Spring 5-15-2015

Effects of Rhes Prenylation on Mouse Cognition in a 3-Nitropropionic Acid Animal Model of Huntington's Disease

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A Thesis

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

Master of Science
in
Psychology

by

Diana A. Hobbs

B.A., Austin College, 2012

May, 2015

Acknowledgement

I would like to thank Dr. Gerald J. LaHoste for his profound guidance throughout the duration of this experiment. Without him, this project would not have been possible. I would also like to thank Dr. Elliott Beaton and Dr. Connie Lamm for serving on my committee and providing valuable feedback and limitless inspiration. Additionally, I would like to thank my esteemed colleagues, Leslie Kelley and Ashley Whitmarsh, for their labored assistance and unwavering support throughout this project. Finally, I would like to thank my friends and family for never doubting me even when I doubted myself. I would be lost without you.

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Abbreviations

3-Hydroxy-3-Methylglutaryl Co-Enzyme A ..... HMG-CoA
3-Nitropropionic Acid..... 3NP
Caudate-Putamen .....CPu
Dorsolateral Prefrontal Cortex ..... dlPFC
Globus Pallidus External.....GPe
Globus Pallidus Internal..... GPi
Huntington's Disease .....HD
Knockout.....KO
Medium Spiny Neurons ..... MSNs
Morris Water Maze .....MWM
Mutant Huntingtin..... mHtt
Ras Homolog Enriched in the Striatum .....Rhes
Saline..... Sal
Simvastatin..... Sim
Substantia Nigra pars Compacta.....SNc
Substantia Nigra pars Reticulata..... SNr
Wild-type Huntingtin ..... wtHtt
Zoledronic Acid ..... ZolA

Abstract

Located on the short arm of chromosome 4, there exists a gene, IT15, responsible for the trinucleotide CAG expansion involved in the autosomal dominant neurodegenerative disorder known as Huntington's disease (HD). The brain region associated with the most atrophy, the striatum, leads to expression of severe motor dysfunction, the hallmark feature of HD. To a lesser degree, the cortex and hippocampus show earlier deterioration indicative of the cognitive deficits that occur prior to motor symptom onset. The brain regions associated with HD-induced neuronal death additionally selectively express the protein Rhes - the combination of Rhes and mutant huntingtin being cytotoxic. Using a 3-nitropropionic acid animal model of HD, we hypothesized that animals with preserved prenylation of Rhes would display cognitive and motor symptomology similar to genetic models of HD while animals administered statins or bisphosphonates would show inhibited Rhes prenylation and delayed cognitive symptoms. Experimental animals, however, did not perform differently than control animals on shallow water variants of the t-maze and MWM.

Huntington's disease, Rhes, prenylation, statins, bisphosphonates, cognition

Introduction

Overview of Huntington's Disease

Neurodegeneration, the progressive loss of neuronal structure and function leading to cellular death, is a common characteristic of many debilitating diseases including Huntington's disease (HD). This disorder produces deleterious effects in the motor, behavioral, and cognitive aspects of the affected individual's life. Three hundred thousand Americans are currently affected by this disease, and an additional 200,000 are at-risk (Huntington's Disease, 2012). This brain disorder was named after Dr. George Huntington following the dissemination of his thorough description of the hereditary ailment in 1872. In his article, "On Chorea," he eloquently depicts the gradual nature of the disease as "…often occupying years in its development, until the hapless sufferer is but a quivering wreck of his former self." Before much was known of the disease, Dr. Huntington described three components: it is hereditary, the symptomology does not manifest until adulthood, and it commonly begets suicide and insanity (Huntington, 1872).

Huntington's disease is an autosomal dominant brain disorder associated with an abnormally long trinucleotide CAG repeat (>36 repeats). Affiliates of the Venezuela Collaborative Huntington's Disease Project, Gusella et al., (1983) mapped the mutation and localized it to chromosome 4. The Huntington's Disease Collaborative Research Group (1993) later isolated the gene, IT15, containing the trinucleotide repeat responsible for the protein product, huntingtin, and its mutated form caused by codon expansion. Kieburtz et al. (1994) further identified a relationship between the number of CAG repeats and the age of HD onset. Due to the autosomal dominant nature of HD, those with an afflicted parent have a 50% chance of inheriting the mutation; therefore, these findings were essential for the early detection of the devastating disorder using genetic testing.

Huntington's disease manifests as a symptom triad including motor, behavioral, and cognitive facets. Full symptomology is typically exhibited when the affected individual reaches adulthood, between 30 and 50 years of age. Motor symptoms include rigid or sporadic movements of the voluntary muscles of the face and extremities. This often leads to displays of unusual facial grimaces, chorea, dystonia, unsteady gait, lack of coordination, and difficulty with speech and swallowing. Following symptom onset, death typically occurs within 15 to 20 years. In 1888, J. Hoffman was the first to observe a juvenile form of the disease that developed from a family afflicted for three subsequent generations (Bates, 2005). In many genetically dominant disorders, the age of symptom onset decreases as the generations of inheritance increase, a term coined "anticipation." Tidley, Frith, Crow, and Conneally (1988) found this to occur more frequently through the male line. Often subtle behavioral and cognitive changes will precede the more obvious motor symptoms and accompany progression of the disease. Early in the progression of disease, these may include irritability, apathy, anxiety, and depression; while thoughts of suicide, confusion, difficulty thinking, planning or organizing, memory loss, and dementia emerge later. Paulsen, Ready, Hamilton, Mega, and Cummings (2001) found that over 50% of patients with HD showed symptoms of dysphoria, agitation, irritability, apathy, and anxiety while fewer than 12% displayed delusions, motor abnormalities, and hallucinations concurrently; however, 98% displayed at least one of these within a given month.

Neuropathology

The neuropathological trademark feature of HD is the progressive neuronal degeneration that occurs within the striatum, the primary input structure of the basal ganglia comprised of the caudate nucleus, the putamen, and the nucleus accumbens. Imaging studies demonstrate the earliest and most robust changes occur in this area, displaying significant decreases in caudate

and putamen gray matter in a dorso-ventral gradient (Kassubek et al., 2004; Fennema-Notestine, et al., 2004; Kipps et al., 2005; and Douaud et al., 2006). Kipps et al. (2005) conducted a two year longitudinal study on patients with the mutant huntingtin gene who had not yet been diagnosed with HD. These patients exhibited early regional gray matter atrophy to the caudate, putamen, external segment of the globus pallidus, and the substantia nigra, all of which are targets of striatal projections. After diagnosis, however, patients showed overall volume reduction in pallidal nuclei, nucleus accumbens, thalamus, hippocampus, and hippocampal gyrus in addition to those regions already described by Kipps (Fennema-Notestine, 2004; Douaud, 2006; Walker, 2007). Mounting evidence supports the idea that reduction is not limited to subcortical structures; there is a more widespread degeneration in the Huntington brain that also includes the cerebral cortex. Various studies using participants with HD show significant reductions in cortical grey and white matter volume as well as increased abnormal white matter signal, i.e. hyper intense white matter voxels falling in the range typical of grey matter (Halliday et al., 1998; Fennema-Notestine et al., 2004; Douaud et al., 2006; and Walker, 2007). The extension of abnormality, both structural and functional, was additionally correlated to increasing numbers of CAG polyglutamine codon repeats, striatal atrophy, loss of GABAergic medium spiny neurons (MSNs), and hippocampal volume (Halliday et al., 1998; Fennema-Notestine et al., 2004; Kassubek et al., 2004; and Kipps et al., 2005). Although generalized atrophy within the striatum occurs by a certain point in disease progression, research has shown differential susceptibility to neuronal degeneration between interneurons (<10%) and projection neurons (>90%) present in this area. The majority of striatal interneurons are relatively impervious to the progression of HD; however, the following four subpopulations of striatal projection neurons show gradual deterioration: striato-globus pallidus external (GPe), striato-

substantia nigra pars reticulata (SNr), striato-globus pallidus internal (GPi), and striato-substantia nigra pars compacta (SNc) (Albin, 1995). Reiner et al. (1988) supported the notion that the striatal neuron projections descend relatively separately to the GP and SN terminals, with early and middle stages of HD showing a higher rate of degradation to those projecting to the GPe and SNr. As the disease progresses, projections to the GPi begin to display noticeable changes until the affected individual reaches the advanced stage where all classes are affected. Despite their names, the GPe and GPi are not homologous structures. The GPi and SNr, on the other hand, are embryologically and functionally homologous with one another and can be thought of as the same structure separated by the fibers of the internal capsule. Likewise, the SNr and SNc are not homologous structures. Names of basal ganglia nuclei were assigned on the basis of adjacency rather than functionality.

Alexander, DeLong, and Strick (1986) introduced the basal ganglia-thalamocortical circuit model that demonstrated the pathway afferent neurons took as they descended from various cortical areas to the striatum, GP, and SN, and funneled through the thalamus to be redirected back to restricted areas of the cortex. The separate and diverse targets of the cortex, the basal ganglia output nuclei, and the thalamus suggested the existence of distinct parallel circuits, of which, Alexander and colleagues (1986) discovered five: two involving motor functions (motor and oculomotor circuit) and three involving non-motor functions (dorsolateral prefrontal [dlPFC], lateral orbitofrontal, and anterior cingulate circuit). This would indicate basal ganglia involvement in a diverse range of behaviors. Medium spiny neurons, the most vulnerable cells to HD degeneration, account for 90-95% of the striatal neuronal population, effectively explaining why this area of the brain is so drastically affected (Walker, 2007; Gil and Rego, 2008). Following the basal ganglia-thalamocortical circuit model, cortical projections

predominantly target these GABAergic neurons, which in turn connect the striatum to output nuclei (Stocco, Lebiere, and Anderson, 2010).

These networks described above can be delineated into two broad pathways which aid in sequencing motor systems: the direct pathway and the indirect pathway which oppose one another (Albin, Young, and Penney, 1989; DeLong, 1990). Only in the presence of dopamine do the basal ganglia-thalamocortical loops exert their functional effects on behavior. A clinical demonstration of this fact is the akinesia that results from progressive loss of dopaminergic neurons in Parkinson's disease. The direct pathway, driven by dopaminergic stimulation of D1 receptors, comprises inhibitory afferents to SNr and GPi output nuclei and results in subsequent disinhibition of the thalamocortical loop. The disinhibition of thalamic activity results in excitation of the cortex and resulting behavior. GABAergic striatal efferent neurons form the initial point in the indirect pathway. These neurons are inhibited by dopaminergic stimulation of D2 receptors, resulting in a net effect of disinhibition of the GPe and subthalamic nuclei. As a result, thalamic output to the cortex is inhibited. This signal is believed to inhibit all cortical activity except that within the target area (Albin, Young, and Penney, 1989; Stocco, Lebiere, and Anderson, 2010). The mutant huntingtin gene, however, produces detrimental effects within the indirect pathway circuitry. Without inhibition to control the surrounding cortical areas, the pathways become unbalanced, resulting in unintended and inappropriate motor and cognitive behavior (Albin, Young, and Penney, 1989).

Cognitive Deficits in Humans

The basal ganglia-thalamo-cortical loop is comprised of several parallel pathways that interconnect and communicate. Output from the striatal-thalamo portion of this system terminates in both motor and non-motor cortical regions, eliciting a variety of behavior.

Neuronal degeneration within this circuit can therefore drastically affect the Huntington disease-diagnosed individual, impacting not only motor but cognitive function as well. It is currently inconclusive whether cognitive deficits originate from the degeneration of striato-cortical or cortico-striatal projections; however, both concepts have been implicated.

Middleton and Strick (2000) suggest that basal ganglia output affects cognition, as pallidal and nigral projections to distinct cortical regions, in particular to the dlPFC, contribute greatly to the heterogeneity of HD symptomology in the cognitive and affective domains. As previously mentioned, the striatum degenerates in a dorso-ventral gradient, indicating that projections from the dorsal striatum are amongst the first to degenerate. As neurons projecting from the dorsal striatum to the dlPFC die, spatial working memory becomes impaired, a phenomena relating to the "Where" functioning attributed to the dorsal cortex (Lawrence et al., 1996; Brandt et al., 2005). Spatial span length in addition to pattern and spatial recognition memory are also affected by the degeneration of the striato-frontal pathway (Lawrence et al., 1998). A large portion of the basal ganglia output nuclei communicates specifically with the dlPFC, the cortical region associated with learning and planning new sequences; however, nine other cortical areas have been implicated as targets of non-motor signal transmission within the basal ganglia-thalamo-cortical circuit (Middleton and Strick, 2000). Imaging studies have shown striatal metabolism to be positively related to verbal learning memory and the Performance Intelligence Quotient, measures that are diminished in patients with HD (Berent et al., 1988). Furthermore, subcortical thalamic nuclei relaying information between the striatum and prefrontal cortex exhibit substantial volume loss that co-varies with cognitive performance, contributing to deficits in psychomotor speed and executive functioning (Kassubek, Juengling, Ecker, and Landwehrmeyer, 2005).

Cognitive impairment is often exhibited before clinical diagnosis and increases in severity while nearing motor symptom onset (delineated by number of CAG repeats) (Lawrence et al., 1998; Duff et al., 2010; O'Rourke et al., 2011; and Stout et al., 2011). Duff et al. (2010) utilized the Mild Cognitive Impairment assessment to evaluate individuals with HD on the following cognitive measures: attention, verbal fluency, psychomotor speed, executive functioning, memory, and visuospatial functioning. Approximately 40% of the participants in the prodromal stage of HD scored below 1.5 standard deviations on at least one of these cognitive domains, rates of which doubled for those closer to symptom onset. Significant deterioration of the cortical ribbon, particularly pyramidal cells in layers III, V, and VI of the dlPFC, transpires early and presents differently throughout the progression of HD (Sieradzan and Mann, 2001; Rosas et al., 2002). Consistent with previous studies, this regional degeneration was associated with impaired learning and planning new sequences.

The frontal lobe has been implicated with preclinical cognitive deficits in numerous studies of HD. Deficits in psychomotor speed, verbal memory, executive function, cognitive flexibility, working memory, visual search, sustained attention, and visuoperceptual ability are reported prior to overt symptoms presented in patients with HD (Rosenberg, Sorensen, and Christensen, 1995; Hahn-Barma et al., 1998; Brandt et al., 2008; O'Rourke et al., 2011; and Stout et al., 2011). Degeneration to the fronto-striatal pathway could explain why these effects are seen before the more obvious motor impairments occur; however, conclusions should be carefully weighed as others have found little evidence for the existence of prodromal frontal lobe deficits in the asymptomatic stage of HD (Blackmore, Simpson, and Crawford, 1995; Rosas et al., 2002).

As HD progresses into the mild and moderate stages, global cognitive impairments continue to be displayed in executive function, visuospatial skill, episodic memory, verbal fluency, psychomotor speed and reasoning, spatial planning, selective attention, and recall and recognition memory (Paulsen, 1995; Backman, 1997; Montoya, 2006; and Rosas et al., 2008). There is mounting evidence of significant frontal lobe involvement, which supports the notion that there may exist a fronto-striatal dementia (Backman et al., 1997). Hodges, Salmon, and Butters (1990) show that advanced stage exacerbates dementia as impairments in information encoding, storage, and retrieval become severe. These deficits have great repercussions on both short- and long-term memory.

Mouse Model

Similar to humans living with HD, longer CAG expansions in animal models are related to the onset and progression of the disease on several fronts: reductions in grey and white matter, cortical thickness, and regional atrophy (Lafore et al., 2001; Sawiak, Wood, Carpenter, and Morton, 2012). Within a rat model of HD, Fusco et al. (1999) found huntingtin protein and mRNA to be abundant not only in the vast majority of striatal neurons, but in corticostriatal neurons as well. Accumulation of mutant huntingtin in the cortical neurons further predicted onset and severity of symptoms as striatal neuronal responses were altered following cortical stimulation (Laforet et al., 2001). These results suggest that corticostriatal neurons with the mutant protein may hold a destructive role towards their downstream striatal neurons. Miller, Walker, Barton, and Rebec (2011) studied symptomatic HD animal models and found altered corticostriatal disruptions, especially during tasks which stimulated synaptic plasticity. While there was no clear trend in corticostriatal dysfunction and HD progression, it can be reasonably hypothesized that such disturbances occurred during the asymptomatic phase and continued

throughout HD advancement. Comparable to findings in preclinical HD human studies, animal models consistently show cognitive impairments that occur before motor symptom onset and progress throughout the course of the disease (Lione et al., 1999; Murphy et al., 2000; Mazarakis et al., 2005; Van Raamsdonk et al., 2005; Cummings et al., 2006; Cummings et al., 2009; and Giralt et al., 2011). Early cognitive deficits often lead to a more global impairment in cognition. Procedural learning, working memory, executive function, impaired recognition memory, sensorimotor gating, and strategy shifting, all of which are impaired in patients with HD, were each found deficient in a mouse model of HD prior to motor symptom onset and progressed to more global cognition deficits (Van Raamsdonk et al., 2005; and Cummings et al., 2006). Additionally, performance on tasks sensitive to frontostriatal and hippocampal function, such as the Morris water maze (MWM), visual cliff avoidance, two-choice swim tank, and the T-maze, was gradually impaired during HD progression (Lione et al., 1999). Changes in synaptic plasticity in the hippocampal and perirhinal cortex, areas involved in spatial and recognition memory, were further displayed (Murphy et al., 2000; Cummings et al., 2006; and Ransome, Renoir, and Hannan, 2012). Overall, altered communication throughout the cortical pyramidal neurons involving both inhibitory and excitatory inputs plays a role in the development and progression of the disease (Cummings et al., 2009).

3-nitropropionic acid model. 3-nitropropionic acid (3NP) is a naturally occurring mitochondrial toxin present in both plants (*Indigofera endecapylla*) and fungi (*Aspergillus flavus*). Its toxic effects are exerted by the suicide inhibitor of succinate dehydrogenase which leads to an irreversible blockade of the Krebs cycle (Alston, Mela, and Bright, 1977). Ingestion of the toxin has been the cause of widespread loss of cattle in the United States, and it is directly related to the moldy sugarcane food poisoning epidemic in China. Among survivors,

gastrointestinal disturbance, encephalopathy, coma, dystonia, and chorieform movements were documented in addition to the development of putamen cell death (Liu, Luo, and Hu, 1992; Ludolph et al., 1991). Since the discoveries of 3NP, it has become a prevalent model in the research surrounding HD.

Ludolph et al. (1991) conducted an extensive review on the history of 3NP and animal studies that exemplified reports of lesioning in the basal ganglia, primarily the striatum, thalamus, hippocampus, spinal tracts, and peripheral nerves. Further evidence was supported by Hamilton and Gould (1987) when they found rats treated with 3NP to develop symmetrical bilateral lesions in the striatum, hippocampus, dentate gyrus, and thalamus. The caudate-putamen (CPu) was affected in all animals, while damage to other structures never occurred alone. Dosing regimens vary widely in the literature; however, it is relatively consistent that subacute 3NP treatment results in broad bilateral neuronal loss to the striatum and hippocampus whereas chronic 3NP treatment results in a more selective dorsolateral striatal lesion (Beal et al., 1993; Guyot et al., 1997). The cortex is relatively preserved except in animals displaying the most severe injury. In these cases, the integrity of cortical tissue laterally surrounding the CPu and rhinal sulcus becomes compromised (Hamilton and Gould, 1987). Furthermore, Chyi and Chang (1999) findings of signal intensity increases in the rat striatum and hippocampus but not the cortex is consistent with the literature on lesioning data. Blum et al. (2003), on the other hand, found lowered succinate dehydrogenase action in both the striatum and outer layers of the cortex, indicating 3NP involvement in both.

Apart from the typical movement dysfunction apparent in 3NP literature, exposure produces cognitive dysfunction. Baboons receiving chronic 3NP showed both spontaneous abnormal movements and significant impairment on the object retrieval detour task. This is a

task sensitive to frontostriatal circuitry, yet lesions were found exclusively in the bilateral striatum with sparing to the cortex and prefrontal cortex (Palif et al., 1996). Mehrotra and Sandhir (2014) found deficits in both motor coordination and performance of a T-maze in rats sub-chronically treated with 3NP. And finally, rats both acutely and chronically treated with 3NP showed impaired memory on the MWM in addition to abnormal walking patterns (Duckworth et al., 1999; Teunissen et al., 2001). These results are consistent with the 3NP lesioning literature as tasks targeting the striatum (T-maze) and hippocampus (MWM) are those that are affected alongside motor abnormalities.

Role of Rhes

The Ras homolog enriched in the striatum (Rhes) is a gene that encodes a small guanine nucleotide (GTP)-binding protein within the brain (Falk et al., 1999). The mRNA and protein product (Rhes) are predominantly expressed in the striatum, with lesser expressions localized to several other brain regions. Amongst them include the nucleus accumbens or ventral striatum, and to a lesser degree, cortical layers II/ III and V, hippocampal pyramidal and granular layers, the dentate gyrus, the piriform cortex, anterior thalamic nuclei, the olfactory tubercle, the inferior colliculus, and the cerebellar granular layer (Spano et al., 2004; Vargiu et al, 2004; and Harrison, LaHoste, and Ruskin, 2008). As identified by Harrison, LaHoste, and Ruskin (2008), Rhes mRNA expresses itself in different brain structures in a developmental pattern. The first strong detection occurs early in development in the anterior thalamic nuclei, hippocampus, and cerebellum, followed in superficial cortical layers then in the striatum mid-to-late development. The most notable expression by adulthood, however, is within the CPu and the shell of the nucleus accumbens (Harrison and LaHoste, 2006).

The pattern in which Rhes mRNA manifests closely resembles the pattern of neuronal degeneration and symptomology found in patients with HD. This would suggest a potential Rhes-mutant huntingtin (mHtt) interaction supporting the HD neuropathology. Subramaniam, Sixt, Barrow, and Snyder (2009) used this theory to answer the following questions: 1) Does Rhes bind to the Htt protein, 2) Does Rhes influence mHtt cytotoxicity, 3) How may Rhes facilitate mHtt cytotoxicity, and 4) Does Rhes influence mHtt aggregation? The authors found overexpressed Rhes bound vigorously with the mHtt protein reducing cell survival by 50%; however, cell survival was not decreased when Rhes was expressed alone, when mHtt was expressed alone, or when wild-type huntingtin (wtHtt) was expressed with Rhes. Interestingly, mHtt, not wtHtt, forms aggregates that are reduced in the presence of Rhes overexpression, increasing the cytoplasmic levels of mHtt and eliciting neurotoxicity. In other words, when Rhes binds to the mHtt protein, SUMOylation (the attachment of a small ubiquitin-like modifier, SUMO, to a protein) occurs leading to a cytotoxic disaggregated soluble product (Steffan et al., 2004). In support, Rhes knock-out (KO) mouse models significantly delayed cortical and striatal neurodegeneration, symptom onset, and dysfunction (Baiamonte et al., 2013; Mealer, Subramanian, and Snyder, 2013). Furthermore, Spano et al. (2004) observed no cognitive impairment amongst Rhes KO mice, an observation that alludes to the Rhes protein's involvement in learning and memory processes outside of its involvement in the striatum. In relation to the 3NP model, Mealer, Subramaniam, and Snyder (2013) replicated the findings that Rhes knockout mice performed better on motor tasks and displayed less dorsolateral striatal neurodegeneration using an acute dosage regimen of 3NP.

Relation to the mevalonate pathway. Common to all complex eukaryotes and several bacteria, the mevalonate pathway, or 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-

CoA reductase) pathway, is an important cellular metabolic pathway involved in converting mevalonate into cholesterol, steroid hormones, lipoproteins, and hydrophobic molecules that post-translationally modify cellular signaling (Buhaescu and Izzedine, 2007). These molecules additionally aid in cell membrane maintenance, protein anchoring, and protein prenylation.

Figure 1 depicts two enzymatic targets for drugs that inhibit Rhes prenylation via the mevalonate pathway. The most upstream target, HMG-CoA reductase, converts HMG-CoA to mevalonate. Statins are well-known HMG-CoA reductase inhibitors and act to suppress the entire mevalonate pathway (Buhaescu and Izzedine, 2007; Sadowitz, Maier, and Gahtan, 2010). In particular, they prevent synthesis of isoprenoid intermediates necessary for lipid attachment posttranslational modification of proteins, i.e. Ras-like proteins (Liao and Laufs, 2005). A target downstream mevalonate, farnesyl diphosphate synthase (farnesyl PP synthase), converts geranyl diphosphate (geranyl PP) to farnesyl diphosphate (farnesyl PP). Bisphosphonates are widely accepted inhibitors of farnesyl PP synthase and therefore can block the downstream prenylation of Rhes (Dunford et al., 2001). Vincenzi et al. (2003) took these a step further and hypothesized that statins might potentiate bisphosphonate activity on the mevalonate pathway. Based on these findings, we decided to use a lipophilic statin, simvastatin (Sim), and a potent nitrogenous bisphosphonate, zoledronic acid (ZolA), alone and combined to investigate the upstream, downstream, and combined effects drug treatment targeting enzymes to inhibit Rhes prenylation would have on mouse behavior.

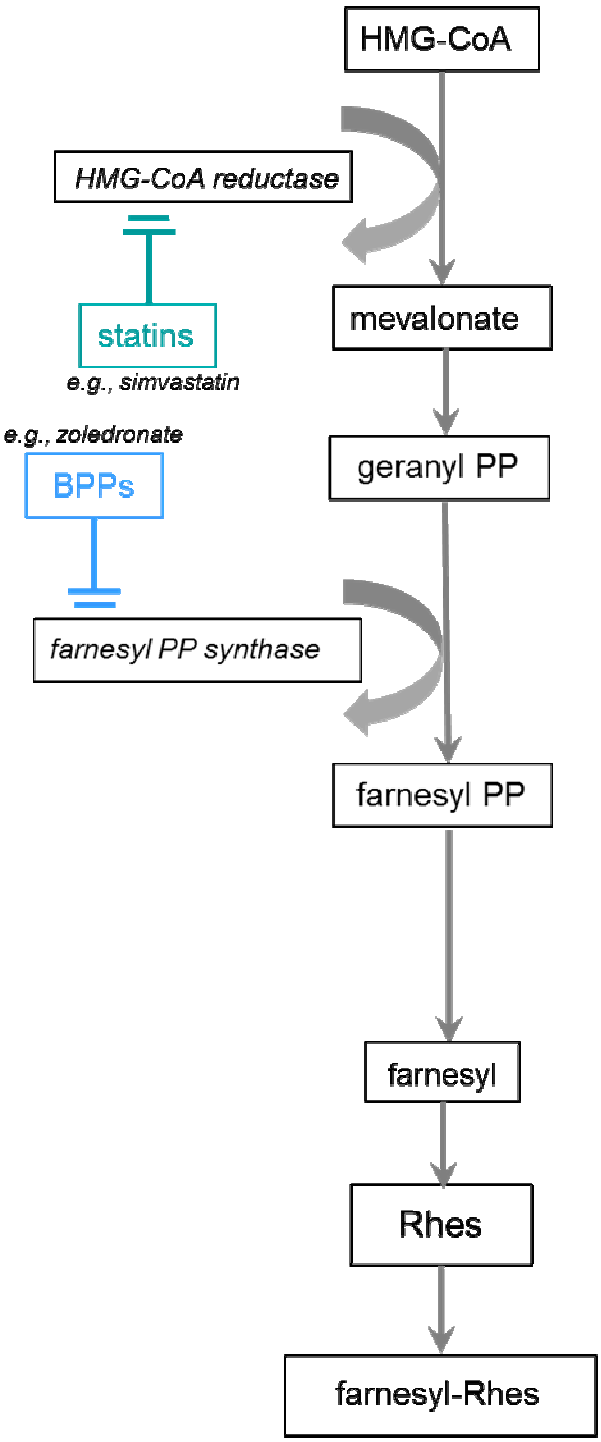

Figure 1. Inhibition of Rhes Prenylation via the Mevalonate Pathway

Note: HMG-CoA reductase: 3-hydroxyl-3-methylglutaryl-Co-enzyme A; BPP: bisphosphonates; and PP: diphosphates.

Hypotheses

The purpose of this study was to test hypotheses regarding the role of Rhes in mediating cognitive dysfunction in a 3NP model of HD. The basis of the hypotheses are provided by two facts: Rhes is a necessary co-factor in the neuronal and behavioral toxicity induced by mutant huntingtin protein or by 3NP; and for its effects to occur Rhes must be prenylated, allowing for its anchorage in the inner surface of the cell membrane. From these two observations we argue that administration of drugs that inhibit prenylation, and thereby decrease the bioactivity of Rhes, will exert a protective effect on the cognitive dysfunction induced by 3NP. We tested the following specific hypotheses:

Hypothesis 1 – Administration of 3NP to mice will impair learning in tasks that require normal functioning of the striatum or hippocampus.

Hypothesis 2 – Pharmacological inhibition of HMG-CoA reductase by administration of a statin (simvastatin) will be protective against the toxic effects of 3NP on cognition.

Hypothesis 3 – Pharmacological inhibition of farnesyl diphosphate synthase by administration of a nitrogenous bisphosphonate (zoledronic acid) will be protective against the toxic effects of 3NP on cognition.

Hypothesis 4 – Combined administration of a statin and a bisphosphonate will be most protective against the toxic effects of 3NP on cognition.

Method

The methods used were designed to measure cognitive dysfunction as the result of striatal damage. Motor dysfunction is the hallmark symptom of diseases involving striatal degeneration, amongst them, Huntington's disease; therefore, it was no surprise that the development of cognitive abnormalities in human HD has been overshadowed by the more obvious motor symptoms. However, the striatum receives extensive input from the entire cerebral cortex and ultimately directs output to the prefrontal lobe, an area known to be critical for cognition. Cognitive symptoms are known to develop *prior to* the appearance of motor symptoms, and we hoped to elucidate these findings using 3-nitroproprionic acid (3NP) to selectively disintegrate striatal neurons in mice and emulate a model of HD. We were additionally interested to see if drug treatment targeting Rhes prenylation would affect cognitive performance in 3NP treated mice.

Animals

Eighty-two male albino Hsd:ICR (CD-1) mice weighing ~30g on arrival were purchased from Harlan Laboratories (Indianapolis, IN). Mice were housed in groups of five under controlled conditions of temperature and humidity, on a normal 12:12 hour light:dark cycle (lights on 0700). Access to food and water was *ad libitum*. Body weight was recorded daily. Animals were housed and handled in strict accordance with the regulations of the U.S. Public Health Service; all conditions and procedures were approved by the University of New Orleans Institutional Animal Care and Use Committee.

Procedure

Drug administration. All drugs were dissolved to the chosen concentrations in sterile phosphate buffered saline (0.1 M PBS) and administered intraperitoneally (i.p.), 10 ml/kg body

weight. 3-Nitroproprionic acid (3NP), a striatal-selective neurotoxin, was dissolved to a concentration of 7.7 mg/ml, then neutralized to pH 7.4 with 10 N sodium hydroxide (NaOH; 1:100) (Mealer et al., 2013) yielding a final concentration of 77 mg/10 ml. Since dosage of 3NP varies with day, volume of injectate was adjusted so as to administer the correct dosage for a given animal. Futhermore, we were constrained by the fact that treatment drugs needed to be given for several days (based on the estimated half-life of Rhes) in order to exert their therapeutic effects and to allow ZolA access to the brain made possible by the disruption of the blood-brain barrier (BBB) caused by 3NP (Duran-Vilaregut et al., 2009). Based on previous studies and these constraints, we adopted the following 3NP regimen. Mice were injected once per day for 7 days using an escalating regimen. The starting dose was 50 mg/kg body weight and increased by 15% each day, yielding rounded numbers of 50, 58, 66, 77, 89, 102, and 118 mg/kg. The cumulative dose was 560 mg/kg (Stefanova et al., 2005).

The statin simvastatin (Sim), and the nitrogenous bisphosphonate zoledronic acid (ZolA) inhibit different enzymes at various points along the mevalonate pathway, ultimately decreasing protein farnesylation. ZolA (1.0 or 0.5 mg/kg) and Sim (40 mg/kg) were diluted in sterile 0.1 M PBS and given as 10 ml/kg. Mice were randomly assigned to receive these drug treatments (Table 1) over the seven day course of injections.

Because of a general toxic effect observed in mice in some drug conditions (40 of 82 animals died prematurely- see Results), drug treatment was terminated prior to the injections of Day 6 and behavior was recorded. We then added mice to the 3NP/Sal group (n = 7). In addition, two new drug treatment groups were added. In both, the dose of ZolA was cut by half (0.5 mg/kg) and administered together with Sim/3NP (n = 9) or Sim/Sal (n = 6).

Table 1

Drug Treatment Groups

Squad	Toxin	Drug Tx	Sample Size
1	Sal	Sal	12
2	3NP	Sal	12
3	3NP	Simvastatin	19
4	3NP	Zoledronate, 1 mg/kg	12
5	3NP	Simvastatin + Zoledronate, 1 mg/kg	12
6	3NP	Simvastatin + Zoledronate, .5 mg/kg	9
7	Sal	Simvastatin + Zoledronate, .5 mg/kg	6
Total			82

Cognitive behavioral tasks. To determine whether our 3NP mouse model of HD developed the same cognitive dysfunction as observed in genetically modified mice with the HD alleles and patients with HD, we used the battery of tests described below. We chose these tests within the context of the brain structures that mediate the behavior required to successfully perform the task.

Apparatus. Modeled after the Oxford paddling pools (Deacon, 2013), the octagonal behavioral apparatus (45.72 cm diameter) was made of clear Lexan and contained eight exit holes (5.08 cm diameter) located medially on each wall face (20.32 x 26.67 cm) 3 cm from the floor. The floor was covered with water (20 - 22 □C) 2 cm deep. The water was deep enough to act as an aversive stimulus, but not deep enough to require swimming. The apparatus was used in

this open-field like structure for the shallow water variant of the MWM; however, a few modifications were necessary for use in the T-maze.

***T-maze*.** In order to assess striatal mediated habit learning, Lexan walls (painted black to reduce visual cues) were inserted into the center of the maze to create a three-armed enclosure in the shape of a "T" (arms: 20.32 x 5.08 x 22.86 cm)**.** The start arm and one of the side arms terminated with a false exit (i.e. the escape arm was blocked), and the remaining arm ended with a true exit connected to a black escape tube (30.48 cm long). Mice were placed in a heated cage after having found the true exit. Following counterbalancing and randomization, left and right true exits were pre-assigned to each mouse and designated for escape throughout experimental testing.

Habituation. Two black walls were extended from the south to north exits to create an enclosed straight path. Mice were placed at the south end and given two 60 second trials to escape through the exit tube located at the north end.

Striatal mediated habit learning. Methodology is based on that used by Guariglia and Chadman (2013). Mice were given 20 trials on the sixth day prior to injections to find the left or right exit pre-assigned to them. In the first five trials, the start arm was located south with the east and west arms serving as either true or false exits. By doing this, we were able to establish either a left or a right body turn necessary for exit per mouse, forcing mice to use an egocentric, striatally-mediated strategy. During the remaining 15 trials, the three armed enclosure was continuously rotated counterclockwise on the N/S/E/W cardinal axis moving the start arm from the south to the east, north, west, and south again. By doing this, we were able to remove the potential for guidance by spatial cues and ensure a habit was formed through body turns. Mice were allotted 60 seconds per trial and were given a rest period in a heated cage following escape.

If they could not find the exit, mice were manually guided to it. Entrances into the false arm or failure to move past the start arm were counted as errors. If a mouse found the true exit by initial body turn, it received a score of 0; if a mouse made any errors, it received a score of 1. The maximum number of errors per mouse was 20.

Morris water maze. This task modeled after the Oxford water paddling pool task (Deacon, 2013) was utilized to assess allocentric spatial learning mediated by the hippocampus. Of the eight exit holes, seven were plugged with a plastic stopper. The remaining true exit was attached to a black tube for escape from the aversive open water field. Mice were placed into a heated cage after finding the true exit. Visually stimulating cues (horizontal, vertical, right diagonal, and left diagonal stripes) were adhered to the north, south, east, and west walls of the maze to aid in spatial navigation. To reduce the potential for cue preference, true exits were only assigned to the walls of the maze that did not contain visual stimuli (northwest, northeast, southwest, and southeast). Following careful counterbalancing and randomization, these four cardinal directions were pre-assigned to each mouse and designated for escape throughout experimental testing.

Hippocampal mediated learning. Methods for acquisition were modeled after those used by Pettan-Brewer and colleagues (2013). On the sixth day prior to injections, mice were given eight trials to navigate to the pre-assigned exit hole using spatial landmarks. Mice were lowered by the base of their tails into the center of the apparatus and allotted 60 seconds to find the escape. If they could not find the exit in that time frame, they were manually guided to it. Following escape, mice were placed on a platform above the water and in front of their deemed exit tube for 30 seconds. This gave them time to notice their surroundings in relation to their escape hole. Each was given an inter-trial rest period in a heated cage following escape and

platform time. If a mouse approached a false exit within heads distance and touched it with its nose or forepaw, it was charged with an error. Mice failing to find the exit within 60s were assigned the ceiling 7 errors/trial (Deacon, 2013). The maximum number of errors per mouse was 56.

Results

We utilized a 3NP model for HD to test whether drug treatment targeting the farnesylation of Rhes would alleviate striatally- and hippocampally-mediated cognitive decline as measured by subjects' performance in a shallow water variant of a T-Maze and a MWM. Before analyzing the data, we examined our independent variables, Drug Treatment group and Number of treatment Injections, and our dependent variables: T-Maze: Amount of Time to complete the task and Task Performance, and MWM: Task Performance and Health (healthy (=1), sickly (=2), or dead (=3)) for theoretical and practical issues that may limit our planned analyses of covariance. The sample included 82 animals, of which 40 subjects were missing data at all measures and omitted from analysis. Due to the high sickness and mortality rate, we conducted analyses on the health of the animals to investigate a toxicity issue. We further screened the variables to evaluate assumptions of normality, homogeneity of variance, linearity, multicolinearity, and homogeneity of regression.

Animal health and drug toxicity

Data were analyzed with analyses of covariance (ANCOVA) in order to investigate whether Health of the animals (dependent variable: healthy, sickly, or dead) was affected by their placement in Drug Treatment groups (independent variable: Table 1) while controlling for the Number of treatment Injections (covariate). Scatter plot and Pearson correlation suggested that Health (dependent variable) and Number of treatment Injections (covariate) were not linearly

related ($p = .250$); therefore, we decided to conduct a one way analysis of variance (ANOVA) to test whether Health (dependent variable) varied across different Drug Treatment groups (independent variable).

One subject was removed from analysis due to a death caused by experimenter error rather than 'natural' means. There were no outliers or signs of abnormal kurtosis and skew; however, Levene's test was significant ($p < .001$). We therefore could not assume homogeneity of variance and conducted Dunnett C post hoc tests to determine where among Drug Treatment groups differences in Health existed. Results indicated significant variation in Health amongst the different Drug Treatment groups [$F(6, 74) = 13.492$, $p < .001$]. Table 2 presents the means, standard deviations, and sample size (n) for each group.

When the means are ordered from low to high mortality, two homogenous subsets emerge with one overlapping mean. There are no significant difference between Sal/Sal and either of the groups in which ZolA was given at a dose of 0.5 mg/kg, even when 3NP was included. Based on homogeneity, these are the "healthy" groups. The group given 3NP in addition to Sim is not significantly different from any other group, indicating a "sickly" group, not healthy, but not close to death. Likewise, the group given 3NP alone was significantly more sickly than the two "healthy" groups administered drugs in combination with Sal but were significantly healthier than the sickest group, 3NP/Sim+1ZolA. A second homogenous group, 3NP-treated animals that were given Sim or ZolA at a dose of 1 mg/kg either alone or in combination constitutes the sickest subset, significantly worse than the healthy subset. Figure 2 represents these findings. The results indicate that, when given in combination with 3NP, Sim and the higher dose of ZolA are highly toxic, especially when given together. One anomaly is that 3NP-Sim was not toxic when combined with the lower dose of ZolA.

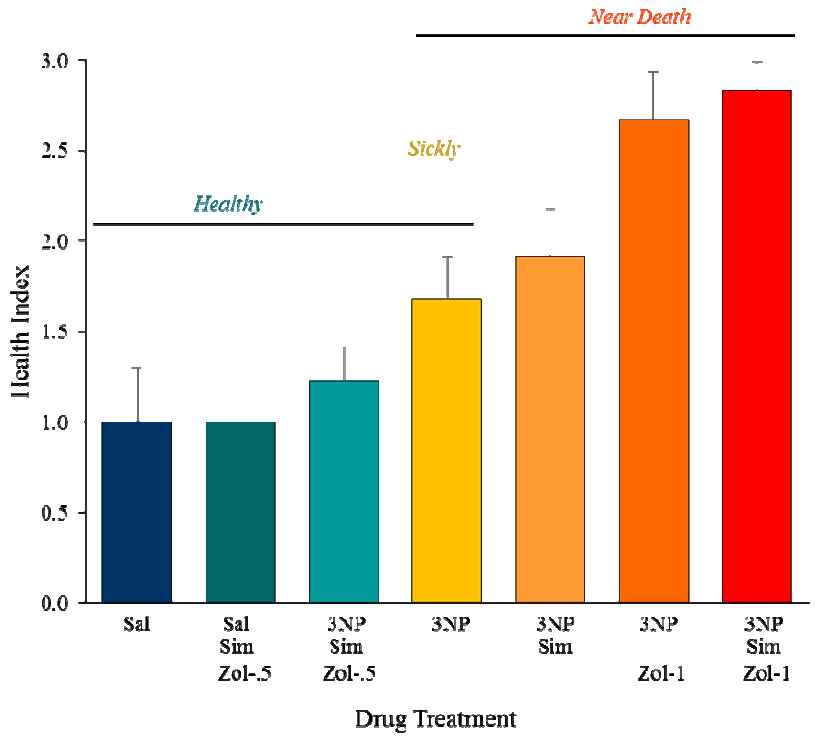

Figure 2. Animal health as a function of drug treatment. Horizontal bars indicate significance to the $p < .05$ level.

Due to the nature of our results, we decided to perform regression analysis on our data to see if the individual Drug Treatments could predict the Health of the animals. Table 3 presents the overall regression results which show that a significant amount of variance (51.4%) in Health was accounted for by the overall model, ($R^2 = 0.514$, $p < .001$). Table 4 summarizes the individual Drug Treatment results and indicates that 3NP (part = -.217, $p < .01$), 1 ZolA (part = -.440, $p < .001$), and 0.5 ZolA (part = .176, $p < .05$) significantly predicted Health of the animals, but Sim (part = -.105, $p = .191$) did not. These findings support the inclination that drug treatments provided differing levels of toxicity and adverse effects to the health of the animals. We can conclude that 3NP in any combination with ZolA at 1 mg/kg is the most toxic, followed by 3NP alone or combined with Sim.

Table 2

Mean Scores and Differences Across Health as a Function of Drug Treatment

	Sal/Sal[1]	3NP/Sal[2]	3NP/Sim	3NP/1Zol[3]	3NP/Sim+1Zol[4]	3NP/Sim+.5Zol[5]	Sal/Sim+.5Zol[6]	F
	N = 11	N = 19	N = 12	N = 12	N = 12	N = 9	N = 6	
	Means (SD)							
Health	1.00 (.00)[1234]	1.68 (.82)[1246]	1.92 (.90)	2.67 (.79)[1356]	2.83 (.39)[12456]	1.22 (.67)[345]	1.00 (.00)[2346]	13.492*

Note: Like superscripts indicate significant differences on post-hoc contrasts, Dunnett C ($p < .05$). * = $p < .001$

Table 3

Summary of Regression Analysis: Drug Treatment Predicting Health

R^2	Adjusted R^2	Std. Error of the Estimate	Change Statistics				
			R^2 Change	F Change	df1	df2	Sig. F Change
0.51	0.49	0.66	0.51	20.12	4	76	.000

Predictors: 3NP, Sim, 1ZolA, 0.5ZolA

Table 4

Summary of Regression Analysis: Drug Treatments Predicting Health

Model	Unstandardized Coefficients		Standardized Coefficients	t	Sig.	Correlations		
	B	Std. Error	Beta			Zero-order	Partial	Part
(Constant)	3.568	.574		6.216	.000			
3NP	-.541	.200	-.239	-2.710	.008	-.469	-.297	-.217
Sim	-.236	.179	-.128	-1.319	.191	-.061	-.150	-.105
1 ZolA	-.986	.179	-.488	-5.506	.000	-.641	-.534	-.440
0.5 ZolA	.532	.242	.224	2.197	.031	.365	.244	.176

Dependent Variable: Health

T-Maze

Data were analyzed using a multivariate analysis of variance (MANOVA) in order to test the hypothesis that Time to Complete the task and Task Performance (the dependent variables) differed as a function of Drug Treatment group (independent variables: Drug Treatment) while controlling for the influence of Number of treatment Injections (covariate).

There was no sign of existing outliers or abnormalities in kurtosis and skew. Further, scatter plot and Pearson correlations suggested that Time to Complete the task and Task Performance (dependent variables) as well as Number of Injections (covariate) were linearly related ($p < .05$), but not to a strong degree (i.e. multicolinearity should not be an issue of concern, $r < .65$); thus, assumptions of normality and linearity posed no threat to MANOVA interpretation. Due to the high degree of association between the dependent variables ($r > .65$), we interpreted univariate results with caution. Box's M test was not significant ($p = .058$), thus Wilks' Lambda criterion was used to determine multivariate main effects of the independent variable and its interaction with the covariate. The non-significant Levene's tests for both Time to Complete the task ($p = .666$) and Task Performance ($p = .321$) allowed us to assume homogeneity of variance. As recommended by Tabachnick and Fidell (2001), homogeneity of regression was tested by creating and examining an interaction term between the Number of Injections and Drug Treatment group in relation to Performance on the T-maze. Because the interaction was non-significant ($p = .264$), we could assume homogeneity of regression.

After adjusting for Number of treatment Injections, results of the MANOVA suggested that Drug Treatment group did not have a significant multivariate effect [$F(10, 68) = 1.429$, $p = .187$, $\eta^2 = .174$, Wilks' Lambda = .683] on corrected Time to Complete task and corrected Task Performance. Univariate analyses of variance (ANOVA's) showed no differences in Task

Performance amongst different Drug Treatment groups when controlling for Number of Injections [$F(5, 35) = 1.401$, $p = .248$, $\eta^2 = .167$]; however, Time to Complete the task varied between Drug Treatment groups when controlling for Number of Injections [$F(5, 35) = 2.946$, $p < .05$, $\eta^2 = .296$]. These results show the strength of the relationship between adjusted Time to Complete the task and Drug Treatment groups was $\eta^2 = .296$, or in other words, 29.6% of the variance in the amount of Time to Complete the task was predicted by Drug Treatment groups when controlling for Number of treatment Injections. To account for type 1 error, LSD post-hoc tests were conducted to see where differences between Drug Treatment groups may have existed. Table 5 summarizes the estimated means, standard deviations, and sample size (n) for each group when controlling for Number of treatment Injections.

When controlling for Number of Injections, we can conclude that subjects in the 3NP/Sim treatment group performed significantly worse on the T-maze than the Sal/Sal treatment group while needing significantly more Time to Complete the task than the Sal/Sal, 3NP/Sim+.5Zol, and Sal/Sim+.5Zol treatment groups. These findings do not support our main hypothesis that the biggest difference would be between the animals in the Sal/Sal and 3NP/Sal treatment groups.

Table 5

Mean Scores and Differences Across the Measures in the T-Maze as a Function of Drug Treatment when Controlling for Number of Injections

	Sal/Sal N = 11	3NP/Sal N = 10	3NP/Sim N = 5	3NP/1Zol N = 2	3NP/Sim+.5Zol N = 8	Sal/Sim+.5Zol N = 6	F
	Means (SD)						
Performance	72.2 (17.66)[1]	64 (20.11)	49 (27.02)[1]	87.5 (10.61)	74.37 (23.97)	79.17 (21.54)	1.401
Completion Time	3.24 (1.73)[1]	3.73 (1.15)	5.43 (1.88)[1]	2.63 (.51)	2.89 (1.16)[1]	2.28 (1.02)[1]	2.946*

Note: Like superscripts indicate significant differences on post-hoc contrasts, LSD ($p < .05$). * = $p < .05$

Morris Water Maze

Originally data were analyzed with analyses of covariance (ANCOVA) in order to test the hypothesis that Performance (dependent variable) differed as a function of Drug Treatment group (independent variables: Table 1) when controlling for the influence of Number of treatment Injections (covariate). Scatter plot and Pearson correlation suggested that Performance (dependent variable) and Number of Injections (covariate) were not linearly related ($p = .453$); therefore, we decided to conduct a one way analysis of variance (ANOVA) to test whether Performance (dependent variable) on the MWM varies amongst Drug Treatment groups (independent variable).

There was no sign of existing outliers or abnormalities in kurtosis and skew. Levene's test was non-significant ($p = .065$), thus we could assume homogeneity of variance. Results indicated no significant differences in Performance existed amongst the different Drug Treatment groups: $F(4, 41) = .845$, $p = .506$, although all groups improved with trials. Table 6 presents the means, standard deviations, and sample size (n) for each group.

Unlike our prediction regarding the effects of striatal damage on the ability to learn the T-maze, we did not find evidence that Performance on the hippocampally-mediated spatial task was influenced by Drug Treatment or the general Health of the animal, nor was there sufficient evidence to suggest the animals learned the task.

Table 6

Mean Scores and Standard Deviations Across Performance on the MWM as a Function of Drug Treatment

	Sal/Sal N = 11	3NP/Sal N = 12	3NP/Sim N = 5	3NP/Sim+.5Zol N = 8	Sal/Sim+.5Zol N = 6	F
			Means (SD)			
Performance	60.71 (11.85)	58.18 (19.27)	49.64 (8.87)	61.61 (12.77)	64.29 (11.52)	.845

Note: Like superscripts indicate significant differences on post-hoc contrasts, LSD ($p < .05$)

Discussion

In this study, we used a mouse model of selective striatal damage by 3NP administration to mimic symptoms presented in the genetic disorder, Huntington's disease. We hypothesized that drug treatments targeting the farnesylation of Rhes would alleviate striatally- and hippocampally-mediated cognitive decline induced by the damaging effects of 3NP and measured by subjects' performance in shallow water variants of a T-Maze and MWM. Overall, we failed to reject the null hypothesis that mice administered 3NP would perform worse than control mice on cognitive measures. In addition, we could not reliably interpret whether drug treatments would prevent prenylation of Rhes and alleviate the hypothesized cognitive decline induced by 3NP administration. Further complicating our measures, we did not anticipate the drugs' severely toxic side effects.

Animal health and drug toxicity

Throughout this experiment, our sample size drastically dropped from 82 subjects to 42. In attempting to identify which factors were having adverse, toxic effects, the following patterns emerged. Animals treated with ZolA at 0.5 mg/kg did not display any toxic or adverse side effects compared to Sal-treated animals. By contrast, when the dose of ZolA was increased to 1 mg/kg and given in combination with 3NP, animals were the sickest and had the highest mortality rate. (ZolA at 1 mg/kg was not given in the absence of 3NP). Thus, in general, two health groups can be discerned based on whether and how much ZolA was given (0-0.5 mg/kg = good health; 1 mg/kg [+ 3NP] = poor health). 3NP alone had an adverse effect on health as indicated by the fact the health of mice given this treatment alone was in between that of the healthy mice and the sickest mice. The presence or absence of Sim does not alter these categorizations (based on homogenous subsets of non-significant differences between group means).

The cumulative dose of 3NP (560 mg/kg) had highly variable results on animal welfare during both our pilot study and main experiment. Stefanova et al. (2005) describe the behavioral effects in both a low dose (430 mg/kg) and a high dose (560 mg/kg) of sub-chronic 3NP treatment on mice – neither of which resulted in death prior to sacrifice. Nearly half of the low dose group never developed motor impairment, thus we opted to use the high dose. These results were consistent to those found in our pilot data of both treatment regimens, and no deaths were recorded. Yet in our main experiment, of the initial set of twelve 3NP/Sal treated mice, four died before we could test them for behavior and five were too sick to perform. Because our sample size was therefore too low to analyze reliably, we added another seven animals to this treatment group; none of these additional animals died or were too sickly to perform prior to testing day. It

is possible that this variability in 3NP-induced toxicity was present in groups that received additional, ameliorative drugs, thereby reducing our power to detect significant effects of these drugs. Every attempt to minimize animal suffering was made; however, in some instances death came suddenly, making euthanasia impossible.

Zoledronic acid at the dose of 1 mg/kg was additionally highly toxic compared to its 0.5 mg/kg counterparts. According to Pfizer's Material Safety Data Sheet (2010), the minimal lethal intravenous (I.V.) dose for mice is >10 mg/kg. Because drugs are more quickly absorbed into the bloodstream via I.V. administration, one would think that our dosage of 1 mg/kg intraperitoneally (I.P.) over six days would be a safer option. Though several studies have used this drug subcutaneously, intravenously, and intraperitoneally at lower doses (Green and Lipton, 2010), we decided to take the risk to ensure the drug would be effective at crossing the BBB. This was a bold, but informative move. Kuiper et al. (2011) however found that ZolA administered at 2 μg/kg in mice adversely suppressed neutrophil activity in a dose-dependent manner which led to cell death and subsequent impaired immune system. Alternatively, the ED50 of ZolA subcutaneously administered was determined at a dose of 0.07 mg/kg (Widler et al., 2002). Because survivability of the 0.5 ZolA administered mice was drastically different than that of the 1 ZolA group, we can conclude that ZolA was toxic at higher doses. This, however, also could have been exacerbated by the differential toxicity in 3NP administration that we observed.

The results indicated that Sim was part of the intermediate sickly group. There is not extensive data to support the toxicity of Sim in animals; however, the Cayman Chemical Company's Material Safety Data Sheet (2010) indicates intraperitoneal LD50 in the rat to be 705 mg/kg – a value much higher than our 40 mg/kg administered. This finding was similar to that of

the 3NP/Sal treated animals, so we may be able to conclude that toxic effects were induced by the selective striatal neurotoxin.

Behavioral Performance

In regards to our first hypothesis, we did not obtain sufficient evidence to reject the null hypothesis. Results indicated that striatally- and hippocampally-mediated cognitive impairments associated with Huntinton's disease were not consistent with our 3NP animal model. This was exemplified in both performance of mice in the shallow water variants of the T-maze and MWM.

Results showed that performance on the T-maze did not differ as a function of drug treatment administration when controlling for number of treatment injections received. Likewise, results indicated that drug treatment administration did not alter performance on the shallow water variant of the MWM. Overall, these were inconsistent with our hypotheses and the literature that the lesioning effects of 3NP in the striatum and hippocampus would lead to striatally- and hippocampally-mediated cognitive decline as evidenced by performance in the T-maze and MWM.

Our current study lacks the histological data necessary to determine definitively whether or not our 3NP dosage regimen did in fact produce lesions similar to those seen in other studies (high-dose, sub-chronic treatment). Colleagues Whitmarsh and LaHoste (paper currently in progress), however, showed 3NP/Sal treated animals performed significantly worse on the rotarod (a common measure of striatal integrity) than control animals. Our cumulative treatment of 3NP may have been too high, mimicking the hypokinetic effects commonly seen in sub-chronic and chronic dosing regimens (Tunez, Tasset, De La Cruz, and Santamaria, 2010). While the rotarod data could point to support of striatal neuronal degeneration, it could also be the mark

of impairment in motor behavior due to adverse effects of the drug treatments. This conclusion is additionally plausible due to the severely compromised health of the animals.

Results vary in support of either of these theories because 3NP/Sim treated animals, in comparison to healthy controls, needed significantly more time to complete the T-maze and performed worse; however, they did not differ in either regard to animals in the 3NP/Sal, or any other, treatment group. Furthermore, performance on the MWM did not differ between mice in any of the treatment groups. We are forced to retain the second null hypothesis that drug treatment inhibiting the prenylation of Rhes would alleviate adverse cognitive symptoms caused by 3NP because there were no significant differences between performance and any of the drug treatments involving 3NP. Because we failed to reject the first null hypothesis, we cannot reliably conclude whether or not Sim or ZolA treatments were effective in disrupting the mevalonate pathway to lead to an absence of Rhes and retardation of cognitive impairment.

It is possible that we did in fact achieve striatal and hippocampal lesions, but one could question whether or not our cognitive tasks were appropriate to test them. In both water and land-based learning mazes, rodents with lesions to the CPu fail to learn egocentric, procedural-type learning and rather employ alternative strategies that are more allocentric or spatial-like while rodents with lesions to the hippocampus will fail to learn allocentrically and rather utilize egocentric strategies (Devan, Goad, and Petri, 1996; Oliveira, Bueno, Pomarico, and Gugliano, 1997; and Pistel et al., 2009). Results, however, indicated that learning did in fact occur across treatment groups in both the T-Maze and MWM. The methodology used in our T-maze was sound to test for egocentric learning. The lack of differences in performance between groups of mice may have then been caused by the short time frame mice were allowed to learn the task, the timing of task conductance within drug treatment regimen, the lack of lesion to the striatum, or a

need to increase the number of animals in each group. Because of positive rotatod data from our colleagues, we strongly believe a lesion to have existed. Due to positive scores across all treatment groups, however, we believe our methodology on trial periods and timing in task administration was appropriate, and we more than likely needed to increase the number of animals in each treatment group. This is especially true considering the vast differences in health between our first group of 3NP/Sal treated animals and our second. The methodology in our shallow water variant of the MWM was a bit more questionable given the results. We did not have a tracking system to include further measures, so the amount of data collected for analysis was minimal. Patterned cue cards attached to the walls could have acted as both spatial references (spatial-hippocampal based) or as visual discriminants (procedural-striatal based). Depending on where lesions existed, it is difficult to predict performance on this task. Saline control mice could have employed either learning strategy, making comparisons to other groups difficult. While the literature supports that 3NP-induced hippocampal lesions do not occur without the presence of striatal lesions, we may be able to conclude that 3NP treated mice were more likely to employ their spatial, cue-based strategies to navigate the maze because it was likely that neurons in the striatum were more compromised than those in the hippocampus. Even if this is the case, however, the lack of differences in performance across the groups treated with 3NP clearly indicated treatment drugs had no effect. It may be the case that differences in cognition did not exist, or that our task needed more training periods or increases in subject number.

Brouillet, Jacquard, Bizat, and Blum (2005) wrote an extensive review on the mechanisms behind 3NP, and we may not have seen differences in performance across treated mice because of them. There are vast toxicity differences between mice and rats, with rats

reacting more adversely to 3NP I.P. injection; and there are further differences within each different strain of rodent. Age is a significant factor as older mice are more susceptible to damage, and males are more affected than females. While we did use the more fragile male rodents, they could have also been more protected from neuronal damage because they were mice and they were relatively young, ~2 months old. This could explain in part the differences between our study and the findings in the literature exemplifying deficits in performance on striatally- and hippocampally-mediated cognitive tasks. The methodology and number of animals used could have been appropriate, with only nature exerting its protective effect.

Whether or not the lack of significant differences in performance on both tasks was due to an absent lesion, inappropriate methods, small sample size, inappropriate timing of task administration, natural protectants, or legitimate neurological outcomes, results showed that there were no differences in performance on cognitive tasks between 3NP treated mice and controls. The sub-chronic dosing of 3NP at 560 mg/kg over 7 days did not produce deficits in cognition, and thus we were unable to test whether or not drug treatment blocking the mevalonate pathway would exert alleviation of the non-existing symptomology.

Future Directions

The central goal of this thesis was to elucidate cognitive dysfunction in a 3NP mouse model of HD and ameliorate abnormalities by inhibiting Rhes prenylation through drug treatments targeting the mevalonate pathway. In understanding how certain molecular pathways are involved with neurodegeneration caused by either genetic means or neurotoxin, we can further aim to find treatments that target them and improve subsequent behavior abnormalities. The implications of studying such models could lead to further treatment applications to diseases that follow the same patterns of neurodegeneration or include the mavelonate pathway. The

mavelonate pathway is a ubiquitous biochemical sequence in cellular signaling. Perhaps the effects of our drugs on system organs obscured any effect in the brain. We were restricted to the use of drugs that crossed the blood-brain-barrier (BBB; either alone or following 3NP) and to drugs that could be obtained feasibly. Future experiments could employ intracerbral administration of potentially ameliorative drugs, thereby by-passing their natural ability to cross the BBB and would require much less drug, thereby allowing the use of otherwise financially prohibitive drugs.

Our study was unique and relied on previously published data for the establishment of experimental parameters. Furthermore, although we controlled for potentially confounding variables, we did not anticipate the degree of variability that we observed. Although we did conduct pilot studies, we could have conducted more provided we were given the time to do so. Future work needs to be done to increase the literature on several aspects. There need to be more defined 3NP dosage regimens for acute, sub-chronic, and chronic conditions in addition to a more cohesive review clarifying differences in dose between rodent species. Cognitive and motor behavior needs to be analyzed based on these data and methodology replicated to unify the deviation from normalcy with the lesioning literature. Further applications to the field would be related to the research on inhibition of enzymatic tartgets in the malevolent pathway leading to prenylation of Rhes and thus retardation of motor or cognitive abnormalities. Clearly the literature is vastly lacking cohesive knowledge in several aspects specific to the selective striatal neuronal degeneration induced by 3NP treatment. Without conducting studies with more reliable methodology, we may never have the literature support we need to generate valid conclusions.

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Institutional Animal Care and Use Committee

UNIVERSITY OF NEW ORLEANS

DATE: **October 8, 2014**
TO: **Gerald LaHoste**
FROM: Bernard B. Rees,
Chair
RE: *IACUC Protocol* # 14-011
Entitled: Ameliorative effect of simvastatin on striatal degeneration and behavioral deficits in an animal model of Huntington's disease

Your application for the use of animals in research (referenced above) has been approved beginning October 8, 2014 and expiring October 7, 2017. The initial approval period is one year. Near the end of this period, you will be asked to complete and submit an annual review in order to continue animal activities.

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

Diana Hobbs graduated *cum laude* from Austin College (2012) with her B.A. in Psychology. She was accepted into the graduate program at the University of New Orleans in 2012 where she is currently pursuing her PhD in applied biopsychology under Dr. Gerald LaHoste. There, Diana is examining the effects of the Rhes protein in animal models of Huntington's disease. Specifically, she is interested in behavioral differences involved in cognition, learning, and memory.