Currently, at least 100 different proteins in a typical mammalian cell contain either a farnesyl or geranyl component, including many GTPases (Hrycyna, Bergo, & Tamanoi, 2011). The possible consequences of global de-prenylation are far-reaching. In other words,

regardless of the efficacy of the current de-prenylation efforts, simvastatin and zoledronate are not, in their nature, specific means to manipulate Rhes activity.

Secondly, as explored above, simvastatin and zoledronate have documented pleiotropic effects beyond their immediate actions of HMG-CoA reductase and FPPS, respectively. Mevalonate intervening drugs not only de-prenylate but prevent the formation of downstream products. Zoledronate's effects on expression downstream of the MVA pathway are still largely unknown. One recent study employed microarray techniques to find that zoledronate treatment influenced, either up- or down-regulating, genes involved in metabolic, cell localization, cell communication, and cell proliferation pathways, respectively (Insalaco et al., 2012). Changes are thought to be representative of early responses because only a 24-hour treatment was used, so longer time courses likely affect change in a different inventory of genes.

As an example, both simvastatin (Bargossi et al., 1994) and zoledronate (Kalyan et al., 2014) inhibit the synthesis of the downstream products. One such product, coenzyme Q10 (CoQ10), is likely a neuroprotective agent and known antioxidant. CoQ10 administration in both transgenic and 3-NP HD models has shown robust benefits from several groups (Naia, 2011). Early HD clinical studies showed promise, but continued investigation does not produce such clear positive effects (Shannon and Fraint, 2015). Perhaps CoQ10 alone cannot produce significant and consistent effects in HD, but supplementation with another pharmacological agent could. Future work targeting the mevalonate pathway must consider this option.

Only recently, interactions between HD related proteins and autophagic factors have been characterized. Wong and Holzbaur (2014) used live cell imaging to follow the autophagosome from its creation at the axonal tip, through the engulfment of its target, and as it moves backwards toward the cell body. They demonstrated that Htt promotes the proper motility of the autophagosome, and without it or in the presence of mHtt, the cell sustains an accumulation of autophagosomes with dysfunctional cargo. In other words, healthy cells' autophagosomes need Htt to properly degrade the components engulfed within. In HD models, there is both a lack of Htt and presence of mHtt, marking autophagy as an exemplary example of dual loss-of-function and gain-of-function and worthy of continued exploration.

Future Considerations

Future studies positioned on the proposed neuroprotective mechanisms of simvastatin and zoledronate in a 3-NP model need to better describe interactive in vivo effects. By staining brain slices with cresyl violet or nissl stain, lesion size in the striatum can be quantified and compared across groups, then used to examine relationship between degeneration and mortality, behavior, etc. Furthermore, in the hypothetical presence of de-prenylation and absence of behavioral amelioration, concurrent supplementation with protective substances, e.g. CoQ10 should be explored.

Currently, how the balance sways between Rhes' pro- and anti-autophagic influences is unknown. Determining what factors push Rhes toward activation of autophagy regulators

is a crucial next step to illuminating why Rhes may have neurotoxic and protective effects in HD. Perhaps incorporation of techniques like live cell imaging can help create a timeline of when Rhes binds to regulators, thus, informing cumulative effects on autophagy. Furthermore, this timeline can be compared between cells with and without mHtt, as its known mHtt influences Rhes binding to at least one regulator.

Future work on Rhes manipulation should aim to utilize the most specific methodology possible for the hypotheses. Pharmacological de-prenylation of Rhes is not the only option for dislocating the possible HD co-factor from the plasma membrane where it binds to a myriad of structures. Using a construct developed in the Snyder lab of Johns Hopkins University, it is possible to create a transgenic mouse model that will have a specific mutation on the Rhes genetic domain, which encodes for farnesylation (Subramaniam et al., 2009). Thus Rhes, and only Rhes, will be detached from membranes and lose signaling effects. This mouse can then be crossbred with HD and wild type mice to look at in vivo effects, which can be directly compared to existing literature (Baiamonte et al., 2013). Furthermore, examining expression of proteins marking apoptotic and autophagic processes, as well as other proposed HD related mechanisms, such as mitochondrial dysfunction, will greatly enhance understanding of how Rhes functions normally and in the presence of mHtt.

Concluding Remarks

The current study shows a lack of behavioral rescue by simvastatin and zoledronate in a 3-NP mouse model of HD. These findings contrast similar, but not exact,

studies finding benefits to treatment with statins and/or bisphosphonates in 3-NP (Ahmed, Darwish, Abdelsalam, & Amin, 2015; Lee et al., 2008). Due to higher mortality in experimental groups receiving one or both of the drugs with 3-NP, an additive toxic effect is suggested. It is unclear whether drug treatment affected Rhes prenylation. If the lack of behavioral improvement is indicative of underlying de-prenylation, peripheral toxicity or pleiotropic influences could be determining factors. Additionally, such findings may support a neuroprotective view of Rhes. If Rhes was not significantly de-prenylated, a neurotoxic view of Rhes remains a predominant theory. This data raises questions about the interplay of the mevalonate pathway, two widely prescribed drugs and Rhes on degradation processes in the brain. Such processes are necessary for cell viability in HD and in the current 3-NP model and should be a focus of future work. Lastly, this project informs future work using 3-NP in CD-1 mice and adds to the simvastatin and zoledronate literature.

At present, Tetrabenazine is the only approved treatment for HD, but serious adverse events limit its benefits. Because this drug causes dopamine depletion, among other central monoamines, possible consequences include the development or worsening of depression and parkinsonism, both of which may co-occur in HD (2006; Frank, 2009). Therefore, HD researchers and advocates should not feel content with the still extremely limited options for those suffering from HD. Animal research is a necessary tool with which to study HD processes, which are overwhelmingly complex. The overwhelming number of mechanisms that have yet to be fully illustrated necessitates fervent pursuit of therapeutic targets like Rhes.

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Vita

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