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Effectiveness of Statin and Bisphosphonate Treatment in a 3NP model of Huntington's Disease

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Effectiveness of Statin and Bisphosphonate Treatment in a 3NP model of Huntington's Disease

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

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Abstract

Recent research suggests that the striatal specific protein Rhes may play a critical role in the behavioral and neurodegenerative process that occurs in Huntington's Disease, but the exact role of Rhes is unclear. Farnesylation of Rhes is required for it to become biologically active. This study examines the influence of pharmacological inhibitors of farnsylation and geranylgeranylation on behavior in a 3NP model of Huntington's.

Keywords: Rhes, Huntington's Disease, AKT, 3NP

1. Introduction

1.1 Huntington's Disease

Huntington's Disease (HD) is a neurodegenerative disorder that involves the unstable expansion of the trinucleotide repeat (CAG) coding for a poly-glutamine stretch on the Huntingtin gene on chromosome 4. This expansion results in a long tract of poly-glutamines in the N-terminus of the huntingtin (Htt) protein. Htt is normally expressed with as many as 34 CAG repeats. However, more than 37 repeats (Walker, 2007) results in the expression of a mutated huntingtin protein (mHtt). The expanded polyglutamine tract has been linked to incorrect protein folding (Fink, 1999), and leads to the abnormal shape of the mHtt proteins. The mHtt protein forms aggregates in the striatum and cortex, consistent with HD pathology. Although aggregates are the primary pathological feature associated with HD, their contribution to toxicity and cell death is debatable.

While HD pathology results in substantial damage to several areas of the brain, including the cortex, and in later stages of the disease the hippocampus and cerebellum (Vonsattel & DiFiglia, 1998), the majority of neurodegeneration is localized in the striatum. Although the Htt protein is expressed throughout the peripheral and central nervous system, the majority of damage from the disease occurs specifically in GABAergic medium spiny neurons (MSNs) which comprise 90-95% of striatal neurons (Graveland, Williams, & DiFiglia 1985; Albin, Reiner, Anderson, Dure, Handelin, Balfour et al., 1992). Additionally, post mortem findings indicate a greater number of aggregates in the lesser damaged cortex than in the degenerated striatum (Gutekunst, Li, Yi, Mulroy, Kuemmerle, Jones, et al., 1999), suggesting the aggregates are not directly associated with cell death.

A member of the Ras protein signaling family, Rhes, is expressed abundantly and selectively in striatal neurons (Falk et al., 1999). Due to its restricted expression, the Rhes protein has become a promising target for understanding the neurodegenerative process in HD. Overexpression of Rhes in cultured cells that also over-express mHtt has been shown to reduce cell survival by 50%, whereas overexpression of either mHtt or Rhes alone did not result in a decrease in cell survival (Subramaniam, Sixt, Barrow & Snyder, 2009). However, when Rhes expression was diminished, the reduction in cell loss was reversed so that cell survival increased. Attenuation of Rhes has also shown promise in animal models of HD. HD mice bred with Rhes knockout (KO) mice show significant delay in the onset of HD symptoms (Baiafonte, Lee, Brewer, Spano & LaHoste, 2013). Recently, Rhes KO mice were shown to be protected from striatal damage (Mealer, Subramaniam & Snyder, 2013) following 3-nitropropionic acid (3NP) treatment, a striatal lesion model of HD. The 3NP model is a mitochondrial complex II inhibitor, which creates a striatal specific lesion, as well as motor and behavioral symptoms similar to HD while sparing neurons outside of the striatum.

1.2 Huntington's Disease and Behavior

Early indications of HD motor disturbances are found in the fingers, toes and small facial muscles. The muscle twitches may not be visible or may even be mistaken as signs of nervousness. The disturbances eventually move from distal to more proximal and axial areas. Hyperkinesia and hypokinesia cause difficulties in walking and standing. Saccadic eye movements are another early abnormality associated with HD as well as chorea, with akinesia and dystonia occurring in later stages of the disease. (Penney et al., 1990).

Psychiatric symptoms of HD usually emerge before motor symptoms, but are often difficult to diagnosis in comparison with outward motor disturbances. However, anxiety and depression are

frequently observed in HD patients (Walker 2007). A recent study matching depressed HD patients with depressed patients from the general population found no significant difference in clinical features of depression based on the Hospital Anxiety and Depression Scale, Beck Depression Inventory, Test for Anhedonia questionnaire and a slightly modified Montgomery Asberg Depression Rating Scale (Scaria & Craufurd, 2014). In addition to depressive symptoms, feelings of guilt, anxiety and low self-esteem are also frequently reported. Ninety-eight percent of HD patients in one study exhibited neuropsychiatric symptoms (Paulsen, Ready, Hamilton, Mega & Cummings, 2014). The most common symptoms were dysphoria, agitation, irritability, apathy and anxiety ranging from mild to severe and independent of cognitive or motor aspects of the disease.

Currently, a diagnostic tool, the HD Behavioral Questionnaire (HDBQ), is being developed in order to capture psychiatric symptoms of HD while taking into consideration motor and cognitive disturbances associated with the disease. The HDBQ is a reliable instrument for screening behavioral changes in individuals with or at risk for HD, and is particularly important as these behavioral changes can affect quality of life for both the patient and caregiver (Corey-Bloom, Herndon, Breen, Huynh & Gilbert, 2014).

Consistent with the striatal neurodegenerative features of HD, presentation of both motor and psychiatric disorders likely involve striatal abnormalities (Crittenden & Graybiel 2011). The cortico-striatal pathway, along with dopaminergic innervations, is the major input to the striatum. HD has been associated with changes to the frontostriatal loop, which may account for a loss of cognitive flexibility (Kirch et al. 2013). Additional changes in synaptic plasticity in the corticostriatal pathway (Cummings, et al. 2007) may underlie early cognitive behavioral deficits by altering striatal information processing and transfer within basal ganglia-thalamo-cortical

loops. Striatal astrocytes and neurons have an increased vulnerability to calcium load, and striatal astrocytes have a lower mitochondrial buffering compared to cortical astrocytes (Oliveira and Goncalves 2009). Striatal GABAergic medium-sized spiny neurons (MSNs), in particular seem to be selectively vulnerable to metabolic stress, which may contribute to the selective loss of these neurons in HD.

The striatum is a part of the larger basal ganglia which includes two circuits, dorsal and ventral, comprised of several smaller areas of the brain. The ventral tegmental area (VTA) and nucleus accumbens (NAc) together form the ventral pathway (Groenewegen, Wright, Beijer, & Voorn, 2006). This circuit of the striatum is associated with the recognition of salient elements in the environment, and may be involved in symptoms such as anhedonia, loss of interest or passive behavior. A second circuit, the dorsal pathway, is associated with downstream regulation of the motor cortex and motor learning, and is formed by the pallidum, substantia nigra pars compacta (SNpc), and subthalamic nucleus (STN).

The MSNs that make up the majority of striatal neurons project to other areas of the brain through two pathways: direct and indirect. MSNs in the direct pathway project to the internal division of the globus pallidus (GPi) and the substantia nigra pars reticulata (SNpr), which in turn project to the ventroanterior and ventrolateral thalamus (VTh). The VTh projects to upper motor neurons in the primary motor cortex (Gerfen & Surmeier, 2011). In addition, the SNpr projects to the deep layer of the superior colliculus, and controls rapid eye movements (saccades), which is often one of the earliest reported motor symptoms of HD (Penney et al., 1990). MSNs in the indirect pathway project to the external segment of the globus pallidus (GPe) which then project to the STN prior to innervating the SNpr.

MSNs receive input from three different sources: dopamine (DA) from the SNpc; glutamate from the cortex and thalamus; and GABA from striatal interneurons and other MSNs (Cha et al., 1999; Luthi-Carter, et al., 2000). Additionally, the direct and indirect pathways express different proteins (Andre, Cepeda & Levine, 2010). The direct pathway primarily expresses DA D1-like receptors (D1 & D5). The indirect pathway highly expresses DA D2-like receptors (D2, D3, D4), which are among the first neurons to die during the early stages of the disease.

NMDA receptors on MSNs are associated with altered calcium homeostasis, mitochondrial membrane depolarization, oxidative stress and caspase activation (Andre, Cepeda & Levine, 2010). NMDA and AMPA receptor function increase with D1-like receptor activity due to several signaling pathways including cAMP and voltage-gated Ca^{2+} channels, and decrease with D2-like receptor activity. Normally DA modulates glutamate-induced excitation in the basal ganglia and cortex, but the balance between these systems is disturbed in HD. Overactivity of glutamatergic neurons is believed to lead to excitotoxicity and striatal changes, while altered dopaminergic neurotransmission has also been implicated in HD pathology including evidence of early stage increases in dopamine and later stage decreases (Cyr, Sotnikova, Gainetdinov, Caron 2006; Stack, et al., 2007; Crook and Houseman, 2012).

1.3 Rhes

Interestingly, Rhes has been implicated in dopaminergic and striatal function through regulation of dopamine supersensitivity (Harrison and LaHoste, 2006). Removal of dopaminergic input to the striatum leads to a reduction of Rhes mRNA, possibly contributing to the supersensitive response to DA observed in the striatal tissue following removal. Rhes related signal transduction applies also to neurons of the indirect striatopallidal pathway, implicating Rhes as a

partner in dopamine transmission, mediated through cAMP/PKA signaling (Errico, et al., 2008). Rhes inhibits behaviors stimulated by D2 receptor stimulation or D1/D2 receptor synergism, and facilitates the D1-specific behavior of grooming. Behaviors including locomotor activity and anxiety are also influenced by Rhes expression (Quintero, Spano, LaHoste, and Harrison, 2008).

1.3.1 Rhes and AKT

Striatal D2 receptors signal through G proteins, and also by promoting the formation of a striatal multi-protein complex which includes β -arrestin2, AKT and Protein Phosphatase 2A (PP2A). Upon formation of this complex, PP2a dephosphorylates and deactivates AKT. AKT, also known as Protein Kinase B (PKB), is a serine/threonine-specific protein kinase that plays a key role in apoptosis, cell proliferation, transcription and cell migration as well as the development of diabetes and cancer (Cantley, 2002; Sarbassov, Guertin., Ali & Sabatini, 2005). AKT activation levels are inversely related to activation of the dopamine receptor pathway (Souza, Romano-Silva, Tropepe, 2011), and AKT has recently been recognized as an important factor in neurotransmission and mental illness (Kitagishi, Kobayahi, Kikuta & Matsuda, 2012). Recent evidence suggests that Rhes interacts with β -arrestin 2 to form the protein complex, and this interaction is necessary for PP2A to dephosphorylate and deactivate AKT (Harrison, Muller, Spano, 2014).

Lithium treatment disrupts the formation of this striatal complex and increases levels of AKT phosphorylation and activation. Rhes KO mice develop a lithium-treated phenotype (Harrison, Muller, Spano, 2014), as they express increased basal AKT phosphorylation following treatment with a D1/D2 agonist that is not further altered by lithium. Although, lithium is primarily recognized as treatment for mood disorders, its attenuating effect on mood requires AKT

phosphorylation (Pan et al., 2011), and it has been successfully used to treat motor symptoms of HD (Sarkar, Krishna, Imarisio, Saiki, O’Kane & Rubinsztein, 2008).

Rhes also influences AKT signaling via another pathway in a seemingly contradictory manner.

Growth factors, such as Insulin-like Growth Factor-1 (IGF-1), activate Phosphoinositide 3-

Kinase (PI3K), which primarily targets AKT for phosphorylation (Cantley, 2002). Insulin

signaling is initiated when IGF-1 binds to its receptor, and activates the tyrosine kinase domain

of the receptor. Insulin receptor substrate-1 (IRS-1) then binds to the activated receptor, and

functions as the binding and activation site for PI3K, beginning the cell survival cascade.

Although Rhes is necessary for dephosphorylation and deactivation of AKT, Rhes also enhances

growth factor-mediated AKT phosphorylation and activation by binding with p85, the regulatory

subunit of PI3K and by directly interacting with AKT and facilitating its movement to the

membrane to initiate signaling (Bang, Steenstra & Kim, 2013), suggesting that Rhes may

function as a critical bridge between PI3K and AKT. The authors further speculate that given the

inverse relationship between DA activation, as well as the relationship between DA

supersensitivity and Rhes expression, DA supersensitivity may lead to a decrease in Rhes

expression and reduced AKT activity.

Insulin signaling promotes cell growth and proliferation via AKT, and involves both Insulin and

IGF-1. The Insulin receptor substrates (IRS1/2) intermediate receptor signaling inside of the cell

for activated cell surface receptors. Like IGF-1 bound to its receptor, Insulin bound to the Insulin

receptor (IR) also activates the tyrosine kinase domain and binding of IRS-1/2. Bound IRS1/2

then activates several downstream pathways including PI3K/AKT (Nemoto et al., 2010;

Ramalingam & Kim, 2014). Activation of the insulin receptor has a direct effect on

neurotransmission and primes synapses for the induction of LTP following the binding of IRS-1

and IRS-2 to PI3K. IRS-1/2 bound with PI3K can then phosphorylate NMDA receptors to increase the opening associated with the Ca²⁺ channel (Hilscher, 2011) to induce LTP.

1.3.2 Rhes and Thyroid Hormone

Rhes is primarily expressed in the striatum, but has also been found in select peripheral tissues, and is dependent on thyroid hormone (TH) for expression (Verma, Kumar & Thapliyal, 2014). Notably, Rhes is also expressed in thyroid tissue and additionally has been shown to inhibit the functional activity of the thyroid stimulating hormone receptor (Agretti et al., 2007), possibly suggesting a role for Rhes in regulation of its own expression. TH is deregulated in HD, and the striatal protein protein μ -crystallin (Crym), a key regulator of TH may link TH deregulation and striatal vulnerability to mHtt, as overexpression of Crym in striatal MSNs is neuroprotective against N-terminal fragment mHtt toxicity in vivo (Francelle et al., 2014). Crym expression is reduced in HD, which may in turn either influence or be influenced by Rhes expression depending on interactions with TH.

TH has also been shown to regulate IGF-1 expression (Harakawa et al., 1990), and inhibit IGF-1 stimulated glucose uptake and cell proliferation (Incerpi et al., 2013), suggesting an additional link to Rhes expression and AKT phosphorylation mediated by insulin signaling. While the majority of HD symptoms result from damage to the central nervous system, the conflicting influence of Rhes on Insulin signaling and subsequent AKT activation and deactivation, may also clarify HD related changes in the peripheral nervous system.

1.3.3 Rhes and Insulin Signaling

Rhes is also expressed in pancreatic β -cells, where it may regulate the secretion of insulin (Chan et al., 2002; Verma, Kumar & Thapliyal, 2014). Pancreatic β -cells release insulin in response to

high levels of glucose. The insulin then circulates through the bloodstream until it binds to an insulin receptor in either the peripheral nervous system or central nervous system. Insulin resistance is a normal occurrence; however, when it co-occurs with impaired insulin secretion it contributes to type 2 diabetes (Withers & White, 1999). Pancreatic β -cells initially increase insulin secretion to compensate for hyperglycemia induced by insulin resistance (Rui et al., 2001). If the hyperinsulinemia and hyperglycemia persist, insulin action may become impaired and diabetes may develop.

In addition to neurological defects, HD is also associated with an increased incidence of diabetes (Podolsky, Leopold & Sax, 1972; Hu, Liang & Yu, 2014). Recently, a case study was published describing a 40 year old type 1 diabetic presenting symptoms of hyperglycemic induced chorea-ballism, or extreme choreatic movement. Although the patient recovered from the chorea-ballism after being treated with fluids and insulin, mild choreatic movement persisted (Hashimoto et al., 2012). Magnetic resonance imaging and DNA analysis revealed HD, suggesting a possible role for the acute influence of insulin on HD motor behavior.

Transgenic mouse models of HD show progressive alterations in glucose levels that eventually result in the development of insulin deficiency and diabetes; altered pancreatic islets; and an age dependent reduction in insulin mRNA expression (Andreassen et al., 2002). The polyglutamine expansion tract in the Htt protein is thought to disrupt expression of transcription factors in pancreatic β -cells and result in insulin deficiency and diabetes (Björkqvist et al., 2005).

Preferential Rhes expression in β -cells further supports a role for the involvement of Rhes in the selective toxicity of the ubiquitously expressed Htt protein. Binding partners of Htt may also interact with Rhes to increase toxic effects. For example, Huntingtin-Associated Protein 1

(HAP1) is also expressed in pancreatic β -cells, and, was the first binding partner identified with Htt. Hap1 aids in intracellular processes which are disrupted by the presence of mHtt. Hap1 KO mice show decreased amounts of insulin-containing vesicles at docking sites in β -cell membranes (Cape, 2010), suggesting HAP1 may be an important target for further understanding HD related impairment of β -cell insulin release and mHtt toxicity.

AKT is also implicated in diabetes, and likely involves the multi protein complex required for AKT dephosphorylation. Loss of β -arrestin 2 results in defective AKT activation. AKT in an inactive dephosphorylated state inhibits insulin signaling, and contributes to insulin resistance (Teruel, Hernandez & Lorenzo, 2001). Peripheral administration of IGF-1 activates AKT, enhances insulin levels and protects against diabetic features in an animal model of Huntington's disease (Duarte et al., 2011).

Insulin and IGF-1 decrease mitochondrial reactive oxygen species (ROS) induced by mHtt, and normalize mitochondrial activity of the antioxidant defense Superoxide Dismutases (SOD).

Insulin signaling also increases AKT phosphorylation and mitochondrial-encoded cytochrome c oxidase II, in mitochondria of insulin- and IGF-1 treated striatal cells (Ribeiro, Rosenstock, Oliveira, Oliveira & Rego, 2014). Insulin/IGF-1-treated mHtt striatal cells also showed reduced apoptotic features.

Insulin signaled AKT activation inactivates proapoptotic proteins such as glycogen synthase kinase-3 (GSK-3 β), Bad, and the forkhead transcription factors (FOX) (Fort et al. 2010) which are activated upon decreased survival signals, increased cellular stress, or both. AKT phosphorylates FOXO so that it becomes a substrate of ubiquitin ligase. Once a protein is ubiquitinated, it is tagged for degradation and broken down by the proteasome.

Ubiquitin ligase deficiency accelerates disease phenotype and increases global aggregate load in an animal model of HD (Maheshwari, et al., 2014). SUMOylation (Small Ubiquitin-like MOdifier), another post-translational modification, may serve as a necessary primer for ubiquitination, and stimulate protein ubiquitination and degradation (Wei & Lin, 2012). SUMOylation cell culture studies show that mHtt is sumoylated by Rhes. Rhes expression decreases mHtt aggregation and elicits neurotoxicity by increasing soluble levels of fragmented mHTT in addition to significantly reducing mHtt ubiquitination (Subramaniam, Sixt, Barrow & Snyder, 2009). Rhes also binds with much stronger affinity to mHtt compared to wild type Htt (Subramaniam, Sixt, Barrow & Snyder, 2009). SUMO was recently shown to be a key regulator of AKT phosphorylation while down-modulation of SUMO diminishes AKT and cell proliferation. Additionally, an AKT SUMOylation mutant displayed reduced activation, and decreased anti-apoptotic and pro-tumoral activities (de la Cruz-Herrera et al., 2014), possibly indicating a normally protective function of Rhes in protein degradation which becomes toxic in the presence of fragmented mHtt.

1.3.4 Rhes and mHtt: Autophagy and Apoptosis

In addition to the ubiquitin-proteasome system, proteins may also be degraded via autophagy, a lysosomal degradation pathway. The mammalian Target of Rapamycin (mTOR) is typically associated with the promotion of cell survival via phosphorylation of AKT, and is an important regulator of autophagy. However, many researchers intensely debate whether autophagy is involved in maintenance and cell survival, or is a necessary component of apoptosis (Levine & Yuan, 2005; Nixon, 2006; Denton et al., 2009). Autophagy and apoptosis may be activated by common upstream signals that sometimes results in combined autophagy and apoptosis, or the cell switching between the two responses in a mutually exclusive manner. Specifically, the

shared apoptotic and autophagic pathways can either link or polarize these processes (Maiuri, Zalckvar, Kimchi & Kroemer, 2007).

mTOR was discovered in mammals after the discovery of yeast Target of Rapamycin (TOR). Like TOR, mTOR consists of two distinct complexes: mTOR Complex1 (mTORC1) and Complex 2 (mTORC2). Rapamycin, an inhibitor of mTOR and TOR activates autophagy, but TORC2 and mTORC2 are insensitive to acute Rapamycin administration (Jacinto et al., 2004). mTORC1 activates the translational regulator S6K (S6 kinase), leading to increased protein synthesis in the presence of nutrients while mTORC2 responds to the presence of growth factors such as insulin by phosphorylating AKT. Moreover, chronic Rapamycin administration impairs glucose tolerance and insulin action, and is mediated by increased mTORC2 signaling and its negative feedback regulation of IRS-1. mTORC1 promotes phosphorylation and down-regulation of IGF-1 while mTORC2 mediates IRS-1 degradation (Sarbassov, et al., 2006; DeStefano & Jacinto, 2013).

Rhes was recently shown to bind to and activate mTORC1, but also appeared to influence mTORC2 in a study of Rhes KO mice (Subramaniam et al., 2013). Rhes KO mice display reduced mTOR, were predicted to inhibit autophagy secondary to mTOR activation. However, a follow up study showed that Rhes behaves in the opposite manner (Mealer, Murray, Shahani, Subramaniam & Snyder, 2014), and actually activates autophagy independent of mTOR by competitively binding to autophagy regulator Beclin-1 (Bcl-1) and decreasing its inhibitory interaction with Beclin-2 (Bcl-2).

The mitochondrial pathway to cell death is regulated by Bcl-2 family proteins which include Bcl-2, BH3 (the BCL-2 homology 3), Bax (BCL-2-associated X protein) and Bak (BCL-2

antagonist/killer). BH3 signals the start of apoptosis while Bcl-2 guards survival and Bax and Bak promote apoptosis (Czabotar, Lessene, Strasser & Adams, 2014). These proteins interact on the mitochondrial membrane to determine apoptosis based on combined signaling. While it is widely believed that Bcl-2 inhibits autophagy by binding to Bcl-1 and blocking its function, recent evidence suggests that Bcl-2 is only indirectly involved in autophagy by inhibiting Bax and Bak, and that in the absence of Bax and Bak, autophagy cannot be stimulated (Lindqvist, Heinlein, Huang & Vaux, 2014). Inhibiting Bcl-2 in the absence of Bax and Bak stimulated autophagy, but also correlated with increased cell death. Additionally, increasing Bcl-2 should sequester Bcl-1 and inhibit autophagy, but no differences in autophagy were found following upregulation of Bcl-2. The authors propose a model where apoptosis induces autophagy, and suggest that autophagy does not significantly induce cell death in the absence of Bax and Bak.

Once the apoptotic threshold is met via signaling of the Bcl-2 family, apoptogenic products are released into the cytosol, such as cytochrome c (COX). Interestingly, COX was increased following AKT phosphorylation by insulin treatment of striatal cells which reduced apoptosis (Ribeiro, Rosenstock, Oliveira, Oliveira & Rego, 2014) suggesting a role for insulin signaling in both proliferation and apoptosis.

Insulin also diminished oxidative damage due to lactate dehydrogenase (LDH), nitric oxide (NO), reactive oxygen species (ROS) and calcium ion (Ca^{2+}) in neuronal cells (Gurzov & Eizirik, 2011). A follow up study in glia cells revealed that Insulin is protective due to activation of AKT, and upregulation of Bcl-2 by preventing Bax and Bax/Bcl-2 ratio, which is associated with apoptosis when the ratio increases. The Bcl-2 family is crucial to pancreatic β -cell mitochondrial apoptosis, and members regulate glucose metabolism and β -cell function (Gurzov & Eizirik, 2011). The combined role of Bcl-2 and Bcl-x is crucial for mitochondrial integrity and

β -cell survival, and involves metabolic signaling, Ca^{2+} homeostasis, and insulin secretion. Further, Bcl-2 and Bcl-x suppress the β -cell response to glucose, suggesting the Bcl-2 family is involved in normal cell function in addition to survival (Luciani et al., 2013). Lithium treatment prevented the decrease of striatal Bcl-2, and attenuated the increase of striatal Bax in a dopaminergic lesion model. Additionally, lithium increased striatal Bcl-2 in both lesioned and control mice (Youdim & Arraf, 2004); further indicating a role for Insulin signaling, AKT phosphorylation, and ultimately Rhes in both normal cell function and pathology.

Given the complex and often conflicting roles of Rhes in cell signaling, it is difficult to determine how decreased Rhes expression is protective in HD. Because Rhes appears to be involved in multiple processes that regulate normal cell function, cell survival and cell death, manipulation of Rhes expression would seem to interfere with these processes in unpredictable ways. Although, mHtt is ubiquitously expressed throughout the body in HD, the majority of damage occurs in the select organs that express Rhes (e.g. striatum, pancreas). Despite localization in the CNS versus the PNS, damage occurring in these organs seems to involve the same cellular processes (e.g. AKT/mTOR), and Rhes is directly involved in both the activation and deactivation of these signals which promote survival and apoptosis.

Many theories link neuronal death in HD to an increase in apoptotic signaling. One theory even suggests that midlife onset of HD may be an evolutionary tradeoff of reduced cancer risk during fertile years as a result of increased apoptotic activity (Turner, Goldacre & Goldacre, 2013). Interestingly, this theory is supported by studies showing reduced risk of cancer in HD (Sørensen & Fenger, 1992; Turner, Goldacre & Goldacre, 2013). Huntingtin is a substrate for the protease apopain8 which belong to a family of cysteine proteases that play a crucial role in the apoptotic pathway. Expansion of the polyglutamine tract may lead to conformational changes to the

huntingtin protein that increase apoptotic properties of mHtt throughout the body (Sørensen, Fenger & Olsen, 1999). Further, the PI3K/AKT pathway, which Rhes directly activates and inactivates, is one of the most frequently targeted pathways for cancer treatment. AKT was originally discovered as an oncogene, and its significance in transformation and cancer was confirmed by studies showing that AKT is frequently amplified and overexpressed in human cancers (Cheng, Lindsley, Cheng, Yang & Nicosia, 2005).

Given the ubiquitous expression of mHTT, select damage to organs with Rhes expression, the role of Rhes in proliferation and apoptosis, the role of mHtt in apoptosis, and increased toxicity of mHTT following SUMOylation, Rhes and mHtt specific toxicity may reflect an interaction of several processes which are normally protective, but destructive when combined. As mentioned previously, fragmentation of mHtt by Rhes increases toxicity compared to aggregation of mHtt (Subramaniam, Sixt, Barrow & Snyder, 2009). Additionally, co-expression of mHtt blocks Rhes induced autophagy (Mealer, Murray, Shahani, Subramaniam & Snyder, 2014), and Rhes induced autophagy is independent of mTOR. Rhes is down-regulated in HD, and because Rhes is crucial for so many cellular processes such as autophagy and cell survival, damage may occur as a result of decreased AKT/mTOR activation as well as inhibition of possible alternate compensatory processes (i.e. mTOR independent activation of autophagy by Rhes) due to its unique interaction with mHtt.

1.4 3NP and Rhes

Administration of 3NP, an irreversible inhibitor of the mitochondrial II complex, succinate dehydrogenase (SD), produces a selective striatal lesion similar to the selective striatal damage observed in HD. 3NP also produces a long-term potentiation (LTP) of glutamatergic NMDA-

mediated synaptic excitation in MSNs (Calabresi et al., 2001). 3NP-LTP involves increased intracellular calcium and is critically dependent on endogenous dopamine acting via D2 receptors, and negatively regulated by D1 receptors.

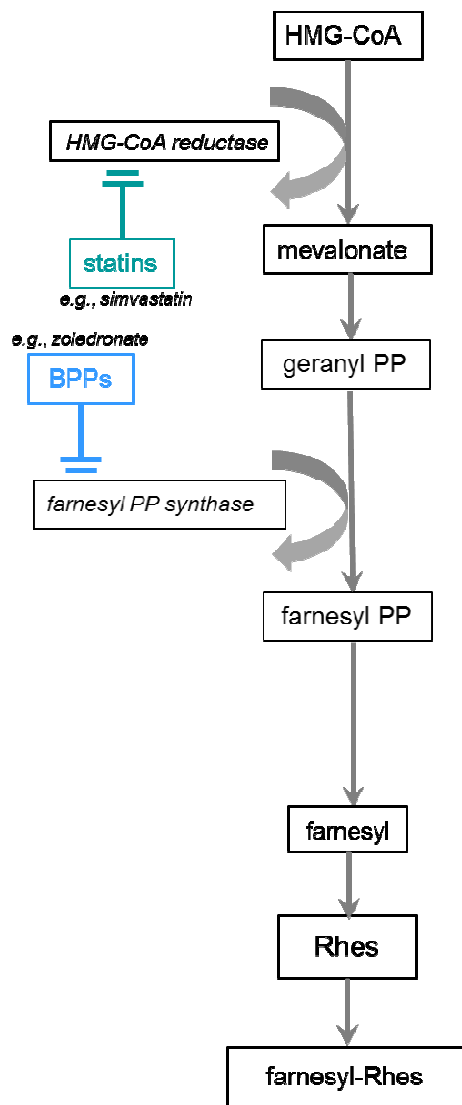
In addition to altered DA and glutamatergic neurotransmission, many similarities exist between the 3NP model and HD. MSNs in HD patient brains show a significant decrease in SD activity, and as seen in several studies of neuroprotection in HD, 3NP appears to involve AKT signaling. Lithium reduced 3NP striatal neurodegeneration in rats by inhibiting activation of calpain (a calcium dependent protease involved in cell function), and reducing intracellular calcium levels (Crespo-Biel, Camins, Pallas, & Canudas, 2009). Calpain knockdown in a HD *Drosophila* model also protects against aggregation and toxicity of proteins in an autophagy dependent manner, as inhibition of calpain by calpastatin increases autophagosome levels and is also protective in a mouse model of HD (Menziez et al., 2014).

Rhes involvement in both AKT and autophagy suggests that manipulation of Rhes expression should have a significant influence on 3NP toxicity; however, because Rhes is involved in activating and deactivating these processes, it is difficult to predict whether the influence would be protective or detrimental. A recent study found that Rhes deletion is neuroprotective in the 3NP model (Mealer, Subramaniam & Snyder2013), indicating that down regulation of Rhes should also be protective.

1.4.1 Protein Prenylation and Rhes

Prenylation is a posttranslational process that occurs when hydrophobic molecules are added to a protein in order to aid in anchoring of the protein to its membrane. Protein prenylation occurs via the mevalonate synthetic pathway that ultimately affects the transfer of a functional farnesyl or

geranyl-geranyl molecule to a target protein. Two of the enzymes that carry out this process are farnesyltransferase and geranylgeranyl transferase. Inhibition of these processes prevent anchoring of the target protein, and result in an inactive form of the protein. Pharmacological inhibition by drugs such as Simvastatin and Zoledronic Acid can disrupt this pathway (see Figure 1.1).



(Figure 1.1 : Overview of the mevalonate pathway.)

Farnesylation of Rhes is required for it to become biologically active. An in vitro study of farnesyl deficiency cause by a mutated form of Rhes showed a significant reduction of Rhes binding to mHtt (Subramaniam, Sixt, Barrow & Snyder, 2009), however, replication of this finding in an animal model of HD has not yet been attempted.

Additionally, this pathway involves cell signaling related to Rhes expression. Inhibition of the mevalonate pathway has been shown to modulate AKT (Kureishi et al., 2000; Larson-Casey, Murthy, Ryan, & Carter, 2014) and impair Insulin/IGF-1 signaling (Martínez-González, Viñals, Vidal, Llorente-Cortés & Badimon, 1997). Interestingly, the drug used in this study to inhibit farnesylation, Simvastatin, has been shown to both activate (Kureishi et al., 2000) and inhibit AKT (Kochuparambil, Al-Husein, Goc, Soliman & Somanath, 2011); while glucose induced insulin secretion was shown to be inhibited by Simvastatin (Yada, Nakata, Shiraishi & Kakei, 1999). The bisphosphonate Zoledronic Acid was also used in this study because it inhibits geranyl-geranylation. Further, Zoledronic Acid has been shown to inhibit AKT and reduce Bcl-2 activity (Caraglia et al., 2007).

1.5 Purpose and Hypothesis

The purpose of this thesis was to further evaluate the influence of Rhes expression on the behavioral and neurodegenerative symptoms associated with HD. Using the 3NP model of HD, behavioral data were collected on animals treated with pharmacological inhibitors of farnsylation and geranyl-geranylation.

Hypothesis: 3NP will induce motor and behavioral deficiencies that will be ameliorated by drugs that inhibit Rhes prenylation.

2. Method

2.1 Animals

Sixty male albino Hsd:ICR (CD-1) mice were purchased from Harlan Laboratories (Indianapolis, IN). Mice weighed approximately 30 g on arrival, and were housed in groups of five. Temperature and humidity were under controlled conditions, and mice were kept on a normal 12:12 hour light:dark cycle (lights on 0700).

Body weight was recorded during daily handling, and access to food and water was *ad libitum*. Animals were housed and handled in strict accordance with the regulations of the United States Public Health Service, and all conditions and procedures were approved by the University of New Orleans Institutional Animal Care and Use Committee.

2.2 Drug Administration

Drugs were dissolved daily prior to injections using sterile saline and administered in a volume of 10ml/kg. 3NP was dissolved to a concentration of 7.7mg/ml and neutralized to pH7.4 with 10 Normal Sodium Hydroxide (Mealer et al., 2013) for a final concentration of 77mg/10 ml. While the concentration of 3NP remained the same throughout the experiment, the volume administered varied based on day of injection. Mice were injected once daily, starting at 50 mg/kg with dosage increasing by 15% each day for a period of 7 days, and a cumulative dose of 560 mg/kg at day 7.

Simvastatin (40mg/kg) and Zoledronic Acid (1.0 mg/kg) or a combination of both drugs, were administered to animals along with 3NP for 7 days following random assignment to drug condition. Due to toxic effects of drug conditions involving 3NP+Saline, 3NP+Zoledronic Acid

and 3NP+Simvastatin+Zoledronic Acid, several animals did not survive to complete drug treatment, and no 3NP+Zoledronic Acid animals were behaviorally tested. Additional groups were added to the study in order to further study possible toxic effects of the drugs, and the dose of Zoledronic Acid was reduced to 0.5mg/kg and administered with either Simvastatin+3NP or Simvastatin+Saline (See Table 1.1). Injections were ceased for the animals remaining in these groups prior to day 7, and behavior was recorded after 5 injections on day 6.

Table 1.1: *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

2.3 Behavioral Tests

Twenty-four hours after the last drug injection all mice were tested using a battery of behavioral tests as described below.

Sucrose Preference Test

This test was used to examine HD-like symptoms such as anhedonia or lack of interest in seeking a reward (access to sucrose-adulterated drinking water). Mice have a bias for the sucrose solution, and a failure to consume the solution is considered to be indicative of anhedonia (Papp, Willner & Muscat, 1991). Mice were presented with two bottles with sipper tubes. One bottle contained water and the other contained a 30% sucrose solution. The bottles were weighed before the test, and placed in the cage with the mouse for one hour. After one hour, the bottles were weighed again, and a score was calculated by dividing the total amount of water intake by the total amount of water intake plus the total amount of sucrose intake and multiplied by 100:

$$\text{Index of Anhedonia (\% water preference)} = 100 \times \left(\frac{\text{vol. water consumed}}{\text{vol. water} + \text{vol. sucrose consumed}} \right)$$

Locomotor Behavior Test

Mice were placed in the center of an elevated open field grid without any enclosure, and observed for 2 minutes. Number of squares crossed was recorded in order to capture any locomotor impairment. During this test, hypokinetic circling behavior was observed and recorded. These behaviors have previously been implicated in motor impairment and altered basal ganglia functioning (Miwa, Nishi, Fuwa & Mizuno, 1998; Löscher, 2010 Nambu et al., 2011).

Elevated Plus Maze

The elevated plus maze is used as a test of anxiety in mice (Pellow, Chopin, File & Briley, 1985). The apparatus is a plus-shaped maze elevated 50 cm off the floor with two closed arms positioned across from each other, two open arms across from each other and a center area. The

four arms were made of strips of Plexiglas 10 cm wide and 50 cm long. They were positioned on supports 50 cm above the floor of the testing room at 90° intervals and meet at a central square (10 cm²). Two of the arms in 180° linear arrangement had walls 40 cm high that were interrupted at the central square. The two orthogonal arms contained no walls. The preference for being in closed arms over open arms is recorded to measure anxiety-like behavior.

3. Results

Data were screened for missing values and unequal sample sizes. Three cases were deleted from Saline+Saline Drug Group, and one case was deleted from the 3NP+Simvastatin Drug Group due to missing data. To create equal sample sizes from the remaining data, 6 cases were selected randomly from each group using SPSS case selection (N=30).

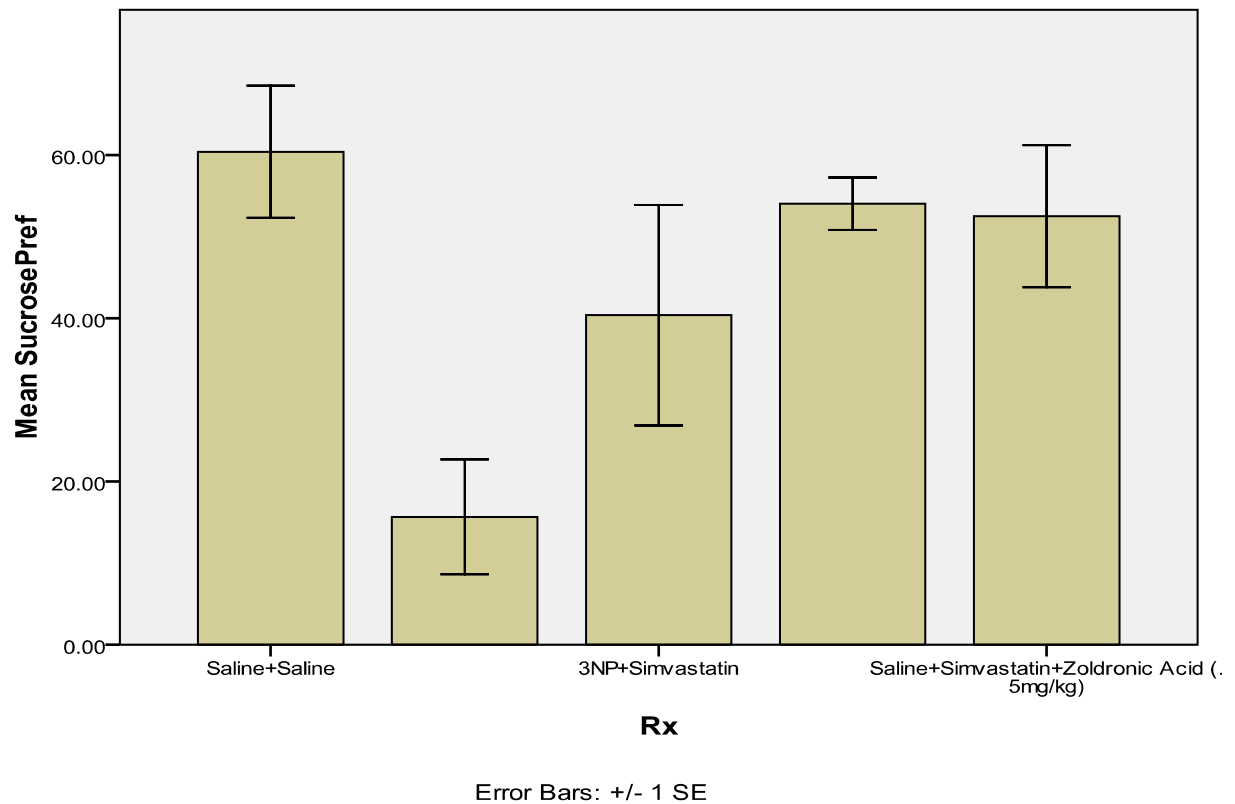
Due to changes in study design, Number of Injections varied between groups, and was used as a covariate with Drug Group in all statistical analyses of behavior.

3.1 Sucrose Preference

Homogeneity of regression showed that the interaction between Drug Group and Number of Injections was not significant ($p=.915$). Levene's Test showed that homogeneity of variance could be assumed ($p=.067$). ANCOVA indicated that Number of Injections was not a significant covariate [$F(1,24)=0.01$, $p=.915$, $\eta^2=.00$], and indicated a strong significant relationship between Drug Group and Sucrose Preference Score [$F(4,24)=3.93$, $p=.014$, $\eta^2=.40$].

Group means from highest to lowest (see Figure 3.1a) were Saline+Saline ($M=69.39$), 3NP+Simvastatin+Zoledronic Acid ($M=54.04$), Saline+Simvastatin+Zoledronic Acid ($M=52.49$), 3NP+Simvastatin ($M=40.36$) and 3NP+Saline ($M=15.65$). 3NP+Saline was

significantly different from Saline+Saline [$t(10)=4.17$, $p=.002$, 2-tailed];
 3NP+Simvastatin+Zoledronic Acid [$t(10)=-4.97$, $p=.001$, 2-tailed]; and
 Saline+Simvastatin+Zoledronic Acid [$t(10)=-3.29$, $p=.008$, 2-tailed].



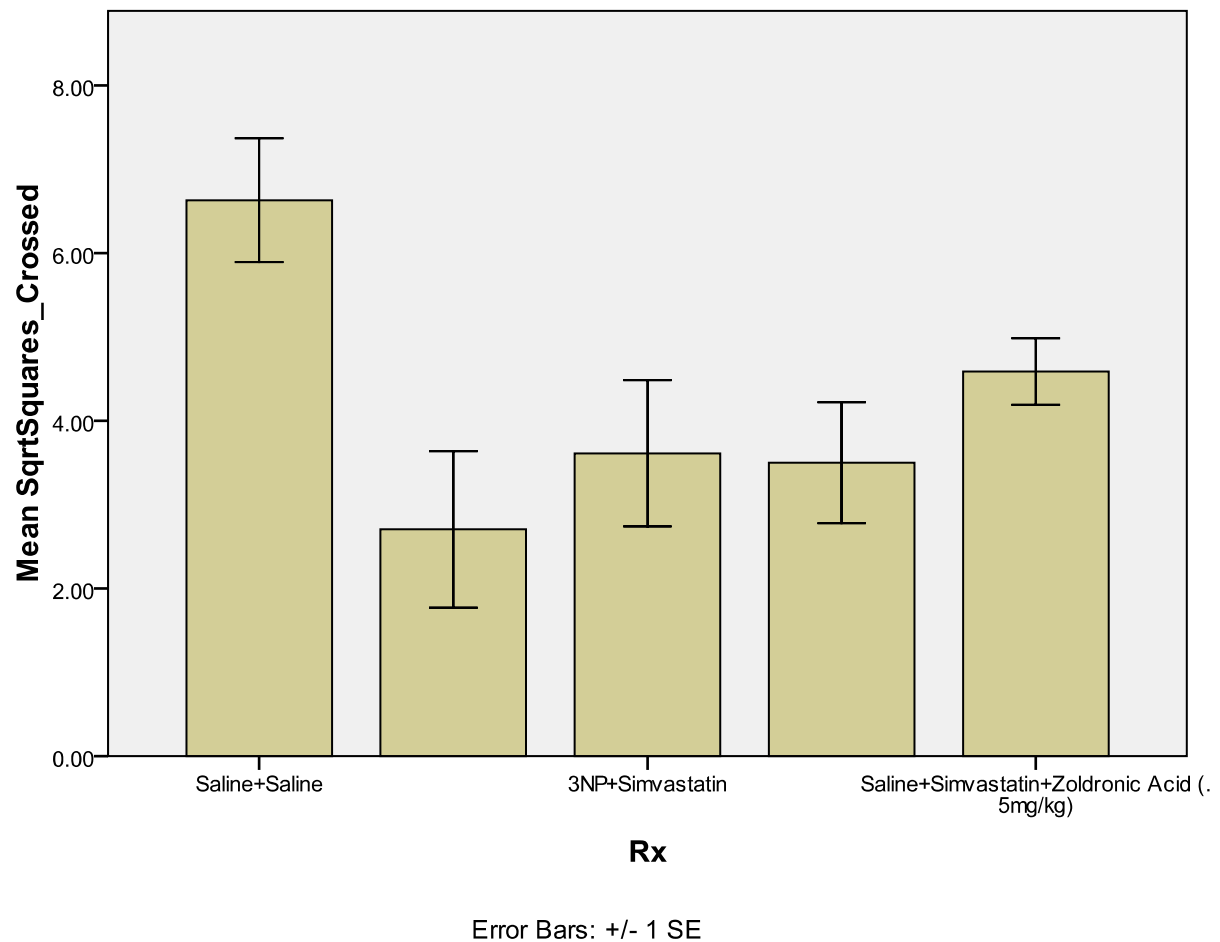
(Figure 3.1a, Sucrose Preference-Results)

3.2 Locomotor Behavior

The variable Number of Squares crossed was positively skewed, and transformed via square root transformation. Homogeneity of regression showed that the interaction between Drug Group and Number of Injections was not significant ($p=.268$), and Levene's Test showed that homogeneity of variance could be assumed ($p=.556$). ANCOVA indicated that Number of Injections was not a significant covariate [$F(1,24)=1.29$, $p=.268$, $\eta^2=.05$], and indicated a strong significant

relationship between Drug Group and Number of Squares crossed [$F(4,24)=4.06$, $p=.012$, $\eta^2=.40$].

Group means from highest to lowest number of squares crossed were Saline+Saline ($M=6.63$), Saline+Simvastatin+Zoledronic Acid ($M=4.59$), 3NP+Simvastatin+Zoledronic Acid ($M=3.49$), 3NP+Simvastatin group ($M=3.61$), and 3NP+Saline group ($M=2.70$). Follow up comparisons showed that the Saline+Saline group was significantly different than the 3NP+Saline [$t(10)=3.30$, $p=.008$]; the 3NP+Simvastatin group [$t(10)=2.64$, $p=.025$]; the 3NP+Simvastatin+Zoledronic Acid group [$t(10)=3.04$, $p=.013$, 2-tailed]; and the Saline+Simvastatin+Zoledronic Acid group [$t(10)=2.44$, $p=.035$, 2-tailed].



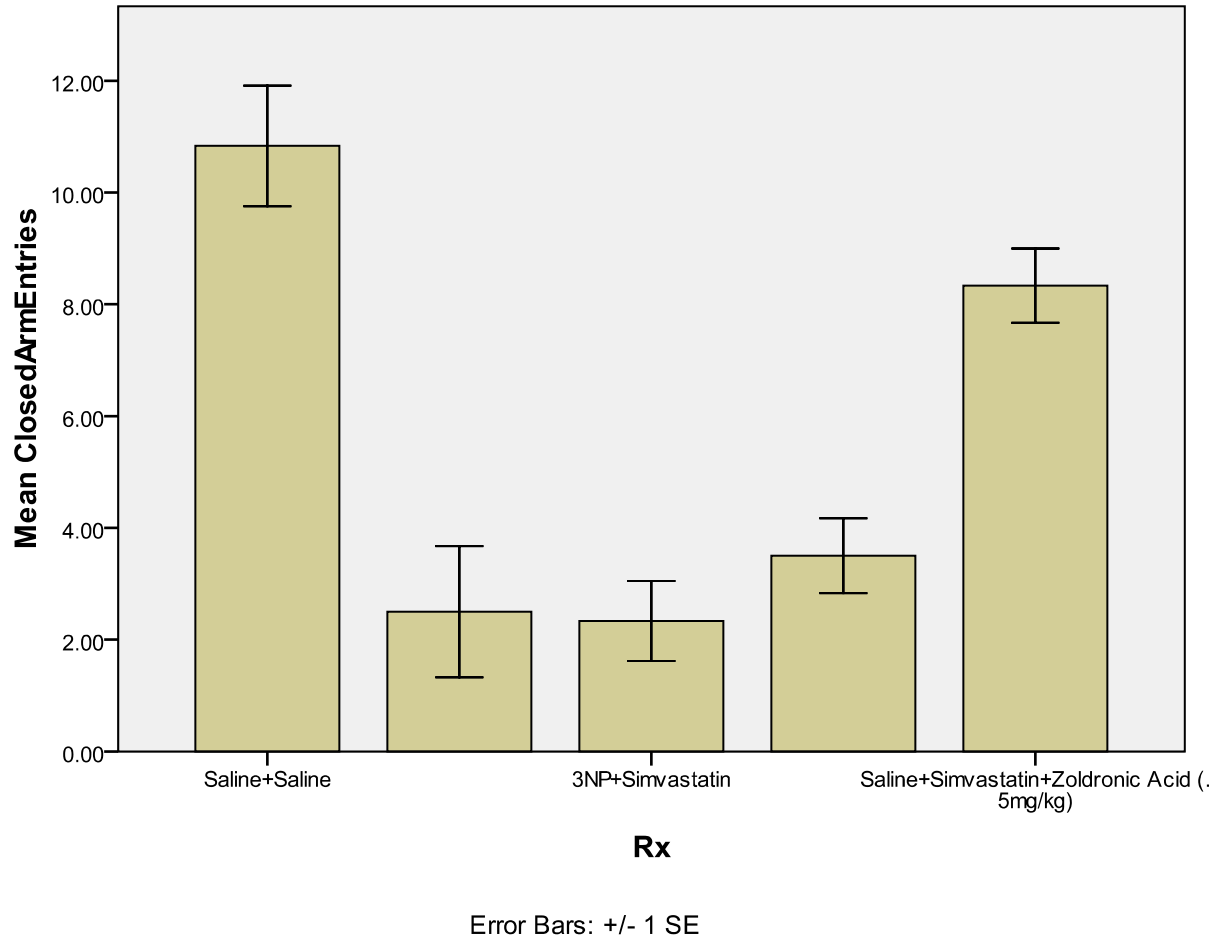
(Figure 3.2a, Locomotor Behavior-Results)

3.3 Elevated Plus Maze

Examination of homogeneity of regression showed that the interaction between Drug Group and Number of Injections was not significant ($p=.240$). Levene's Test showed that homogeneity of variance could be assumed ($p=.252$). ANCOVA indicated that Number of Injections was not a significant covariate [$F(1,24)=1.45$, $p=.240$, $\eta^2=.06$], and indicated a strong significant

relationship between Drug Group and Number of Closed Arm Entries [$F(4,24)=19.42$, $p=.000$, $\eta^2=.76$].

The highest average number of closed arm entries from highest to lowest were Saline +Saline ($M=10.83$), Saline+Simvastatin+Zoledronic Acid ($M=8.33$), 3NP+Simvastatin+Zoledronic Acid ($M=3.50$), 3NP+Simvastatin group ($M=2.33$), and 3NP+Saline group ($M=2.50$). Follow up comparisons showed that the Saline+Saline group was significantly different from the 3NP+Saline group [$t(10)=5.22$, $p=.000$]; 3NP+Simvastatin group [$t(10)=6.57$, $p=.000$]; and the 3NP+Simvastatin+Zoledronic Acid group [$t(10)=5.77$, $p=.000$]. There was also a significant difference between the 3NP+Saline group and Saline+Simvastatin+Zoledronic Acid [$t(10)=-4.32$, $p=.002$], and 3NP+Simvastatin and Saline+Simvastatin+Zoledronic Acid groups [$t(10)=6.14$, $p=.000$].



(Figure 3.3a, Closed Arm Entries-Results)

An additional ANCOVA was conducted using Closed Arm Entries as the independent variable, and the dichotomous variable Circling Behavior (0=no, 1=yes) observed during the locomotor test as a covariate with Drug Group. Examination of homogeneity of regression showed that the interaction between Drug Group and Circling Behavior was not significant ($p=.240$), and Levene's Test showed that homogeneity of variance could be assumed ($p=.157$). ANCOVA indicated that Circling Behavior was a significant covariate [$F(1,24)=10.68$, $p=.003$, $\eta^2=.31$], and indicated a strong significant relationship between Drug Group and Closed Arm Entries when controlling for Circling Behavior [$F(4,24)=17.37$, $p=.000$, $\eta^2=.74$].

4. Discussion

These results suggest that prenylation inhibitors are not protective against 3NP induced behavioral changes in anxiety, as the significant differences between treatment groups appear to reflect motor impairment. Additionally, these treatments seem to exacerbate motor symptoms as reflected by the locomotor test and elevated plus maze. Surprisingly, the 3NP+Simvastatin+Zoledronic Acid and 3NP+Simvastatin groups showed similar motor impairment, but sucrose preference results indicate that 3NP induced anhedonia was attenuated in the 3NP+Simvastatin+Zoledronic Acid group only. The combined motor impairment with attenuated anhedonia may indicate DA specific alterations in the 3NP+Simvastatin+Zoledronic Acid group as DA is believed to mediate sucrose preference (Towell, Muscat, & Willner, 1987) and may not represent deficits in non-dopaminergic neurotransmission.

There may be several possible reasons for the detrimental effect of the drugs on motor behavior. First, as this was a simple behavioral study, we cannot be certain that the drugs acted to prevent the anchoring of Rhes without follow up studies examining striatal tissue. Second, even if the drugs were successful in preventing the anchoring of newly translated Rhes, there may still be an effect of previously anchored Rhes. Again, the only way to be certain of this would be a follow up study examining Rhes expression in brain tissue.

Additionally, while the 3NP model replicates many features of HD, there are several studies that indicate a difference in cell survival processes. For instance, insulin and insulin growth factor signaling which are normally protective against HD damage are detrimental in the 3NP model. (Escartin, Boyer, Bemelmans, Hantraye & Brouillet, 2007). However, the authors reported no

change in COX activation following IGF-1 treatment. This is contradictory to another study showing an increase in COX following IGF-1, mediated protection of HD striatal cells (Ribeiro, Rosenstock, Oliveira, Oliveira, & Rego, 2014), suggesting that 3NP model cell survival mechanisms may be different than HD.

A fourth possible explanation may be that decreasing Rhes expression is not protective in HD. Although these findings seem to contradict the protective effect of Rhes KO in a 3NP model (Meale, Subramaniam, & Snyder, 2013), they support other recent findings that suggest reduced Rhes expression actually enhances the HD phenotype (Lee, et al., 2014). Additionally, given the multiple roles for Rhes in AKT activation and cell function, it is likely that simply decreasing Rhes expression results in a deficit in cellular processing of both apoptosis and autophagy.

The dual role of Rhes in AKT signaling may be explained by a recent finding in pancreatic cancer cells. Specifically, in pancreatic cancer cells, inhibition of either AKT or mTOR induces autophagy, but only inhibition of AKT leads to caspase-mediated apoptosis while inhibiting AKT in combination with inhibition of autophagy blocked cell death. These results suggest that AKT regulates both autophagy and apoptosis through different paths, and mTOR mediates autophagy but is not directly involved in cell death. Autophagy plays a role in cell survival by AKT, but only when mTOR independent pathways are simultaneously activated (Muilenburg, Parsons, Coates, Virudachalam & Bold, 2014), such as the mTOR independent functions of Rhes in autophagy and AKT activity (Mealer, Murray, Shahani, Subramaniam & Snyder, 2014).

Due to the localization of Rhes and HD damage in both pancreatic and striatal cells these finding may explain some important contradictions as a result of the elusive functions of Rhes. Rhes activates and inhibits AKT, and was also recently found to activate autophagy independent of

mTOR. Therefore, Rhes may regulate signals that determine proliferation (i.e. AKT activation/phosphorylation), autophagy (i.e. AKT and mTOR), apoptosis (i.e. AKT deactivation/dephosphorylation) and/or autophagy with cell death. (i.e. mTOR independent activation). This contradictory process may explain the protective effect of combined Lithium and Rapamycin treatment for HD (Sarkar, Krishna, Imarisio, Saiki, O'Kane & Rubinsztein, 2008). Additionally, the lithium treated phenotype of Rhes KO mice may also explain why the KO model was protective against 3NP while targeting Rhes expression is not.

5. Conclusion

Simply decreasing Rhes expression appears to be detrimental to behavioral outcomes in the 3NP model, and a follow up histological study is needed to be certain that the drugs used in this study prevented Rhes prenylation.

Although Rhes is a crucial target for HD research, future studies may benefit from investigating striatal specific gene expression changes that may in turn influence Rhes function, and possibly explain the delayed onset of symptoms. For example, given the relationship between Rhes, growth factors, and AKT, age-related methylation of neurotrophic factors and their receptors may lead to altered cellular function.

In particular, Glial derived neurotrophic factor (GDNF) may be a promising target. GDNF is mainly expressed in the striatum, and is crucial for striatal function and the survival of DA terminals where it is retrogradely transported from the striatum (Chermenina et al., 2006). Excitation of glutamate receptors mediates GDNF expression in the striatum (Ho, Gore, Weickert & Blum, 1995) and expression is altered following lithium treatment (Tunca et al., 2014). Upregulation of GDNF has been shown to be protective in both a genetic and 3NP mouse

model of HD (Ebert, Barber, Heins & Svendsen, 2010; Lee et al., 2006), and exerts its protective effect via PIK3 (Anitha et al., 2006). Additionally, agonism of the GDNF receptor may enhance insulin releasing cells (Srinivasan, 2014), suggesting GDNF may be a promising target to include in future Rhes research.

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